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CONTENT

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USAGE OF RICE MILK IN PROBIOTIC YOGHURT PRODUCTION

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ABSTRACT

There are previous studies focusing on the production of probiotic and fermented dairy products made using vegetable based raw materials like oats and soy, however there is a limited number of studies on the usage of rice milk in fermented dairy products. Four different types of yoghurt samples were produced and stored for 21 days at 4°C. Physical, chemical, microbiological and sensory characteristics of the samples were performed at the 1st, 7th, 14th and 21st days of the storage. It was determined that rice milk increased the viscosity values but decreased the values of the texture, whey separation and the chemical and microbiological properties of yoghurts. Acetaldehyde, acetoin, acetone and diacetyl of carbonyl compounds were detected as main flavor components of yoghurt samples. In the sensory analysis, scores decreased as the rice milk proportions in yoghurt was increased and the panelists reported that P1 sample (25% rice milk + 75% cow's milk) was the closest sample to the control sample (100% cow's milk). Generally speaking, samples containing rice milk did not give good results. However, P1 samples were the most favored products among the samples containing rice milk as they were the closest product to the control group. The consumption of such products is continuously increasing as the customers' tendency to consider them as functional products rather than traditional food products increase.

1. Introduction

The content of yoghurt, which is produced with lactic acid fermentation using *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* and has a rich content in terms of carbohydrates, protein, fat, vitamins, calcium and phosphate, show similarities with milk, however, differences occur due to fermentation (Shahani & Chandan, 1979; Caglar & Çakmakçı, 1999). The positive effects of yoghurt-like fermented dairy products on human health have been determined. Yoghurt, which is suitable for lactose intolerant individuals, is also easy to digest (Dewit, Pochart & Desjeux, 1988; Marteau et al., 1990; Rosado, Solomons & Allen, 1992). Due to its bacterial content, it stimulates the growth of other useful bacteria in the body and shows antagonistic effects with antimicrobial substances produced against various pathogens (Hayaloglu & Konar, 1998).

Depending on the changing customer preference and advancing scientific researches, yoghurt production using different starter cultures has become widely a subject of interest in the recent years. Studies also focus on the production of yoghurt using cultures with probiotic properties and hence having supporting health effects, but generally focused on the effects of using different yoghurt cultures on the common qualities of yoghurt. Increasing population of the world makes the

efficient use of natural sources in human nutrition essential. As the welfare and life standards of the countries rise, phytonutrients leave their place to more quality and rich in terms of protein foods of animal origin. Among the zoological nutrients, milk products take an important place (Tamime & Marshall, 1997; Ziemer & Gibson, 1999; Tamime & Robinson, 1999; Yerlikaya, Akpınar & Kılıç, 2013).

Among the milk products, yoghurt and similar fermented dairy products have high digestibility and contain starter cultures protecting the microflora which have inhibitor activities against harmful microorganisms and show anti-tumor, anti-carcinogenic and anti-cholesterol activities, also they can be safely consumed by individuals with lactose intolerance. Also, fermented dairy products which have important functions as protein source of animal origin, contain balanced amounts of carbohydrates, protein and fat, high amounts of calcium for the healthy development of bones and are an important group of nutrients with low calorie, high nutritional value, refreshing properties and being ready to be consumed anywhere, anytime (Granato et al., 2010; Ozer & Kirmaci, 2010; Soccol1 et al., 2010; Divya et al., 2012).

There are previous studies focusing on the production of probiotic and fermented dairy products made using vegetable based raw materials like oats and soy, however there is a limited number of studies on the usage of rice milk in fermented dairy products. In our study, we aimed to produce fermented dairy product by using rice milk or cow milk-rice milk mixture instead of milk with multi-functional properties, containing amino acids and nitrogen essential for growth and development and bioactive peptides which are recently found to have specific functional properties. In researches, knowing the functional properties of food components, enable us to produce nutritive, healthy and resistant foods with good taste, flavor and consistency (Faccin et al., 2009; Ramos et al., 2011; Coda et al., 2012).

For this purpose, in order to increase its commercial acceptance and enhance the sensory properties, rice milk, an important raw food source considered worldwide, was used in probiotic yoghurt production. In Turkey, there are limited amount of studies focused on the production of such products. Besides, food formulations and diets are needed for individuals with various health problems. With this perspective, we aimed to use rice milk, which is a fermented product added into cow milk and newly introduced in Turkey, with probiotic adjunct cultures used combined with standard yoghurt culture to enhance its physical, chemical, microbiological and sensory properties.

2. Materials and methods

2.1. Materials

Milk used in this study was obtained from Ege University Menemen Research and Application Farms. Beneo (Mannheim, Germany) Nutriz, rice bran formula was obtained from Artisan Gida San. Tic. Ltd. Sti. For the preperation of rice milk, 13.6 g of rice bran was diluted in 100 mL of water. MYE 96-98 starter culture for yoghurt production containing *S. thermophilus* and *L. bulgaricus* was obtained from Maysa Gida San. Tic. A. S. In addition to the yoghurt culture, *Lactobacillus gasseri* ATCC 4963 and *Bifidobacterium longum* DSM Lafti B22 strains were used. Filling and packaging were done with packages obtained from Ege University Faculty of Agriculture Menemen Farms and Ege University Faculty of Agriculture Department of Dairy Technology.

2.2. Methods

Yoghurt samples were encoded as C: 100 % cow milk probiotic yoghurt; P1: 25 % rice milk - 75 % cow milk probiotic yoghurt; P2: 50 % rice milk - 50 % cow milk probiotic yoghurt; P3: 75 % rice milk - 25 % cow milk probiotic yoghurt.

2.3. Probiotic yoghurt production

In probiotic yoghurt production, raw milks were treated according to Turkish Food Codex Communiqué on Fermented Milk. Production was conducted in Ege University Faculty of Agriculture Department of Dairy Science Pilot Production Plants. Mixtures containing only cow milk and three different proportions of cow milk-rice milk (C, P1, P2 and P3) were pasteurized (90°C minutes) in different containers. Following this process, milks were cooled to fermentation temperature (42-43 °C) and inoculated with preactivated lactic cultures with 2 % proportions. Inoculated milks were incubated at 42°C. pH values of yoghurt samples were measured and incubation was ended at pH 4.6. Samples were kept at room temperature for 15 minutes and taken to cold storage at +4°C. Physical, chemical, rheological, microbiologic and sensory properties of samples were measured on the 1st, 7th, 14th and 21st days of the storage.

2.4.Physical analysis

At physical analyses, syneresis rate, viscosity and some textural properties were analyzed.

2.4.1.Syneresis

Volumetric method is used for the determination of syneresis rate. Yoghurts samples were taken with a constant volume (40 mL) ice cream scoops in one go (in order to keep the coagulum as intact as possible) and put onto the filter papers placed in a cone which was fixed over a cylindrical graduate. The amount of syneresis was measured in mL at 30th, 60th and 90th minutes (Gönç, 1986).

2.4.2. Apparent viscosity

For the viscosity analysis, Brookefield DV II Pro+ Viscometer (Brookfield Engineering Lab Inc., Stoughton, Mass., U.S.A.) was used. Samples were mixed 20 times (10 times clockwise, 10 times counter clockwise) and measured at 10 rpm in +8°C in mPa (Aryana et al., 2007; Yerlikaya, Akpınar & Kılıç, 2013).

2.4.3.Texture analysis

Brookfield Texture Analyzer (TA - CT3, Brookfield Engineering Laboratories, Inc., Middlebore, MA, USA) (Probe: TA4/1000) was used for the texture analysis (Lee & Yoo, 2011). Samples were analyzed in 150 mL beakers at +8°C. Texture analyzer CT3 was calibrated before use. Parameters for the calibration; Used probe; TA 4/1000, pretest speed: 2 mm/s, test speed: 1 mm/s, spinning speed: 1 mm/s, shape: cylinder, sample length: 76 mm and sample depth: 45 mm.

2.5.Chemical analysis

Dry matter and fat contents of the samples were measured according to TS 1330. Titrable acidity values were measured according to TS 1330 in ºSH (Soxhelet-Henkel) and lactic acid % (Anonymous, 1999). Total nitrogen content of yoghurt samples were analyzed with Kjeldahl method. Protein contents were calculated by multiplying the nitrogen content by 6.38 (Anonymous, 1999; Barbano et al., 1990).

2.5.1.DL Lactic acid

Lactic acid analysis was performed with Assay Proceolurs K-DLATE 12/11 kit (Megazyme, Wicklow, Ireland) (Amatayakul et al., 2006).

2.5.2.Proteolysis

OPA solution was prepared daily and 25 mL 100 milimoles of Sodium tetraborate, 2.5 mL 20 % Sodium dodecyl sulphate, 40 mg OPA solved in 1mL methanol and 100 μm beta-mercaptoethanol were added and diluted in 50 mL water. As substrates, milk proteins (usually 5-100 μg protein in 10-50 μL) were added to 1 mL OPA. Samples were placed in quartz cuvettes of the standard spectrophotometer and kept in room temperature for 2 minutes and absorbance values were read in 340 nm (Church et al., 1983).

2.5.3.Aroma compounds

The volatile compounds of yoghurt samples were determined with a solid-phase-micro extraction (SPME) method using a fiber (57348-U, Supelco Inc., Bellefonte, PA, USA) coated with the sorbent material, divinylbenzene/Carboxen/Polydimethyl-siloxane, and GC-MS (Trace GC Ultra/ISQ, Thermo Scientific, U.S.A.) equipped with flame ionization detector. Prior to GC-MS analysis, yoghurt samples stored at -20°C were conditioned to room temperature. The yoghurt samples (10 mL) were placed into a 20 mL headspace vial containing a micro stirring bar and a PTFE silicone septum was sealed with an aluminum crimp seal. Before extraction, stabilization of the headspace in the vial was obtained by equilibration for 30 min at 60°C. Then SPME fiber was inserted into headspace of the vial and waited for 30 minutes at 60° C for the absorption of volatile compounds. After equilibration time, the fiber was inserted into the GC injector port and held for 5 minutes for desorption of absorbed molecules at 250 °C. The volatile compounds were separated by using 30 m × 0.2 μm i.d. TR-5MS column (Thermo Scientific, U.S.A.) with 0.25 μm film thicknesses. Carrier gas (He) flow rate was 1 mL/min. Oven temperature was programmed as: 40 °C for 5 min then the temperature was raised to 100 °C (4 °C/min) to a final temperature 240 °C (10°C/min) and hold that temperature for 1 min. Volatile compound fractions were expressed as percentage area. The volatile compounds were defined by using the library of GS/MS (NIST and WILEY). Two replicates of each yoghurt sample were analyzed.

2.6.Microbiologic analysis

2.6.1.Preparation of dilutions

8.5 g NaCl were diluted in 1 L pure water. 90 mL of this solution were taken to special glass bottles and 9 mL to test tubes. Bottles and tubes were sealed and sterilized at 121 ºC, 1.1 atm pressure for 15 minutes. Homogenized 10 g probiotic yoghurt samples were added to 90 mL of saline solution and stirred. 1mL of this solution was added to 9 mL saline containing test tubes. Finally, dilutions were prepared in appropriate proportions.

2.6.2.Lactobacillus delbruecki subsp. bulgaricus counts

For *L. bulgaricus* counts, MRS-Agar (Merck, Germany) fixed at pH 5.2 with 1.0 M HCL was used. 1 mL of dilutions were taken to petri dishes and approximately 15-20 mL of MRS-Agar were added and mixed. After gelation of the media, petri dishes were incubated upside down at 43ºC for 3 days in anaerobic conditions. Following the incubation, white colored colonies were counted and determined as *L. bulgaricus* count in CFU/g (Tharmaraj & Shah, 2003).

2.6.3.Streptococcus thermophilus counts

For the determination of *S. thermophilus* counts were determined with M-17 Agar (Merck, Germany) according to pour plate technique. 1 mL of dilutions was taken to petri dishes and approx. 15-20 mL of pre-liquefied M17-Agar at 40 – 45oC were added until forming a thin layer and mixed. After gelation of the media, petri dishes were incubated upside down at 370C for 3 days in aerobic conditions. After the incubation, round shaped yellowish colonies were counted as *S. thermophilus* in CFU/g (Dave & Shah, 1996; Donkor *et al.,* 2006).

2.6.4.Lactobacillus gasseri counts

For *L. gasseri* counts, MRS-Agar (Merck, Germany) fixed at pH 5.2 with 1.0 M HCL was used. After diluted in proper proportions, 1 mL of dilutions was taken to petri dishes and approx. 15-20 mL of MRS-Agar was added until forming a thin layer and mixed. After gelation of the media, petri dishes were incubated upside down at 43ºC for 3 days in anaerobic conditions. Following the incubation, white colored colonies were counted and determined as *L. gasseri* count in CFU/g (Tharmaraj & Shah, 2003).

2.6.5.Bifidobacterium longum counts

For *Bifidobacterium longum* counts, Rogosa Agar was used by adding tetramethybenzidin at 37ºC for 72 hours at microaerophilic conditions. 1 mL of appropriate dilutions was taken to petri dishes and approx. 15-20 mL of Rogosa Agar were added until forming a thin layer and mixed. After gelation of the media, petri dishes were incubated upside down at 37 ºC for 3 days in aerobic conditions. Following the incubation, round shaped yellowish colonies were counted and determined as *B. longum* count in CFU/g (Lapierre, Undeland & Cox, 1992; Vinderola & Reinheimer, 1999).

2.7. Sensory analysis

In order to evaluate the consuming quality of the probiotic yoghurts, sensory analysis were made. For this purpose grading method was used in sensory analysis (Bodyfelt F.W., Drake M.A., Rankin, 1998; Uysal, Kınık & Kavas, 2004). Grading sensory analysis was made with a trained group of panelists consisting the academic staff and graduate students of Ege University Faculty of Agriculture Department of Dairy Technology. For the grading, evaluation criteria specified in TS-1330 were considered. According to these criteria, evaluation forms graded between 1 and 5 were given to the panelists and were asked to fill for the evaluation.

2.8. Statistical analysis

In the study, 4 different yoghurt types were produced in 2 repetitions. For the statistical evaluation of results Analysis of Variance was used and for the determination of different groups Duncan Test was applied. Accordingly, statistical analysis software SPSS version 19.0 was used.

3. Results and discussions

3.1.Physical Properties of Probiotic Yoghurt Samples

3.1.1.Syneresis

The average syneresis values of probiotic yoghurt samples were given in Table 1. On the 30^{th} minute of the measurements, the highest syneresis rate was determined in P2 sample on the 1^{st} day (18.50 mL), while the lowest syneresis rate was in K sample (12 mL) on the 14^{th} and 21^{st} days. As a result of the analysis of variance, the difference between the storage days were significant ($p<0.05$). In the samples containing rice milk, the syneresis rate was lower in P3, compared to the two other samples. Among all the results, difference between P1 sample and K sample was not significant ($p>0.05$). On the 60^{th} minute of the measurements, the highest syneresis rate was in P2 sample on the 1^{st} day (17 mL) while the lowest syneresis rate was seen in K sample (13 mL) on the 21^{st} day. As a result of the analysis of variance, the difference between the samples according to days were be significant ($p<0.05$). On the sample groups P1, P2, P3 differences were associated with rice milk proportions. The difference between K and P1 on the 1^{st} day was not significant ($p>0.05$), whereas on the 7^{th}, 14^{th} and 21^{st} days, the differences between K sample and samples containing rice milk were significant ($p<0.05$).

At the end of the 90^{th} minute, syneresis values varied between 18.50 mL – 26 mL. The highest value was obtained in P2 on the 21st day and the lowest in control sample on the 14^{th} and 21^{st} days of the storage. As a result of the analysis of variance, the difference between the samples according to days were significant ($p<0.05$). The differences between P2 and P3 on the 7^{th} day, K and P1 on the 21^{st} day and, P2 and P3 on the 21^{st} day were statistically not significant ($p>0.05$). The syneresis rates usually had a decline on the 30^{th}, 60^{th} and 90^{th} minutes since the 1st day of the storage. This continued until the last day of the storage. The decline in the syneresis rates in yoghurt samples during storage period were also reported in some of the studies (Atalay, 1994). This was associated with the water holding capacity of proteins, and reported that the water holding capacity of proteins increased as the pH levels decreased down to 4.00. Atamer & Sezgin (1987) also reported a decline during storage periods as the pH dropped to 4.00.

Table 1. The avarage syneresis values of probiotic yoghurt samples (ml) (n=2).

		Storage Period (Day)			
Time (Minute)	**Sample**	**1**	**7**	**14**	**21**
30	**K**	13.25 ± 0.35^{aX}	13.00 ± 0.00^{aX}	12.00 ± 0.00^{aY}	12.00 ± 0.00^{aY}
	P1	13.00 ± 0.00^{a}	12.50 ± 0.70^{a}	11.50 ± 0.70^{a}	11.50 ± 0.70^{a}
	P2	18.50 ± 0.70^{bX}	17.25 ± 0.35^{bXY}	16.75 ± 1.06^{bXY}	16.00 ± 0.00^{bY}
	P3	15.00 ± 0.00^{c}	14.50 ± 0.70^{c}	14.00 ± 0.00^{c}	14.00 ± 0.00^{c}
60	**K**	16.25 ± 0.35^{aX}	15.50 ± 0.70^{aX}	13.50 ± 0.70^{aY}	13.00 ± 0.00^{aY}
	P1	17.00 ± 0.00^{a}	17.00 ± 0.00^{b}	16.75 ± 1.06^{b}	16.50 ± 0.70^{b}
	P2	24.00 ± 0.00^{bX}	23.50 ± 0.70^{cXY}	22.50 ± 0.70^{cYZ}	22.00 ± 0.00^{cZ}
	P3	21.50 ± 0.70^{cX}	20.00 ± 0.00^{dY}	20.00 ± 0.00^{dY}	20.00 ± 0.00^{dY}
90	**K**	19.50 ± 0.70^{a}	19.00 ± 0.00^{a}	18.50 ± 0.70^{a}	18.50 ± 0.70^{a}
	P1	21.00 ± 0.00^{b}	21.00 ± 0.00^{b}	20.75 ± 1.0^{b}	20.50 ± 0.70^{a}
	P2	26.00 ± 0.00^{cX}	25.00 ± 0.70^{cX}	25.00 ± 0.00^{cX}	24.00 ± 0.00^{bY}
	P3	24.00 ± 0.00^{d}	23.00 ± 1.06^{c}	23.00 ± 0.00^{d}	22.75 ± 1.06^{b}

Table 2. The average viscosity values of probiotic yoghurt samples (Pa.s)

	Sample	**Storage Period (day)**			
		1	**7**	**14**	**21**
Viscosity (Pa.s)	**K**	3.52 ± 0.33^{aXY}	3.29 ± 0.03^{aY}	4.32 ± 0.61^{aX}	3.79 ± 0.14^{aXY}
	P1	3.22 ± 0.13^{a}	3.22 ± 0.11^{a}	3.20 ± 0.05^{b}	3.36 ± 0.31^{a}
	P2	1.09 ± 0.08^{bX}	1.43 ± 0.24^{bXY}	1.53 ± 0.08^{cY}	1.63 ± 0.13^{bY}
	P3	0.85 ± 0.03^{b}	0.83 ± 0.09^{c}	0.82 ± 0.15^{c}	0.89 ± 0.25^{c}

3.1.2.Apparent viscosity values

The average viscosity values of probiotic yoghurt samples were given in Table 2. At the end of the 21 days of the storage period, viscosity of the samples were found between 0.89 and 3.43 Pa. The highest value was obtained in control sample (4.22) on the 14th day, and the lowest in P3 (0.87) again at the 14th day of the storage. The difference between the storage days were found to be significant ($p<0.05$).

On the first day the difference between K and P1 and the difference between P2 and P3, on the 7th day the difference between K and P1, on the 14th day the difference between P2 and P3 and on the 21st day the difference between K and P1 were found to be statistically not significant ($p>0.05$). As seen on the table, since the first day, the viscosity of probiotic yoghurt samples increased. During the storage period, the values for syneresis decreased, while the viscosity values increased. In other words, for obtaining the firm structure, syneresis decreased while the viscosity increased. The reason for this, as Akin & Konar (1999) explained, is the increase in the water holding capacity of proteins during the storage period and tightening of the gel structure during the storage period.

Table 3. Textural properites of probiotic yoghurt samples

Parameter	Sample	Storage Period (day)			
		1	7	14	21
Hardness (g)	K	233.25±76.01^{a}	241±95.45^{a}	242.5±96.87^{a}	194±125.86
	P1	124.75±34.29^{ab}	124.25±39.24^{ab}	125±41.71 ab	106.75±67.52
	P2	66.75±21.56^{b}	68.5±23.33^{b}	67.5±26.16 b	65.75±18.03
	P3	39.25±2.47^{b}	39±2.82^{b}	38.75±1.76 ^{b}	40±0.70
Consistency (mj)	K	21.08±5.72^{a}	22.20±8.32^{a}	22.53±8.00 ^{a}	18.95±12.31
	P1	12.51±4.49^{ab}	12.68±4.86^{ab}	12.89±4.99 ^{ab}	10.81±8.29
	P2	5.98±2.18^{b}	6.16±2.49^{b}	6.11±2.63 ^{b}	6.00±2.15
	P3	3.78±0.17^{b}	3.76±0.29^{b}	3.73±0.29 ^{b}	3.85±0.11
Cohesion Force (g)	K	39 ± 10.60^{a}	29.25 ± 41.36	48.75 ± 21.56	44.75 ± 23.68
	P1	35 ± 14.84^{ab}	23.25 ± 30.05	23 ± 31.11	24 ± 33.94
	P2	11 ± 14.14^{ab}	0.5 ± 0.70	10.25 ± 14.49	0.50 ± 0.70
	P3	4.0 ± 5.65^{b}	1.0 ± 0	1.25 ± 1.761	0.25 ± 0.35
Cohesiveness (mj)	K	3.38 ± 1.08	2.5 ± 3.52	3.26 ± 1.64	3.37 ± 0.96
	P1	3.56 ± 2.05	2.75 ± 3.88	2.3 ± 3.24	2.42 ± 3.42
	P2	1.32 ± 1.86	0 ± 0	1.46 ± 2.06	0 ± 0
	P3	1.02 ± 1.43	0 ± 0	0.43 ± 0.60	0 ± 0

Table 4. Dry matter, fat and protein content of probiotic yoghurt samples

	Sample	Storage Period (Day)	
		1	21
Dry matter	K	16.43 ± 0.05^{a}	16.30 ± 0.06^{a}
	P1	16.13 ± 0.15^{a}	16.14 ± 0.10^{a}
	P2	15.47 ± 0.28^{b}	15.32 ± 0.28^{b}
	P3	14.30 ± 0.16^{c}	14.34 ± 0.24^{c}
Fat	K	3.25 ± 0.06^{a}	3.14 ± 0.02^{a}
	P1	3.15 ± 0.01^{b}	3.11 ± 0.01^{ab}
	P2	3.05 ± 0.01^{c}	3.07 ± 0.01^{bc}
	P3	2.94 ± 0.01^{d}	2.90 ± 0.01^{d}
Protein	K	3.43±0.05^{a}	3.42±0.03^{a}
	P1	3.28±0.02^{b}	3.31±0.04^{b}
	P2	2.88±0.01^{cX}	2.82±0.02^{cY}
	P3	2.53±0.01^{dX}	2.57±0.02^{dY}

3.1.3. Texture

Firmness is the force to be applied to a food material in order to provide a certain deformation. In our study, the average firmness values of probiotic yoghurt samples were given in grams (g) and shown in Table 3 with the standard deviations. The firmness values of samples varied between 41.13 g and 240.88 g. The highest value was obtained in control sample on the 14th day and the lowest in P3 on the 14th day of the storage. The difference between the different storage days were not significant ($p>0.05$). In the samples containing rice milk, the firmness values decreased as the ratio of the mixture increased. During the storage period, firmness values of the samples decreased. Regarding the increase in denaturation of whey proteins and heat process applied, it was determined that, as hydrophilic properties increased up to a certain level, the firmness increased and the storage period also caused an increase in firmness. Additionally, low acidity affected the water holding capacity

of proteins and the firmness negatively. Water holding capacity of proteins decreased in high acidity as well, causing shrinkages in gel formation and syneresis. Between pH 4.6 - 4.0 water holding capacity increases and syneresis does not occur (Atamer & Sezgin, 1986). Homogenization also affects the firmness of yoghurt structure, the firmness increase as the homogenization pressure applied increase.

Consistency is the energy needed to initiate the flow. The average cohesiveness values of probiotic yoghurt samples with standard deviations were given in mj (milijoule) in Table 3. Consistency values of probiotic yoghurt samples varied between 3.77 – 24.41 mj. The highest value was obtained in control sample on the 14th day, and the lowest in P2 on the 14th day of the storage. The difference between the storage days were not significant ($p>0.05$). Herrero & Requena (2005), in their study focusing on production of yoghurts from goat milks fortified with whey concentrate in a ratio of 1 %, reported that whey concentrate has a positive effect on the structure and consistency of yoghurt products. In a similar study, using whey concentrates caused an increase in the firmness and dry matter of the yoghurt products, and consistency deformations due to over softening of yoghurt samples fortified with 15 % whey concentrate occurred and weak flavor formation was observed (Tosun, 2007).

The average cohesiveness values of probiotic yoghurt samples with standard deviations were given in Table 3These textural values varied between 1.16 and 41.82 g. The highest value was obtained in control sample on the 14th day, and the lowest in P3 on the 21st day of the storage. The cohesion force of K sample increased from the 1st day of the storage, where P1, P2 and P3 samples decreased.

Cohesiveness is the work done to break the attraction force of the surface (tongue, tooth, palate or probe) in contact. Cohesiveness values of probiotic yoghurt samples varied between 0.30 – 5.08 mj. The highest value was obtained in P1 on the 1st day, and the lowest in P2 and P3 on the 7th and 14th days of the storage The difference between the storage days were not significant ($p>0.05$). K and P1 had higher values compared to others, where P2 and P3 had changing values with descents and ascents.

3.2.Chemical Properties of Probiotic Yoghurt Samples

3.2.1.Total dry matter

Total dry matter analysis of the probiotic yoghurt samples were made at the 1st and 21st days of the storage. The results with standard deviations were given in Table 4. The difference between the dry matter contents of probiotic yoghurt samples were statistically not significant on the 1st and 21st days of the storage ($p>0.05$). Dry matter contents of samples varied between 14.30 – 16.43 %. The results obtained were similar to those of Akalin (1993) and Dave & Shah (1997a; 1997b) (%15.30-15.80). The dry matter contents of the yoghurt-like fermented dairy products vary in a wide range. The dry matter results reported by other researchers and our current results share partial similarities but some differences. The types of raw milk used in the production, dry matter, rice milk, the process applied during the production and degree of cultures to ferment lactose are the factors that affect the dry matter contents.

3.2.2.Fat

Fat content of the probiotic yoghurt samples were measured at the 1st and 21st days of the storage. The results with standard deviations were given in Table 4. Fat contents of samples varied between 3.25 – 2.90 %. The difference between the fat contents of probiotic yoghurt samples were statistically not significant at the 1st and 21st days of the storage ($p>0.05$).

3.2.3.Total protein

Total protein analysis of the probiotic yoghurt samples were made at the 1st and 21st days of the storage. The results with standard deviations were given in Table 4. At the end of the 21 days of the storage period, protein

contents of the samples found between 2.53 and 3.43 %. The difference between the protein contents of probiotic yoghurt samples on the 1st and 21st days of storage found to be significant ($p<0.05$). The protein contents of K and P1 samples were found complying with the rates (3% minimum) specified in Fermented Milks Regulations, where P2 and P3 were found lower than the rates specified. In previous studies protein contents were found between 2.66 % and 8.38 % (Yaygın, 1981; Akin & Konar, 1999; Küçüköner & Tarakçı, 2003; Ayar, Sert & Kalyoncu, 2006). Protein values found in our study had similarities with these results.

3.2.4.Titratable acidity

Titratable acidity values of probiotic yoghurt samples in lactic acid % and (°SH) were given below.

Lactic acid

The average lactic acid % values of probiotic yoghurt samples with standard deviations were given in Table 5. On the 1st, 7th, 14th and 21st days, the difference between lactic acid (%) values were found insignificant ($p>0.05$). Although the differences between storage days were not significant, lactic acid levels of the samples increased during the storage period. Lactic acid (%) values of probiotic yoghurt samples varied between 0.41 and 1.14 % during the storage. According to the Turkish Food Codex Fermented Milks Regulations, lactic acid levels shall be between 0.6 - 1.5 %. The levels of K, P1 and P2 have complied with the regulations and kept their compliance during storage period. The highest level of lactic acid was usually measured in sample K, where the lowest was P3. In all the samples, as a result of periodical increase during the storage period, total acidity values increased. This was associated with the continuous acid production by culture bacteria. The change in acidity after incubation is important in terms of determining the shelf life of the products. Also, the increase in the titratable acidity may be due to the increase in the levels of protein, phosphate, citrate, lactate and some minerals, as well as dry matter (Tamime & Robinson, 1999). In a study on yoghurts produced from soy milk, lactic acid levels in samples containing soy milk were reported lower (Lee & Yoo, 2011). In a similar study, syneresis due to the increase in lactic levels in yoghurts affected the consumability of the yoghurts significantly. It was reported that lactic acid levels in yoghurts produced exclusively from soy milk were considerably lower than those of produced from cow milk, additionally; lactic acid levels in soy milk yoghurts were increased to standard yoghurt levels by adding ingredients such as milk powder (Granta & Morr, 1996).

Soxhelet Henkel (°SH) acidity

The average °SH values of probiotic yoghurt samples were given in Table 5, with standard deviations. °SH values varied between 16.5 and 45.5. The highest value was obtained in control sample on the 21st day, while the lowest in P3 on the 1st and 7th days of the storage. Although the °SH levels of samples increased during the storage, the effect of the storage period were not significant ($p>0.05$). Among the groups, the difference between the °SH levels were significant ($p<0.05$). Only on the 7th day, the difference between K and P1 samples were not significant. Various researchers reported the °SH levels in their studies between 39.19 and 53.55 °SH (Yaygin, 1981; Akin & Konar, 1999; Akalin, 1993; Sarı, 2005; Tosun, 2007; Yalçınkaya,2002). Akın & Konar (1999), in their study, produced fruit added/flavored yoghurts from cow and goat milks and stored for 15 days and reported an increase in titratable acidity during the storage period.

3.2.5.DL Lactic acid

Lactic acid, which is formed as a result of fermentation of lactose, has three isomers, ; L(+) which rotates the light clockwise direction, D (-) which rotates the light counterclockwise direction and DL with no optical activity. This is related with the location

of the hydroxyl groups on the 2nd carbon atom. If the hydroxyl group is on the right, called D(-), and if on the right, it is called L(+). Lactic acid isomers vary depending on the starter cultures used in the production of dairy products. In the studies, it is found that *L. casei*, *S. thermophilus*, *L. lactis*, *L. cremoris*, *L. diacetylactis*, *Bifidobacterium bifidum* produce L(+), *L. bulgaricus* D(-) and *Lactobacillus helveticus* and *Lactobacillus acidophilus* DL lactic acid.

Lactic acid isomers have positive effects on human health as well as maintaining the typical formations desired in dairy products. One of the two stereo isomer forms of lactic acid, L(+) lactic acid, is formed as an intermediary product of human metabolism, and then it is partially hydrolyzed to CO_2 and H_2O, and used as an energy source, also partially used in glycogenesis for the formation glycogens. Therefore, L(+) lactic acid is defined as physiologic lactic acid. On the contrary, D(-) lactic acid is metabolized slowly and insufficiently in the organism, causing a burden for the organism.

DL lactic acid values of probiotic yoghurt samples were given in Table 5. DL values of samples varied between 0.44 mg and 1.21 mg. The highest value was obtained in P3 on the 1st day and the lower in control sample on the 21st day of the storage. As a result of the analysis of variance, the difference between the storage days were found to be significant ($p<0.05$). The difference between K and P1, P2 and P3 at the 1st day and the difference between K and P1 samples at the 14th day were statistically not significant ($p>0.05$).

Table 5. Some physic-chemical properties of probiotic yoghurt samples

Parameter	Sample	Storage Period (day)			
		1	7	14	21
Acidiy (Lactic acid%)	K	1.11 ± 0.01^{a}	1.09 ± 0.05^{a}	1.13 ± 0.10^{a}	1.14 ± 0.05^{a}
	P1	0.90 ± 0.07^{b}	0.95 ± 0.00^{a}	0.95 ± 0.03^{b}	0.93 ± 0.03^{b}
	P2	0.66 ± 0.053^{c}	0.64 ± 0.08^{b}	0.70 ± 0.03^{c}	0.70 ± 0.00^{c}
	P3	0.41 ± 0.01^{d}	0.41 ± 0.05^{c}	0.43 ± 0.00^{d}	0.43 ± 0.03^{d}
Acidity (ºSH)	K	44.50 ± 0.70^{a}	43.50 ± 2.12^{a}	45.00 ± 4.24^{a}	45.50 ± 2.12^{a}
	P1	36.00 ± 2.82^{b}	38.00 ± 0.00^{a}	38.00 ± 1.41^{b}	37.00 ± 1.41^{b}
	P2	26.50 ± 2.12^{c}	25.50 ± 3.53^{b}	28.00 ± 1.41^{c}	28.00 ± 0.00^{c}
	P3	16.50 ± 0.70^{d}	16.50 ± 2.12^{c}	17.00 ± 0.00^{d}	17.00 ± 1.41^{d}
Acetaldehyde (ppm)	K	16.81 ± 2.15^{W}	11.5 ± 0.61^{aX}	8.89 ± 0.43^{aY}	6.82 ± 0.46^{aZ}
	P1	16.71 ± 2.09^{W}	11.427 ± 0.21^{aX}	9.05 ± 0.41^{abY}	6.75 ± 0.24^{aZ}
	P2	15.5 ± 1.16^{W}	10.96 ± 0.08^{abX}	8.22 ± 0.35^{bcY}	6.44 ± 0.20^{abZ}
	P3	14.46 ± 1.00^{W}	10.625 ± 0.16^{bX}	7.72 ± 0.63^{cY}	6.11 ± 0.38^{bZ}
DL Lactic acid (mg/100g)	K	0.92 ± 0.05^{aW}	0.77 ± 0.02^{aX}	0.50 ± 0.03^{aY}	0.44 ± 0.02^{aZ}
	P1	1.03 ± 0.08^{abW}	0.84 ± 0.01^{bX}	0.59 ± 0.03^{aY}	0.51 ± 0.03^{bZ}
	P2	1.11 ± 0.09^{bcW}	0.94 ± 0.04^{cX}	0.69 ± 0.05^{bY}	0.56 ± 0.03^{cZ}
	P3	1.21 ± 0.04^{cW}	0.98 ± 0.04^{cX}	0.81 ± 0.08^{cY}	0.65 ± 0.01^{dZ}
Proteolytic activity (OPA Value)	K	0.93 ± 0.02^{bcX}	1.06 ± 0.02^{aW}	0.88 ± 0.01^{abY}	0.742 ± 0.02^{bZ}
	P1	0.99 ± 0.03^{aX}	1.02 ± 0.05^{aX}	0.85 ± 0.04^{aY}	0.66 ± 0.02^{cZ}
	P2	0.97 ± 0.03^{abX}	0.90 ± 0.07^{bX}	0.91 ± 0.01^{bX}	0.80 ± 0.04^{abY}
	P3	0.89 ± 0.04^{cX}	1.10 ± 0.02^{aY}	0.92 ± 0.03^{bX}	0.81 ± 0.05^{aZ}

3.2.6.Proteolytic activity

OPA (ortho-phthalaldehyde), (ortho-phthalaldehyde) is the chemical compound with the formula $C_6H_4(CHO)_2$. The molecule is a dialdehyde, consisting of two formyl (CHO) groups attached to adjacent carbon centers on a

benzene ring. The molecule was first described in 1887 when it was prepared from α,α,α',α'-tetrachloro-ortho-xylene.α,α,α',α'-tetrachloro-ortho-xylene. Related to ortho-phthalaldehyde are the meta- and para-isomers, which are respectively named isophthalaldehyde and terephthalaldehyde. It is sensitive to UV illumination and air oxidation. In dairy industry, proteolysis occurring during the production of milk and milk products have both positive and negative effects. The negative effects of proteolysis on milk and dairy products during storage is related to heat resistant alkaline milk proteinases, storing milk for an extended length of time and heat resistant proteinases produced by psychotropic microorganisms. Therefore OPA is essential regarding the determination of proteinase and proteolysis tracking. OPA values of probiotic yoghurt samples were given in Table 5. OPA levels of samples varied between 0.66 – 1.10. The highest value was obtained in P3 on the 7th day, and the lowest in P1 on the 21st day of the storage. As a result of the analysis of variance, the difference between the storage days were found to be significant ($p<0.05$). Also, the difference between different periods were significant ($p<0.05$). The difference between K and P2, P1 and P2, K and P3 among each other on the 1st day, K, P1 and P3 among each other on the 7th day, K and P1, K, P2 and P3 among each other on the 14th and K and P2, P2 and P3 among each other on the 21st day were not significant, whereas the difference between all the samples among each other were statistically significant ($p<0.05$).

Table 6. Microbiological properties of probiotic yoghurt samples (CFU/g)

Bacteria	Sample	Storage Period (day)			
		1	7	14	21
***Lactobacillus bulgaricus* (CFU/g)**	**K**	8.48 ± 0.12^{aX}	7.45 ± 0.09^{aY}	7.44 ± 0.04^{aY}	6.57 ± 0.05^{aZ}
	P1	5.92 ± 0.03^{bX}	5.58 ± 0.08^{bY}	5.24 ± 0.06^{bZ}	5.06 ± 0.07^{bZ}
	P2	5.65 ± 0.04^{cX}	5.47 ± 0.06^{bX}	5.23 ± 0.07^{bY}	4.92 ± 0.08^{bZ}
	P3	4.94 ± 0.04^{dX}	4.66 ± 0.06^{cY}	4.31 ± 0.05^{cZ}	4.25 ± 0.04^{cZ}
***Streptococcus thermophilus* (CFU/g)**	**K**	9.75 ± 0.09^{aX}	8.33 ± 0.12^{aY}	8.2 ± 0.02^{aY}	8.13 ± 0.04^{aY}
	P1	8.57 ± 0.07^{bX}	8.4 ± 0.07^{aXY}	8.21 ± 0.07^{aYZ}	8.06 ± 0.07^{aZ}
	P2	7.27 ± 0.07^{cX}	6.42 ± 0.02^{bY}	6.43 ± 0.02^{bY}	6.36 ± 0.05^{bY}
	P3	6.94 ± 0.05^{dW}	5.94 ± 0.02^{cX}	5.66 ± 0.06^{cY}	5.19 ± 0.02^{cZ}
***Lactobacillus gasseri* (CFU/g)**	**K**	7.34 ± 0.03^{aY}	7.60 ± 0.02^{aX}	7.58 ± 0.00^{aX}	7.67 ± 0.06^{aX}
	P1	7.48 ± 0.02^{aY}	7.31 ± 0.07^{bYZ}	7.15 ± 0.04^{bZ}	7.75 ± 0.14^{aX}
	P2	6.17 ± 0.10^{bY}	6.09 ± 0.04^{cY}	5.29 ± 0.03^{cZ}	6.41 ± 0.00^{bX}
	P3	6.9 ± 0.11^{cX}	6.67 ± 0.07^{dY}	6.62 ± 0.02^{dY}	6.96 ± 0.02^{cX}
***Bifidobacterium longum* (CFU/g)**	**K**	8.77 ± 0.07^{aX}	8.67 ± 0.06^{aX}	8.15 ± 0.01^{aY}	7.6 ± 0.02^{aZ}
	P1	8.93 ± 0.02^{bW}	8.41 ± 0.21^{aX}	7.93 ± 0.03^{bY}	7.29 ± 0.04^{bZ}
	P2	7.33 ± 0.01^{cW}	6.51 ± 0.01^{bX}	6.19 ± 0.04^{cY}	6.85 ± 0.04^{cZ}
	P3	6.40 ± 0.00^{dW}	5.91 ± 0.00^{cX}	5.23 ± 0.02^{dY}	5.07 ± 0.01^{dZ}

3.2.7.Aroma compounds

Flavor is one of the most important properties of food products and is an important factor determining its acceptability and preference. The sensory properties of dairy products depend largely on the relative balance of flavor compounds derived from fat, protein or carbohydrate in the milk types (Cheng, 2010; Routray & Mishra, 2011). During storage, the volatile constituents in yoghurt may differ depending on the culture, mix formulation, milk type and the storage conditions. Table 6 summarizes the main aroma compounds identified in yoghurts produced from cow milk and cow/rice milk mixtures. Quantitatively, the major volatile compound in

the headspace and contributing to the flavor of yoghurt samples appeared to be 2-3 butanedione, acetoin, methyl benzene, 3,4 dihydroxyphenethyl alcohol and isoamyl hexanoate. Ketones and aromatic compounds mentioned above are common constituents of yoghurt samples as volatile compounds. However, some methyl ketones were not detected in all yoghurt samples. Even though di ketone di acetyl (2,3 butanedione) significantly varied from sample to sample. Monnet & Corrieu (2007) indicated that di ketones in yoghurt come only from pyruvate, since thermophilic starter cultures are not able to metabolize citrate. S. thermophilus strains process an α-acetolactate synthase and acetohydroxy acid synthase which produce α-acetolactate and 2-hydroxyacetolactate respectively from pyruvate (Cheng, 2009; Güler & Park, 2011). These two α-acetoacids are generally metabolized into neutral compounds to protect pH homeostasis by decarboxylation. Also, they could be converted into branched-chain amino acids such as valine, leucine or isoleucine. Also, diacetyl/2-3 butanedione was negatively related to acetoin as mentioned earlier (Warsy, 1983; Güler et al., 2009; Güler & Park, 2011). This may be sourced to the reduction of diacetyl to acetoin. There were significant differences in acetoin concentration in yoghurt samples. This compound is derived from β-oxidation of saturated free acids depending on the lipolytic activity of yoghurt starters (Tsau, Guffanti & Montville, 1992; Güler & Park, 2011; Routray & Mishra, 2011).

Acetaldehyde, acetoin, acetone and diacetyl of carbonyl compounds are main flavor components of yoghurt. But, many researchers indicated the importance of acetaldehyde for a favorable flavor in yoghurt (Tamime & Deeth, 1980). Heating the milk at high temperatures, increase in dry matter content, milk or milk powder addition, type of milk and properties of yoghurt bacteria have effects on the acetaldehyde content (Yaygın, 1981). The average acetaldehyde values of probiotic yoghurt samples are given in Table 5. Acetaldehyde levels of samples varied between 6.11 and 16.81 ppm. The highest value was obtained in control sample on the 1st day, and the lowest in P3 on the 21st day of the storage. The difference between the samples according to 21 days of storage were significant ($p<0.05$). Acetaldehyde contents tended to decrease since the 1st day of storage. It is reported that the decrease in the acetaldehyde contents during the storage period is related to reduction of acetaldehyde to ethyl alcohol (Tamime & Deeth, 1980).

Robinson et al. (1977) reported that high quality yoghurt contains 27.6 ppm acetaldehyde, whereas Rasic & Kurmann (1987) reported that this value should be between 23-41 ppm. Beyatli & Tunail (1980) reported that the acetaldehyde levels of yoghurts in Turkish markets varied between 12.28 and 34.72 ppm and in yoghurts produced with selective cultures according to their acid production and proteolytic activity changed between 19.14-32.21 ppm. In another study, it was reported that acetaldehyde levels in bio-yoghurts produced with *S. thermophilu*s and *L. acidophilus*, for a favorable flavor, should be between 3-5 ppm (Sezgin, Yıldırım & Karagül, 1994). Quantitavely, the major volatile compound in the headspace and contributing to the flavor of set type yoghurt and Turkish yoghurt appeared to be acetaldehyde which was mentioned by other researchers (Kneifel et al., 1992; Ott, Germond & Chaintreau, 2000). The acetaldehyde contents of yoghurt samples have changed during storage. During storage, these non-regular changes in acetaldehyde may depend on the culture, mix formulation and storage conditions (Brauss et al., 1999; Tamime & Robinson, 1999). Also the lower concentration of acetaldehyde may be related to the nonstarter lactic acid bacteria. There were significant variations in hexanal and 2 heptanal concentrations in yoghurts. Level of hexanal concentration decreased during storage in P1 yoghurts and P3 yoghurt samples, however, hexanal concentration increased until the 7th day then reduced until 21st day of storage in control group. In comparison of all samples,

hexanoic acids initially increased in all samples after the first day, then increases were observed only in control samples during all storage days. The carbonyl compounds cover aldehydes, ketones etc. also related to the fat contents of fermented milks. Stelios et al. (2007) found that carbonyl compounds, especially ketones, increased in yoghurts depending on the increase in fat content and storage time. 2-3 Pentanedione was changed in all samples during storage. The highest 2-3 pentanedione level was observed in control compared to P2 sample. However, the lowest 2-3 pentanedione level was found in P2 sample at 1st and P3 sample at 21st day of storage.

The other main aromatic volatile compounds such as methyl benzene, methyl 2 benzoate showed significant differences between yoghurt samples. These volatile aromatic compounds may be derived from oxidation of carboxylation or naturally occurred depending on the activity of yoghurt strains. Additionally, Stelious et al. (2007) mentioned that these volatiles may be related in yoghurts depending on the composition of milk and storage time. According to our results; differences between studies may be associated with the factors including the starter culture, synergistic effects of the microflora, fermentation conditions and the composition parameters of milk used in yoghurt production. Furthermore, the applied analytical method may also be a source of divergent volatile compound concentrations.

3.3.Microbiological Properties of Probiotic Yoghurt Samples

3.3.1.Lactobacillus bulgaricus counts

The average *L.bulgaricus* counts of probiotic yoghurt samples were given in Table 7. The average *L. bulgaricus* counts of probiotic yoghurt samples varied between 4.25 – 8.48 log CFU/g. The highest value was obtained in control sample on the 1st day, and the lowest in P3 on the 21st day of the storage. The difference between the storage days were found to be significant ($p<0.05$). *S. thermophilus* and *L. bulgaricus* present in the traditional yoghurt culture have a symbiotic living and during the fermentation, first *S. thermophilus* and then *L. bulgaricus* get active. Our results were similar to those by Medina & Jordano (1994), Akalin (1993) and Donkor et al. (2006).

3.3.2.Streptococcus thermophilus counts

The average *S. thermophilus* counts of probiotic yoghurt samples were given in Table 7. The average *S. thermophilus* counts of probiotic yoghurt samples varied between 5.19 – 9.75 log CFU/g. The highest value was obtained in control sample on the 1st day and the lowest in P3 on the 21st day of the storage. The difference between the storage days were significant ($p<0.05$). The *S. thermophilus* counts obtained in our study was similar to those reported by Scmazny & Reinartz (1982), Akalin (1993), Vinderola et al. (2000), Oliveira et al. (2002), and lower than those reported by Fenderya (2002) and Mada (1981). These differences may be due to type and ratio of culture used (DVS or liquid), strains, incubation temperature and microorganisms present in culture combination, production methods, and media used in determination of microorganism counts. Considerably lower live counts determined in fermented dairy products produced with liquid cultures than those of produced with freeze dried cultures may cause different results in different stages of the researches.

3.3.3.Lactobacillus gasseri counts

The average *L. gasseri* counts of probiotic yoghurt samples were given in Table 7. The average *L. gasseri* counts of probiotic yoghurt samples varied between 5.29 – 7.75 log CFU/g. The highest value was obtained in P1 on the 1st day and the lower in P2 on the 21st day of the storage. As a result of the analysis of variance, the difference between the storage days were found to be significant ($p<0.05$).

3.3.4.Bifidobacterium longum counts

The average *B. longum* counts of probiotic yoghurt samples were given in Table 7. The

average *B. longum* counts of probiotic yoghurt samples varied between 5.07 – 8.93 CFU/g. The highest value was obtained in P1 on the 1st day and the lowest in P3 on the 21st day of the storage. The difference between the storage days were significant ($p<0.05$). *Bifidobacterium* ssp. counts in our studies were similar to those by Mada (1981) and Kim et al. (1992), lower than those reported by Sonoike et al. (1986) and Dave & Shah (1997a). These differences may be due to the different strains used in production, different production methods (cystein, ascorbic acid addition etc.), inoculation ratios, the temperature and duration of incubation and different microorganisms found in the production. Also, Dave & Shah (1997a) reported that, polysaccharide production by *S. thermophilus* during fermentation may suppress the growth of Bifidobacterium ssp.

3.4.Sensory Evaluations of Probiotic Yoghurt Samples

The sensory analysis of samples was performed using grading method according to TSE criteria. An ideal yoghurt is clean, with a bright appearance, having a milkish color (pale yellowish in none homogenized, porcelain white in homogenized), no cracks or gas bubbles, consistent, a viscose structure after stirring, low syneresis and characteristic odor and flavor (Anonymous, 1999).

3.4.1.Appearance

Our probiotic yoghurts were graded out of 5 and evaluated according to their state of being clean, bright, milk colored, having no syneresis, cracks and gas bubbles and being homogenous. The average appearance values of probiotic yoghurt samples were given in Table 8. The appearance values for sensory properties of probiotic yoghurt samples varied between 2.11-5.00. The highest value was obtained in control sample on the 21st day, and the lowest in P3 on the 1st day of the storage. The difference between the different storage days were not significant ($p>0.05$). K and P1 had close grades, where P2 and P3 had lower. In all the samples, appearance grades were the highest on the 21st day of the storage. Rasic & Kurman (1987) reported that protein hydration and gel formation which effects the appearance occured after a length of time.

3.4.2.Consistency

Consistency evaluations were graded out of 5, according to their smoothness and meaty consistency, viscose structure after stirring, having no syneresis and easy dispersion in the mouth. The average cohesiveness values of probiotic yoghurt samples were given in Table 8. The consistency values for sensory properties of probiotic yoghurt samples varied between 1.67 – 4.82. The highest value was obtained in control sample at the 21st day, and the lowest in P3 at the 1st day of the storage. The difference between the different storage days were not significant ($p>0.05$). K and P1 had close grades, where P2 and P3 had lower. Although the panelists have been trained, a slight vision of flaw in appearance might have stimulated the panelist for grading low. Therefore, our consistency values show a great range.

3.4.3.Odor

Odor evaluations were graded out of 5 according to the characteristic odor of yoghurt. The average cohesiveness values of probiotic yoghurt samples were given in Table 8. The odor values for sensory properties of probiotic yoghurt samples varied between 3.05-5.00. The highest value was obtained in control sample at the 21st day, and the lowest in P3 at the 21st day of the storage. The difference between the storage days were found to be not significant ($p>0.05$).

3.4.4.Flavor

Flavor evaluations were graded out of 5 according to the characteristic flavor of yoghurt. The average cohesiveness values of probiotic yoghurt samples were given in Table 8. The flavor values for sensory properties of probiotic yoghurt samples varied between 2.11 – 4.86. The highest value was obtained in

control sample at the 14th day, and the lowest in P3 at the 1st day of the storage.

The difference between the storage days were not significant ($p>0.05$). There were limited sources on the evaluation of sensory properties of probiotic yoghurts in the literature, usually results on sensory properties and flavor components concentration of yoghurt products were available. Akalin (1993), reported that the products that gained the highest flavor and odor scores were Bio-yoghurt and Bifi-yoghurt, where products showed no significant consistency and appearance differences, but the researcher determined a decrease in sensory evaluation grades at the 28th day of the storage, similar to our results.

Table 8. Sensorial properties of probiotic yoghurt samples

Sensory Criteria	Sample	Storage Period (day)			
		1	7	14	21
Appearance	**K**	4.72±0.21^{a}	4.78±0.04^{a}	4.86±0.01^{a}	5.00±0.00^{a}
	P1	4.33±0.58^{a}	4.55±0.27^{a}	4.57±0.20^{ab}	4.6±0.15^{ab}
	P2	3.20±1.30^{ab}	3.28±0.65^{b}	3.50±0.70^{bc}	3.96±0.45^{b}
	P3	2.11±0.36^{b}	2.48±0.15^{b}	2.64±0.30^{c}	2.78±0.50^{c}
Consistency	**K**	4.38±0.24^{a}	4.63±0.06^{a}	4.65±0.31^{a}	4.82±0.25^{a}
	P1	4.14±0.50^{a}	4.42±0.28^{a}	4.35±0.30^{a}	4.57±0.20^{a}
	P2	2.93±1.32^{ab}	2.92±0.54^{b}	2.92±0.70^{b}	3.46±0.45^{b}
	P3	1.67±0.45^{b}	2.01±0.15^{c}	2.35±0.10^{b}	2.21±0.30^{c}
Odour	**K**	4.81±0.26^{a}	5.06±0.08^{a}	4.85±0.20^{a}	5.00±0.00^{a}
	P1	4.37±0.35^{ab}	4.52±0.03^{b}	4.64±0.10^{a}	4.57±0.00^{a}
	P2	3.59±0.83^{ab}	3.81±0.00^{c}	3.26±0.02^{b}	3.92±0.30^{b}
	P3	3.05±0.27^{b}	3.41±0.31^{c}	3.21±0.10^{b}	3.03±0.25^{c}
Flavor	**K**	4.58±0.28^{a}	4.81±0.26^{a}	4.86±0.01^{a}	4.67±0.05^{a}
	P1	4.31±0.34^{ab}	4.22±0.12^{a}	4.32±0.05^{a}	4.03±0.25^{a}
	P2	3.30±0.53^{b}	3.34±0.30^{b}	2.85±0.80^{b}	3.14±0.40^{b}
	P3	2.11±0.36^{c}	2.30±0.18^{c}	2.85±0.00^{b}	2.42±0.40^{b}
General Evaluation	**K**	4.52±0.03^{a}	4.85±0.14^{a}	4.78±0.10^{a}	4.87±0.17^{a}
	P1	4.14±0.41^{a}	4.35±0.11^{a}	4.42±0.00^{a}	4.39±0.45^{ab}
	P2	3.11±0.99^{ab}	3.22±0.38^{b}	2.78±0.70^{b}	3.53±0.65^{bc}
	P3	2.06±0.26^{b}	2.33±0.31^{c}	2.20±0.11^{b}	2.57±0.40^{c}

3.4.5.General evaluation

General evaluation was obtained by calculating the average values of all sensory parameters of the samples. The average cohesiveness values of probiotic yoghurt samples with standard deviations were given in Table 7. The general evaluations for sensory properties of probiotic yoghurt samples varied between 2.06 - 4.87. The highest value was obtained in control sample at the 21st day, and the lowest in P3 at the 1st day of the storage., The difference between the different storage days were found to be not significant ($p>0.05$). Among all the groups, control group had the highest points, where rice milk added samples had a decline with inverse proportions with their rice milk content. The reasons why samples with rice milk had lower points (especially P2 and P3) are that the rice milk has a sweet flavor and it cannot provide the desired consistency and appearance in yoghurt. This sweet flavor can be sensed slightly in samples with lower rice milk content (P1 and P2), whereas it was felt intensely in sample with high rice milk content (P3).

Table 7. Aroma compounds of probiotic yoghurt samples

Compounds	RT (min)	Control				P1				P2				P3			
	Days	1	7	14	21	1	7	14	21	1	7	14	21	1	7	14	21
2-Ethyl-N-methyl-1-hexanamine	1.53	1.61	1.85	0.90	1.22	1.61	0.91	1.84	1.68	3.78	2.59	1.61	1.50	2.79	1.96	2.05	1.43
Acetaldehyde	1.61	2.39	2.11	1.57	1.63	1.52	1.89	1.49	1.58	2.92	1.69	2.15	1.59	0.75	0.93	0.91	0.65
2-Fluoropropan-1-ol	1.70	ND	ND	ND	0.94	0.71	0.94	0.36	0.57	1.69	0.80	1.19	1.09	1.39	1.32	1.70	1.30
Methyl acetoacetate	1.80	3.97	3.87	3.75	4.89	2.76	3.20	2.74	3.47	3.54	2.22	2.21	3.92	1.90	1.86	1.92	1.74
Acetic acid	2.16	4.06	3.06	2.52	3.05	2.30	3.93	3.03	4.48	1.38	2.29	3.98	2.13	1.23	1.18	ND	ND
2,3-Butanedione	2.23	7.88	6.95	7.62	9.32	4.53	6.93	5.82	8.15	8.59	5.04	5.12	6.32	4.45	5.71	5.27	3.58
Dichloromethyl ethyl sulfone	2.51	6.46	6.35	6.78	5.52	4.38	3.93	6.18	5.82	12.11	5.67	5.42	7.24	3.52	4.52	10.04	4.44
Triphenylborane–Sodium hydroxide	2.90	2.54	2.50	2.88	2.29	2.18	1.78	2.55	2.84	3.12	2.49	1.99	4.50	2.10	2.14	3.07	2.52
3,3-Difluoro-2-propen-1-ol acetate	3.33	3.42	2.75	3.03	2.03	2.99	3.51	3.74	4.15	3.13	2.73	3.67	3.72	2.71	2.36	1.10	1.57
2,3-Pentanedione	3.50	5.42	4.82	5.11	6.39	3.37	4.47	3.99	4.51	2.19	2.72	3.20	3.85	2.27	2.92	3.85	3.20
Acetoin	3.81	9.68	7.41	6.87	6.87	6.41	9.80	7.59	9.76	3.59	3.98	4.11	4.16	3.02	1.89	0.82	1.88
Methyl benzene	5.22	16.13	14.55	17.75	14.74	13.64	10.31	13.81	16.17	18.99	16.52	18.42	16.65	11.38	12.97	24.39	14.31
Ethyl butyrate	6.48	2.67	2.08	2.34	2.52	2.18	2.24	2.43	2.60	2.36	3.08	2.79	3.06	2.41	1.96	3.15	2.35
Hexanal	6.56	5.2	10.05	6.87	5.75	5.94	3.74	3.16	2.22	4.03	1.09	1.16	2.44	5.69	3.97	2.33	1.85
2-Heptanone	10.28	2.8	2.50	3.48	3.39	1.87	1.82	2.18	2.00	1.58	2.20	2.11	2.80	1.50	1.68	1.67	2.25
Heptanal	10.84	0.74	1.50	1.26	0.86	1.18	0.74	1.30	0.83	2.06	0.67	0.89	1.18	1.42	0.90	0.81	0.80
Methyl 2-[(trimethylsilyl)oxy]benzoate	13.47	4.62	4.39	3.68	4.67	7.71	7.82	7.86	7.33	4.90	4.77	5.71	5.02	12.26	8.99	6.62	13.60
Hexanoic acid	14.38	3.75	3.91	4.17	4.71	3.42	4.08	3.34	3.55	2.22	2.84	2.33	2.31	1.66	1.21	1.97	1.79
2-Nonanone	18.65	1.98	1.82	2.29	2.13	1.14	1.24	1.24	1.18	1.39	1.42	1.55	1.49	1.67	1.32	1.36	1.68
3,4-dihydroxyphenethyl alcohol	19.44	5.04	3.76	2.82	4.98	10.46	13.33	10.82	6.48	6.99	14.57	13.72	12.49	20.58	19.88	14.57	19.69
Isoamyl hexanoate	20.54	3.39	4.99	5.10	4.19	8.11	5.18	5.66	3.97	3.03	8.50	7.20	5.14	6.38	8.87	4.80	8.22
Pentyl 2-methylvalerate	20.60	4.41	6.42	6.69	5.56	9.50	6.06	6.95	4.91	3.97	10.03	8.03	6.22	7.56	10.26	5.33	9.26
Octanoic acid	21.45	1.85	2.34	2.53	2.35	2.06	2.14	1.91	1.73	2.43	2.08	1.44	1.19	1.37	1.19	2.25	1.92

4. Conclusions

Dairy industry is focusing on development of new production methods or active marketing strategies in order to meet the various flavor and health claiming expectations of consumers and to increase the dairy product consumption per capita. Production of food products from rice milk is newly developing and the effects on human health are the subject of scientific searches.

This study aimed to add a new fermented dairy product to the dairy technology in our country, inform the public regarding its dietetic and therapeutic benefits, giving the consumer a choice for a healthier diet and increase the consumption per capita. Also we considered that our study may be a guide for further studies and selections of starter culture for similar productions. Generally speaking, samples containing rice milk did not gave good results. However, P1 samples were the most favored products among the samples containing rice milk as they were the closest product to the control group. The consumption of such products is continuously increasing as the customers' tendency to consider them as functional products rather than traditional food products increase. The demand on convenience foods with single portions with no extra process required for consumption is rapidly increasing. While drinking fermented dairy products market were developing, custom labeled products are entering the market, bringing the competition to a climax. The place of functional foods is maintained by the hand of brandization causing safety and consumer loyalty.

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DETECTION OF ADULTERATION IN RAW COW MILK SUPPLIED IN THE QAZVIN PROVINCE, IRAN, DURING (2015-2016)

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ABSTRACT

Milk is the most appropriate source of food required for growth of infants and Children and for preservation of health in adults, It supplies nutrients like proteins, carbohydrates, fat, vitamins and minerals in moderate amounts in an easily digestible form. Improving milk quality; in addition to maintaining the health satisfaction of milk and its products consumer, is really important. Milk adulteration is a very common food fraud and is a big social problem both in the backward and advanced countries. Apart from the ethical and economical issue, it also causes serious health problems**.** So, given the importance of above facts, this study aims to detect the some of common adulteration was conducted in raw cow milk collected from Qazvin province. A total 61 raw milk samples were collected from 15 collection center of raw milk during the different seasons (2015-2016). The results showed that, 4.9% of the milk samples were adulterated with water. Formalin, hydrogen peroxide and salt were detected as 16.4, 11.5 and 34.4 % in the milk samples, respectively. None of the samples were contaminated with bicarbonate. The number of samples positive containing salt, formalin, hydrogen peroxide, added water and bicarbonate were as 21, 10, 7, 3 and 0, respectively. Positive cases of formalin, salt and hydrogen peroxide in the first six months were higher than the second six months. However, the only positive cases of formalin was significant between warm and cold seasons ($P<0.05$)**.** Milk used for human consumption can be adulterated with cheaper materials or hazardous chemicals. Thus, more analysis is essential to create awareness among the consumers regarding malpractices and negligence in milk production, especially in the warm seasons.

1.Introduction

Milk is a complex compound made up of protein in colloidal suspension form, fat emulsion and a lactose in a actual solution, it also contains vitamins, enzymes minerals and organic compounds such as lactic and citric acids. Therefore, milk is almost a complete food that can be providing lots of human nutritional needs (Harding, 2003; Karim, 2008).

Milk is one of the most consumed dairy products, with high nutritional value. Thus, its use is recommended for all age groups (Harding, 2003; Karim, 2008). Milk is needed for growth and maintenance of health, However it can be used as a vehicle for the transmission of chemicals and other impurities (Nirwal et al., 2013). According to definition provided by the International Milk Commission, raw milk delivered to the factory must be fresh, pure, clean, and free of colostrum and with natural taste and smell. Also Milk should be nothing added or taken from it (including extracting fat, adding water, salt, sugar, dried milk, baking soda, hydrogen peroxide and formalin

(Barbano et al., 2006). Milk can primarily contaminate with microbial or chemical agents(drugs, mycotoxins, preservatives, insecticide, and etc.). It can also contaminate secondarily with milking equipment, employees, and various processes and at the stage of transition to the factory. On the other hand, there is a variety of adulterations in milk that their recognition is also useful for consumer (Tipu et al., 2007).

Milk adulteration is a very common food fraud and is a big social problem both in the backward and advanced countries. Apart from the ethical and economical issue, it also causes serious health problems associated with the milk health, subsequently health of consumers which are added to milk in order to lower microbial load and hide defects (Barbano et al., 2006; Das et al., 2016). Sometimes milk is adulterated with low value Ingredient like water or whey for the purpose of increasing the profits and reducing the cost of its production. Adding water or 'liquid-whey' to milk is a very common practice by the milk supplier to increase its volume. Diluted milk reduces its nutritional value. Also, if the water is contaminated, for example, with chemicals or pathogens, it causes serious health problems in consumers. In addition to, added water changes specific gravity of the milk and its natural color gets destroyed. To compensate specific gravity by the milk supplier's different types of salt and sugar is added to milk which in turn can be dangerous for the consumer. Sometimes to maintain the natural color of milk a small amount of coloring matter is added (Das et al., 2016). Other the common adulteration include in starch, chlorine, hydrated lime, sodium carbonate, formalin, hydrogen peroxide, ammonium sulfate, urea, boric acid and various antibiotics which are used for different purposes (Das et al., 2016; Tipu et al., 2007).For example, neutralizers like sodium hydroxide, sodium carbonate or sodium bicarbonate to neutralize the acidity of the milk are added so that the consistency and shelf life of the milk is increased. Hydrogen peroxide is added to milk to lengthen its freshness. It can damage the digestive system cells and lead to gastritis and inflammation of the intestine. Urea is added to adjust the solids-non- fat of milk, to provide whiteness and giving actual appearance to milk.it causes pain in lower abdomen, muscle cramps, irregular heartbeat, numbness and weakness in hands and feet, chills and shivering fever (Singuluri and Sukumaran 2014). Some of the common adulteration, the aim of adding them and their effects on human health is shown in table1 (Afzal et al., 2011; Ramya et al., 2015). Keeping in view the above facts, determination of milk adulteration is very important.

Therefore, the present study was conducted to detect some of the common adulteration using standard methods in raw cow milk samples collected from raw milk collection centers in Qazvin province of Iran.

Table1. Some of the common adulteration, the aim of adding them and their effects on human health (Barbano et al., 2006; Das et al., 2016; Tipu et al., 2007)

Types of adulteration	Goal of addition	Effects on human health
Hydrogen peroxide	Slow down microbial growth and delay spoilage of milk	The effect on the digestive system cells and creating gastroenteritis
Formalin	As microbial growth inhibitor is added to the milk	Causes diarrhea, vomiting and abdominal pain. in high doses may cause decreased the body temperature, weak irregular pulse, shallow respiration, ,unconscious and blindness
Bicarbonate	neutralize the high acidity of milk	Cause disruption in the activity of growth hormones and reproduction

Salt	Hiding added water to the milk, set the density and freezing point of milk	Taking too much salt increases the risk of cardiovascular diseases
Added water	Increase the volume of milk	Decrease the nutritive value of milk and following that nutritional disorders in humans. If contaminated poses a health risk especially for sensitive people.
Urea	To adjust the solids-non- fat of milk, to provide whiteness and giving actual appearance to milk	causes pain in lower abdomen, muscle cramps, irregular heart beat, numbness and weakness in hands and feet, chills and shivering fever
Starch	To adjust the density of diluted milk	It can cause diarrhea. Can be dangerous for diabetic patients.

2. Materials and methods

2.1. Samples Collection

A total of 61 samples of raw milk from raw milk collection centers mainly on Qazvin city during four seasons (The number of samples in each of the season's autumn, winter and summer 15 and in spring 16) were collected. The samples transported under appropriate conditions to Health and Food Safety Laboratory of Qazvin University of Medical Sciences and Some common Adulteration (including added water, salt, bicarbonate, formalin and hydrogen peroxide) were analyzed in the samples collected.

2.2. Detection of Adulterations

2.2.1. Water detection

Added water Adulterations was determined through specific gravity using Thermolac-densimete (Mahmoudi and Norian, 2015).

2.2.2. Sodium chloride detection

To detect salt, 5 ml of silver nitrate 34.1 g/ l was mixed with a few drops of 5% potassium dichromate and then 1 ml of milk were added to it. Appearance of yellow color indicates presence of sodium chloride (Tipu et al., 2007).

2.2.3. Hydrogen peroxide detection

For determining Hydrogen peroxide 5 ml of milk were poured in a test tube and 5 drops of a solution of 2% para-phenylenediamine were added to it, then was stirred well. The blue color indicates the presence of hydrogen peroxide in milk (Tipu et al., 2007).

2.2.4. Bicarbonate detection

For bicarbonate 10 ml milk samples were taken in test tube and 10 ml 95 % ethyl alcohol were added. Five drops of rosolic acid (1% ethanol) were added and mixed well. Pink color indicates presence of carbonate (Tipu et al., 2007).

2.2.5. Formalin detection

Formalin was detected by the method hehner's test (Tipu et al., 2007).

2.3. Statistical Analysis

All the experiments were performed in triplicate. Statistical analysis was analyzed in SPSS-19 using Chi-square test at significance level $P<0.05$.

3. Results and discussions

The results of this study (Table 2) showed that respectively 34.4% and 4.9% of the milk samples had salt and extra water. Formalin and hydrogen peroxide adulteration was present in 16.4% and 11.5% samples. No sample was found to be adulterated with bicarbonate**.** Positive cases of salt and hydrogen peroxide in the first six months were higher than the second six months (Figure 1). However, only positive cases of formalin were significant between warm and cold seasons ($P<0.05$).

Milk in its natural form is one of the most important and completes natural food that is consumed by people of all ages as various dairy products. Given the properties of milk, control of this valuable product and measuring its

ingredients are completely essential for milk producers and the dairy industry. Because, milk quality has effect directly on milk processing and quality of dairy products (Nirwal et al., 2013).

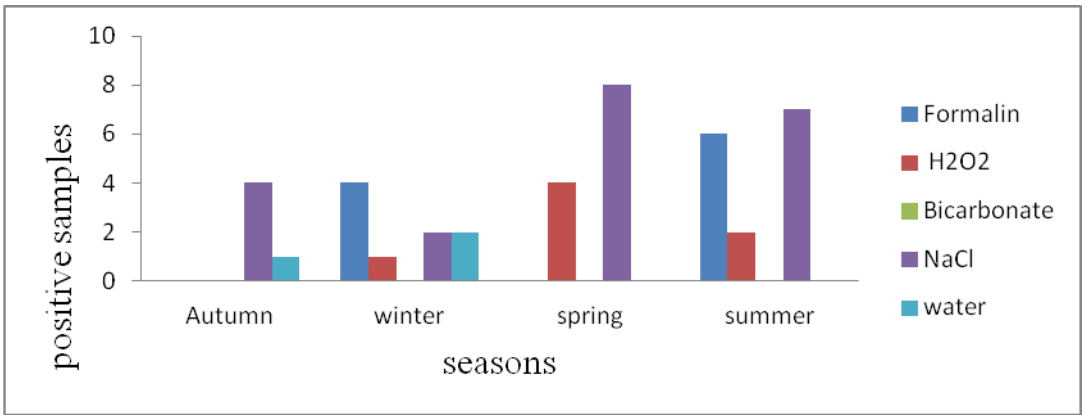

Figure 1. Comparing the Adulteration in milk samples during difference seasons

Table 2. Presence of adulterant (% positive sample) in the raw cow milk collected from Qazvin province (2015-2016)

		Various Adulteration				
seasons	**Sample size (n)**	**Formalin (%)***	**hydrogen peroxide (%)**	**salt (%)**	**Bicarbonate (%)**	**Added water (%)**
Autumn	15	0(0%)	0(0%)	4(26.7%)	0(0%)	1(6.7%)
Winter	15	4(26.7%)	1(6.7%)	2(13.3%)	0(0%)	2(13.3%)
spring	16	0(0%)	4(25%)	8(50%)	0(0%)	0(0%)
summer	15	6(40%)	2(13.3%)	7(46.7%)	0(0%)	0(0%)
Total	61	10(16.4%)	7(11.5%)	21(34.4%)	0(0%)	3(4.9%)

*There was significant difference between cold and warm seasons ($P<0.05$).

Table 3. The percentage of some adulteration in several countries

Country	**Type of sample**	***Various Adulteration***							
		(N)	**Formalin**	**H_2O_2**	**salt**	**Neutralizing**	**water**	**urea**	**Reference**
India	Raw milk	50	6(12%)	13(26%)	27(54%)	_	_	12(24%)	(Ramya et al., 2015)
India	Raw milk	50	16(32%)	16(32%)	41(82%)	13(26%)	_	30(60%)	(Singuluri and Sukumaran 2014)
Iran	Raw milk	60	0(0%)	0(0%)	6(10%)	7(11.6%)	24(40%)	_	(Mahmoudi et al., 2015)

Iran	Raw milk	112	0(0%)	2(1.74%)	0(0%)	0(0%)	18(16.6%)	_	(Mahmoudi and Norian, 2015)
Sudan	Raw milk	240	5(2.08%)	1(0.41%)	_	_	_	_	(El Zubeir and El Owni, 2009)
Pakistan	Pasteurized	60	(27%)	(3%)	_	_	(96%)	(86%)	(Faraz et al., 2013)
Pakistan	Milk	100	11(11%)	9(9%)	8(8%)	11(11%)	74(74%)	7(7%)	(Barham et al., 2015)
Bangladesh	Raw milk	50	5(10%)	0(0%)	_	10(20%)	50(100%)	_	(Chanda et al., 2013)
Brazil	UHT milk	100	(44%)	(30%)	_	_	_	_	(Souza et al., 2011)

On the other hand, environmental factors and livestock characteristics affect the quality of raw milk. Thus, maintaining quality of raw milk in order to maintain its competitive place in the market is critical and any change on its composition is serious threat to producers of milk (price cuts), dairy industry (production costs), and consumers (dietary and health aspects (Smit et al., 2000). At the time of buying milk, consumers have right to receive healthy and adulteration -free milk. Adulteration of milk may be intentional or unintentional occur in the process of production. The first type is intended to increase the margin of profit and second type of adulteration may be incidental contamination which is usually due to ignorance, negligence or lack of proper facilities (Kamthania et al., 2014; Nirwal et al., 2013). Consumers are often become victim of diseases by consuming adulterated milk. Diseases of the kidneys, skin, eye, heart problems and even cancer are some of the common disease caused by consuming adulterated milk (Das et al., 2016). For example, adding of carbonate in milk causes digestive problems such as stomach ulcer, diarrhea, colon cancer and Impaired in the balance of body fluids. Hydrogen peroxide with disruption in the operation of body's natural antioxidant system causes early aging. Chlorides in milk also causes imbalance in blood acid, base, and pH (Mahmoudi and Norian, 2015). So, the aim of this study was to determine and assess adulteration in raw cow milk supplied in the Qazvin province. Results obtained from this study showed that numbers of positive raw milk samples for formalin, hydrogen peroxide and salt in the warm seasons (Spring and summer)is higher in compared to the cold seasons(autumn and winter). In the warm seasons, due to high temperature and difficulties in Maintenance of milk under standard conditions the possibility addition of neutralizing materials and microbial growth inhibitors to hide the acidity and spoilage of milk is more. Assessment of raw milk adulteration in Bangladesh showed that 100% of the milk samples had added water and 20% and 10% of milk samples was adulterated with sodium bicarbonate and formalin, respectively. The results of the study above showed that, there is a positive relationship between temperature and the amount of adulteration. So that, the numbers of positive cases containing formalin and bicarbonate were more in the warm months compared to cold months that its consistent with our results (Chanda et al., 2013).

The results of the assessment of physicochemical properties and adulteration in the samples of raw cow milk collected from 14 semi-industrial dairy farms across Province in Qazvin, Iran during 2011 showed that respectively 1.78% and 0.89%of milk samples were contaminated with hydrogen peroxide and hypochloride. Added water was also16.7%. In mentioned study all samples in terms of formalin, carbonate and salt were negative (Mahmoudi and Norian, 2015). Density of raw milk is in the range of 1.028- 1.034 kg/m3 at

15°C. This means that weight of a liter of milk at 15°C is between 1028 to 1034 grams. Taking fat of milk increases the specific gravity of milk. Conversely, added water reduces the specific gravity of milk. In this study specific gravity 4.9% of the samples were lower than the normal range that was being related to the cold seasons. But, the specific gravity of milk was no significant difference in cold and warm seasons (Karim, 2008).The survey of 240 raw cow milk samples in Sudan (2009) showed that 5 samples were contaminated with formaldehyde and 1 sample was contaminated with hydrogen peroxide (El Zubeir and El Owni, 2009). Results of a study by Singuluri and Sukumaran (2014) in India on 50 raw cow milk samples showed that 41(82%), 16(32%) and 13(26%) of samples were contaminated with salt, hydrogen peroxide and neutralizing, respectively (Singuluri and Sukumaran 2014).

Investigation of adulteration in raw cow milk samples collected from East Azerbaijan Province of Iran showed that of the 60 samples collected, 7(11.6%) and 6(10%) of samples were contaminated with Carbonate and salt respectively. Also, 24(40) % of samples had Added water. But formalin and hydrogen peroxide was negative in all samples (Mahmoudi et al., 2015).

A study conducted by Ramya et al (2015) showed that 12 percent of the samples were contaminated with formalin and 26 percent with hydrogen peroxide. Also 5% of the samples were containing sodium chloride (Ramya et al., 2015). In the present study the extent of adulteration with formalin is 16.4%, which was close to the results mentioned study.

Results of a study by Faraz et al. in Pakistan on 60 milk samples collected from canteens of educational institutes and public places showed that 97% of samples had added water. Urea, formalin and hydrogen peroxide were detected as 87%, 27% and 3% of samples, respectively (Faraz et al., 2013). The percentage of some adulteration in several countries is shown in table 3.

In comparison with the above mentioned studies, water adulteration in this study was lower. In contrast, adulteration percent of formalin in the current study was more than aforementioned studies. However, the case of contamination with hydrogen peroxide (11.5%) was also remarkable.

4. Conclusions

The obtained data from this study and previous studies indicate that milk adulterations are quite common in many regions worldwide developing countries or backward countries. Adulteration of milk may be intentional or unintentional occur in the process of production. To prevent milk adulteration, providing training and necessary instructions is essential for the distributors of milk and animal breeders. Milk is an essential food for humans, especially for children, pregnant women and patients. Therefore, consumption of lower quality milk can create serious health problems in human. At the time of buying milk, consumers have right to receive healthy and adulteration -free milk.so, it seems that the only remedy to this problem is to create awareness among consumers about their rights.

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IMMOBILIZATION OF PROBIOTIC BACTERIA WITH BANANA FLOUR AND EFFECT ON QUALITY OF SYNBIOTIC ICE CREAM AND SURVIVAL UNDER SIMULATED GASTROINTESTINAL CONDITIONS

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ABSTRACT
This study is to determine the effect of supplementation of free and immobilized cells on physicochemical and microbiological properties of ice cream and to investigate the survival of cells under simulated gastrointestinal conditions. Probiotic *Lactobacillus casei* TISTR 1463 and *Lactobacillus acidophilus* TISTR 1338 were used while banana flour were used as prebiotic and immobilization support. Immobilization of cells with banana flour was prepared that ratio of banana flour with suspension cells culture was 1:4 (w/v). Immobilized cells were used separately as adjuncts in producing ice cream. In parallel, ice cream with free cells were also produced. The results revealed that the addition of immobilized cells had affected on overrun values and resulted in a decrease in melt down rate, lightness and greenness. However, addition of immobilized cells had no effect on pH values. Freezing storage at -18ºC for 50 days had no caused effect on a significant decrease in the viability of free and immobilized cells ($p \leq 0.05$). Moreover, the viable cell number of ice cream added free and immobilized *L. casei* and *L. acidophilus* was maintained 8.60 and 8.47 log CFU g^{-1} of storage, respectively. Cells immobilization did not significantly influenced the survival of probiotic cultures ($p > 0.05$). Free cells and immobilized cells incorporated in ice cream samples survived when exposed to simulated gastric and intestinal juices. The addition of immobilized cells with banana flour have potential for being used in synbiotic ice cream and capacity to resist acid stress and to grow in the presence of bile salts.

1. Introduction

Functional foods are any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains, when consumed in usual diet. Probiotics and prebiotics are current examples of functional food ingredients (Siró *et al.*, 2008). A food product containing both probiotics and prebiotics is named as functional food or synbiotic. There is a synergy between probiotics and prebiotics in synbiotic products. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the consumer (Homayouni *et al*., 2008). Species of *Lactobacillus casei* and *Lactobacillus acidophilus*, normal components of the intestinal microbiota, are usually employed as probiotic for dairy products. The efficiency of added probiotic bacteria depends on the dose level, temperature and type of dairy foods, their viability must be maintained throughout the product's shelf-life and the gut environment (EL-Sayed *et al.*, 2014).

The therapeutic value of probiotic bacteria normally depends on the viability of these bacteria. Therefore, FAO/WHO (2002) has suggested that a minimum of 10^6 to 10^7 probiotic bacterial cells should be alive at the time of consumption per gram of the product. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of populations of bacteria in the colon (FAO/WHO, 2001). Prebiotics might also enhance the growth and survival of probiotics in foods (Akin, 2005). There are certain carbohydrates with well-known as prebiotic particular some non-digestible fructo-oligosaccharides, resistant starches and inulins. Bananas are mainly produced in tropical and subtropical developing countries and is one of the most popular fruits in the world. Banana flour obtained from unripe banana has been reported as a high amount of total dietary fiber (DF) (56.24 g 100 g^{-1}), which consisted of resistant starch (RS) (48.99 g 100 g^{-1}), inulins (0.05 g 100 g^{-1}) and DF without RS or inulins (7.2 g 100 g^{-1}) (Menezes *et al.*, 2011). Indigestible compounds in unripe banana that are capable of selectively stimulating the growth of probiotic bacteria. Moreover, unripe banana flour contained chemical composition of 2.5 to 3.3% protein, 0.33 to 0.82% fat, 2.6 to 3.5% ash, 61.3 to 76.5% total starch, bioactive compounds like phenolic acids and moderate antioxidant activity (da Mota *et al.*, 2000; Aurore *et al.*, 2009; Menezes *et al.*, 2011). Synbiotic formulation containing food products are used for the development of therapeutic foods. Ice cream can fulfil such a function by serving as a means of delivering probiotics and prebiotics (Akalın and Erişir, 2008; EL-Sayed *et al.*, 2014). The ice cream matrix might be a good vehicle for probiotic cultures, due to its composition, which includes milk proteins, fat and lactose, as well as other compounds and pH near to neutral and it could supplement with prebiotic efficiently (Cruz *et al.*, 2009). It is interesting to add the banana flour in ice cream to enhance functional properties as a source of prebiotic for stimulation on growth of probiotic. The several advantages of using fruit fibre in ice cream production can be the improvement of the structure of ice cream due to their fibrous framework and melting properties, reduction of recrystallization, resulting in prolonged shelf life and allowing freezing at higher overrun (Dervisoglu and Yazici, 2006). Banana flour, in this respect, is acceptable to be a suitable source of dietary fibre and an ingredient for ice cream production. Nevertheless, some authors have reported that the freezing process may seriously damage the cells, which could defeat the potential advantages of probiotics (Santivarangkna *et al.*, 2008). In order to overcome this problem, cell immobilization can be applied to increase the survival of probiotic cultures in frozen dairy products (Homayouni *et al.*, 2008).

Immobilization of the microorganisms by the adsorption of bacterial cells onto supports are hypothesized to be a promising prospect for introducing viable probiotic bacteria in foods because the supports matrix can provide a physical barrier against harsh environmental conditions such as freezing and those encountered during passage through the gastrointestinal tract (Li *et al.*, 2014; Fijałkowski *et al.*, 2016), thus potentially preventing cells loss. Various materials have been used as supports for cell immobilization including fruit segments, dietary fiber, bacterial cellulose and montmorillonite (Michida *et al.*, 2006; Phuapaiboon *et al.*, 2013; Li *et al.*, 2014; Fijałkowski *et al.*, 2016). Unripe banana flour is good choice for immobilization materials because it consists in part of resistant starch and inulin, which is prebiotic carbohydrate and can be used as potential immobilization supports for probiotic bacteria. Also, the immobilization technique is simple (Michida *et al.*, 2006). In previous study, the immobilization of probiotic with prebiotic has been used successfully to improve the survival rate of probiotics (Chen *et al.*, 2005). However, cell survival during freezing storage and its ability to protect the probiotic organisms against gastrointestinal conditions in ice cream

containing immobilized cells with banana flour as a prebiotic substance have not yet been investigated. So the present study an attempt was made to assess the effect of supplementation of immobilization certain probiotics with banana flour and free cultures on the viability of probiotics and on physicochemical and sensory properties in ice cream over a period of 50 days storage at -18^{o}C. Another aspect was evaluated for their ability to improve the survival of probiotics during exposure to simulated human gastrointestinal conditions.

2. Materials and methods

2.1. Production of banana flour

Unripe Thai banana Musa (AA group) "Kluai Khai" (KK), Musa (ABB group) "Kluai Nam Wa" (KN) and Musa (AAA group) "Kluai Hom Thong" (KH) were obtained from a local market in the province of Khon Kaen, Thailand. Three varieties of banana were steamed by autoclaving at 121^{o}C for 15 min. Steamed banana were then peeled, cut into 2 mm slices and immediately dipped for 4 min in sodium metabisulfite solution (1,500 ppm) which adjusting the pH to 3.3 with citric acid. Banana fruit slices were dried at 50^{o}C for 5 h in a dryer. The dried slices were ground in a pulverizing machine for 90 s to pass through 80 mesh sieve, collected and stored at room temperature in a sealed plastic bag before use.

2.2. Probiotic strain used

The probiotic strains used in this study were freeze-dried pure strains of *Lactobacillus casei* TISTR 1463 and *Lactobacillus acidophilus* TISTR 1338. Two probiotic bacteria were obtained from Thailand Institute of Scientific and Technological Research, Thailand. These strains were propagated individually in Lactobacillus MRS Broth (Himedia, India) and incubated at 37°C for 24 h. Then they were kept at low temperature (4°C) and were twice subcultured to obtain an active culture for testing.

2.3. Preparation of free cells and cell immobilization with banana flour

Free cells and cell immobilization were prepared in accordance with the previous procedure described by Phuapaiboon *et al.* (2013) with modification. 2.5% (v v^{-1}) *of L. casei* and *L. acidophilus* were separately grown statically in 160 mL of Lactobacillus MRS broth at 37°C for 21 h, which corresponded approximately 10^{9} CFU mL^{-1}. The cells were centrifuged at 1,250 rpm for 20 min. The pellets were then washed two times with sterile saline solution (0.85%, w v^{-1}). These *L. casei* and *L. acidophilus* cells were used as free cells and immobilized cells with banana flour. Immobilization of cells was achieved as follows: the cells were resuspended in 40 mL of sterile saline solution. After that, KK, KN and KH flour were separately added to sterile saline solution that ratio of banana flour with sterile saline solution was 1:4, slowly stirring to probiotic cells immobilized with banana flour and incubated at 37°C for 3 h without agitation. When immobilization was complete, the immobilized cells were then used as adjuncts in ice cream production.

2.4. Ice cream production

Pasteurized milk (65.20%; CP-Meiji Co., Ltd., Thailand) and UHT whipping cream (8.69%; Anchor, New Zealand) were bought from local supermarket, Maha Sarakham, Thailand. The dry matter content in the milk was adjusted by adding skim milk powder (6.76%; Fonterra, New Zealand) and granular sugar (11.59%; MitrPhol Sugar Co., Ltd., Thailand). Corn flour (1.93%; McGarrett, Thailand), guar gum (0.03%; E412), egg yolk (0.97%) were used as stabilizer. Free cells and immobilized cells with banana flour (4.83%) added for probiotic and synbiotic ice cream. Ice cream was formulated based on 35 to 39.5% total solid for a batch.

The milk and cream were mixed and temperature was increased to 50°C; the blend of skim milk powder, corn flour and along with sugar were added and temperature was increased to 68°C for 15 min. The resultant of

mixture was cooled and added egg yolk and guar gum. The ice cream mix was blend for 10 min then was pasteurized at 82°C for 30 min, cooled to 4°C and stored for 24 h for aging at this temperature. Free and immobilized probiotic cells were separately added after cooling at 4°C, to keep about 10^8 CFU mL^{-1}. Then, the mixtures was frozen/aired, in ice cream maker (Nemox Gelatissimo, Italy). The ice creams obtained were put in rectangular plastic packing and stored in freezer -18°C for 50 days.

2.5. Physicochemical analysis

The pH of ice cream was measured using a digital pH-meter (model 220/225, Mettler Toledo). The overrun of the final product was determined using the following formula (Homayouni *et al.*, 2008).

$$\text{Overrun} = \frac{\text{Weight of unite mix-weight of equal of ice cream}}{\text{Weigh of equal volume of ice cream}} \times 100$$

Color analysis of stored ice cream in freezer for 10 days was conducted with the benchtop HunterLab UltraScan XE spectrocolorimeter equipped with Universal software Version 2.2.2 (UltraScan XE, Hunter Lab, USA) coupled to a personal computer. Instrument was calibrated using their standard white tile as specified by the manufacturer. The measurement was made in the Hunter L a b color space and the D65 standard illuminant was used throughout. Meltdown was measured on each sample of ice cream in terms of initiation of drip loss and % drip loss based on the method described by Bund and Hartel (2013). Approximately 50 g of ice cream was cut and placed on a wire mesh screen (12 holes cm^{-1}) in a room control at 19°C. Each sample was allowed to drip for up to 30 min and the weight of the dripped portion was recorded every 5 min. The % drip loss was plotted against time to compare melting characteristic of ice cream samples. All analysis were done in triplicate.

2.6. Enumeration of probiotic bacteria

The enumeration of viable free and immobilized probiotic bacteria in ice cream was determined during storage at day 0 and day 10, 20, 30, 40 and 50 of frozen storage. Ten grams of each probiotic ice cream were aseptically transferred into a sterile stomacher bag, diluted with 90 mL of sterile 0.1% (w v^{-1}) peptone water (Himedia, India), and homogenized for 1 min in a stomacher. Subsequent serial dilutions were made and viable cell numbers enumerated using the spread plate technique. The counts of *L. casei* and *L. acidophilus* were enumerated on MRS agar (Himedia, India) incubated aerobically at 37°C for 72 h. (Christiansen *et al.*, 1996). Plates containing 30 to 300 colonies were enumerated and recorded as Log_{10} CFU g^{-1} of sample.

2.7. Sensory analysis

Sensory analysis to compare ice cream with free probiotic cells and ice cream with immobilized probiotic cells with banana flour was performed with 30 untrained panelists using a hedonic scale. The panelist received samples of approximately 25 g of each ice cream and were asked to evaluate the overall liking using a structured 9 points hedonic scale (1 = dislike extremely and 9 = like extremely) for the properties such as color and appearance, body and texture, flavor and overall acceptance. Samples were stored at -18°C in a freezer prior the tests and randomly served, in individual disposable plastic cup codified with three random digits.

2.8. Survival under simulated gastrointestinal condition

2.8.1. Preparation of simulated gastric and intestinal juices

The simulated gastric and intestinal juices were prepared according to Brinques and Ayub (2011). Simulated gastric juices were prepared by suspending pepsin (1:10,000 MP) in sterile sodium chloride solution (0.5%, w v^{-1}) to a final concentration of 3 g L^{-1} and adjusting the pH to 2.5 with concentrated HCl or sterile 0.1

mol L^{-1} NaOH using a pH meter. Simulated intestinal juices were prepared by suspending pancreatin (from porcine pancreas, Sigma-Aldrich) in sterile sodium chloride solution (0.5%, w v^{-1}) to a final concentration of 1 g L^{-1}, with 4.5% bile salts (Biomark Laboratories, India) and adjusting the pH to 8.0 with sterile 0.1 mol L^{-1} NaOH. Both solutions were sterile-filtered through a 0.22 µm membrane.

2.8.2. Measurement of cell tolerance to gastrointestinal

In simulated gastric juices tolerance, one gram of stored ice cream in freezer for 50 days was put in sterile wide mouth tubes and mixed with 5 mL of simulated gastric juices for 10 s. The mixture was then incubated at 37ºC for 2 h (Brinques and Ayub, 2011). After incubation, number of viable cells of *L. casei* and *L. acidophilus* were enumerated by plating serial dilutions (0.1% (w v^{-1}) peptone water) on MRS agar at 37ºC for 72 h. The results expressed on a Log_{10} CFU mL^{-1} of sample. In bile tolerance measurements, the growth of *L. casei* and *L. acidophilus* when previously exposed to simulated gastric juices was evaluated. After simulated gastric juices treatment, the surviving *L. casei* and *L. acidophilus* were collected by centrifugation (1,250 rpm, 20 min) and wash once with 0.1% (w v^{-1}) peptone water. They were resuspended in 1.13 mL simulated intestinal juices and incubated at 37ºC for 2 h. Simulated intestinal juices tolerance of *L. casei* and *L. acidophilus* cells was determined by comparing the viable count on MRS agar after incubation of 37ºC for 72 h. The results expressed on a Log_{10} CFU mL^{-1} of sample.

2.9. Statistical analysis

The experiment was conducted in a completely randomized factorial design with three replications. The results were submitted to analysis of variance (ANOVA) to determine significant differences ($p \leq 0.05$) between of difference types of treatments, using SPSS 13 software (SPSS Inc., Chicago, IL, USA).

3. Results and discussions

3.1. Physicochemical measurements

The initial pH of ice creams containing immobilized *L. casei* with KK, KH and KN flour (6.37±0.04, 6.26±0.02 and 6.34±0.06, respectively at 0 day) slightly increased to6.52±0.01, 6.45±0.01 and 6.38±0.01, respectively during frozen storage 50 days (Figure 1).

Table 1. Effect of the addition of immobilized *Lactobacillus casei* and *Lactobacillus acidophilus* with KK, KH, KN flour and free *L. casei* and *L. acidophilus* cells on the physical properties of ice cream (means±SD)

Parameter	Culture								
	Lactobacillus casei TISTR 1463					*Lactobacillus acidophilus* TISTR 1338			
	KK flour	KH flour	KN flour	free cells		KK flour	KH flour	KN flour	free cells
Overrun values (%)	46.21 ±1.06^{e}	44.95 ±0.10^{de}	42.45 ±0.40^{abc}	44.53 ±0.40^{cd}		43.55 ±0.20^{d}	43.50 ±1.60^{bcd}	41.71 ±0.60^{a}	41.82 ±0.80^{ab}
Color									
L	83.97 ±0.06^{a}	84.56 ±0.45^{a}	84.43 ±0.16^{a}	85.72 ±0.21^{b}		85.59 ±0.31^{b}	85.60 ±0.43^{b}	85.73 ±0.03^{b}	87.04 ±0.05^{c}
a	0.13 ±0.06^{g}	0.01 ±0.06^{f}	-0.18 ±0.01^{e}	-0.39 ±0.01^{c}		-0.71 ±0.01^{b}	-0.28 ±0.02^{d}	0.07 ±0.01^{fg}	-0.86 ±0.01^{a}
b	14.15 ±0.69^{c}	14.33 ±0.58^{c}	12.64 ±0.16^{ab}	15.76 ±0.55^{d}		14.00 ±0.00^{c}	13.72 ±0.43^{c}	11.76 ±0.14^{a}	13.59 ±0.19^{bc}
Melting rate (% drip loss min^{-1})	1.18	0.69	1.52	1.70		0.74	1.04	0.79	1.85
R^2	0.95	0.97	0.95	0.90		0.98	0.98	0.92	0.96

Means values within the same row with different superscript letters are significantly different ($p \leq 0.05$).
L = lightness, a = redness (+)/greenness (-), b = yellowness

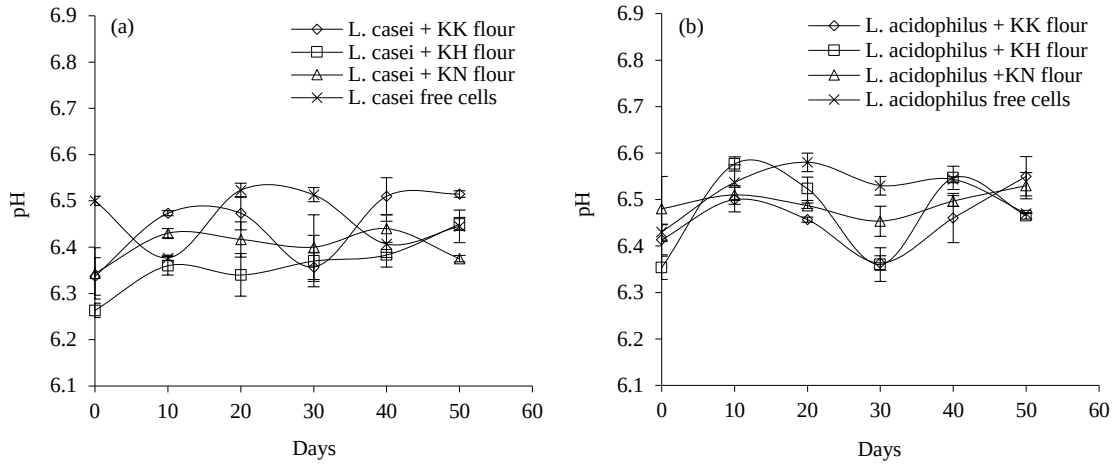

Figure 1. Variation of the pH of ice cream (a) containing immobilized *Lactobacillus casei* with banana flour and free *L. casei* cells and (b) containing immobilized *Lactobacillus acidophilus* with banana flour and free *L. acidophilus* cells during storage for 50 days at -18ºC

According to ice creams containing immobilized *L. casei*, the initial pH of ice cream containing immobilized *L. acidophilus* with KK, KH and KN flour (6.41±0.03, 6.35±0.03 and 6.48±0.07, respectively) slightly increased to 6.55±0.04, 6.47±0.01 and 6.53±0.03, respectively during storage. However, ice cream receiving free *L. casei* and *L. acidophilus* cells were not difference between pH at 0 day and 50 days, and showed a lower initial pH than ice cream supplemented immobilized cells with banana flour.

This is probably due to adding of immobilized probiotic cells with banana flour in aged ice cream mix increased acidity that inulin content in banana flour of cells immobilization had stimulated the metabolic activity of starter bacteria and improved development of acidity (Vatanasuchart *et al.*, 2012).

This effect was much more evident for initial pH of the ice cream containing immobilized cells with KH flour while similar pH values at day 50 were determined in all types of ice cream.

The physical propertied of synbiotic ice cream are shown in Table 1. Overrun values of all ice cream ranged between 41.71±0.60 to 46.21±1.06% similar to those found in literature for artisanal ice creams (40 to 50%). The data shows also to the overrun values which refer to overrun slightly significant different. The highest overrun value was also obtained in ice cream mix containing immobilized *L. casei* with KK flour ($p \leq 0.05$), indicating its responsibility for the increased air incorporation. The overrun value increased when immobilized with KK flour was used in the manufacture, in contrast to KN flour and free cells. No significant differences were found between ice cream containing immobilized cells with KK flour and ice cream containing immobilized cells with KH flour, and between ice cream containing immobilized cells with KN flour and ice cream with free cells. This is probably due to the addition of KH and KK flour resulting in an increased viscosity of ice cream mix and affect the increase in overrun. These findings in agreement with the results of Akın (2005) of the ice cream with inulin and different sugar and Akalın and Erişir (2008) of the ice cream with inulin and oligofructose. The unripe banana flour presented high amylose content and high amount of total dietary fiber, which consisted of resistant starch, inulin and dietary fiber without resistant starch or inulin (Menezes *et al.*, 2011; Vatanasuchart *et al.*, 2012; Liao and Hung, 2015).

Color measurements for the samples with free cells were compared to samples containing

immobilized cells with banana flour (Table 1). Lightness/darkness (L) value of all samples ranged from 83.97 to 87.04 that ice cream with free cells was slightly lighter compared to ice cream containing immobilized cells with banana flours. According to ice cream supplemented with immobilized *L. casei* with banana flour, ice cream supplemented with immobilized *L. acidophilus* with KK, KH and KN flour showed no significant difference in lightness. There were marginally significant difference in red/green (a) values of all ice cream types that red/green values ranged between 0.13 and -0.86. Yangilar (2015) reported red/green value between 1.62 and -1.05, which is in agreement with the results obtained in this study. All of ice cream taken into consideration were found to have positive yellowness value while yellowness value slightly decreased with addition of immobilized *L. casei* with banana flours and immobilized *L. acidophilus* with KN flour. Boonkong *et al.* (2014) reported that the addition of unripe banana "Hom Thong" decreased the color properties and these results are in agreement with the results of the present study.

Melting profiles of the ice creams containing free cells and immobilized cells with banana flours in terms of % drip loss are shown in Figure 2.

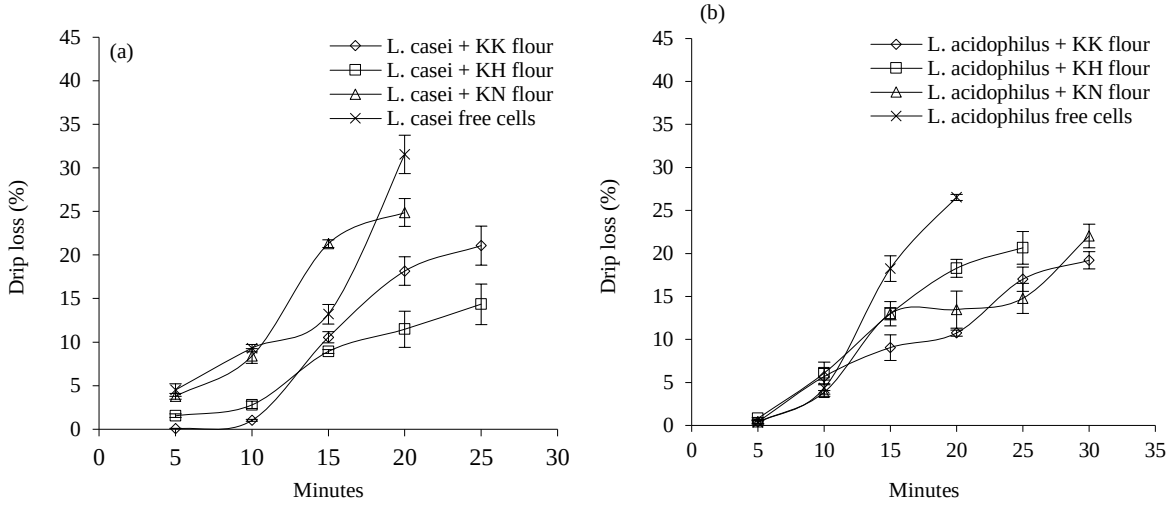

Figure 2. Percent drip loss of ice cream (a) containing immobilized *Lactobacillus casei* with banana flour and free *L. casei* cells and (b) containing immobilized *Lactobacillus acidophilus* with banana flour and free *L. acidophilus* cells

As heat penetrates the ice cream slab, initiation of meltdown occurs as the ice crystals begin to melt, followed by drainage of diluted serum phase through the remaining ice cream structure in order to be recorded as drip-through (Bund and Hartel, 2013). The % drip loss of free or immobilized ice cream samples increased with increasing the time of the test. In the case of *L. casei* (Figure 2a), the quickest meltdown was observed for the ice cream receiving free cells, followed by ice cream receiving immobilized cells with KN, KK and KH flour, respectively. Accordingly, in the case of *L. casei* (Figure 2b), the quickest meltdown was observed for the ice cream containing free cells, followed by ice cream containing immobilized cells with KH, KN and KK flour, respectively.

Melting rates of the ice creams were determined using the linear portion of the melting curves of averaged data for the respective samples (Table 1). Ice cream receiving immobilized *L. casei* with KH flour and immobilized *L. acidophilus* with KK and KN flour showed the lowest melt rate (0.69, 0.74 and 0.79% drip loss min^{-1}, respectively) compared with those receiving free cells. It was

suggested by Akin *et al.* (2007) and Akalin and Erişir (2008) that the reason for slower melting of ice cream with added inulin might be the ability of inulin to prevent water molecules from moving freely, hygroscopic properties and control ice recrystallization like a stabilizer agent. The gelling properties of inulin improve the consistency of mix and retard the melting of the product. Results of the present study indicate that the length of time until the first dripping was prolonged as a consequence of banana flour content in the ice cream samples. Yangılar (2015) reported that ice cream samples with green banana pulp flour had longer dripping times. These findings are in agreement with the findings in the present study. However, ice cream supplemented free *L. acidophilus* cells showed the highest melt rate (1.85% drip loss min^{-1}), part of which may be attributed to the slightly lower freezing point depression of the ice cream and also to potential differences in the fat network in the ice creams. This study has verified that the highest values for the overrun and the most remarkable improvement in the melt down characteristics were obtained in ice cream containing immobilized probiotics with KK and KH flour.

3.2. Viability of free cells and immobilized cells in ice cream during storage periods at freezing temperature

Survivability of two proven probiotic *L. casei* and *L. acidophilus* were enumerated on day 0 and the end of 50 days of storage showed a slightly different. Generally, the cell numbers of free and immobilized strains slightly varied with the increased in the storage period. The viable counts presented in Figure 3.

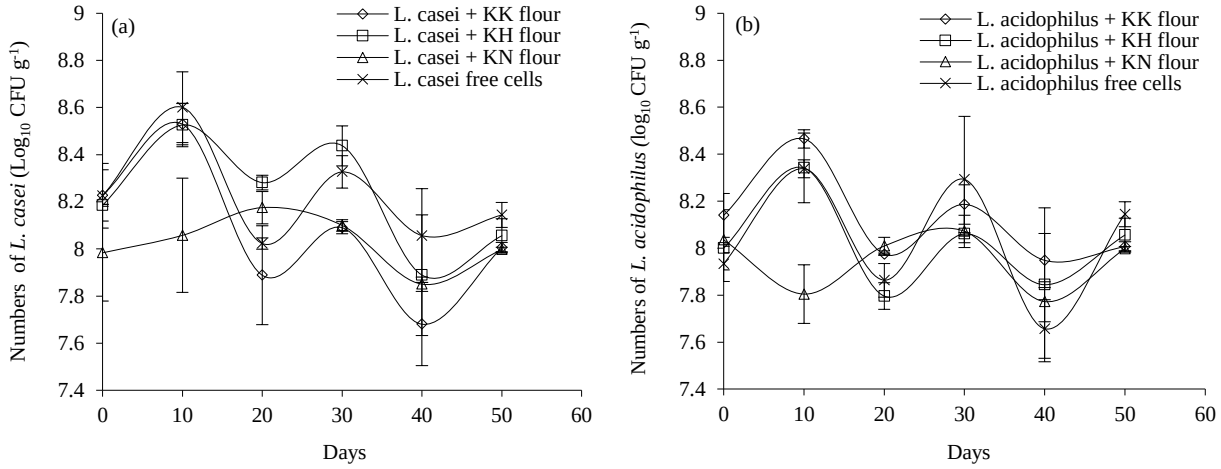

Figure 3. Viable cell numbers of ice cream (a) containing immobilized *Lactobacillus casei* with banana flour and free *L. casei* cells and (b) containing immobilized *Lactobacillus acidophilus* with banana flour and free *L. acidophilus* cells during storage for 50 days at -18ºC

Free *L. casei*, the cell numbers decreased from 8.23 to 8.15 log CFU g^{-1} (approximately 0.02 log units) from day 0 to 50 days of storage, where in immobilized *L. casei* with KK flour, the cell numbers decreased from 8.23 to 8.01 log CFU g^{-1} (approximately 0.04 log numbers), also immobilized *L. casei* with KH flour cells count decreased from 8.18 to 8.06 log CFU g^{-1} (approximately 0.02 log units), moreover immobilized *L. casei* with KNflourcells count increased from 7.98 to 8.00 log CFU g^{-1} (approximately 0.18 log units). At the end of 50 days of storage, ice cream with free microorganism revealed mean counts of *L. casei* of 8.15±0.05 log CFU g^{-1}, and no significant difference ($p>0.05$) were detected between population of *L. casei* in ice cream with KK, KH and KN flour (Figure 3a). The *L. acidophilus* count showed an average 0.01 log reduction in free state from 7.93 to 7.87 log CFU g^{-1} during day 0 to 50 days, wherein immobilized with KK flour of the same strain

showed a decreased count from 8.14 to 7.97 log CFU g^{-1} (about 0.03 log units), also immobilized with KH flour cells count increased from 8.00 to 8.06 log CFU g^{-1} (about 0.01 log units), moreover the immobilized with KN flour showed a decreased from 8.40 to 7.84 log CFU g^{-1} (about 0.01 log units) respectively. Although significant differences ($p \leq 0.05$) were detected between *L. acidophilus* counts in ice cream containing immobilized cells with KK, KH flour and free *L. acidophilus* cells on day 50, the difference were less than 0.19 log CFU g^{-1} (Figure 3b). Immobilized *L. casei* with KN flour and immobilized *L. acidophilus* with KH flour cells survived freezing better than those of the samples when compared within the same strain, possibly depending on the more conducive structure consists of resistant starch and inulin in banana flour to cell viability during storage. However, during freezing those of the samples, the counts of both viable bacteria slightly decreased between 0.01 and 0.02 log units and is in agreement with Davidson *et al.* (2000) and Alamprese *et al.* (2002) who reported that starter culture bacteria in ice cream did not change significantly during storage. To provide health benefits, a minimum of 10^6 to 10^7 CFU g^{-1} of probiotic bacteria must be presented in food in the moment of consumption (FAO/WHO, 2002). In this study, after 50 days of storage, the number of *L. casei* and *L. acidophilus* in all types of ice cream were above 10^8 and 10^7 CFU g^{-1}, respectively. This results shows that the dairy ice cream is an adequate vehicle for probiotic incorporation even without immobilization of microorganisms. Di Criscio *et al.* (2010) also observed high population of probiotic bacteria in ice cream stored for 16 weeks at -20ºC. The high rate of survival of probiotic microorganisms in this study, especially free cells, can be justified by the high total solids (35 to 39.5%), protein and fat, that could protect or even immobilization the probiotics. Furthermore, the ice cream pH (6.38 to 6.55), is considered favorable for the survival of probiotic microorganisms. Therefore, in the preparation of synbiotic ice cream by using *L. casei* and *L. acidophilus* appears to promote beneficial effects for the consumers.

3.3. Sensory Properties of ice cream

Sensory analysis of probiotic and symbiotic ice cream which contained free and immobilized strains was showed in the Table 2.

Table 2. Sensory properties of ice cream samples containing immobilized *Lactobacillus casei* and *Lactobacillus acidophilus* with KK, KH, KN flour and free *L. casei* and *L. acidophilus* cells (means±SD)

Sensory property	Score			
	KK flour	KH flour	KN flour	free cells
Lactobacillus casei				
Color and appearance	6.43±1.67^{a}	7.46±1.11^{b}	6.83±1.15^{ab}	7.03±1.27^{ab}
Body and texture	5.36±1.60^{a}	7.53±1.35^{b}	5.43±1.50^{a}	5.76±1.79^{a}
Flavor	6.00±1.55^{a}	7.13±1.52^{b}	6.13±1.43^{a}	6.13±1.33^{a}
Taste	6.30±1.72^{a}	7.40±1.42^{b}	6.26±1.46^{a}	6.46±1.52^{a}
Over all acceptance	6.33±1.56^{a}	7.76±1.19^{b}	6.30±1.49^{a}	6.53±1.36^{a}
Lactobacillus acidophilus				
Color and appearance ^{ns}	6.53±1.27	6.66±1.66	6.26±1.22	6.80±1.32
Body and texture ^{ns}	6.20±1.75	5.73±1.80	6.10±1.42	6.60±1.33
Flavor ^{ns}	5.86±1.61	6.46±1.67	6.06±1.31	6.63±1.40
Taste ^{ns}	6.03±1.45	6.56±1.65	6.20±1.47	6.83±1.23
Over all acceptance ^{ns}	6.16±1.62	6.66±1.44	6.46±1.22	6.90±1.12

Means values within the same row with different superscript letters are significantly different ($p \leq 0.05$).
^{ns} = non-significant ($p > 0.05$)

In the case of *L. casei*, there were no significant differences ($p>0.05$) in the color and appearance between ice cream with immobilized cells with KK, KH, KN flour and ice cream with free cells. It was expected that the addition of immobilized cells in ice cream could produce a slightly different color in the product. However, it was found that the addition of immobilized cells with KH flour in ice cream exhibited higher the body and texture, flavor and over all acceptance than ice cream with added immobilized cells with KK, KN flour and free cells ($p\leq0.05$). The body and texture, flavor and over all acceptance perception of ice cream with KK, KN flour and free cells showed no significant differences ($p>0.05$). The overall acceptability of free and immobilized synbiotic ice cream samples in storage period were ranged between 6.30 and 7.76. In the case of *L. acidophilus*, data showed that the addition of free and immobilized probiotic strains in ice cream had no effect on sensory properties of ice cream samples. The overall acceptability of free and immobilized synbiotic ice cream samples in storage period were ranged between 6.16 and 6.90. The addition of immobilized cells had no significant effect on the sensory properties of ice cream in which using the banana flour as prebiotic compound. Overall acceptability in terms of body and texture, flavor and taste of all samples were good and no marked off flavor was found during the storage period. None of ice cream was judged to be crumbly, weak, fluffy or sandy. This results agreement with EL-Sayed *et al.* (2014) who found that the addition of microencapsulated probiotic in ice cream had no significant effect on the sensory properties. Also, this result near to Yangilar (2015) who found that no significant differences in the means values of color, body and texture, resistance to melting, creaminess, mouth feel, gumming structure, banana flavor and unacceptable taste score of control and samples of ice cream with the addition of 2% banana pulp flour throughout the storage period. The acceptability of ice cream sample produced using 2% green banana pulp flour had the highest score from panelists.

3.4. Survival under simulated gastrointestinal conditions

Gastrointestinal tract is the location where viability of probiotic bacteria is mostly affected. In this study, the survival of free and immobilized strains (*L. casei* and *L. acidophilus*) in each ice cream after storage at -18°C for 50 days was determined after 2 h incubation in simulated gastric juices (pH 2.5).

Table 3. Effect of simulated gastric and intestinal juices on the survival for immobilized *Lactobacillus casei* and *Lactobacillus acidophilus* with KK, KH, KN flour and free *L. casei* and *L. acidophilus* cells (log_{10} CFU g^{-1}) (means±SD)

Conditions	Viable counts								
	Lactobacillus casei TISTR 1463					*Lactobacillus acidophilus* TISTR 1338			
	KK flour	KH flour	KN flour	free cells		KK flour	KH flour	KN flour	free cells
Initial cells number	8.01 ±0.02^{fgh}	8.06 ±0.07^{gh}	8.00 ±0.01^{fgh}	8.15 ±0.05^{gh}		7.97 ±0.03^{fgh}	8.06 ±0.01^{gh}	7.84 ±0.01^{defg}	7.87 ±0.01^{efg}
Gastric juices	7.33 ±0.04^{abc}	7.53 ±0.03^{bcde}	7.39 ±0.07^{bc}	7.33 ±0.01^{abc}		6.98 ±0.15^{a}	7.22 ±0.17^{ab}	7.16 ±0.07^{ab}	6.99 ±0.09^{a}
Intestinal juices	7.65 ±0.63^{cdef}	8.31 ±0.22^{h}	7.30 ±0.08^{abc}	7.86 ±0.20^{efg}		7.49 ±0.02^{bcd}	7.41 ±0.12^{bc}	7.46 ±0.08^{bc}	7.28 ±0.08^{abc}

Means values within the same row and column with different superscript letters are significantly different ($p\leq0.05$).

Result from Table 3 show that viable free and immobilized cells of *L. casei* and *L. acidophilus* were significantly affected by simulated gastric juices in the eight ice cream samples. Each probiotic demonstrated a small reduction in viability compared to before

treatment. Initially, all samples tested had an average of 7.84 to 8.15 log CFU g^{-1} of viable cells and after exposure to simulated gastric juices, all samples tested had an average of 6.98 to 7.53 log CFU g^{-1} of viable cells. The number of viable cells *L. casei* decreased; the delta log CFU g^{-1} was 0.68 for KK flour, 0.53 for KH flour, 0.61 for KN flour, 0.82 for free cells. However, there was a decrease viable cells *L. acidophilus* of 0.99, 0.84, 0.68 and 0.88 log for KK, KH, KN flour and free cells, respectively. The lowest decrease of probiotic cells after exposure to simulated gastric juices was observed with immobilized *L. casei* with KH flour. From the result (Table 3), viability of strain *L. casei* after exposure to simulated gastric juices was higher than that of strain *L. acidophilus* in the ice cream product. The viability of *L. casei* after exposure to simulated gastric juices was highest with ice cream supplemented with immobilized cells with KH flour (7.53 log CFU g^{-1}) and a somewhat lowest viable count from immobilized *L. acidophilus* with KK flour (6.98 log CFU g^{-1}) and free *L. acidophilus* cells (6.99 log CFU g^{-1}). This is in agreement with the study of Martoni *et al.* (2007) observed small losses of cell viability at pH 2.5 with 1.09 log CFU g^{-1} reduction, when *L. plantarum* 80 BHS^{+} was exposed to simulated stomach conditions for 4 h. Ranadheera *et al.* (2012) report that, after 180 min of exposure gastric juices, *L. acidophilus* LA-5 in goat's milk ice cream showed variation in the viability at pH 2, 3 and 4 with 3.56, 0.14 log reduction and 0.06 log increasing, respectively.

The same authors reported that the use of ice cream as a carrier food matrix provided a relatively positive influence on viability retention of each probiotic (*L. acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Propionibacterium jensenii* 702) during gastric transit with simulated gastric juices at pH 2.0 compared to plain and stirred fruit yogurts. dos Santos Leandro *et al.* (2013) observed that the viable *L. delbrueckii* UFV H2b20 incorporated in three ice cream formulations (low fat, fat free and high fat) was not significantly affected by incubation in PBS buffer (pH 3.0) for 3 h. The survival rate of probiotic bacteria in stomach will thus increase in the presence of food, which affects the pH value and may protect the probiotic bacteria from the effects of pepsin and acid in the stomach.

Bile resistance is one of the basic characteristic of probiotic bacteria. The composition of human intestinal juices is not exactly the same as that of the simulated intestinal juices. In this study, *L. casei* and *L. acidophilus* survived simulated gastric juices (pH 2.5), incubated in simulated intestinal juices (pH 8.0), for the assessment of theirs tolerance to bile. After 2 h incubation, effects of simulated intestinal juices on the growth of *L. casei* and *L. acidophilus* were observed (Table 3).

The growth of *L. casei* and *L. acidophilus*, incorporated in eight ice cream samples, in simulated intestinal juices was not significantly affected. Viability was maintained throughout the incubation period at the same order of 10^7 CFU g^{-1}. There was a significant increase of 0.78, 0.53 and 0.51 log for immobilized *L. casei* with KH flour, free *L. casei* cells and immobilized *L. acidophilus* with KK flour, respectively. This ability to grow in the simulated intestinal juices demonstrates that *L. casei* and *L. acidophilus* resists very well to ice cream storage. The use of immobilized cells with banana flour does not affect the protection of this stress conditions except for the ice cream containing immobilized *L. casei* with KH flour. Resistance of *L. casei* and *L. acidophilus* to the action of simulated intestinal juices seems to depend on the physicochemical properties of the cellular envelopes. This is in agreement with similar studies done by other workers. dos Santos Leandro *et al.* (2013) reported that *L. delbrueckii* UFV H2b20 has potential for being used in ice cream and capacity to growth in the presence of 0.3% (w v^{-1}) bile salts. Kimoto-Nira *et al.* (2010) showed that the survival of strain *Lactococcus lactis* G50 after 3 h incubation with 0.3% bile was highest in

lactose-containing broth.

Bifidobacterium animalis strains Bb-12 and Bo, and *L. brevis* strain LMG 6906 were found to survive better during in the gastrointestinal tract when administered with Requeijão, a Portuguese whey cheese, suggestion a protective role by the whey cheese matrix (Madureira *et al*, 2015). Ice cream may protect bacteria in a stress acid and bile salt by acting as a buffer, or by simple physical "encapsulation" with a matrix of ice cream and fat for example (dos Santos Leandro *et al*, 2013). In the present study, fat contents of ice cream samples were approximately 10% respectively. The high fat percentage in ice cream may have provided protection to probiotic by reducing their exposure to gastric and bile juices in present study. Moreover, the ice cream contained additional ingredients such as skim milk powder and stabilizers (corn flour, guar gum and egg yolk). These ingredients may also have provided some protection towards probiotic survival during simulated gastric and intestine transit in the present study by acting as a protective cover against gastric and small intestinal juices.

4. Conclusions

This study showed that probiotic cells alone (*L. casei* or *L. acidophilus*) or immobilized probiotic cells with banana flour was successfully used in the ice cream production. The addition of immobilized probiotic cells with banana flour affected overrun values and melting rate. The immobilized of *L. casei* and *L. acidophilus* with KK, KN and KH flour did not interfere in the survival of microorganisms in ice cream during 50 days of storage at -18ºC. The numbers of probiotic bacteria in all types of ice cream were above 10^8 CFU g^{-1} at the end of 50 days of storage. Moreover immobilization of probiotic cells had no effect on sensorial acceptability of synbiotic ice cream. The survivability of both free and immobilized *L. casei* and *L. acidophilus* was slightly affected by simulated gastric juices and while the simulated intestinal juices maintain the viability of *L. casei* and *L. acidophilus* under test condition. All two probiotics have demonstrated significantly acid and bile tolerance when they were incorporated in ice cream. Based on the results, it can be concluded that immobilized probiotic cells with banana flour can be used in the dairy ice cream formulation successfully.

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PHYSICAL, RHEOLOGICAL AND SENSORY PROPERTIES OF NON-ALCOHOLIC MALT BEVERAGE FORMULATED WITH DATE SYRUP

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ABSTRACT

Non-alcoholic malt beverage is popular in many countries in particular in Muslim countries which alcoholic beverages are banned. This beverage is usually prepared in a manner similar to beer, but excluding fermentation and it is generally produced using barley malt. In this study, malt beverage sweetened with 0:100, 25:75, 50:50, 75:25 and 100:0 ratios of date syrup to sucrose and their physicochemical, rheological and sensory properties were evaluated during six months of refrigerated storage. According to results pH and Brix were not changed and significant difference ($p<0.05$) was observed in reducing sugar, color and viscosity compared with the control. Based on our findings increasing the proportion of date syrup in beverages could elevate reducing sugar. The amount of total sugar was significantly different in the samples with 75%, 50% and 25% but not in the sample with 100% date syrup. From the sensory aspect, malt beverage with 75% date syrup exhibited the highest overall acceptability. In overall, beverages formulated with date syrup had a desirable characteristics and malt beverage with 75% date syrup was the best formula from both the technological and sensory aspects.

1.Introduction

As the well-known carbohydrate, sucrose is present in various food products in which it affects their taste, texture and consistency. However, sucrose is often preferably replaced with other sweeteners since that it is the main cause of obesity, as well as diabetes and dental cavities. There are several known intense sweeteners which provide little or no energy intake. Since intense sweeteners are amongst the most controversial food additives due to suspicions of adverse health effects (Kroger *et al.*, 2006), only few of them are allowed to be used in modern food industry (Zygler *et al*., 2009). On the other hand, there is a wide range of natural sweeteners, which besides providing sweetness to the product, they contain various bioactive compounds, such as vitamins, minerals or polyphenols that are known to exhibit positive health effects (Sherman, 2003) and contribute to the concept of functional foods.

Though not widely used, several natural alternatives to sugar are available. These natural non-refined sugar alternatives potentially contain beneficial bioactive compounds, especially polyphenolic compounds, known and appreciated for their antioxidant properties. Some of these natural sugar substitutes include plant saps/syrups (e.g. maple syrup and agave nectar), syrups made from raw sugar and grains (e.g. molasses, barley malt, and brown rice syrup), honey and fruit or vegetable sugars (e.g. date sugar and carrot).

Date fruit from date palm (*Phoenix dactylifera* L.) is one of the nutritious foods

that is cultivated and found in the Canary Islands and northern Africa, the Middle East, Iran, Pakistan, India, and California (Homayouni *et al.,* 2015). The importance of the date in human nutrition comes from its rich composition of carbohydrates (70–80%), salts and minerals, dietary fiber, vitamins, fatty acids and amino acids (El-Nagga and Abd El-Tawab, 2012).

Date juice concentrate products are condensed products made out of date extract by water evaporation. They differ in appearance, taste and consistency depending on the type of the raw juice and the degree of concentration. Three main products can be distinguished, including date spread, date syrup and date liquid sugar (Barreveld, 1993).

Date syrup is thick and dark brown syrup and it is the most commonly derived date product. It contains various components such as carbohydrates, proteins, lipids, pectin, salts and minerals (Alanazi, 2010).

Glucose and fructose are the major sugars in date syrup and total sugar contents reach up to 88%. Beside its nutritional compounds, it is rich in antioxidants and the components responsible for antioxidative effect include flavonoids, phenolic acid, ascorbic acid and carotenoids (Al-Hooti *et al*., 2002; Abbès *et al*., 2011).

Non-alcoholic malt beverages are consumed in many countries. There are many people who avoid alcohol because of their health concerns, and in Muslim countries alcoholic beverages are religiously banned. Malt drinks are classified based on the alcohol content as alcoholic (more than 1.2%), low alcoholic (0.5-1.2%) and with no alcohol (less than 0.5%). Non-alcoholic malt beverages are produced as non-fermentative or fermentative products. Malt drinks are generally produced by dissolving wort granulates in water, filtration, and adding pure hop aroma, followed by carbonation. The drink has some health benefits such as protection against coronary heart diseases, cancers and ulcers (Bamforth, 2002). Malt beverages is usually produced using barley malt and in some cases from other sources such as wheat, oats, rye and sorghum (Briggs *et al*., 2004).

Malt beverage is usually sweetened with sucrose. Date syrup can be used as a substitution of sucrose in non-alcoholic malt beverage. Date syrup is considered to have functional properties such as sugar replacer.

Most of the carbohydrates in date syrup are in the form of fructose and glucose, which are easily absorbed by human body (Myhara *et al*., 1999; Al-Farsi *et al*., 2005).

The aim of this study was to investigate the possibility of using date syrup as a sweetener in malt beverages. Various ratios of sucrose and date syrup were used for the production of malt beverages, and their effects on physicochemical, rheological and sensory properties of malt beverages were evaluated.

2.Materials and methods

Barley malt and hops pellets were obtained from Sard Sahra Company (Tabriz, Iran). Sucrose was purchased from Khoy sugar factory (Khoy, Iran) and date syrup from Shahd Bab Pars factory (Tabriz, Iran).

The physicochemical properties of date syrup are shown in table 1.

Materials used for producing beverages are mentioned in table 2.

Table1. Date syrup physicochemical properties

Brix (%)	pH	Fructose/Glucose
70±2	4.5	0.9-1.1

2.1.Sample preparation

Barley malts were milled using a roller mill, then steeped with hot water in a mash tank. The slurry was heated according to a regular heating program and malt extract was filtered to produce thick, sweet liquid called wort. The wort was then boiled for an hour and date syrup and sugar were added together to the boiling wort at the ratios of 0:100, 25:75, 50:50, 75:25 and 100:0. Hops pellets were also added to the extract. The wort was then chilled using heat exchanger plate to avoid fermentation and filtered through the three

successive polypropylene membrane filters with the pore sizes of 5, 1 and 0.1 µm (Bamforth, 2003). The brix and pH of extracts were adjusted to 6 and 3.8 and they were transferred to 300 ml green Polyethylene Terephthalate (PET) bottles and 1.5 gr solid CO_2 was added to each of them. Upon completion of gas dissolution, drinks were pasteurized at 70°C for 30 minutes and stored in refrigerator at 4°C for further analysis.

Table 2. Malt beverages formulation with different ratios of sucrose and date syrup

Malt beverages	Malt (Kg)	Date syrup (kg)	Sucrose (kg)
100% date syrup	2.5	4.2	0
75% date syrup	2.5	3.15	0.625
50% date syrup	2.5	2.1	1.25
25% date syrup	2.5	1.04	1.875
0% date syrup (control sample)	2.5	0	2.5

2.2.Physicochemical and rheological evaluation of malt drink

Physicochemical specifications of the malt drinks including brix (Abbe refractometer, 2WAJ, England) and pH (knick pH meter, England) were determined according to standard methods of American Official Analytical Chemists (AOAC, 2002). Total and reducing sugars were measured according to Lane-Eynon method (AOAC, 2002). Acidity was also measured by titration. The titratable acidity was determined using titrimetry (Sadler and Murphy, 2010). Malted roasted beverage samples were titrated against NaOH (0.1N) using phenolphthalein as indicator to a faint pink endpoint and total acidity expressed as percent of citric acid equivalent.For the determination of the color of industrial worts, beers and liquid adjuncts, the method EBC 8.3 was used by which the absorbance of the sample was measured at a wavelength of 430 nm and the color obtained by multiplying by a factor of 25 (White, 1995).Rheological measurement were carried out with Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria). The temperature was maintained constant at 25 ˚C by a peltier system. The shear rate range was 2-100 s^{-1} and test were performed in 3 replicate of all the samples.

2.3. Sensory evaluation

Trained laboratory-based panel of 8 members (n = 8; 4 females and 4 males, ages 22 to 37) who were graduate students of the Department of Food Science and Technology (Tabriz University of Medical Sciences, Tabriz, Iran) with previous experience in sensory evaluation assessed beverages in an acceptance test (Stone and Sidel, 2004). Beverages were presented to the panelists in identical containers labeled with a different 3-digit code. Samples were analyzed for color, taste, mouthfeel and overall acceptability. A 5-point hedonic scale was provided to the panelists that ranged from 5 = very good to 1 = undesirable. Mean scores for each attribute were calculated for comparison of the samples. Panelists were instructed to rinse their mouths with drinking water before evaluating subsequent samples. A serene atmosphere of good lighting and ventilation, as well as quietness was provided for panelists to conduct the evaluation (Tortoe *et al*., 2012).

2.4.Statistical analysis

The experiment was set up in a 2-way factorial analysis using five samples of beverages and 10 storage times. Analysis of variance was used for data analysis (SPSS 19; IBM SPSS Statistics, Chicago, IL). When F-values were significant ($P<0.05$) in ANOVA, Duncan's multiple range test was used to compare treatment means. Pearson correlation coefficient analyses were used to evaluate potential relations between all parameters

experimented. The data reported in all of the tables are the means of triplicate observations.

3.Results and discussions

Table 3. Evaluation of pH content of the examined samples during 6 months of storage

Sample/ day	1	7	14	21	28	60	90	120	150	180
100%	3.8	3.8	3.8	3.79	3.79	3.79	3.79	3.79	3.78	3.78
75%	3.8	3.8	3.8	3.79	3.79	3.79	3.79	3.79	3.78	3.78
50%	3.8	3.8	3.79	3.79	3.79	3.79	3.79	3.79	3.78	3.78
25%	3.8	3.8	3.79	3.79	3.79	3.79	3.79	3.79	3.78	3.78
0%	3.8	3.8	3.8	3.79	3.79	3.79	3.79	3.79	3.78	3.78

3.1.pH

The pH of malt beverages within 1, 7, 14, 21, 28, 60, 90,120, 150 and 180 days of storage were shown in table 3. Results indicated that pH was almost constant and change of pH during the storage time was not significant ($p>0.05$). Hosseini *et al.* (2012) found the same result and pH was constant in 100% malt beverage after 6 month storage. Since no fermentation occurred in non-alcoholic malt beverages, pH did not change during the time. If pH was changed, it could be referred to microorganisms' growth and the beverages were not completely pasteurized. Because the beverages were pasteurized at 70^{o}C for 30 minutes no microbial growth occurred and therefore pH did not change.

This result also indicated that substitution of sucrose by date syrup did not leave any undesirable effects on pH.

3.2.Brix

Brix of malt beverages which was adjusted to 6 stayed constant during storage time, Hosseini *et al.* (2012) had the same result and Brix was constant in 100% malt beverage after 6 months storage. As a result, substitution of date syrup to sucrose did not have any effect on Brix too.

3.3.Reducing sugars

Nonalcoholic malt beverages in which date syrup was used, generally had significantly higher ($p<0.05$) reducing sugar (Figure 1). Yaseen *et al*. (2013) reported that muffins which had high concentration of date syrup had high reducing sugar. Most of the carbohydrates in date syrup are in the form of fructose and glucose (Myhara *et al*., 1999: Al-Farsi *et al*., 2005), which are reducing sugars. As a result, of rising the proportion of date syrup, reducing sugar was significantly increased in the beverages. Fructose and glucose are easily absorbed by human body (Myhara *et al*., 1999: Al-Farsi *et al.,* 2005) and fructose feeding does not directly cause an increase in plasma insulin whereas sucrose feeding does (Mayes, 1993), so beverages with date syrup could have more benefits than beverages with sugar especially for diabetics (Homayouni Rad *et al.,* 2013).

3.4.Total sugar

Samples with 75%, 50% and 25% date syrup showed significant difference to the sucrose containing samples. However, there was no significant difference between the sample with 100% date syrup and sucrose containing sample (Figure 2). Hosseini *et al.* (2012) indicated that total sugar was significantly decreased in 100% barely malt drinks after 6 months but our results showed that the total sugar was constant during 6 months of storage.

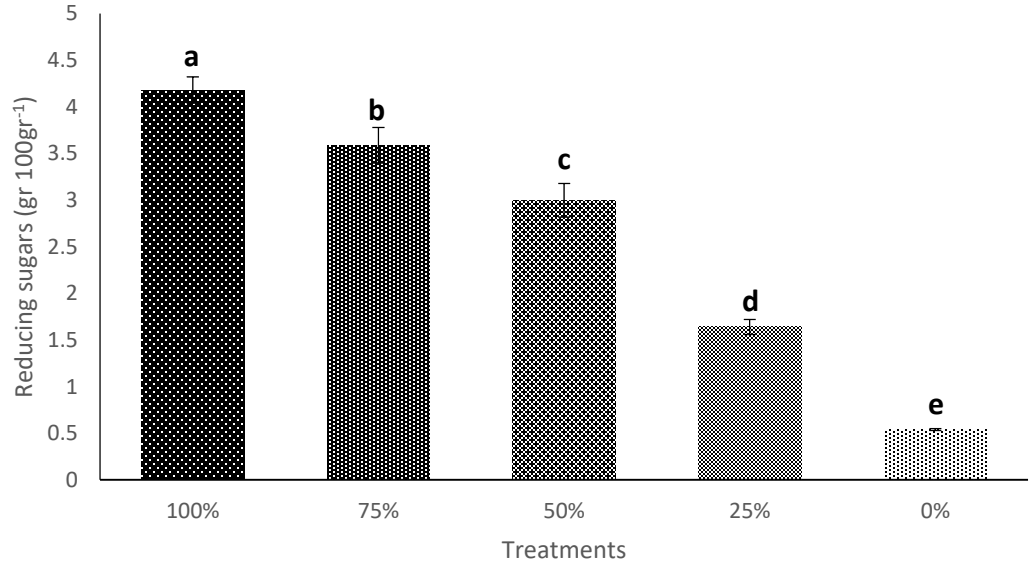

Figure 1. The amount of reducing sugars in malt beverages containing 0, 25, 50, 75 and 100% date syrup Means with the same letter are not significantly different (P>0.05) by Duncan's Multiple Range Test for variable

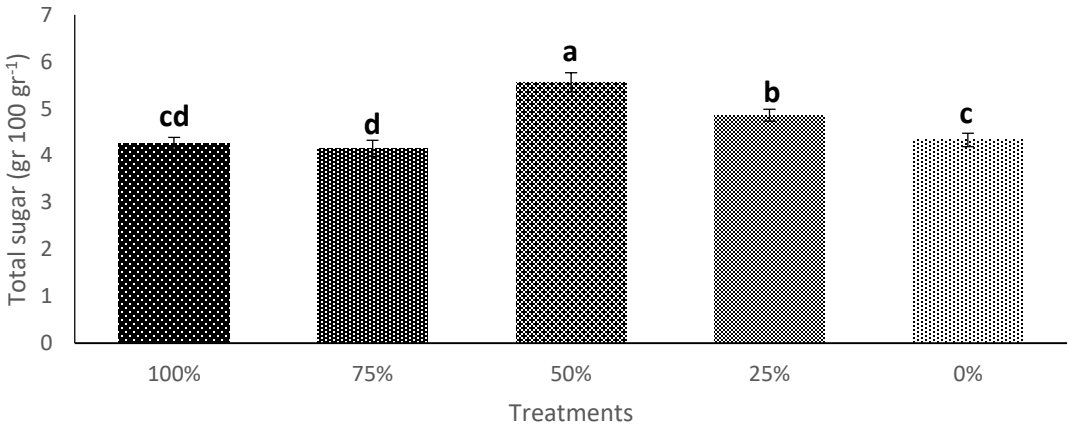

Figure 2. The amount of total sugar in malt beverages containing 0, 25, 50, 75 and 100% date syrup. Means with the same letter are not significantly different (P>0.05) by Duncan's Multiple Range Test for variable

Table 4. Color of nonalcoholic malt beverages with different concentrations of date syrup replaced to sucrose

	1	7	14	28	60	90	120	150	180
100	9.32^{Aa}	9.31^{Aa}	9.31^{Aa}	9.32^{Aa}	9.325^{Aa}	9.31^{Aa}	9.325^{Aa}	9.32^{Aa}	9.35^{Aa}
75	9.187^{Ab}	9.23^{Ab}	9.209^{Ab}	9.187^{Ab}	9^{Ab}	9.25^{Ab}	9.225^{Ab}	9.256^{Ab}	9.27^{Ab}
50	8.437^{Ac}	8.44^{Ac}	8.44^{Ac}	8.432^{Ac}	8.45^{Ac}	8.441^{Ac}	8.436^{Ac}	8.437^{Ac}	8.45^{Ac}
25	7.762^{Ae}	7.768^{Ae}	7.765^{Ae}	7.77^{Ae}	7.72^{Ae}	7.747^{Ae}	7.76^{Ae}	7.755^{Ae}	7.775^{Ae}
0	7.33^{Ad}	8.356^{Ad}	8.365^{Ad}	8.351^{Ad}	8.325^{Ad}	8.33^{Ad}	8.351^{Ad}	8.329^{Ad}	8.375^{Ad}

a-e Different superscripted letters in the column indicate statistical significance between the samples ($p<0.05$).
A-E Different superscripted letters in the row indicate statistical significance during the time ($p<0.05$).

3.5.Color

There was significant difference in samples color compared with the control, but no significant change in beverages color was observed during the storage (Table 4). The color of beverages with 100% date syrup was 9.3 according to EBC color scale.

Significant decrease in color was observed as the proportion of date syrup was decreased ($p<0.05$) (Figure 3).Malt beverage color is generally affected by the hops and malt components. Millard reactions produce melanoidins, creating yellow, orange, red and brown pigments (Shellhammer and bamforth, 2008). Date syrup color contributed to the color groups, degradation products of reducing sugars, melanoidines and iron-polyphenolic complexes (Mostafa and Ahmed, 1981). The color of Beverages with 100% and 75% date syrup Due to date syrup color and more reducing sugars were higher than control sample.

Malt beverage color is also affected by the processing steps. Color is also under the effect of temperature, boiling time, pH of the extract, concentration of free amino nitrogen and sugars (Shellhammer and bamforth, 2008).

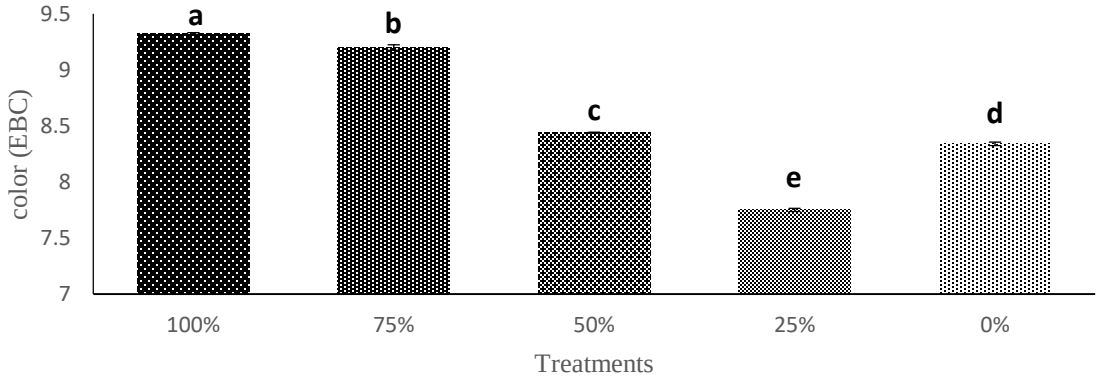

Figure 3. The amount of color of malt beverages containing 0, 25, 50, 75 and 100% date syrup. Means with the same letter are not significantly different (P>0.05) by Duncan's Multiple Range Test for variable

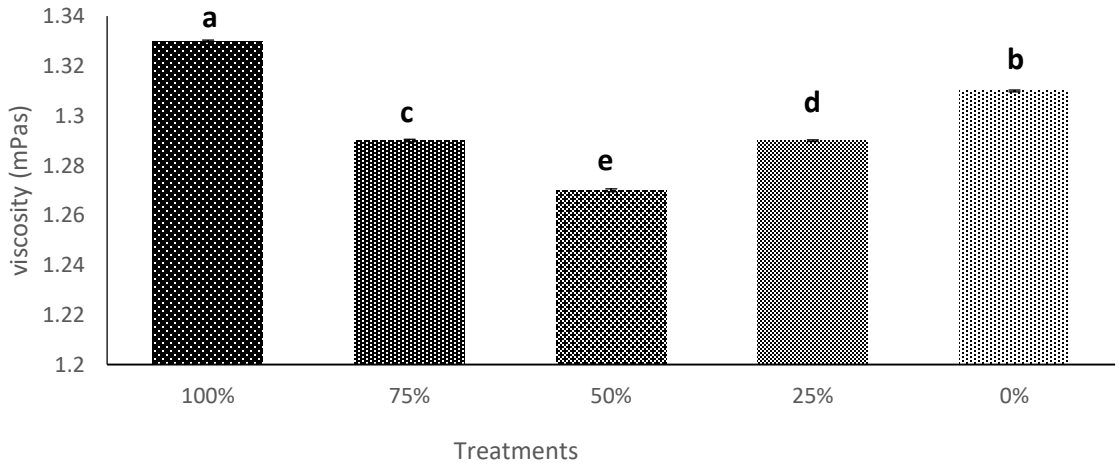

Figure 4. The viscosity of malt beverages containing 0, 25, 50, 75 and 100% date syrup. Means with the same letter are not significantly different (P>0.05) by Duncan's Multiple Range Test for variable

Vandehaegan *et al*. (2007) reported color increase during 1 year of storage of fermented malt beverage, but our results indicated that the color of malt beverages was not significantly increased over 180 days of storage (Table 4).

3.6.Viscosity and rheological properties

Results showed that there was a significant difference between samples viscosity and the control sample ($p<0.05$) (Figure 4). The highest viscosity was related to sample with 100% date syrup. The most important features of malt beverage are the storage and release of Co_2 and foam stability. Foam stability increased as viscosity increased.

Although we proved significant difference between samples viscosity and the control sample ($p<0.05$) but the overall rheological behavior of beverages were stable. (Mita *et al.*, 1977). The non-alcoholic malt beverages showed Newtonian behavior, as indicated by the linear dependence of the shear stress on the shear rate shown in Figure 5.

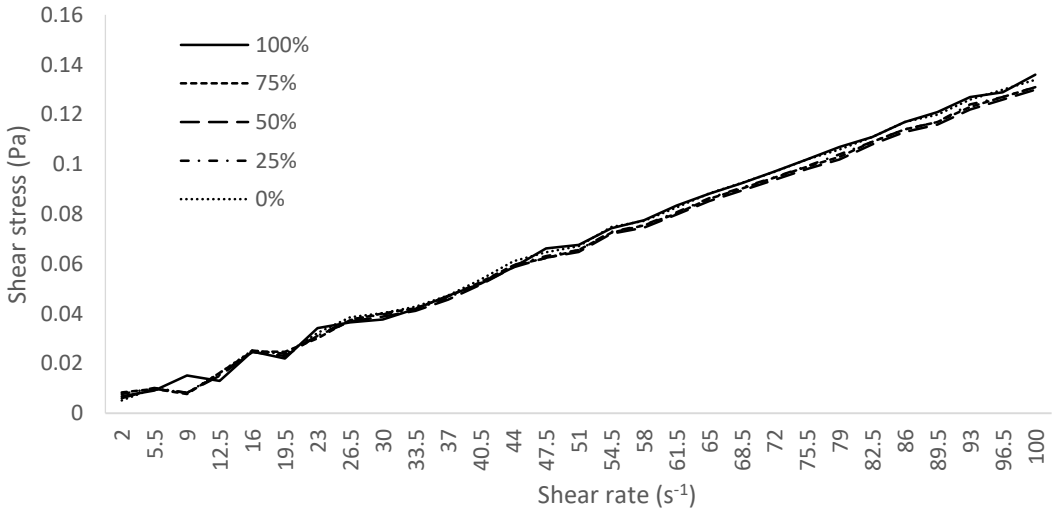

Figure 5. Rheograms obtained for malt beverages containing 0, 25, 50, 75 and 100% date syrup

3.7.Sensory evaluation

Using sucrose alternatives in food industry requires that such alternatives should not cause significant changes in the sensory characteristics of a product (Bolini-Cardello *et al.*, 1999). Table 5 presents the average sensory evaluation results of samples with different date syrup concentrations in terms of color, taste, mouthfeel and overall acceptability. It was found that samples prepared with 100% and 75% date syrup received the highest overall scores among other samples, but samples with 75% date syrup were chosen as the best. Samples with 100% and 75% date syrup were more desirable than samples with 100% sucrose because they had date syrup desirable taste and these beverages were the most acceptable by the panelists.

Among the beverages produced in this study, control beverage was scored the best in terms of color (Figure 6). Color of beverages with 100% and 75% date syrup were the most similar to control beverage. In general, the overall acceptability ranking was in accordance with the ranking of taste and mouth feel indicating that these attributes were analyzed by panelists. According to this fact, beverage with 75% date syrup exhibited the highest overall acceptability and beverage with 25% date syrup, characterized with the poorest taste scores and the lowest acceptability. Figure 6 reveals that beverage with 100% date syrup was scored highest in terms of mouthfeel and taste though beverage with 75% date syrup gained the highest acceptance.

The results of this study indicate the potential of date syrup to completely replace sucrose and achieve the desired sweetness of malt beverage.

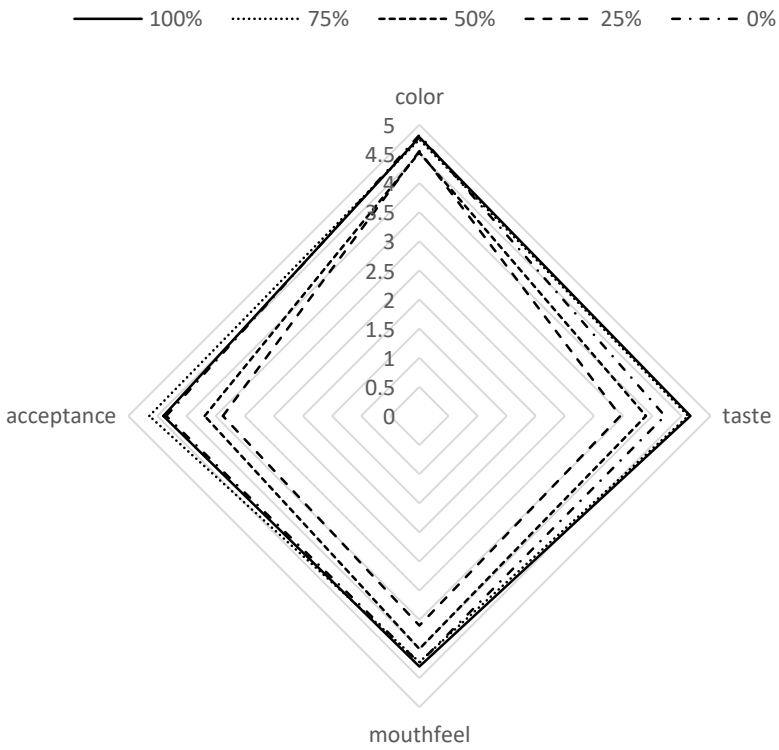

Figure 6. Sensory properties of formulated non-alcoholic malt beverage with 0, 25, 50, 75 and 100% date syrup as sweetener

Table 5. Sensory evaluation for nonalcoholic malt beverage with different concentrations of date syrup replaced to sucrose

Date syrup %	color	taste	mouthfeel	acceptance
100%	4.787^{a}	4.65^{a}	4.3^{a}	4.4^{b}
75%	4.75^{a}	4.613^{a}	4.225^{a}	4.637^{a}
50%	4.525^{b}	3.888^{c}	4^{b}	3.688^{c}
25%	4.55^{b}	3.45^{d}	3.6^{c}	3.375^{d}
0%	4.825^{a}	4.213^{b}	4.238^{a}	4.35^{b}

a-e Different superscripted letters in the column indicate statistical significance between the samples ($p<0.05$).

4.Conclusions

The present study displays the potential of producing malt beverage, by using sucrose substitutes such as date syrup, as well as natural ingredients as sources of sweeteners. So effects of four concentrations (25, 50, 75 and 100%) of date syrup on physicochemical, rheological and sensory properties of malt beverages were investigated. results indicated that substitution of sucrose by date syrup did not leave any undesirable effects on pH and brix of the beverages during 6 month. The amount of the reducing sugar (Fructose and glucose) was significantly increased with rising the proportion of date syrup However, there was no significant difference between the sample with 100% date syrup and sucrose containing sample in the amount of total sugar. Color of beverages with 100% and 75% date syrup were higher than control sample but no significant change in beverages color was observed during the storage. The highest viscosity and as a result the foam stability was related to sample

with 100% date syrup. According to our findings, beverage with 75% date syrup exhibited the highest overall acceptability. Generally beverages formulated with date syrup had desirable characteristics and sensory evaluation results suggested the sample containing 75% of date syrup as the most accepted sample.

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EFFECT OF NISIN ON QUALITIES OF PORK MEATBALLS DURING REFRIGERATED STORAGE

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ABSTRACT

Pork meatballs are one of popular ready-to-eat meat products, especially in Asia. The shelf-life of this product is only 1 week at 4°C; therefore, antimicrobials, such as benzoic acid, sorbic acid, nitrite, are usually added into the meatballs during manufacturing. Nisin, a bacteriocin produced from *Lactococcus lactis*, is an alternative natural antimicrobial, which can be safely digested in human digestive tract. The objective of this research was to investigate the efficacy of nisin (6.25, 12.5 and 25.0 mg/kg) on qualities of pork meatballs during refrigerated storage (4±2 °C), compared with control (no nisin added). All samples were examined for microbial growth (total mesophilic aerobic bacteria and total psychrotrophic aerobic bacteria), color, texture, chemical compositions and sensory qualities during storage. All nisin treated samples contained significantly lower total mesophilic and psychrotrophic aerobic microbial counts than those of the control sample during storage *($p<0.05$)*. Samples treated with 25.0 mg/kg nisin was the most efficient in inhibiting total mesophilic and psychrotrophic aerobic microbial growth during 15 of storage at 4°C without affecting other qualities of the meatballs, when compared with those of the control sample.

1. Introduction

Pork is an excellent source of protein, vitamins, and minerals for human diet. The consumption of pork is approximately 30% world consumption of all meat, which includes pork, bovine meat, poultry meat, and ovine and caprine meat (Bruinsma, 2015). Bacon, sausage, pork chops, ham, and meatball are examples of products processed from pork in the global market. In Asia, one of popular meat products is an emulsified meatball, which is produced by different processing techniques in different countries. The difference between the Asian emulsified meatballs and western meatballs is the addition of emulsifiers, which provides softer texture in emulsified meatballs (Hsu and Yu 1999). Meat and meat products usually have a short shelf life since meat is rich in nutrient that enhances the growth of meat spoilage microorganisms and food-borne pathogens; thus, appropriate food preservation is used to maintain its quality and safety (Aymerich *et al*., 2008).

Nisin is a polypeptide with low molecular weight (3.3 kDa) synthesized by *Lactococcus lactis* subspecies lactis. It is an effective bacteriocin against Gram-positive bacteria, including *Bacillus cereus*, *Bacillus sporothermodurans*, and *Clostridium botulinum* (Thomas and Delves-Broughton, 2005; Carballo *et al*., 2012), as well as bacterial spores (Ray, 1992). According to Joint FAO/WHO Codex Alimentarius Commission (2015), nisin is

allowed in heat-treated processed meat, poultry, and game products in whole pieces or cuts at the maximum level of 25 mg/kg. In the United States, nisin has affirmed as generally recognized as safe (GRAS) and it is approved for inhibiting the outgrowth of *Clostridium botulinum* spores and toxin formation in pasteurized cheese spreads and pasteurized cheese spread with fruits, vegetables, or meats (U.S. Food and Drug Administration, 2015). In Mexico and Peru, nisin is also approved as a permitted additive in any food (Cleveland *et al.*, 2001).

In this study, the efficacy of nisin on quality attributes of pork meatball was investigated during storage at 4±2 °C.

2. Materials and methods

2.1. Materials

The commercial food-grade ingredients, except ice and sodium tripolyphosphate (STPP), were purchased from a local market and the following formulation were used for preparing pork meatballs in this study: 70.0%(w/w) pork tenderloin, 10.0%(w/w) pork back fat, 14.6%(w/w) ice, 2.7%(w/w) sugar, 2.0%(w/w) salt, 0.5%(w/w) ground black pepper, and 0.2%(w/w) STPP (SD Bni (Cn) Co., Ltd., China). Ice was prepared by freezing sterile water.

2.2. Sample preparation

To prepare a control, pork tenderloin, back fat and ice were ground and mixed with sugar, salt, ground black pepper, and STPP by a food processor (HR-7628/00, Philips Electronics (Thailand), Co., Ltd., Thailand). The meat mixture was cooled at -18°C for 30 min and kneaded for 15 min by hand in order to obtain a homogenous mixture. The mix was then formed into 2±0.5 cm diameter meatballs before cooking at 80±2°C for 20 min. The cooked meatballs were immediately cooled with ice water and stored in a resealable storage bag (18 x 20 cm^2) (Big C Supercenter Public Co., Ltd., Thailand) at 4°C until analysis.

The procedures for preparing treated samples were similar to those for the control, except food-grade nisin (Shandong Freda Biotechnology Co., Ltd., China) (6.25, 12.5 and 25.0 mg/kg meatballs), which had been dissolved in sterile water before freezing into the ice.

The preparation process was independently repeated on 3 separate days as replication.

2.3. Microbiological analysis

Total mesophilic aerobic bacteria and total psychrotrophic aerobic bacteria were determined by the pour-plate and spread-plate methods (Colak *et al.*, 2008) respectively at 0, 3, 6, 9, 12, and 15 days of storage. 10 g of each sample was aseptically weighted and homogenized with 90 ml of sterile peptone water (Merck, KGaA, Germany) for 1 min using a stomacher (Funke-Gerber, Germany) at 230 rpm. The homogenized samples were serially diluted (1:10) in sterile peptone water. 1 ml of sample from serial dilutions was pour-plated in duplicate on plate count agar (PCA) (Merck, KGaA, Germany) incubated at 35°C for 48 h to count total mesophilic aerobic bacteria. The same decimal dilutions were spread-plated on PCA and incubated at 7°C for 10 days to count total psychrotrophic aerobic bacteria. After incubation, colonies were counted and microbiological data was expressed as logCFU/g.

2.4. Color

Color values of the meatball surface at 0, 5, 10, and 15 days of storage were directly determined with a colorimeter (Color Quest 45/0, Hunter Associates Laboratory, Inc., Reston, VA) at room temperature. The instrument was calibrated using a standard white and black reflector plates. Three meatballs per treatment were measured and four readings were made in each replicate by changing the position of the sample. The results were expressed as lightness (L*) and total color difference (ΔE). ΔE was calculated using measured L*, a* (a positive a* represents red and a negative a* represents green) and b* (a positive b* represents yellow and a negative a* represents blue) as follows:

$$\Delta E = \sqrt{(L_0 - L^*)^2 + (a_0 - a^*)^2 + (b_0 - b^*)^2} \quad (1)$$

where subscript "0" refers to the color coordinates reading of control sample at 0 day of storage used as a reference. ΔE was used to describe the color change as compared to the reference and the larger ΔE indicates greater color change from the reference (Saricoban and Yilmaz, 2010).

2.5. Texture

Texture of meatballs was measured by compressing samples with texture analyzer TA.XT2i (Stable Micro Systems Ltd., United Kingdom) as described by Petersson *et al.* (2014) at 0, 5, 10, and 15 days of storage.

2.6. Chemical compositions

Chemical analysis of the control and the best treatment (from microbiological analysis, color and texture) at the beginning of storage was performed following AOAC (2012) including moisture, lipid, protein, fat, ash, and carbohydrate contents.

2.7. Sensory evaluation

Sensory evaluation was performed to determine the acceptability of the best treatment (from microbiological and instrumental analysis) compared to the control sample among 30 untrained panelists screening from juniors and seniors in Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Thailand. The control and treated samples at 0 and 7 days of storage were evaluated for appearance, firmness, flavor, and overall acceptance using a nine-point hedonic scale, including 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely. All samples were labeled with random 3-digit codes and presented in random order.

2.8. Statistical analysis

All analyses were run in triplicate. Data were analyzed by analysis of variance (ANOVA) using IBM SPSS Statistics 21 (IBM Corporation, Armonk, NY). Duncan's multiple range test was used to determined significant differences among means (significance was defined at $p < 0.05$).

3. Results and discussions

3.1. Microbiological analysis

Mesophilic and psychrotrophic aerobic bacteria counts of meatballs are presented in Figure 1 and 2 respectively.

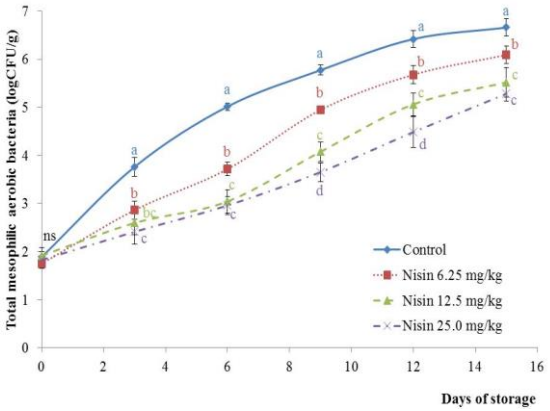

Figure 1. Total mesophilic aerobic bacteria of control and nisin treated samples during storage at 4°C. Different letters indicate significant difference at the same day of storage *(p<0.05)*. ns indicates no significant difference at the same day of storage *(p≥0.05)* (n=3, error bars: standard deviations).

Compared with control, all treatments significantly inhibited the growth of bacteria in meatballs during storage (*p<0.05*). The slowest growth of bacteria was found in the sample treated with 25.0 mg/kg nisin. Nisin is effective against Gram-positive bacteria by forming pores at the cytoplasmic membrane. These pores disrupt the proton motive force and the pH equilibrium leading to leakage of ions, hydrolysis of ATP, and eventually cell death. Nisin can also inhibit cell wall biosynthesis by binding lipid II, a peptidoglycan precursor (Bauer and Dicks, 2005; de Arauz *et al.*, 2009; Deegan *et al.*, 2006).

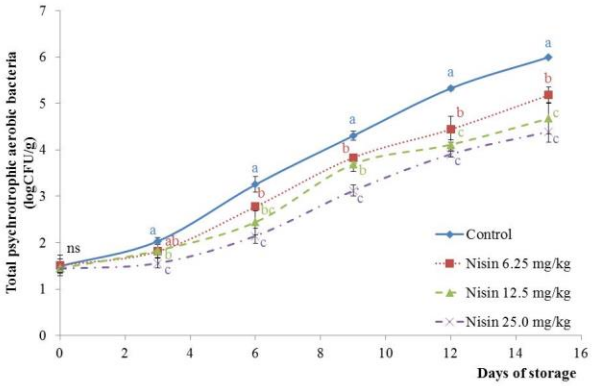

Figure 2. Total psychrotrophic aerobic bacteria of control and nisin treated samples during storage at 4°C. Different letters indicate significant difference at the same day of storage *($p<0.05$)*. ns indicates no significant difference at the same day of storage *($p\geq0.05$)* (n=3, error bars: standard deviations).

Recently, Vongsawasdi *et al.* (2012) have reported that 15 µg/ml nisin could improve microbial safety against *Staphylococcus aureus* from approximately 7log CFU/g to 5.70 and 6.43log CFU/g at 4±2°C (on day 12) and 10±2°C (on day 6) respectively in fish balls (94% fish meat, 2.8% salt, 1.9% sugar, 1% ice, and 0.3% other spice assortment). Intarapichet and Gosaarak (2008) found that coating pork meatballs (78.6% lean pork, 15% ice, 4% tapioca starch, 2% salt, and 0.4% sodium phosphate) with crude bacteriocin supernatant produced by *Lactococcus lactis* TISTR 1401 showed effective antimicrobial activity against total aerobic bacteria and *Enterobacteriaceae* during storage at 4 °C for 12 days. A mixture of lactoferrin (200 µg/g) and nisin (100 µg/g) has been recommended for inhibition of mesophilic aerobic bacteria, psychrophilic bacteria, lactic acid bacteria, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* spp. Sulfite-reducing anaerobic bacteria, and yeast and mold on Turkish-style meatballs (Tekirdağ köfte), produced from 84% ground veal, 8% toasted bread crumbs, 3% onion rind, 2% red pepper, 2% salt, 0.5% garlic clove rind, 0.4% cumin, and 0.1% ground black pepper, during 12 days of storage at 4°C (Colak *et al.*, 2008).

3.2. Color

Lightness (L*), and total color difference (ΔE) of control and nisin treated samples are presented in Table 1 and 2 respectively. All samples, including control, had no significant difference in L* during storage (*$p\geq0.05$*). Intarapichet and Gosaarak (2008) also reported no significant difference in L* of pork meatballs with and without crude bacteriocins from *Lactococcus lactis* TISTR 1401 added.

Table 1. Lightness (L*) of meatballs during storage at 4°C.

Treatment	Lightness (L*)			
	0 day of storage^{ns}	5 days of storage^{ns}	10 days of storage^{ns}	15 days of storage^{ns}
Control^{NS}	65.43 ± 0.67	66.07 ± 0.11	65.13 ± 0.65	64.67 ± 1.11
Nisin 6.25 mg/kg^{NS}	65.50 ± 0.52	65.20 ± 1.70	65.03 ± 1.37	65.07 ± 0.45
Nisin 12.5 mg/kg^{NS}	65.62 ± 0.88	65.60 ± 0.23	65.52 ± 0.31	65.44 ± 0.95
Nisin 25.0 mg/kg^{NS}	66.40 ± 0.43	65.88 ± 0.45	65.66 ± 0.38	65.55 ± 0.95

NS indicates no significant difference at different days of storage in the same treatment (*$p\geq0.05$*).
ns indicates no significant difference among different treatments at the same day of storage (*$p\geq0.05$*).

Similarly, all samples, as well as control, were not significantly different in ΔE after preparation (*$p\geq0.05$*); however, ΔE of control and samples treated with 12.5% and 25% nisin slightly increased with storage time (*$p<0.05$*) due to slight decrease and increase in a* and b* respectively during storage (data not shown). Intarapichet and Gosaarak (2008) also found that a* of pork meatballs increased during storage. Moreover, the decrease of b* with storage time in Turkish-style meatballs (lean beef, 5% toasted bread crumbs, 5% onion, 1.8% salt, 0.4% black pepper, 0.3% red pepper, and 0.3% cumin) has been recently reported by Gök

and Bor (2012). The color change in emulsified meat products can be affected by the structural proteins and meat pigments, as well as their denaturation (Grossi *et al.*, 2012). Discoloration of meat products is also influenced by the formation of metmyoglobin and lipid oxidation (Ding *et al.*, 2015).

Table 2. Color Difference (ΔE) of meatballs during storage at 4°C.

Treatment	Color Difference (ΔE)		
	5 days of storage^{ns}	10 days of storage^{ns}	15 days of storage
Control	$^{B}1.02 \pm 0.16$	$^{AB}1.19 \pm 0.48$	$^{A}2.24^{a} \pm 0.81$
Nisin 6.25 mg/kg^{NS}	1.14 ± 0.85	1.32 ± 1.11	$1.26^{ab} \pm 0.32$
Nisin 12.5 mg/kg	$^{B}0.86 \pm 0.48$	$^{B}1.13 \pm 0.29$	$^{A}1.89^{b} \pm 0.19$
Nisin 25.0 mg/kg	$^{B}0.59 \pm 0.15$	$^{AB}0.91 \pm 0.08$	$^{A}1.30^{b} \pm 0.37$

A-B indicate significant difference at different days of storage in the same treatment (*p<0.05)*.
NS indicates no significant difference at different days of storage in the same treatment (*p≥0.05)*.
a-b indicate significant difference among different treatments at the same day of storage (*p<0.05)*.
ns indicates no significant difference among different treatments at the same day of storage (*p≥0.05)*.

3.3. Texture

Firmness of control and nisin treated samples are presented in Table 3. Firmness in all samples, as well as control, was not significantly different at the same day of storage (*p≥0.05*); however, it significantly increased with storage time (*p<0.05*). The similar results were also found in the study of pork sausages (60.02% ground cured lean meat, 20.95% konjac gel, 11.76% pork fat, 3.00% corn flour, 2.00% sodium caseinate, 0.87% sugar, 0.75% pepper, 0.37% sodium tripolyphosphate, 0.12% monosodium glutamate, and 0.16% spice mix), which were significantly firmer during storage at 4-5°C for 4 weeks (*p<0.05*) (Akesowan, 2008). The increase of firmness of meat products during storage may happen due to the change of the gel/emulsion matrix, resulting in the increase of binding strength of the protein-protein and protein-water interactions (Belitz and Grosch, 1999).

3.4. Chemical compositions

Since the sample containing 25.0 mg/kg nisin showed the best results from microbiological analysis without affecting color and texture of the meatballs, it was selected for chemical analysis and sensory evaluation to compare with the control sample. Chemical compositions of control and treated sample with 25.0 mg/kg nisin during storage at 4°C are presented in Table 1. There was no significant difference between control and treated sample in every chemical composition at the first day of storage (*p≥0.05*). Similarly, Colak *et al.* (2008) reported nisin had no significant effect on moisture content and water activity (a_w) in Turkish-style meatballs (Tekirdağ Köfte) (*p≥0.05*).

Table 3. Firmness of meatballs during storage at 4°C.

Treatment	Firmness (N)			
	0 day of storage^{ns}	5 days of storage^{ns}	10 days of storage^{ns}	15 days of storage^{ns}
Control	$^{D}23.12 \pm 0.37$	$^{C}27.18 \pm 0.64$	$^{B}29.02 \pm 0.34$	$^{A}36.62 \pm 1.08$
Nisin 6.25 mg/kg	$^{D}23.64 \pm 0.61$	$^{C}27.58 \pm 0.29$	$^{B}28.54 \pm 0.45$	$^{A}35.71 \pm 0.56$
Nisin 12.5 mg/kg	$^{C}23.52 \pm 0.92$	$^{B}27.82 \pm 0.59$	$^{B}29.04 \pm 0.64$	$^{A}35.56 \pm 1.36$
Nisin 25.0 mg/kg	$^{D}22.82 \pm 0.84$	$^{C}27.19 \pm 0.64$	$^{B}28.55 \pm 0.44$	$^{A}35.26 \pm 0.64$

A-D indicate significant difference at different days of storage in the same treatment (*p<0.05*).
ns indicates no significant difference among different treatments at the same day of storage (*p≥0.05*).

Table 4. Chemical composition of control and sample treated with nisin after preparation.

Chemical Compositions (%)	Control	Sample treated with nisin 25.0 mg/kg
Moisture^{ns}	67.73 ± 0.33	67.66 ± 0.58
Protein^{ns}	22.40 ± 0.28	22.29 ± 0.21
Fat^{ns}	5.38 ± 0.30	5.41 ± 0.46
Ash^{ns}	2.87 ± 0.08	2.84 ± 0.05
Carbohydrate^{ns}	1.62 ± 0.12	1.79 ± 0.73

ns indicates no significant difference among different treatments at the same day of storage *($p \geq 0.05$)*.

3.5. Sensory evaluation

The acceptance test was conducted in order to evaluate the level of acceptability on the treated sample among untrained panelists (n=30), compared with the control sample. Scores from acceptance test are presented in Figure 3.

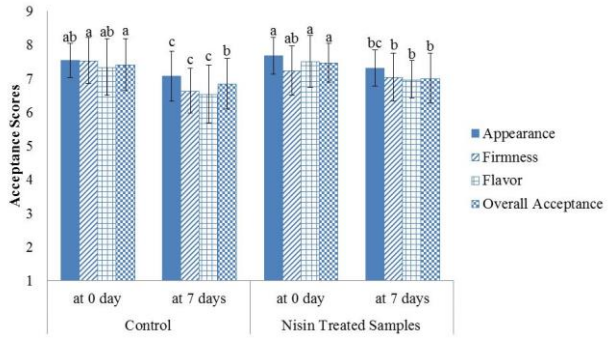

Figure 3. Acceptance scores of control and nisin treated samples (25.0 mg/kg) at 0 and 7 days of storage at 4°C. Different letters indicate significant difference at $p<0.05$ (n=30, error bars: standard deviations).

There was no significant difference between the control and the treated sample in appearance, firmness, flavor, and overall acceptance at the beginning of storage ($p \geq 0.05$). Both samples obtained scores above '7' or 'like moderately' at the beginning of storage. These results could ensure that nisin did not affect the sensory qualities of the samples. In the application of nisin in buffalo meat sausage, Sureshkumar *et al.* (2010) also found that nisin did not affect the sensory qualities of samples, including appearance, flavor, juiciness, and overall acceptability at the beginning of storage. Moreover, Colak *et al.* (2008) reported no significant difference between Turkish-style meatballs (Tekirdağ Köfte) with and without nisin added in sensory scores, including color, odor, flavor, appearance, texture, and overall acceptability, at the beginning of storage ($p \geq 0.05$). After 7 days of storage, all samples had significantly lower in sensory scores than those at the beginning of storage while the nisin treated sample had significantly higher in flavor and firmness than control ($p<0.05$).

4. Conclusions

Nisin can be safely used in pork meatballs and the recommended concentration of nisin in this meat product was 25.0 mg/kg since it efficiently reduced the changes in microbial growth (both mesophilic and psychrotrophic bacteria) without affecting color, firmness, chemical compositions, and sensory characteristics of meatballs when compared to the control sample.

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STUDIES ON PHYSICAL AND MORPHOLOGICAL CHARACTERISTICS OF GREEN GRAM *(VIGNA RADIATA)* SEED, SPROUTS AND TREATED SPROUTS

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ABSTRACT

Physical and morphological characteristics represents quality of raw material, various physical properties of green gram seeds, soaked seeds, sprout and treated (10 and 20 min soaked in essential oil) sprouts were evaluated as a function of moisture content in the range of 10.24 to 89.7% d.b. The average length, width and hundred grain mass of green gram seeds, soaked seeds, sprouts and treated sprouts were 5.85± 0.2, 4.49 ±0.4mm, 6.3±0.1g, 7.01±.01, 5.93± 0.2mm,8.2±0.1g, 9.69±.01, 6.23 ±.01mm, 11.4±0.1g and 9.69±.01, 6.23 ±.01mm, 11.4±0.1g respectively. The geometric mean diameter increased from 5.85mm to 9.69mm, bulk density increased from 0.42 to 0.43 g/ml, tap density increased from 0.48 to 0.49g/ml, car's index increased from 8.16 to 12.2%, angle of repose increased from 30.96° to 33.02° and The static coefficient of friction varied from 0.48 to 0.36 against different six material surfaces and varied from 0.48 to 0.50 in treated samples due to significant effect ($p < 0.05$) of moisture content within the studied moisture range. Attended studies showed morphological characteriscs of green gram seeds at different seeding densities like 50g, 75g and 100g/500ml water, significant difference ($p < 0.05$) observed in sprout length, hypocotyl length and hypocotyl weight over different seeding densities. Significant decrease observed in sprout length, hypocotyl length and hypocotyl weight 20.6±0.1,15.3±0.4mm, 5.02±0.01g for 50g/500ml, 15.14±0.08, 9.80±0.5mm, 3.2±0.11g for 75g/500ml, mm,10.34±0.3, 5.01±0.2mm, 1.1±0.05g for 100g/500ml after changing the densities for seeding.

1. Introduction

In India, cultivation of the green gram (*Vigna radiata*) legume is about 3.08 million hectares with an annual production of 1.31 million tonnes (Indian Government, 1997). Green gram is a important source of protein, which contains 22.51% protein content (Kochhar and Hira, 1997). Physical properties are important to design equipment and facilities for handling, processing and storage. Fraser *et al*. (1978) reported that, for fababeans, there was an increase in the hundred grain mass, angle of repose and coefficient of static friction with increase in moisture content. Various physical properties including thousand grain mass, roundness, sphericity, volume, elective diameter, bulk density, true density, porosity and coefficient of friction were of pigeon pea evaluated by Shepherd and Bhardwaj (1986). Dutta *et al*. (1988) determined various properties of chickpea including shape, hundred grain mass, sphericity, roundness, size, volume, surface area,true density, porosity, bulk density,

coefficient of static friction and angle of repose in the moisture range of 9.24 to 31.3% d.b. physical properties of chickpea and green gram in the moisture range of 7.6 to 31.5% d.b. determined by Gupta and Prakash (1990) and reported that the mean diameter and Hundred grain mass increased but the bulk density decreased with an increase in moisture content. Static and dynamic coefficients of friction, dimensions, bulk density, porosity, projected area, and terminal velocity, of lentil seeds in the moisture range of 6.3-32.1% d.b was evaluated by Carman (1996). Literature study presents very little data on the physical and morphological properties of green gram. However, detailed measurements of the principal dimensions and the variation of physical properties of green gram at various levels of moisture content have not been investigated.

The development and growth of hypocotyl takes place during germination where the hypocotyl evidently grows in length. During germination, seeds undergo pronounced physical and morphological changes. Germination procedure provides optimal conditions for the growth of seeds which is also optimal for the growth of microorganism. Incorporation of natural antibacterial agents such as essential oils of cinnamon, clove, cumin etc added with the aim of reducing the microbial contamination on seeds and sprouts is of high significance from food safety point of view. Very few data is available on the physical properties of such treated samples.

The objective of this study was to investigate some moisture dependent physical properties like dimension size, hundred grains mass, bulk density, tap density, car's index, angle of repose and coefficient of static friction as well as to investigate the effect of seeding density, during sprouting in a chamber, on morphological characteristics (Deshpande *et al*., 1993).

This is significant because the seeding density that produces sprouts with the optimum morphological and physical characteristics can be exploited for commercial production of sprouts.

2. Material and methods

2.1. Experimental design

Green gram grain was procured from local market. Initial grain moisture content was found to be 10.24% d.b. The grain was cleaned manually to remove foreign matter and broken or immature grain. Then seeds were soaked and sprouted for different analytical purpose, which showed moisture content 89.7% and 86.2% respectively for soaked and sprout. All physical properties were carried out at different moisture content.All other analysis was carried out in triplicates and the values were reported as mean±SD. Statistical analysis of the data was carried out using SPSS version 20.0.

2.2.Physical characteristics measurement

2.2.1.Determination of color

Determination of color was analysed by hunter colorimeter where samples were analysed using 6cm diameter, 3.8cm height of cells and 0.5 breadth cuvette where L* value means white(100) to black(0), a*value means red(+) to green(-)and b value means yellow (+) to blue (-) according to standard procedure (AOAC 1990).

2.2.2. Determination of seed dimensions

To determine the average size of the grain, selected kernels of green gram seeds, soaked seeds and green gram sprouts and their two dimensions were measured using vernier caliper (P. M. Nimkar *et al.,* 2001) & (Mohsenin, 1986).

2.2.3. Determination of hundred seed mass

Hundred grain mass was determined by measuring hundred kernels of green gram seeds, soaked seeds and sprouts, measuring mass of that on weighing balance as per the standard procedure. (P. M. Nimkar *et al.,*2001) & (Mohsenin, 1986).

2.2.4. Determination of effect of moisture content on grain size and hundred seed mass

Determination of effect of moisture content on hundred grain mass and grain size were carried out on the basis of moisture content and changes occurred in grain dimensions and weight. (P. M. Nimkar *et al.*,2001)

2.2.5. Determination of bulk and Tap density

The bulk density of grains based on the volume occupied by the bulk sample was measured using a standard described procedure and Tap density defines as the ratio of weight of sample to the weight of volume obtained after 100 times tapping. (P. M. Nimkar *et al.*,2001) & (Mohsenin, 1986).

2.2.6. Determination of car's index

The car's index is determined by tap density and bulk density values. (P. M. Nimkar *et al.*,2001) & (Mohsenin, 1986).

$$\frac{\text{Tap density} - \text{bulk density}}{\text{Tap density}} \times 100 \quad (1)$$

2.2.7.Determination of angle of repose

For determination of the angle of repose, apparatus consisted of plywood box of 300mm by 300mm by 300mm, a funnel with a 10cm circular disc fitted inside and discharge gate at at the bottom of the box.

After filling the box with a grain sample, the gate was quickly removed. The height of grain pile retained on the circular disc was measured and used to determine the angle of repose. (P. M. Nimkar *et al.*,2001)

2.2.8. Determination of Coefficient of static friction

The static friction coefficients against plywood, aluminium, zinc alloy, galvanized iron and stainless steel were determined using a cylinder of diameter 75 mm and depth of 50 mm filled with seeds.

With the cylinder resting on the surface, the surface was raised gradually until the filled cylinder just started to slide down (P. M. Nimkar *et al.*,2001).

2.3.Morphological parameters measurement

2.3.1. Determination of Sprout length, Hypocotyl length and Weight at different seeding densities.

Determination of seeding densities were carried out at three different weight criteria i.e. 50g/500ml, 75g/500ml, 100g/500ml (w/v) and effect of seeding densities on sprout length, hypocotyl length and weight were observed.(Ahmad *et al*., 2010)

2.4.Statistical analysis

All experiments were performed in triplicate. The data were expressed as the mean ± SD. The data were statistically analyzed by independent sample T test using SPSS programme version 20.0 to express the significance of difference ($P < 0.05$) between means

3. Results and discussions

3.1. Physical characteristics measurement

3.1.1. Determination of color

Germinated seeds were analysed for color and considered as control and essential oil treated samples of different concentration were considered as treated samples.

For analysis of green gram sprouts green tile were used which shows color value for control sample was L*(55.33), a*(-20.02) and b*(17.9).

No significant difference ($P > 0.05$) was observed in color analysis of control and treated green gram sprouts of all concentrations as shown in Table 1.

Table 1. Color analysis of control and treated samples

	10 min soaked samples			20 min soaked samples		
Sample	L*	a*	b*	L*	a*	b*
Standard value	56.64	-21.69	11.05	56.64	-21.69	11.05
Control	55.33^{A}	-20.02^{A}	17.9^{A}	55.33^{A}	-20.32^{A}	17.93^{A}
1%	54.15^{A}	-20.36^{A}	18.1^{A}	54.85^{A}	-20.16^{A}	18.41^{A}
1.8%	53.32^{A}	-20.42^{A}	17.2^{A}	53.42^{A}	-20.12^{A}	17.69^{A}
2.5%	53.19^{A}	-20.19^{A}	19.6^{A}	53.09^{A}	-20.09^{A}	19.46^{A}
3.5%	52.15^{A}	-19.15^{A}	17.2^{A}	52.15^{A}	-19.45^{A}	17.08^{A}
4%	53.69^{A}	-19.36^{A}	19.4^{A}	53.69^{A}	-19.56^{A}	19.13^{A}

*Values are means ± standard deviations. ^{A} means within the same column values do not statistically differ from each other (p> 0.05).

3.1.2. Seed dimensions

Table 2shows the mean and standard error of measurements of each dimension of green gram seed, soaked seeds and sprouts.

The result shows the significant difference (P < 0.05) in length and width (mm), due to change in moisture content level from 10.24 to 86.2% d.b.

Table 2. Seed dimensions

Samples	Moisture content (%) d.b	Major axis L (mm)	Minor axis B (mm)
Seed^{A}	10.2±0.05^{A,a}	5.85± 0.2^{A,b}	4.49±0.4^{A,c}
Soaked^{B}	89.7±0.05^{B,a}	7.01±.01^{B,b}	5.93± 0.2^{B,c}
Sprout^{C}	86.2±0.05^{C,a}	9.69±.01^{C,b}	6.23 ±.01^{C,c}

*Values are means ± standard deviations. $^{A\text{-}C,a\text{-}c}$ means within the same column (upper case) and row (lower case) labelled with the same letter do not statistically differ from each other (p> 0.05).

3.1.3. Determination of hundred seed mass and effect of moisture content on it and grain size

There is significant difference (P < 0.05) observed in hundred seed mass due to increase in moisture content. Green gram has a relatively small grain size, compared with other commonly grown pulse crops; for example at moisture content of 10.24% d.b., the hundred seed mass for green gram seed, soaked, sprout and hypocotyl weight is 6.3±0.1, 8.2±0.1, 11.4±0.1 and 1.16±0.05 respectively shown in Table 3and effect of moisture content on hundred seed mass and grain size shown in Fig 1.

3.1.4. Determination of bulk, tap density and car's index

The bulk density and tap density of green gram sprout is 0.43 and 0.49g/ml respectively. There is no significant differences (P > 0.05) observed in bulk and tap density of the control samples and treated samples. Car's index was determined by using mean values of bulk density and tap density was 12.24% of control sample and there is no significant difference (P > 0.05) obtained in values of control and treated samples as shown in Table 3.

Table 3.Determination of hundred seed mass, bulk, tap density, car's index and angle of repose

Sample	Hundred seed mass (g)	Bulk density (g/ml)	Tap density (g/ml)	Car's index (%)	Angle of repose (°)
Seed ^{A}	6.3±0.1 ^{A}	0.42±0.005 ^{A}	0.48±0.005 ^{A}	8.16±0.005 ^{A}	30.96±0.005 ^{A}
Soaked ^{B}	8.2±0.1 ^{B}	0.42±0.005 ^{B}	0.48±0.005 ^{B}	6.25±0.005 ^{B}	30.96±0.005 ^{B}
Sprout ^{C} (control)	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.2±0.05 ^{C}	33.02±0.005 ^{C}
10 min soaking					
1%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	34.03±0.005 ^{C}
1.8%	11.4±0.1	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	34.03±0.005 ^{C}
2.5%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	34.03±0.005 ^{C}
3.5%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	34.03±0.005 ^{C}
4%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	34.03±0.005 ^{C}
20 min soaking					
1%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	35.01±0.005 ^{C}
1.8%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	35.01±0.005 ^{C}
2.5%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	35.01±0.005 ^{C}
3.5%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	35.01±0.005 ^{C}
4%	11.4±0.1 ^{C}	0.43±0.005 ^{C}	0.49±0.005 ^{C}	12.24±0.05 ^{C}	35.01±0.005 ^{C}

*Values are means ± standard deviations. $^{A, B, C}$ means within the same column labelled with the same letter do not statistically differ from each other (p> 0.05).

Table 4.Coefficient of static friction

Coefficient of static friction, μ					
Samples	Plywood	Zinc alloy	Galvanized iron	Aluminium	Stainless steel
Control	0.48±0.01^{A,a}	0.45±0.005^{A,b}	0.42±0.008^{A,c}	0.38±0.007^{A,d}	0.34±0.007^{A,e}
10 min soaking treatment					
1%	0.50±0.005^{B,a}	0.48±0.005^{B,b}	0.44±0.005^{B,c}	0.41±0.016^{B,d}	0.36±0.005^{B,e}
1.8%	0.50±0.005 ^{B,a}	0.48±0.005 ^{B,b}	0.44±0.005 ^{B,c}	0.41±0.016 ^{B,d}	0.36±0.005 ^{B,e}
2.5%	0.50±0.005 ^{B,a}	0.48±0.005 ^{B,b}	0.44±0.005 ^{B,c}	0.41±0.016 ^{B,d}	0.36±0.005 ^{B,e}
3.5%	0.50±0.005 ^{B,a}	0.48±0.005 ^{B,b}	0.44±0.005 ^{B,c}	0.41±0.016 ^{B,d}	0.36±0.005 ^{B,e}
4%	0.50±0.005 ^{B,a}	0.48±0.005 ^{B,b}	0.44±0.005 ^{B,c}	0.41±0.016 ^{B,d}	0.36±0.005 ^{B,e}
20 min soaking treatment					
1%	0.53±0.007^{C,a}	0.52±0.005^{C,b}	0.48±0.007^{C,c}	0.43±0.005^{C,d}	0.38±0.006^{C,e}
1.8%	0.50±0.005 ^{C,a}	0.52±0.005 ^{C,b}	0.48±0.007 ^{C,c}	0.43±0.005 ^{C,d}	0.38±0.006 ^{C,e}
2.5%	0.50±0.005 ^{C,a}	0.52±0.005 ^{C,b}	0.48±0.007 ^{C,c}	0.43±0.005 ^{C,d}	0.38±0.006 ^{C,e}
3.5%	0.50±0.005 ^{C,a}	0.52±0.005 ^{C,b}	0.48±0.007 ^{C,c}	0.43±0.005 ^{C,d}	0.38±0.006 ^{C,e}
4%	0.50±0.005 ^{C,a}	0.52±0.005 ^{C,b}	0.48±0.007 ^{C,c}	0.43±0.005 ^{C,d}	0.38±0.006 ^{C,e}

*Values are means ± standard deviations. $^{A,C,a-e}$ means within the same column (upper case) and row (lower case) labelled with the same letter do not statistically differ from each other (p> 0.05).

3.1.5. Determination of angle of repose and coefficient of static friction

The experimental results for the angle of repose with respect to moisture content are shown in Table 3, the values were found toincrease from 33.02° to 35.01° from control to treated samples. Significant difference was observed ($P < 0.05$) due to soaking treatment which increases moisture content of samples. Coefficients of static friction for green gram, determined with respect to five different structural surfaces, are listed in Table 4.It is observed that the coefficient of static friction was greatest against plywood (0.48±0.01) followed by zinc alloy (0.45±0.005), galvanized iron (0.42±0.008), aluminium (0.38±0.007) and the least for stainless sheet (0.34±0.007) of control samples. It was observed that moisture had more effect than the material surface on the coefficient of static friction. This is owing to the increased adhesion between the grain and the material surface at highermoisture values. The significant difference ($P < 0.05$) observed in control and treated samples.

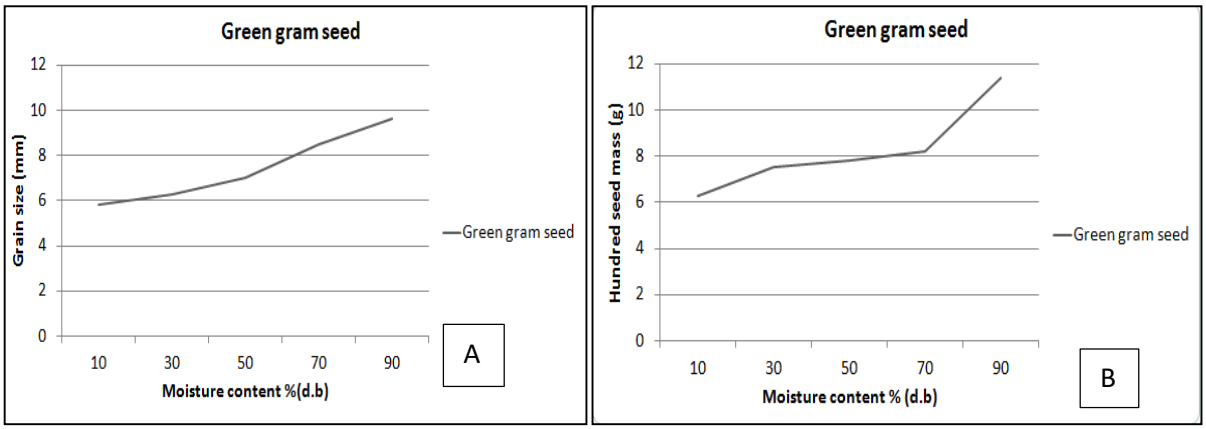

Figure 1. Effect of moisture content on grain size and hundred seed mass A-Grain size; B-Hundred seed mass

3.2. Morphological parameters measurement

Based on the data gathered in the experiment, there was a significant ($P < 0.05$) decrease in the length of sprouts as seeding density was increased (Fig 2). Initially, the sprout length showed linear decrease when seeding densities were increased from 50g/500ml to 100g/500ml. There were also significant decreases of hypocotyl length as shown in the Table 5.This decrease caused due to insufficient water for the growth of sprouts as it becomes dense after increasing the weight of seeds.

Table 5. Morphological parameters measurement

Seeding densities	Sprout length (mm)	Hypocotyl length (mm)	Hypocotyl weight (g)
$50g/500ml^{A}$	$20.6±0.1^{A,a}$	$15.3±0.4^{A,b}$	$5.02±0.01^{A,c}$
$75g/500ml^{B}$	$15.14±0.08^{B,a}$	$9.80±0.5^{B,b}$	$7.33±0.15^{B,c}$
$100g/500ml^{C}$	$10.34±0.3^{C,a}$	$5.01±0.2^{C,b}$	$10.23±0.11^{C,c}$

*Values are means ± standard deviations. A-C,a-c means within the same column (upper case) and row (lower case) labelled with the same letter do not statistically differ from each other ($p> 0.05$).

A- 50g/500ml B- 75g/500ml C- 100g/500ml

Figure 2. Morphological parameters (Different seeding densities and its effect on sprout length)

4. Conclusions

As shown in this study, moisture content had significant effect on physical properties, as moisture content increased length, width of seed increases, even moisture having significant effect on hundred seed mass and coefficient of static friction, as it increases these parameters also increases, and different seeding densities also shows significant difference in morphological characteristics such as sprout length, hypocotyl length, hypocotyl weight.

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OPTIMIZATION OF EXTRACTION OF POLYSACCHARIDES FROM WHITE HYACINTH BEAN USING PLACKETT-BURMAN AND STEEPEST ASCENT EXPERIMENT

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ABSTRACT
The effects of cellulase, pectinase and xylanase on the polysaccharide yield were studied by single-factor tests. Then the Plackett-Burman and steepest ascent experiment were conducted according to the results of single-factor tests. Finally, the optimum conditions of extracting polysaccharide from white hyacinth bean were obtained so as to conduct the further experiment. The results showed that pH (p=0.0599), cellulose (p=0.0756) and ratio of water to material (p=0.0951) are important factors of extracting polysaccharides. And the factors that has positive effect on the polysaccharides yield are: cellulose, pectinase, xylanase, ratio of water to material, extraction time and pH. While the extraction temperature has negative influence to polysaccharides yield. The optimum conditions of polysaccharides extraction were: pH was 7.8, the addition amount of cellulose was 2.7% and the ratio of water to material was 62. The polysaccharides yield was the 3.23% under these conditions.

1.Introduction

Oxidation is a very important biological process in living organisms for the energy production. However, the excessive reactive oxygen species (ROS) such as hydrogen radical, and hydroxyl free radical would be produced if the balance between prooxidant conditions and the antioxidant defense was broken.(Kenji et al., 2001). There are much evidence showed that the excessive free radicals would cause some damage to human such as aging, cancer, inflammation, liver injury, heart disease and skin damage (Enrique and Kelvin, 2000; Abe, 1998; Jorge and Bruce, 1995; Meerson et al., 1982). It has been suggested that the polysaccharides extracted from several plants showed excellent antioxidant activities with nontoxic property (Kardosova and Machová, 2006; Qiuhong et al., 2014; Ge *et al*., 2014). White hyacinth bean is a healthy plant seeds, presently, it is widely cultivated throughout the tropics and subtropics. In many areas, which produce hyacinth beans, the young pods are boiled as vegetable while the dry seeds are cooked with rice (Achmad 2006). Polysaccharide is an important constituent of white hyacinth bean, which may contribute to the antioxidant activity. However, there is less literature about the polysaccharide from white hyacinth bean. The Plackett–Burman method offers a design where n variables are studied in n+1 experimental runs. These experimental designs are excellent screening methods, because the required number of experimental runs are very few, which could save time, chemicals, glassware and manpower (Carvalho, *et al.*, 1997; Kumar *et al.*, 2013; Srinivas *et al.*, 1994; Usha *et al.*, 2011). In addition, the design is orthogonal in nature, implying that the effect

of each variable worked out is pure in nature and not confounded with interaction among variables. Experimental design and data analysis using appropriate software makes the analysis easier (Naveena, *et al.*, 2005).

In our present study, the effects of cellulase, pectinase and xylanase on the polysaccharide yield were studied by single-factor tests. Then the Plackett-Burman and steepest ascent experiment were conducted according to the results of single-factor tests. Finally, the optimum conditions of extracting polysaccharide from white hyacinth bean were obtained so as to conduct the further experiment.

2. Materials and methods

2.1. Materials

The white hyacinth beans were purchased from Xi'an, Shannxi Province, China. The samples were cleaned and dried before extraction. Cellulose, pectinase and xylanase were obtained from ruiyang-biotech (Jiangsu, China). All other chemicals were of analytical grade.

2.2. Extraction of polysaccharides from white hyacinth bean

The polysaccharides were obtained with the water extraction and alcohol precipitation method added different enzymes. The samples were pulverized to powder and passed through a 60 mesh sieve before extraction. The dried powered was extracted by hot water (60℃-95℃) in water bath. The enzyme was added at the fifth minutes of insulation. After extraction, the extra enzyme was killed at high temperature and the suspension mixture was separated by centrifugation. The supernatant was concentrated and ethanol was added to precipitate the polysaccharide. Then the solution was filtered by filter paper, and then added 50 fold of water to dissolve and centrifuged, Finally, diluted the supernatant at appropriate multiple and determined the polysaccharide yield at OD490nm.

2.3. Calculation of polysaccharides yield

The content of polysaccharides in the extract was determined by phenol-sulfuric acid method. The yield of polysaccharides in white hyacinth bean was calculated by the following equations:

The content of polysaccharides:

$$C=(A-0.1147)/0.0102 \quad (1)$$

Where C was the polysaccharides content in 1ml supernatants and A was the absorbance of polysaccharides.

The total content of polysaccharides:

$$D=C\times N\times 50 \quad (2)$$

Where D was the total content of polysaccharides N was the dilution multiple and 50 means the polysaccharides were dissolved in 50 fold of water.

The yield of polysaccharides:

$$Y=D\times 10^6/1\times 100\% \quad (3)$$

Where Y was the yield of polysaccharides and 1 was the 1g polysaccharide.

3. Results and discussions

3.1. Standard curve of glucose

Different volumes of 0.2mg/ml glucose (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7ml) were added to colorimetric tube and added water to 2ml. Then 1ml 5% phenol and 5ml concentrated sulfuric acid were added to the mixture. After shaking vigorously and standing for 30 minutes, the absorbance was measured at 595 nm. Water was used as a reference material. The standard curve and regression equation were obtained according to the absorption value and concentration of glucose. The result was shown in Fig.1.As is shown in Fig.1, the regression equation was y=0.0102x+0.1147, R2=0.9958, which indicated that there was a good linear relationship between the absorbance and concentration of glucose.

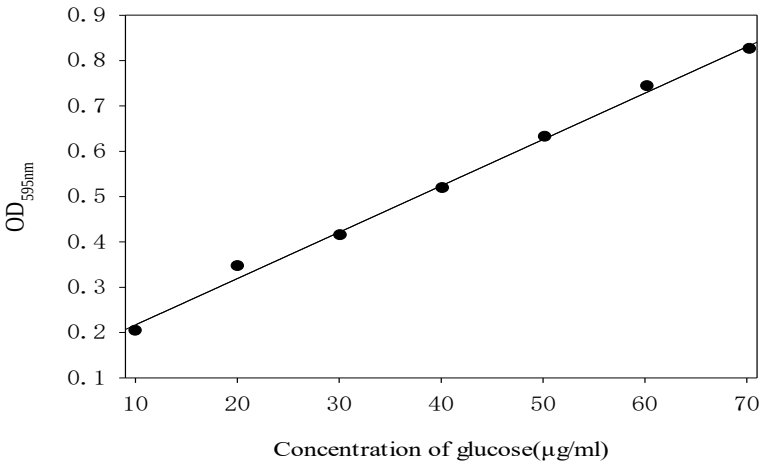

Figure 1. The standard curve of glucose

3.2. Effects of the ratio of water/material, addition amount of three enzymes, temperature, pH and time on the polysaccharide yield

The effects of several important factors (the ratio of water to material, addition amount of enzymes, temperature, pH and time) on the polysaccharides yield of white hyacinth bean were studied by single-factor tests. The three different enzymes (cellulase, pectinase and xylanase) were added to improve the polysaccharides yield. The ratio of water to material was from 40 to 60, the range of addition amount of three enzymes was 1%-3%, the pH was from 3 to 7, the temperature was 30℃-70℃ and the extraction time was chosen from 1h to 3h. The polysaccharides yield of white hyacinth bean under different conditions was measured. The results were shown in Fig.2-Fig.6.

Fig. 2 showed that when added cellulase, the polysaccharides yield increased as the increasing of the ratio of water to material. Specifically, the yield of polysaccharides increased gradually from 1.3% to 2.1%. For the pectinase, we can see easily that the
Polysaccharides yield also increased as the increasing of the ratio of water when the ratio of water to material was at the range of 40-55. While the yield of polysaccharides decreased rapidly after 55. As for xylanase, the polysaccharides yield increased as the increasing of the ratio of water to material when the ratio of water was at the range of 40-50. While after peaking at 50, the yield of polysaccharides was decreased rapidly.

From Fig. 3, we can see clearly that the polysaccharides yield increased as the increasing of the addition amount of cellulase at the initial stage, then the yield of polysaccharides decreased when the addition amount exceed 2%. As for PM, the polysaccharides yield had a similar trend with CM. The polysaccharides yield presented an uptrend when the addition amount of pectinase increased, then decreased rapidly when the addition amount exceeded 2%. Also, Fig. 3 presented that the polysaccharides yield increased as the increasing of the addition amount of xylanase, while the yield of polysaccharides began to decrease when the addition amount exceed 2.5%.

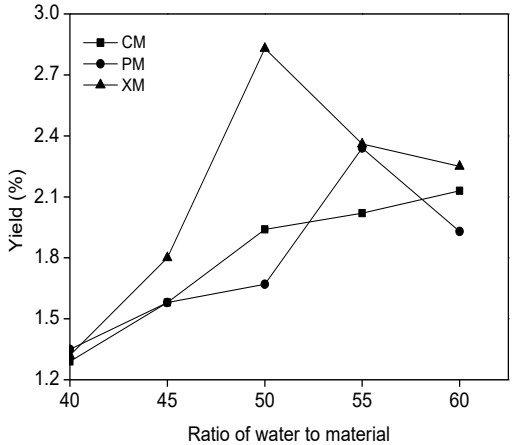

Figure 2. Effect of the ratio of water/material on the polysaccharides yield with cellulase(CM), pectinase(PM) and xylanase(XM)

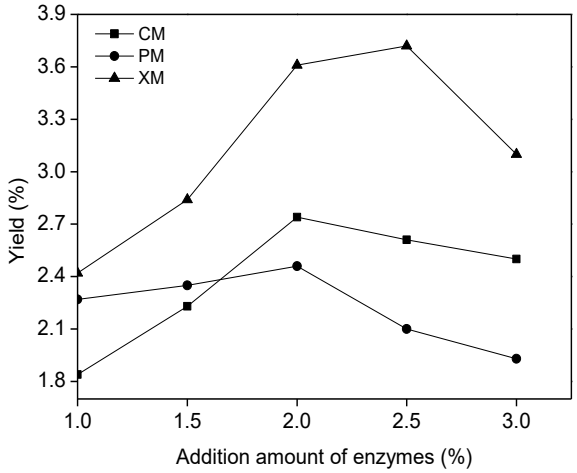

Figure 3. Effect of addition of cellulase(CM), pectinase(PM) and xylanase(XM) on the polysaccharides yield

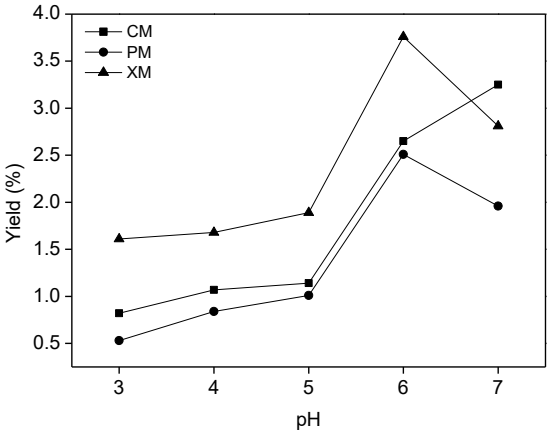

Figure 4. Effect of pH on the polysaccharides yield with cellulase(CM), pectinase(PM) and xylanase(XM)

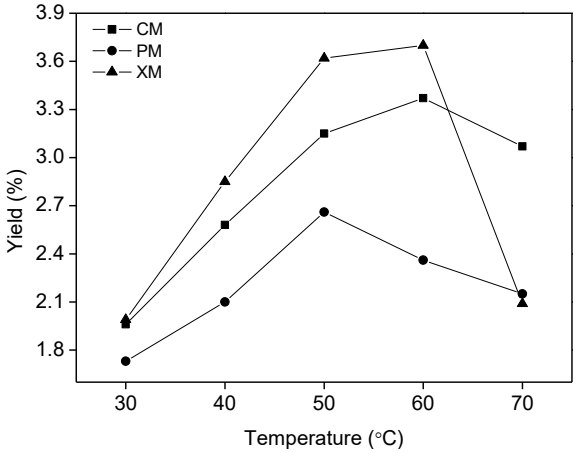

Figure 5. Effect of temperature on the polysaccharides yield with cellulase(CM), pectinase(PM) and xylanase(XM)

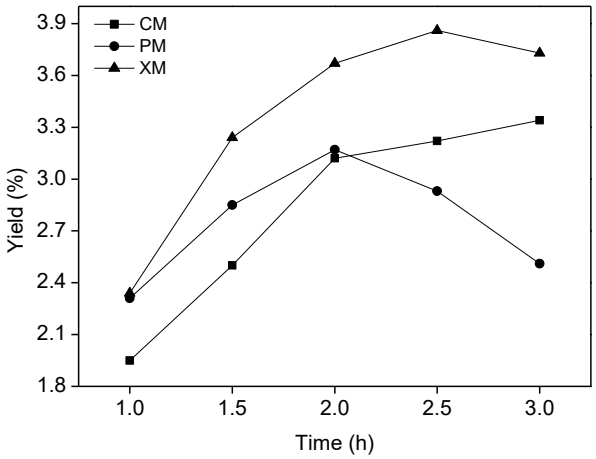

Figure 6. Effect of time on the polysaccharides yield with cellulase(CM), pectinase(PM) and xylanase(XM)

Fig. 5 showed that the optimum temperature to extract polysaccharides with cellulase was 60℃. The polysaccharides yield increased as the increasing of temperature before 60℃, while the polysaccharides yield decreased after peaking at 60℃. The speed of chemical reaction accelerate as the increment of temperature, however the enzyme is actually protein and it would denature at the high temperature. For the pectinase, the polysaccharides yield showed an uptrend when the temperature increased before 50℃, While the polysaccharides yield decreased after peaking at 50℃. As is shown in Fig. 5, 50℃was the optimum temperature to extract polysaccharides with xylanase. The polysaccharides yield increased as the increasing of temperature before 50℃. Then tended to be stable at the range of 50℃-60℃, while the yield of polysaccharides decreased after 60℃.

As is shown in Fig. 6, the polysaccharides yield (CM) increased rapidly as the increasing of extraction time at the initial stage. But the increasing of polysaccharides yield was not noticeable any more after 2h, which is because that the reaction efficiency reached the maximum at 2h. For the PM, the polysaccharides yield increased when the extraction time increased at the initial stage, while the increasing of polysaccharides yield was not noticeable after 2h. From Fig. 6, it can

be also suggested that the polysaccharides yield (XM) increased as the increasing of time before 2.5h and then tended to be stable.

3.3.Screening of the main factors using Plackett-Burman design

Plackett-Burman design (N=12) was conducted to choose the main influence factors according to the results of single-factor test. Seven factors are cellulase (X1), pectinase (X2), xylanase (X3), ratio of water to material (X4), pH value (X5)e, extraction temperature (X6) and extraction time (X7). The response value was the polysaccharides yield. Each factor choose two levels (high and low), the optimum extraction condition of single-factor test was regarded as the low level, and 1.2 times low level was conducted as high level. The coded values of each factor were shown in table 1, the experimental design and results were presented in table 2. The analysis of results was shown in table 3.

Table 1. The factors level coding table of Plackett-Burman

Level	Factors						
	Cellulase (%)	Pectinase (%)	Xylanase (%)	Ratio of water	pH	Extraction temperature /℃	Extraction time /h
-1	2.0	2.0	2.0	50	6.0	50	2.4
1	2.5	2.5	2.5	60	7.5	60	3.0

Table 2. The experimental design and results of Plackett-Burman

Run	Model	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Polysaccharides yield /%
1	−−+−+++	-1	-1	1	-1	1	1	1	1.83
2	+−+++−−	1	-1	1	1	1	-1	-1	3.39
3	−+−+++−	-1	1	-1	1	1	1	-1	2.83
4	−+−−+−+	-1	1	-1	-1	1	-1	1	2.12
5	−−−+−−+	-1	-1	-1	1	-1	-1	1	2.6
6	−+++−−−	-1	1	1	1	-1	-1	-1	1.89
7	+−−+−++	1	-1	-1	1	-1	1	1	2.31
8	−−+−−+−	-1	-1	1	-1	-1	1	-1	1.42
9	++−−−+−	1	1	-1	-1	-1	1	-1	1.61
10	+−−−+−−	1	-1	-1	-1	1	-1	-1	3.28
11	+++++++	1	1	1	1	1	1	1	4.27
12	+++−−−+	1	1	1	-1	-1	-1	1	2.64

Table 3. Result of ANOVA of variables for polysaccharides yield (Y1)

Source	Estimation value	Error	t-value	P>|t|
pH (6-7.5)	0.328	0.126	2.6	0.060
Cellulase (2, 2.5)	0.100	0.042	2.38	0.076
Ratio of water (50, 60)	1.829	0.840	2.18	0.095
Temperature (50, 60)	-0.688	0.840	-0.82	0.459
Time (2.4, 3)	0.034	0.050	0.67	0.540
Xylanase (2, 2.5)	0.014	0.042	0.34	0.750
Pectinase (2, 2.5)	0.011	0.042	0.26	0.806

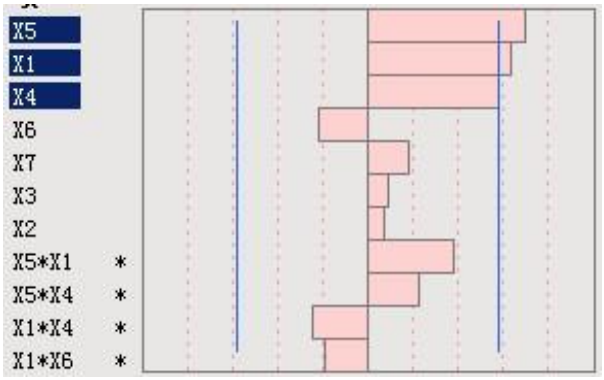

Figure 7. Screening results of the main factor of Plackett-Burman design

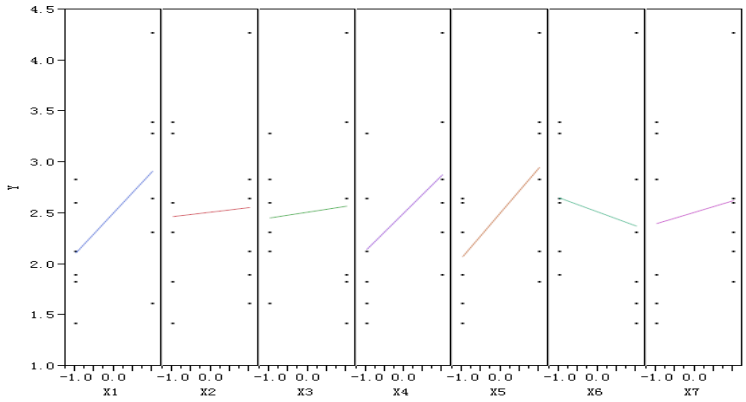

Figure 8. 95% CI interval graph of factor

The effect of each factor on the polysaccharides yield was presented by p value. The smaller p value, the more noticeable influence to the polysaccharides yield. As is shown in table 3, the results of each factor to the polysaccharides yield were: pH (X5) > cellulase (X1) > ratio of water to material (X4)> extraction temperature (X6) > extraction time (X7)> xylanase (X3) > pectinase (X2). In the statistics, the significant factors are those reliability exceed 95% ($0.01<p<0.05$), and the important factors are those reliability exceed 90% ($0.05<p<0.1$). From table 3, pH (X5, p=0.0599), cellulose (X1, p=0.0756) and ratio of water to material (X4, p=0.0951) are important factors of extracting cellulose (X1), pectinase

(X2), xylanase (X3), ratio of water to material (X4), extraction time (X7) and pH (X5). While the extraction
temperature (X6) has negative influence to polysaccharides yield.polysaccharides. Furthermore, fig. 7 and fig. 8 showed that the factors that have positive effect on the polysaccharides yield are:

3.4. Steepest ascent experiment of extraction of the polysaccharides

The steepest ascent experiment (pH, cellulose and ratio of water to material) was conducted according to the result of P-B experiment. Three factors all have positive effect to the polysaccharides yield and should be increased. The step sizes were designed according to the effect value of three factors. The extraction temperature was 50℃ and the extraction time was 3h. the experimental design and results were presented in table 4.

Table 4. Experimental design and results of steepest ascent

Run	pH	Cellulase (%)	Ratio of water	Temperature /℃	Time /h	Polysaccharides yield /%
1	7.5	2.5	60	50	3	3.12
2	7.8	2.7	62	50	3	3.23
3	8.1	2.9	64	50	3	3.00
4	8.4	3.1	66	50	3	2.76
5	8.7	3.3	68	50	3	2.57

As is shown in table 4, the polysaccharides yield reached the highest at the second experiment. Hence, the optimum conditions of polysaccharides extraction were: pH was 7.8, the addition amount of cellulose was 2.7% and the ratio of water to material was 62. The polysaccharides yield was the 3.23% under these conditions.

Yu (Yu, *et al.*, 1998) studied the effect of extraction time, temperature, and pH on ganoderma polysaccharides yield. And showed that the best extraction time was 4h, the polysaccharides had a better hydrolysis when the temperature was during 37℃-40℃, and the best range of pH was 4.2-4.6. Gao (2014) used renninum and cellulase to extract polysaccharides from Lilium davidii var. unicolor Salisb. And found that the optimum conditions were: the addition amount of renninum cellulase were 1.5% and 2%, extraction time was 20mins, temperature was 55 ℃. And the polysaccharides yield reached 28.751% under these conditions. Chen (Chen, *et al.*, 2012) extracted the polysaccharides from garlic with combined-enzyme method. And gained the optimum conditions extraction: the extraction temperature was 55℃, extraction time was 90mins, pH was 4, and the ratio of water to material was 1:30. The polysaccharides yield was highest under these conditions.

3.5. Discussion

The effects of cellulase, pectinase and xylanase on the extraction of polysaccharides from white hyacinth bean were analyzed by measuring the variation of polysaccharides yield. The polysaccharides yield would change as the variation of the amount of enzymes. This is because that the enzymatic hydrolysis increased as the increasing of the enzyme amount. And because cellulase could hydrolyze cellulose so the cell wall was cracked and polysaxcharide was vastly discharged due to the increasing amount of enzyme. However, the polysaccharides yield would decrease after the maximum, which may because that the white hyacinth bean would not make full use too many amount of enzyme, which would cause the waste of extra enzyme and the decreasing of polysaccharides yield (Gao, 2014; Will, 2000; Han, 2003; Shi Q, 2006). In addition, the ratio of water could affect the polysaccharides yield.

Which is because that the binding of extraction solvent and effective components is physical process. When the amount of extraction solvent increased, the contact of extraction solvent and effective components also increased. Hence, the polysaccharides yield would have a rising trend. However, high ratio of water to material caused the decreasing of enzyme concentration per unit, the speed of enzymatic reaction decreased and the polysaccharides yield would also decreased (Chen, *et al.*, 2012). pH and temperature had similar influence on the polysaccharides yield, the polysaccharides yield increased as the increment of pH and temperature. After the maximum, the result would decrease. Which is may because that the enzyme would denatured due to the high pH or temperature (Yu, 1998; Chen, 2012). In addition, the polysaccharides yield would increase as the increment of the time at the beginning. This is because the enzymatic hydrolysis accelerated as the increasing of extraction time, hence, the polysaccharides yield also increased. But the increasing of polysaccharides yield was not noticeable any more after the maximum, which is because that the reaction efficiency reached the peak at that time.

Chen (Chen, *et al*, 2015) optimized the sugar alcohol and proteins for Lactobacillus bulgaricus LB6 during the process of freeze drying using a Plackett-Burman design and founded the optimum conditions for freeze drying of Lactobacillus bulgaricus. Chen (Chen, *et al.*, 2014) Optimized the immobilization of β-galactosidase using Plackett-Burman design and steepest ascent method. And the immobilized enzyme activity increased about 37.60% than the previous single factor experiment. Chen (Chen, *et al.*, 2014) screened the emulsification conditions on microcapsulation of B. bifidum BB01 and BB28 by Plackett-Burman design. which indicated that Plackett-Burman design is indeed a good experiment method for optimization. Jiang (Jiang, *et al.*, 2015) optimized the processing of polysaccharide extraction from Spirodela polyrrhiza using Plackett-Burman design and Box-Behnken response surface methodology. And found that these two methods are effective and stable.

4. Conclusions

Single-factor test and Plackett-Burman design were conducted to analyze the effect of three different enzymes on the extraction of polysaccharides. The results showed that pH, cellulose and ratio of water to material are important factors of extracting polysaccharides. While the extraction temperature has negative influence to polysaccharides yield. The optimum conditions of polysaccharides extraction were: pH was 7.8, the addition amount of cellulose was 2.7% and the ratio of water to material was 62. The polysaccharides yield was 3.23% under these conditions.

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PHYSICAL, MECHANICAL, THERMAL AND ANTIMICROBIAL PROPERTIES OF ACTIVE EDIBLE FILM MADE FROM CRESS SEED GUM INCORPORATED WITH *ZATARIA* ESSENTIAL OIL

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ABSTRACT
An active edible film based on cress seed gum (CSG) and different concentrations of *Zataria multiflora* essential oil (ZEO) was developed, and the film's barrier, physical, thermal, microstructural and antimicrobial properties were investigated. Water vapor permeability was found to be in range 0.95-3.21×10^{-10} g m^{-1} s^{-1} Pa^{-1}. As expected, the increase in ZEO concentration increased percent of elongation at break *(EB%)* the extensibility, but decreased tensile strength (TS) and water solubility (WS). *S. aureus* was found to be the most sensitive bacterium to ZEO, followed by *B. cereus* and E. coli. Scanning electron microscopy (SEM) was carried out to explain structure–property relationships. A reduction of the glass transition temperature (Tg), as determined by differential scanning calorimetry (DSC), was caused by addition of ZEO into the CSG-films.

1.Introduction

Interest in antimicrobial edible films has risen recently due to increased consumption of fresh-cut produce. In order to control undesirable microorganisms on foods, antimicrobial substances can be incorporated in edible films (Labuza and Breene, 1989; Chen et al., 1996; Padget et al., 2000; Chung et al., 2001; Jouki et al., 2014a; Jouki et al., 2014b). *Zataria multiflora* Boiss is a member of Labiatae family that grows in some area of Iran such as central and southern parts and has various therapeutic effects such as antiseptic, antispasmodic, antiviral, antibacterial and antifungal properties (Misaghi and Basti, 2007). Thymol and carvacrol, are the main constituents of this essential oil (Misaghi and Basti, 2007; Ali et al., 2000). Gum and polysaccharides are hydrophilic biopolymers with high molecular weight that are soluble in water.

Because gum is polymeric in nature, its high molecular weight and interaction
between chains increases viscosity, which makes them suitable for viscous solutions used to form edible film (Cha and Chinnon 2004; Mikkonen et al., 2007; Khazaei et al., 2014). *Lepidium sativum* is an annual herb, belonging to the Cruciferae family, which grows widely in the Middle East, Europe and USA. It is commonly named as Garden Cress, Pepper Grass, Garden Pepper Cress, Water Cress, and Pepper Wort (Duke et al., 2002). The potential of cress seed gum (CSG) as antibacterial, anti-asthmatic, diuretic, aphrodisiac, and abortifacient has been reported in some publications (Duke et al., 2002; Sahraiyan et al., 2013). Recently we investigated the possibility of producing the edible film from CSG with glycerol as plasticizer in the different

concentrations, and reported the physical, mechanical, barrier, thermal and microstructure properties of CSG-films (Jouki et al., 2013a).

The characteristics of biopolymer-based films are determined mainly by chemical nature of the biopolymer. The development of edible films based on biopolymers has attracted attention mainly due to their friendliness to the environment and their potential substitution for some petrochemicals in the food packaging industry (Zhong, and xia, 2008). The aim of this study was characterization of CSG-based edible films containing different concentrations of *zataria multiflora* boiss essential oil (ZEO).

2. Materials and methods

2.1. Materials

Magnesium nitrate, sodium chloride, Tween 80 and glycerol (Gly) (98% reagent grade) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cress seeds used in this study were purchased from a local supermarket (Tehran, Iran). Brain Heart Infusion (BHI) (broth and agar) and MRS Broth were bought from Merck Co (Darmstadt, Germany). *Zataria multiflora* boiss essential oil supplied by Yas-e-sepidevash Company, (Tehran, Iran)

2.2. Bacterial strains

Staphylococcus aureus (ATCC 25923), *Bacillus cereus* (PTCC 1154), *Escherichia coli* (ATCC 25922), *Lactobacillus plantarum* (ATCC 8014) were provided by Iranian Research Organization for Science and Technology (Tehran, Iran).

2.3. Preparation of active films

Film solution was prepared according to the method described by Jouki et al. (2013a). Briefly, it was prepared by slowly dissolving 1% gum and glycerol as a plasticizer in 35% (w/w) based on CSG weight were prepared under constant stirring (750 rpm) at 45 ±2 °C for15 min. Tween 80 at level of 0.2% (v/v) of EO was added in film forming solutions to assist essentials oil dissolution, and then ZEO were incorporated into the film solutions at various concentrations of 0% (as control), 1, 2, 3 and 4% (v/v) of film solutions. The solution was homogenized (IKA T25- Digital Ultra Turrax, Staufen, Germany) at 12,000 rpm for 5 min to obtain an emulsion. Finally, the film solutions were casted onto the sterile plastic petri dishes and dried at room temperature (25±2 ◦C) and room relative humidity (37±2%) for 24 h. The obtained films were peeled from plates and conditioned at 25±2 °C in desiccators containing saturated solutions of $Ca(NO_3)_2$, $6H_2O$ (50±2% relative humidity, RH) for at least 48 h prior to tests.

2.4. Characterization of active films

2.4.1. Scanning electron microscopy (SEM).

Microstructure of the cross-sections of the film was examined by scanning electron microscopy (EM-3200, KYKY, China). Film specimens were fractured by immersion in liquid nitrogen and mounted on copper stubs perpendicularly to their surface. Samples were gold coated and observed, using an accelerating voltage of 27 KV.

2.4.2. Differential scanning calorimetry (DSC)

The thermal properties of films were estimated according to the method described by Jouki et al. (2013a) by differential scanning calorimetry (DSC) (Model F3 200 DSC Netzsch, Germany). Samples were scanned at a heating rate of 10 °C/min between temperature ranges of −100 and 200°C. Tg was identified as the midpoint temperature of the shift in the baseline due to the change in heat capacity upon glass transition.

2.4.3. Film Thickness

Film thickness was determined using a digital micrometer (Mitutoyo 689037, Detroit, MI) for six randomly chosen portions of film. The average values for these six portions were used to identify the other physical and mechanical properties of the film.

2.4.4. Water solubility

The solubility in water of the different films was measured from immersion assays under constant agitation in of distilled water for 6 h

according to method of Jouki et al. (2013b). The tests were conducted in triplicate.

2.4.5. Mechanical properties

Mechanical properties (tensile strength (TS), percentage of elongation at break (EB) and elastic modulus (EM)) were measured according to the American Society for Testing and Material method D882-91(ASTM, 1996) using a Testometric Machine M350-10CT (Testometric Co. Ltd., Rochdale, Lancashire, England). Five film specimens (100±25 mm) of each group were clamped between tensile grips and Tensile strength and elongation were recorded during extension at 30 mm/min, with an initial distance between the grips of 50 mm.

2.4.6. Water vapour permeability

A modified ASTM standard method E96-95 (ASTM, 1995) expressed by Jouki et al. (2013a) was used to measure water vapor permeability (WVP) of the samples. Initially, waterless sodium chloride was poured into a glass container (with an opening area of 0.00263 m^2) and the surface was covered with unblemished and sealed using parafilm and melted paraffin. To keep the gradient RH of75% moving through the film, calcium chloride (0% RH) was used inside the glass containers and a saturated solution of sodium chloride (75% RH) in the desiccator. The changes in the weight of the container were measured over time using a balance, sensitive to ±0.1 (mg); graph containing the weight changes versus time was prepared and a linear regression was fitted to data (R^2= 0.999). Eqs. (1) and (2) were used to evaluate and calculate the water vapor transmission rate (WVTR) and WVP of films:

$$WVTR = \frac{\Delta m}{A.\ \Delta t} \quad (1)$$

$$WVP = \frac{WVTR.\ X}{\Delta p} \quad (2)$$

where $\Delta m/\Delta t$ is the weight of moisture gain per unit of time (g/s), A is an area of the exposed film surface (m^2), X is the film thickness (mm), and Δp is the difference of partial pressure (Pa).

2.4.7. Antimicrobial activity of the films

Antibacterial activity testing was carried out using agar diffusion method. Disks (6 mm diameter) cut from the films were placed on BHI agar (Merck, Germany) plates, previously surface spreaded with the inoculum containing indicator microorganisms in the range of 10^8 CFU/mL. The plates were then incubated at 37 ◦C for 24 h (Seydim and Sarikus, 2006). Antimicrobial activity was evaluated by measuring the diameter of inhibitory zone (mm) surrounding film disks against the test organisms.

2.5. Statistical analysis

Data for each test were statistically analyzed. The analysis of variance (ANOVA) was used to evaluate the significance in the difference between factors and levels. Comparison of the means was done by the Duncan's multiple range test. The statistical analysis of the data was performed using SPSS statistical software version 18 (SPSS Inc., Chicago, IL).

3. Results and discussions

3.1. Antibacterial activity

The antibacterial activities of films incorporated with *zataria multiflora* boiss essential oil (ZEO) against Gram-negative and Gram-positive bacteria are shown in Table 1.

This method is based on the measurement of the clear zone caused by growth inhibition produced by a film disk containing the antimicrobial agent when putting in direct contact with a bacterial culture (Weerakkody et al., 2010). Control films (without ZEO) did not show an inhibitory zone in the bacterial strains tested. Films containing 1% of ZEO were not effective against *Escherichia coli* but exhibited an inhibitory effect on the growth of *Bacillus cereus* as evidenced by minimal growth around the film cuts. At 2% a greater inhibitory effect was observed on *Escherichia coli* followed by *Bacillus cereus* and *Staphylococcus aureus* with zone area of 10.14 mm, 9.71 mm and 4.11 mm, respectively. The antibacterial efficiency of ZEO can be related to the concentration and proportion of phenolic compounds (Jouki et al.,

2014b). Eftekhar et al. (2011) reported that Zataria multiflora essential oil contained 25 constituents of which the major components were carvacrol (50.57%), thymol (13.38%) and p-cymene (8.27%). They also reported that *zataria multiflora* boiss essential oil showed strong antibacterial activity against all pathogenic bacteria strains.

Table 1. Inhibition zone diameters yielded by CSG-film disks with various concentrations (0, 1, 2, 3 and 4% v/v) of *zataria multiflora* boiss essential oil (ZEO).[A]

Test organisms	Inhibition zone diameters(mm)				
	ZEO concentration (% v/v)				
	0	1	2	3	4
Escherichia coli	0.0^{c}	0.0^{c}	10.14±0.17^{b}	12.78±0.21^{a}	13.65±0.21^{a}
Lactobacillus plantarum	0.0^{e}	0.3±0.02^{c}	1.86±0.02^{b}	2.40±0.02^{ab}	3.69±0.02^{a}
Bacillus cereus	0.0^{e}	3.06±0.02^{d}	9.71±0.01^{c}	15.14 ±2.20^{a}	16.2±0.01^{a}
Staphylococcus aureus	0.0^{e}	2.6±0.51^{d}	4.11±0.23$_{c}$	6.02±0.23^{b}	9.47±1.11^{a}

[A] Data reported are average values ± standard deviations. Values (n = 6) within each row with different letters are significantly different (P < 0.05).

3.2. Scanning electron microscopy

SEM micrographs representative of CSG-films with different ZEO concentrations are presented in Figure 1. The control film displayed a compact, smooth and continuous microstructure with no irregularities. The film images showed that adding ZEO caused a heterogeneous structure in which oil droplets were entrapped in the continuous polysaccharide network (Fig. 1b). CSG-films incorporated with ZEO concentrations of 2, 3 and 4% showed a loose texture with sponge- like structure, with pores distributed throughout the film matrix (Fig. 2c, d and e). The existence of holes or pores might be related to the volatility of the pennyroyal essential oil. These results are in agreement with those of Jouki et al. (2014a), who reported these spaces were filled by essential oil that had evaporated from the film surface.

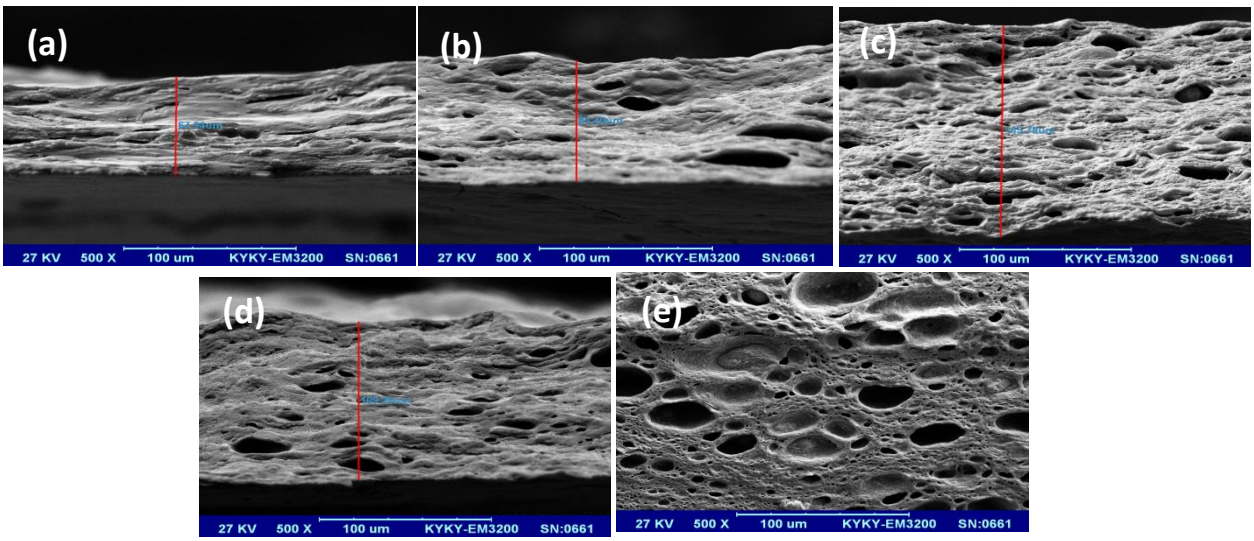


Figure 1. SEM micrographs of the cross-sections of the CSG-films. (a) 0% ZEO, (b) 1% ZEO, (c) 2% ZEO, (d) 3% ZEO, (e) 4% ZEO.

3.3. Thermal properties

The thermal behavior of the control and ZEO-incorporated CSG-films was investigated using DSC. The DSC thermograms of the films with different ZEO concentrations are shown in Fig. 2. As Ghanbarzadeh and Oromiehi (2009) mentioned the glass transition temperature (Tg) is the temperature at which the material undergoes a structural transition from an amorphous solid (glassy) state to a more viscous rubbery state. Below Tg, films are rigid and brittle, whereas above it films become flexible and pliable. Control film had Tg of -30.6±4.5 °C. Addition of ZEO decreased Tg values (-44.8 to -61.5). The films incorporated with ZEO had slightly higher Tm than the control films; this could be attributed to the larger molecular weight and more hydrophobic nature of ZEO compared to glycerol (Jouki et al., 2014c). Jouki et al. (2014a) reported that the addition of thyme essential oil in QSM-films decreased significantly ($p < 0.05$) Tg and increased Tm. The DSC curve of the film containing 3 and 4% ZEO showed two Tm peaks (Fig. 2D and 2E). Jouki et al. (2014c) noted that DSC curve of the QSM-based film incorporated with oregano essential oil (OEO) showed also two endothermic peaks at 167 and 230 °C.

3.4. Physical Properties

Effects of ZEO on the physical properties of CSG-films are presented in Figure 3. Thickness of films varied between 0.067 and 0.122 mm (Fig. 3a). The films prepared with ZEO showed lower moisture content and solubility than the control film; these decreased significantly ($P<0.05$) as ZEO content increased.

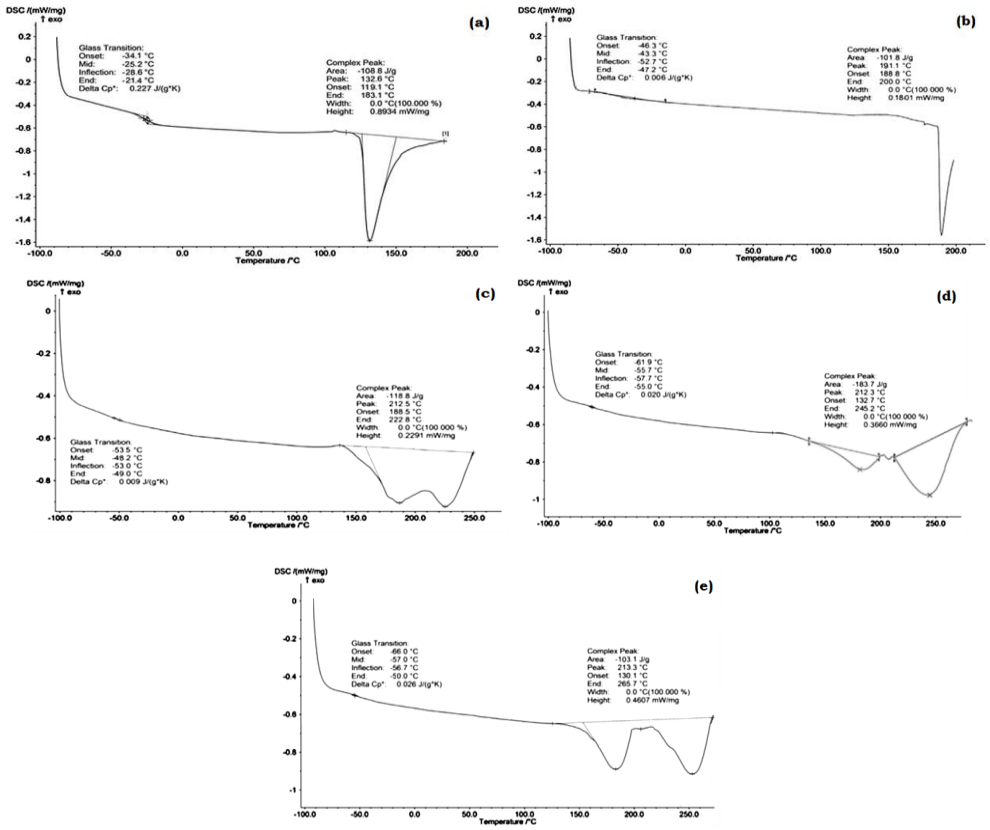


Figure 2. Representative examples of DSC curves of control film (a), CSG+1%ZEO (b), CSG+2%ZEO (c), CSG+3%ZEO (d), CSG+4%ZEO (e).

Lower moisture content with minimum solubility (14.02% and 35.22%, respectively) was achieved for films formulated with 4% essential oil. Jouki et al. (2014c) reported that these results could be attributed to a decrease in the hydrophilic nature of the films, as well as interaction between the components of essential oil and the hydroxyl groups of film, which would reduce availability of hydroxyl groups for interaction with water molecules, consequently leading to a more water-resistant film. Similar results were found by Hosseini et al. (2009) for chitosan films incorporating cinnamon.

WVP of ZEO-incorporated films were increased from 0.95 to 3.21×10^{-10} $gs^{-1}m^{-1}Pa^{-1}$ as the concentrations of ZEO ($P < 0.05$). Similar results were found by Jouki et al. (2014c) in QSM-based films containing oregano essential oils. They showed that increase of OEO concentration leads to the increase of WVP values. Hosseini et al. (2009) stated that, although the hydrophobic nature of essential oils could affect the hydrophilic or hydrophobic property of the film, the physical factors had a dominant influence on the water vapor transmission rate through the film.

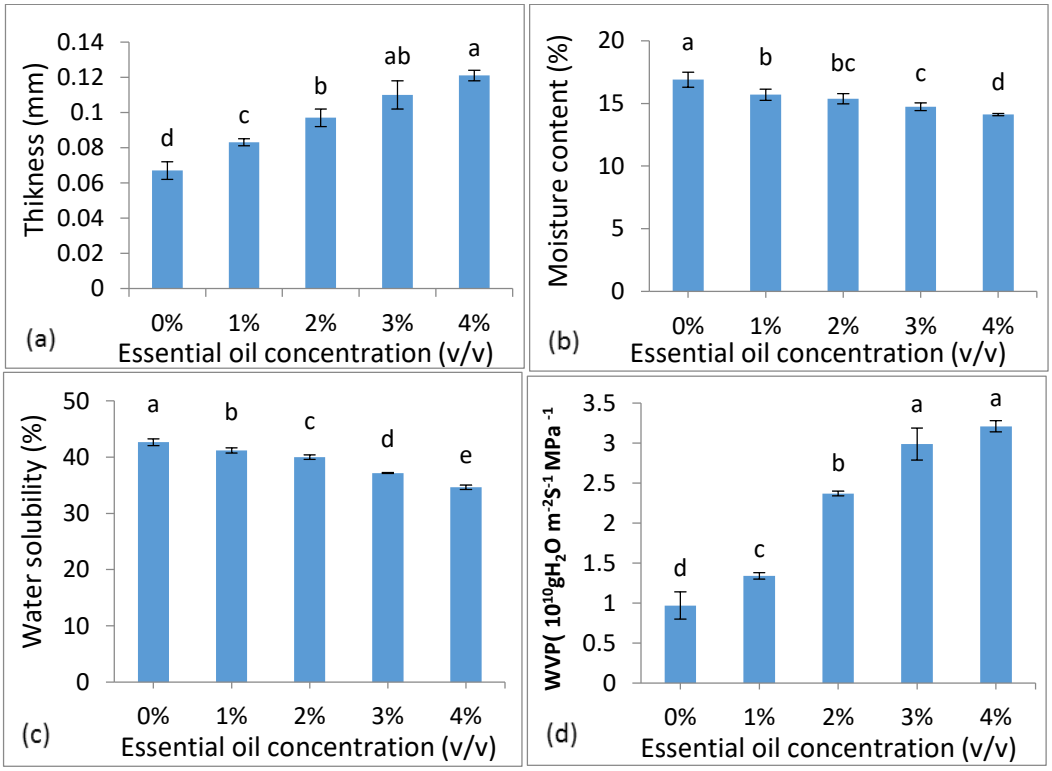
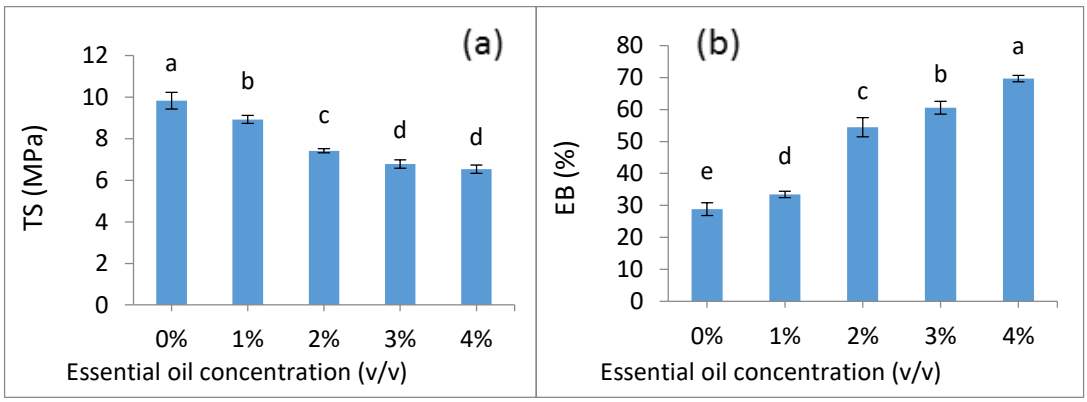


Figure 3. Physical properties of CSG-films incorporated with various ZEO concentrations. (a): thickness, (b): moisture content, (c): water solubility, (d): WVP.



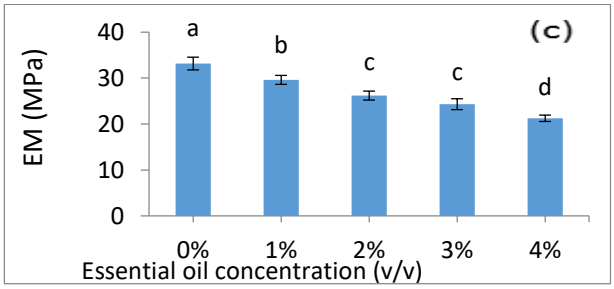


Figure 4. Physical properties of CSG-films incorporated with various ZEO concentrations. (a): Tensile Strength, (b): Elongation at Break, (c): Elastic Modulus.

3.5. Mechanical properties

Results of the mechanical tests are shown in Figure 4. The presence of ZEO in films caused significant differences in TS and EB. Film without ZEO had a tensile strength of 9.83 MPa. Tensile strengths were weaker for films containing ZEO than for the control film, significantly ($P<0.05$) decreasing as essential oil concentration increased. Conversely, EB of CSG-films increased significantly ($P<0.05$) from 28.8% to 69.65%. Because EO is liquid at room temperature, it will be present in the film in the form of oil droplets that can easily be deformed, enhancing the film's extensibility, so it can act as a plasticizer, reducing TS and increasing % EB of the films (Fabra et al., 2008; Jouki et al., 2014c). Similar results were obtained by Rojas-grau et al. (2007), who observed an increment in the elongation percentage in films of alginate and apple puree added with oregano, cinnamon and lemongrass EOs. Jouki et al. (2014a) showed that increase of TEO concentration in QSM films leads to the increase of % EB. The Elastic modulus (EM) of control films (33.17 MPa) was significantly greater than most of the films containing ZEO (Fig. 4c). Jouki et al. (2014a) stated that the addition of essential oils resulted in a film matrix that was less dense, which facilitated the movement of the polymer chains and improved the film flexibility.

4. Conclusions

The properties of CSG-based films were affected by the addition of thyme essential oil. The results of this work showed that CSG-based films prepared with *Zataria multiflora* essential oil exhibited highest inhibition against *Bacillus cereus* and *Escherichia coli.* ZEO significantly reduced Tensile strength and Young's modulus, while increased the elongation at break of the films. Our results pointed out that the incorporation of ZEO as a natural antibacterial agent has potential for using the developed Cress seed gum edible films as an active packaging.

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QUALITY CHARACTERISTICS AND STORAGE STABILITY OF GLUTEN-FREE COATED CHICKEN NUGGETS

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ABSTRACT
The main objective of this study was to produce gluten-free chicken nugget to meet celiac consumer's demands for gluten-free food with high nutritional value. The effects of cellulose, egg powder, whey powder, pectin and their combination using with gluten free wheat flour on the physicochemical characteristics, color, texture and storage stability were investigated. Control group was manufactured with wheat flour which contains gluten. After nuggets were pre-fried, the cooked samples were stored at 4 °C for 21 days and then nuggets were deep fat fried. The results indicated that the addition of whey powder in the formulation was resulted in decreased oil uptake and increased pick up and water holding capacity values ($p<0.05$). The coating pick-up, oil and moisture content were found to be directly proportional to batter viscosity. The lowest TBARS values were determined in the samples manufactured with wheat flour or whey powder at the end of the storage period ($p<0.05$). All groups except gluten-free wheat flour group had similar color values. The use of gluten-free wheat flour resulted in a higher ($p<0.05$) L* values. Texture analysis indicated that incorporation of gluten significantly ($P<0.05$) improved hardness and fracturability. This study demonstrated that 2% whey powder can be used in gluten free nugget manufacture without posing any quality problems.

1.Introduction

Celiac disease is a chronic inflammatory small intestinal disease occurring in genetically predisposed individuals because of an immune response to gluten from wheat, barley and rye (Alaedini and Green, 2005). Fasano et al. (2003) have reported that celiac disease is considered to affect 1% of the population, though in the USA, the majority is currently undiagnosed. In Europe, the prevalence of celiac disease has been estimated to be 1 in 300 to 1 to 5000 persons (Collin et al., 1995). The only therapy of celiac disease is dietary, a gluten-free diet.

Celiac consume several types of gluten-free products. These products fall into the categories of naturally occurring gluten-free foods (fruits, vegetables, and unprocessed meat, fish and poultry) and gluten-free substitute foods (pasta, bread, cereals, crackers and snack foods) in which wheat flour is replaced by gluten-free flours (Lee et al., 2007). However, celiac need to beware of meats and poultry with added ingredients that make them into ready-to-cook or processed meat products which may contain gluten.

Wheat gluten is one of the most commonly used ingredients for different purposes in meat products. A desired property of gluten is its ability to bind fat and water while at the same time increasing the protein content; this makes

gluten attractive for various types of application in meat, fish, and poultry products. Furthermore, gluten improves the utilization of beef, pork and lamb meats by a restructuring process, which converts less desirable fresh meat cuts into more palatable steak-type products. Gluten has also proven as a satisfactory binder for turkey meat pieces because of its ability to produce intact loaves with good slicing qualities. In processed-meat products, gluten is an excellent binder in poultry rolls, canned 'integral' hams, and other non-specific loaf type products, where it also improves slicing characteristics and minimizes cooking losses during processing (Day et al., 2006).

Wheat flour also plays an important role in batter system of coated products. It contains considerable amount of proteins (gluten) that are necessary to form elasto-plastic batter (Amboon et al., 2012). The trend of using batter and breading on chicken has increased approximately 100% since1980s. The per capita consumption of battered and breaded products in the United States increased about three folds from 1982 to 1992, and the annual consumption of these products in Europe, Japan, Oceania, and other Pacific Rim countries is approximately 2 billion pounds (Mukprasirt et al., 2000). Consumption of breaded and battered foods especially poultry, fish, seafood, cheese, and vegetables has become very popular over the last few years (Dogan et al., 2005). Traditional batter basically consists of flour, salt, and water, although the proportions of the basic ingredients are variable. Wheat flour is the most common flour used in batters since it contains considerable amount of proteins that are necessary to form elasto-plastic batter (Xue and Ngadi, 2006). Although gluten allergy in susceptible population has been increasing in recent years, research to produce on gluten-free meat products has very limited. Devatkal et al. (2011) and Jackson et al. (2006) reported the use of rice starch and sorghum flour in the formulation of wheat-free chicken nuggets.

The purpose of present study was to evaluate the quality characteristics of gluten-free chicken nuggets prepared by gluten-free ingredients (cellulose, egg powder, whey powder, pectin and their combination) to develop gluten-free ready to eat meat product for celiac consumer who demand gluten-free meat products.

2. Materials and methods

2.1. Materials

Fresh skinless and boneless broiler chicken breast meat (*Pectoralis major*) was obtained from a local slaughterhouse for each of three replications on separate production days.

Table 1. Batter formulations

Ingredients (%)	Control	GF	C	E	W	P	M
Wheat flour	49	-	-	-	-	-	-
Gluten-free wheat flour	-	49	48	48	48	48	48
Corn flour	49	49	48	48	48	48	48
Salt	1	1	1	1	1	1	1
Leavening agent	1	1	1	1	1	1	1
Cellulose	-	-	2	-	-	-	0.5
Egg powder	-	-	-	2	-	-	0.5
Whey powder	-	-	-	-	2	-	0.5

GF: gluten-free wheat flour, C: cellulose, E: egg powder, W: whey powder, P: pectin, M: mixed of cellulose, egg powder, whey powder and pectin

The ingredients used for the experiments were: cellulose (Danisco A/S, Aarhus, Denmark), egg powder (Lick, A.B Foods Inc., Turkey), whey powder (Maybi, Malraka Alliance Milk and Milk Products Inc., Turkey), high methoxyl pectin (Danisco A/S, Aarhus, Denmark), wheat flour and corn flour (Selva Gıda San. A.Ş., Konya, Turkey), gluten-free wheat flour (Poensgen GbR Special Diet Bakery, Eschweiler, Germany), sunflower oil (Yonca Gıda San A.Ş., Turkey), leavening agent (Dr. Oetker Gıda San. Tic. A.Ş., Turkey). The formulation of experimental groups are presented in Table 1. Gluten analysis of each experimental group was performed. As expected, gluten was only determined in control group (8.68 % ±0.6).

2.2. Sample preparation

Raw meat was trimmed off visible fat and connective tissue. Then each chicken breast was cut (parallel to the fibre direction) using regular knife to have 12±1 g uniform shaped (5x2x1 cm) samples. Basic batter formulation was consisted of gluten-free wheat flour, corn flour, salt and leaving agent. Experimental groups were formulated with cellulose (S) or egg powder (E) or whey powder (W) or pectin (P) or a combination of those (M) as presented in Table 2.

Table 2. Chemical composition of wheat, gluten free wheat and corn flours and chicken meat used in the study

	Moisture (%)	**Protein** (%)	**Ash** (%)	**Fat** (%)
Wheat flour	12.4±0.3	8.9±0.2	0.7±0.1	1.6±0.8
Gluten-free wheat flour	10.5±0.4	0.2±0.1	0.1±0.1	1.3±0.6
Corn flour	14.7±0.8	7.1±0.4	0.6±0.1	3.1±0.7
Chicken meat	74.8±0.6	21.4±1.1	1.3±0.1	4.8±0.4

Batter with wheat flour was used as a control group. Batter formulations were composed of a 5:6 solid to water ratio. In batter preparation, the dry ingredients were blended and mixed with distilled water at room temperature (25±1 ºC) with a hand mixer at the lowest speed (Arzum ARK55 MS, Turkey) for 2 min. Meat sample for each experimental group was immersed individually into the corresponding batter for 60 s and allowed to drip for 30 s. Deep fat pre frying was conducted at a temperature of 180±1 °C for 30 s in commercial bench-top deep fryer (TEFAL, Sarcelles, France). Fried samples were cooled at room temperature and stored at 4 °C for 21 days. After the storage period, samples were deep fat fried at 180±1 °C for 2 min. Four pieces were deposited into the frying oil each time. A 500 mL sunflower oil was used and changed after frying process of each experimental group is completed.

2.3. Viscosity and water holding capacity analysis

Flow behavior and time dependency of batters were determined at 25±1 °C by a parallel plate rotational viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA). The batter was allowed to equilibrate for 5 min and tested using a 1 mm gap. The sample was sheared at a programmed rate linearly increasing from 0 to 200 s^{-1} in 300 s. Time dependency of the batter was evaluated by measuring the apparent viscosity under constant shear rate of 100 s^{-1} for 300 s. In order to evaluate the water holding capacity of the batter, the amount of water released after centrifugation was quantified as described by (Sanz et al., 2004). Thirty grams of batter samples at 25 °C was placed in 50 mL centrifuge tubes and centrifuged at 17,300 x g for 10 min. After centrifugation, the supernatant solution was removed and weighed. Water holding capacity is expressed as the percentage of water released.

2.4. Batter pick-up analysis

The amount of batter adhering to a coated chicken was determined as described by Baixauli et al. (2003) after deep fat frying. The amount of batter adhering to the sample during immersion coating before frying was considered as the batter pick-up and calculated as the weight of coating picked up by a product divided by the non-coated product weight and multiplied by 100.

2.5. TBARS analysis

Evaluation of oxidative stability was performed by measuring the formation of thiobarbituric acid reactive substances (TBARS). TBARS values of samples were determined as described by Kilic and Richards (2003) at manufacturing day for raw samples, after the pre-frying and deep fat frying process and during of storage. This method requires addition of EDTA and propyl gallate to the trichloroacetic acid (TCA) extraction solution to prevent the development of TBARS during the analytical procedure. Sample (1 g) was blended into 6 mL of extraction solution. The samples were homogenized for 15 s. The homogenate was filtered through Whatman 1 filter paper. Filtrate (1 mL) was mixed with 1 mL of thiobarbituric acid (TBA) and vortexed. The mixture was heated at 100 °C for 40 min. After cooling, the sample was centrifuged at 2000 × g for 5 min. Absorbance was determined at 532 nm against a blank containing 1 mL TCA extraction solution and 1 mL TBA solution. The TBARS values were expressed as μmol TBARS per kg meat.

2.6. Chemical composition

pH measurements were carried out using a standard electrode attached to Orion Model 420 digital pH meter (Orion, Boston, USA). The pH was determined after mixing a 10 g sample with 90 mL distilled water and equilibrating for 10 min. Fat, ash, protein and moisture contents were determined in raw and deep fat fried samples (AOAC, 1995, Aoac, 2000). Wet gluten and dry gluten contents of batter were determined according to the approved AACC methods (AACC, 2000).

2.7. Color determination

Color measurement was taken with a Hunterlab model Precise Color Reader TCR 200 (BAMR Corp., Claremont, South Africa) colorimeter. Three readings were taken and averaged for each of the three replications. L*, a*, b* values were determined after the pre-frying and deep fat frying process and during of storage. The results were expressed in accordance with the CIELAB system with reference to illuminant D65 and with a visual angle of 10°. The CIELAB parameters (L*, a*, b*) for all samples were determined following the recommendations of the *Commission Internationale de L'Eclairage* (CIE, 2004). Color differences (ΔE^*_{ab}) were calculated as the Euclidean distance between two points in the three-dimensional space defined by L*, a*, and b*: $\Delta E^*_{ab} = [(L^*-L^*_0)^2 + (a^*-a^*_0)^2 + (b^*-b^*_0)^2]^{1/2}$ where L^*_0, a^*_0, b^*_0 are the parameters corresponding to the control samples.

2.8. Texture profile analysis

Texture profile analysis (TPA) were performed using a TA.XT2 Texture Analyzer (Stable Micro Systems, Godalming, UK) to determine hardness (g) and fracturability (g). The samples were wrapped with stretch film, and held for equilibration to room temperature (20 ºC). Test conditions were: aluminum rectangular probe (5 cm • 4 cm); test speed 5 mm/s; pre-test speed 2 mm/s, post-test speed 2 mm/s; compression 70% and 50 kg load cell.

2.9. Sensory evaluation

The degree of difference and descriptive sensory analysis were performed at the Department of Food Sciences at the Suleyman Demirel University by a group of twenty, non-smoker panelists (7 males and 13 females, between 21 and 29 years old) experienced in the sensory evaluation of foods, using procedures described in Ift Guideline (1981). Each panelist was seated in individual booth with white illumination and water was provided for rinsing

the mouth between samples. Sample from each treatment was randomly chosen, presented in dishes coded with random three-digit numbers, reheated 40 s at microwave oven and served to the panelists. The samples were evaluated for color and color intensity, textural attributes (ease of fracture, firmness, coating crunchiness and greasiness), flavor and off-flavor, odor and the overall acceptability.

2.10. Statistical analysis

Entire experiment was repeated three times, duplicate samples for each parameter were analyzed to get a total number of six observations (n = 6) for each parameter and mean ± standard deviation values were reported. Data collected for physicochemical properties and sensory attributes were analyzed by the statistical analysis system. The statistical evaluation of the results was performed using the SPSS 22.0.0 (SPSS Inc., Chicago, USA). The generated data were analyzed by analysis of variance (ANOVA). Differences among mean values were established using the Duncan test and were considered significant when $p<0.05$.

3. Results and discussions

3.1. Viscosity, batter pick-up and water holding capacity

Changes in batter apparent viscosity with shear rate at 25 °C is presented in Fig. 1.

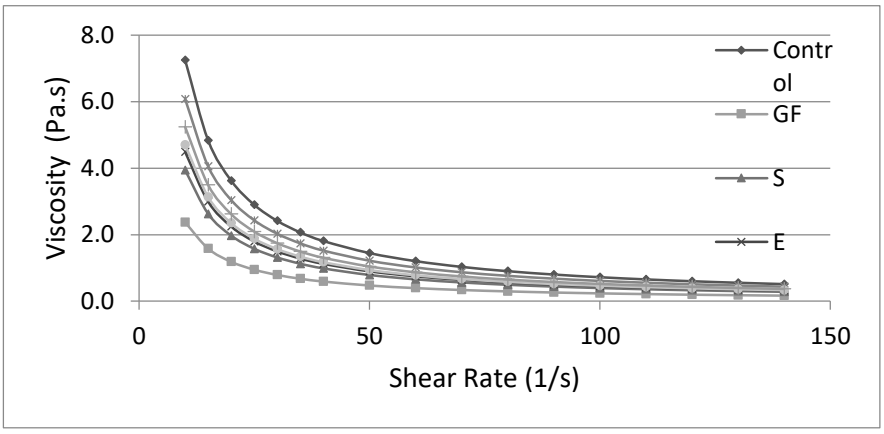

Figure 1. Change in batter viscosity with shear rate for different batter formulations

Batter viscosity is the most important factor affecting flow behavior of the batter before frying and influences the quality and quantity of batter pick-up, appearance, texture, and handling property of the coated product (Xue and Ngadi, 2006, Chen et al., 2008). The results indicated that control and GF groups showed the highest and the lowest apparent viscosity among all treatment groups, respectively ($p<0.05$). Chicken nugget formulations which contained cellulose or egg powder or whey powder or pectin or their combination had a higher ($p<0.05$) viscosity values than that of GF group. Especially, the use of whey protein resulted in significant increase ($p<0.05$) in the batter viscosity compared to that of the GF group. This study revealed that coating pick-up, water holding capacity and oil uptake was found to be directly related to batter viscosity. It was previously reported that as viscosity increases, more batter remains on the sample (Nasiri et al., 2012). This could be attributed to the ability of wheat gluten to absorb water and viscosity building, resulting in decreased free water in the batter system (Day et al., 2006). Dogan et al. (2005) reported that the viscosity development of the dry components is related to water binding capacities in batter formulation. Water holding capacity, batter pick-up and oil uptake values for different batter formulations are presented in Fig. 2.

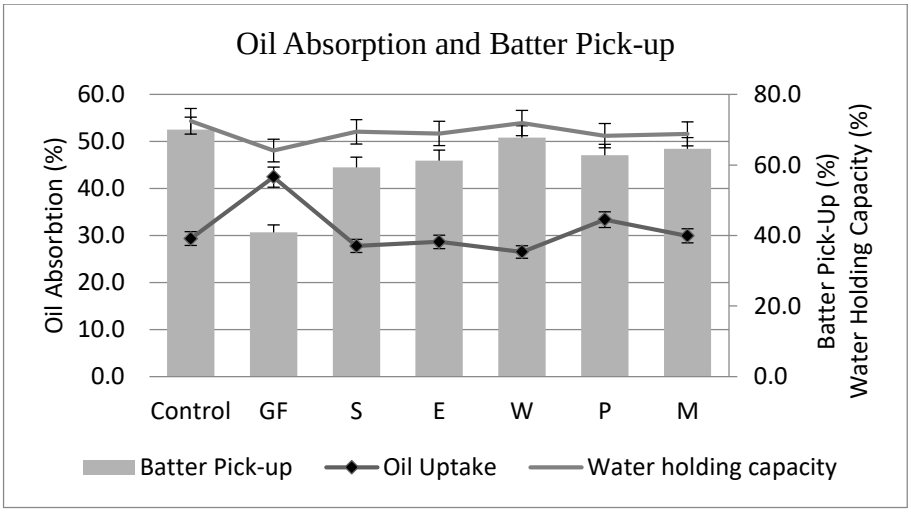

Figure 2. Oil absorbtion, batter pick up and water holding capacity values for different batter formulations

Results showed that control and W groups had the highest batter pick-up and GF group had the highest oil uptake values ($p<0.05$). In addition, cellulose, egg powder, whey powder and pectin were found to be significantly ($p<0.05$) effective for enhancing batter pick-up and oil uptake. Furthermore, the use of whey protein in the formulation caused a significant ($p<0.05$) increase in coating pick-up compared with that of gluten-free wheat flour. Previous studies have reported that the addition of methylcellulose, hydroxypropyl methylcellulose, gums, modified starches and proteins in batter systems has successfully reduced oil uptake and increase batter pick-up in coated fried products such as chicken pieces, fish, vegetables, cheese, and cereal products. (Sahin et al., 2005, Akdeniz et al., 2006, Akdeniz et al., 2005, Fiszman and Salvador, 2003). Coating pick-up is an important index in the coated food industry and generally around 30-50% (Loewe, 1993). Essentially, mechanisms that enable the formation of oil barrier films or increase the water holding capacity may reduce oil uptake (Dana and Saguy, 2006). The proteins in batter provide structure and increase the consistency of raw batters and this is reflected by a rise in viscosity, coating pick-up values and final yield in the fried products (Fiszman and Salvador, 2003, Nasiri et al., 2012).

Water binding capacities of batter was reported to have a significant effect on the rheological characteristics of batter and textural properties of coated food (Dogan et al., 2005). In present study, the lowest water holding capacity ($p<0.05$) was obtained (Figure 2) when batter formulation was prepared with gluten free flour which might be due to its protein content and lower water binding capacity.

3.2. Chemical composition

pH values of chicken nuggets after the pre-frying and deep fat frying process and during of storage are presented in Table 4. Results indicated that pH values did not vary significantly among the treatment groups. Thus, the use of gluten-free wheat flour, cellulose, egg powder, whey powder and pectin was not found to be a significant factor affecting final pH of chicken nuggets. pH values of all treatment groups showed an increasing trend during the storage period. An increase in pH in all treatment groups was also observed after deep fat frying. Our results are in agreement with previous studies on chicken nugget (Allen et al., 1998, Perlo et al., 2006). An increase in pH after deep fat frying and storage in our study could be attributed to the formulation and/or the protonation of some basic amino acid residue side chains which became exposed due to

protein denaturation (Allen et al., 1998, Perlo et al., 2006).

Table 3. Chemical composition* of coated chicken products

Groups	Moisture (%)	Protein (%)	Ash (%)	Fat (%)
Control	39.5±0.5^{a}	22.6±2.3^{ab}	1.3±0.1^{a}	8.3±0.2^{c}
GF	30.1±0.5^{d}	21.3±0.4^{bc}	1.2±0.1^{ab}	11.9±1.2^{a}
S	35.5±0.5^{c}	20.2±1.7^{c}	1.2±0.1^{ab}	10.1±0.2^{b}
E	35.8±0.8^{c}	23.3±1.3^{a}	1.3±0.1^{a}	11.7±0.4^{a}
W	37.9±0.4^{b}	22.5±1.7^{ab}	1.1±0.2^{bc}	9.4±0.2^{bc}
P	35.7±0.2^{c}	23.5+1.8^{a}	1.3±0.1^{a}	11.5±0.5^{a}
M	36.1±0.4^{c}	22.3+0.9^{ab}	1.2±0.1^{ab}	9.8±0.1^{b}

*All values are the mean ± standard deviation of three replicates.
a, b, c, d (↓) Different letters within a column are significantly different ($p<0,05$)

Table 4. pH values* of coated chicken products

		Storage (+4 °C)			
Groups	Pre-fried	7 days	14 days	21 days	Deep-fat fried
Control	6.00±0.04^{bc}	6.16±0.03^{ab}	6.22±0.02^{a}	6.22±0.00^{b}	6.48±0.02^{a}
GF	5.94±0.02^{c}	6.27±0.04^{a}	6.13±0.00^{c}	6.11±0.02^{d}	6.34±0.00^{b}
S	6.01±0.03^{bc}	6.11±0.03^{b}	6.20±0.03^{ab}	6.17±0.01^{c}	6.38±0.06^{ab}
E	5.98±0.04^{c}	6.14±0.06^{b}	6.14±0.007^{c}	6.10±0.02^{d}	6.41±0.07^{ab}
W	6.06±0.02^{ab}	6.11±0.01^{b}	6.16±0.02^{bc}	6.36±0.02^{a}	6.48±0.01^{a}
P	6.10±0.02^{a}	6.12±0.07^{b}	6.02±0.02^{d}	6.02±0.00^{e}	6.36±0.02^{b}
M	6.10±0.01^{a}	6.16±0.04^{ab}	6.07±0.01^{d}	6.09±0.02^{d}	6.31±0.02^{b}

*All values are the mean ± standard deviation of three replicates.
a, b, c, d (↓) Different letters within a column are significantly different ($p<0,05$).

The fat, ash, moisture and protein content of deep-fat fried samples are present at Table 3. Protein and ash content of the samples had shown non-significant differences among all treatment groups. It was determined that chicken nugget samples contained about 22.2 % protein and 1.2 % ash. A significant differences were determined for fat and moisture content of samples among groups ($p<0.05$). Control group had the highest moisture and the lowest fat content among all chicken nugget groups ($p<0.05$). On the other hand, GF samples had the lowest ($p<0.05$) moisture content which may explain the reason determining low apparent viscosity in batters of GF group (Fig. 2). Due to less water-binding capacity of gluten-free wheat flour compared to cellulose, egg powder, whey powder and pectin, there was more free water available to facilitate the evaporation of water during deep fat frying. Deep fat frying is a process of simultaneous heat and mass transfer. Heat is transferred from the oil to the food and oil penetrates the crust through the pores created by the evaporation of water from the food. There was an opposite relationship between moisture loss and oil uptake, so higher oil content corresponded with lower moisture content (Nasiri et al., 2012). For this reason, oil uptake is largely determined by the moisture content of the food. Furthermore, moisture analysis results demonstrated that high moisture content of coated products is related to water binding capacity of cellulose, egg powder, whey powder and pectin (Salvador et al., 2008, Jezek et al.,

2009, Baixauli et al., 2003, Dogan et al., 2005). Due to their high water binding capacity, cellulose, egg powder, whey powder and pectin added batter can control moisture loss and so the oil uptake during frying.

3.3. TBARS analysis

The TBARS values of chicken nuggets after the pre-frying and deep fat frying, and during storage period presented in Table 5.

Table 5. TBARS values* of coated chicken (µmol/kg) during the fermentation and storage period

		Storage (+4 °C)			
Groups	Pre-fried	7 days	14 days	21 days	Deep-fat fried
Control	2.19±0.10^{c}	5.49±0.21^{d}	6.42±0.14^{e}	7.09±0.35^{d}	8.71±0.17^{d}
GF	3.34±0.14^{a}	6.37±0.14^{bc}	8.90±0.04^{a}	9.63±0.16^{a}	12.89±0.21^{a}
S	2.68±0.12^{b}	6.63±0.17^{ab}	7.68±0.24^{c}	7.98±0.24^{c}	10.80±0.31^{b}
E	2.72±0.41^{b}	6.95±0.20^{a}	8.22±0.08^{b}	7.96±0.17^{c}	10.62±0.38^{b}
W	2.20±0.06^{c}	5.78±0.09^{d}	6.93±0.19^{d}	7.17±0.08^{d}	8.95±0.10^{d}
P	3.19±0.02^{a}	6.73±0.22^{ab}	8.29±0.38^{b}	8.85±0.14^{b}	10.84±0.26^{b}
M	3.09±0.16^{ab}	6.84±0.21^{a}	7.35±0.09^{bc}	8.72±0.02^{b}	9.36±0.16^{c}

*All values are the mean ± standard deviation of three replicates.
a, b, c, d (↓) Different letters within a column are significantly different (p<0,05).

Results showed that there were differences in TBARS values among treatment groups in the samples ($p<0.05$). Control and W group had lower TBARS values than that of other treatment groups at pre-frying stage and during storage period ($p<0.05$). On the other hand, GF group which manufactured with gluten-free wheat flour had the highest TBARS after deep-fat frying and 14 days of storage ($p<0.05$). Lipid oxidation in deep fat fried foods could be related with the pick-up rate and oil uptake of batter (Xue and Ngadi, 2007). Additionally, Xue and Ngadi (2007) have indicated that denaturation and gelatinization during the frying process is one of the key factors for oil absorption. TBARS values of chicken nuggets obtained in this study support this approach. GF groups had the lowest batter pick-up and the highest oil uptake values as well as the highest TBARS during frying and storage period. Whereas, control and W group had the lowest oil uptake and TBARS during frying and storage period. Furthermore, S and P groups which contain hydrocolloids with a film-forming property and thermal gelation had a lower oil uptake and TBARS values than the GF group. Many studies stated that oil absorption and oxidation of food can be controlled by small changes in the composition of the external layers. Loewe (1993) indicated that rice, corn, and soy flours have also been used to reduce oil uptake. Shih and Daigle (1999) reported a 69% reduction in oil absorption with using rice flour. Similar results were reported for some hydrocolloids such as protein, pectin, cellulose and starch (Gamonpilas et al., 2013, Dana and Saguy, 2006, Primo-Martin, 2012, Marquez et al., 2014).

3.4. Color analysis

The results of CIE L*a*b* and $\Delta E^{*}_{a,b}$ values (Table 6) showed that there were significant differences among color values of all treatment groups after the pre-frying and deep fat frying, and during storage period. The L*, a* and b* values of chicken nugget samples after pre-frying showed non-significant differences among treatment groups except L* values of gluten-free wheat flour group which was higher than other groups ($p<0.05$). A significant decrease in the L* values and increase in a* and b*values for all samples was determined after the deep fat frying process ($p<0.05$). Gluten free wheat flour group had lower L* and higher a* values ($p<0.05$) after the deep fat frying compared to other treatment groups. These result showed that the nuggets manufactured with gluten-free wheat flour became darker and

redder. This may be related with lower protein content and lower batter pick-up of GF group. Furthermore, the results revealed that ingredients used (cellulose, egg powder, whey powder and pectin) in the formulation had non-significant effect on L*, a* and b* values in deep fat fried chicken nuggets.

Table 6. Color properties of coated chicken products

			Storage (+4 °C)			
	Groups	**Pre-fried**	**7 days**	**14 days**	**21 days**	**Deep-fat fried**
L* value	Control	71.68±3.61^{b}	66.74±2.64^{b}	74.68±1.50^{bc}	68.41±1.59^{bc}	65.42±1.57^{a}
	GF	78.65±1.60^{a}	75.98±2.43^{a}	84.74±4.39^{a}	75.57±1.89^{a}	54.04±1.23^{f}
	S	70.25±1.12^{bc}	66.75±2.90^{b}	75.64±3.01^{b}	68.93±3.93^{bc}	62.74±2.08^{bc}
	E	73.26±1.03^{b}	70.26±2.18^{b}	70.79±2.96^{cd}	72.58±1.71^{ab}	57.96±2.18^{e}
	W	66.83±1.84^{c}	77.17±3.16^{a}	69.78±1.72^{d}	67.51±3.26^{c}	59.27±0.86^{de}
	P	69.32±1.26^{bc}	68.63±2.78^{b}	74.91±2.78^{bc}	72.35±2.86^{ab}	64.83±0.78^{ab}
	M	70.92±4.31^{b}	67.21±2.73^{b}	71.06±3.06^{bcd}	69.14±3.52^{bc}	61.55±0.44^{cd}
a* value	Control	0.34±1.61^{ab}	-0.10±1.43^{b}	-2.05±1.18^{b}	-1.35±1.83^{b}	7.89±1.28^{bc}
	GF	1.55±1.60^{a}	4.66±1.37^{a}	0.83±1.24^{a}	-0.46±2.83^{ab}	11.31±1.65^{a}
	S	-3.12±1.88^{c}	-1.08±0.28^{b}	-1.07±0.88^{ab}	0.15±1.37^{ab}	0.80±0.86^{de}
	E	-0.72±0.81^{b}	1.04±0.98^{b}	-2.06±2.17^{b}	-2.24±0.43^{b}	3.19±1.05^{cd}
	W	-0.29±1.36^{ab}	6.22±0.32^{a}	0.83±0.30^{a}	1.31±1.53^{a}	5.82±0.86^{c}
	P	-0.65±0.99^{b}	-0.20±1.08^{b}	-1.64±1.91^{b}	-2.26±0.19^{b}	3.60±1.35^{cd}
	M	-1.25±0.53^{bc}	0.14±1.37^{b}	-2.22±0.40^{b}	-1.70±0.80^{b}	3.02±1.95^{cd}
b* value	Control	16.81±1.78^{d}	17.69±2.72^{b}	17.51±2.25^{cd}	17.05±0.65^{c}	32.13±1.90^{a}
	GF	18.35±1.76^{cd}	19.03±2.15^{b}	19.83±1.82^{bc}	16.11±1.07^{c}	24.17±0.70^{d}
	S	20.49±2.67^{abc}	18.60±1.50^{b}	20.52±1.39^{b}	17.63±1.76^{bc}	26.55±0.82^{cd}
	E	19.83±2.24^{bcd}	17.96±0.88^{b}	19.79±1.46^{bc}	19.36±1.82^{ab}	29.38±1.55^{abc}
	W	21.68±1.80^{ab}	22.79±3.12^{a}	24.32±0.74^{a}	20.68±1.12^{a}	30.98±1.19^{ab}
	P	17.71±1.56^{cd}	16.95±1.46^{b}	16.81±1.01^{d}	17.91±1.03^{bc}	27.34±2.19^{c}
	M	23.15±1.21^{a}	20.84±3.49^{ab}	19.56±1.70^{bc}	17.96±1.71^{bc}	28.94±2.52^{bc}
$\Delta E^{*}_{a,b}$	Control	0	0	0	0	0
	GF	7.24	10.48	10.73	7.28	7.29
	S	5.26	1.34	3.32	1.71	9.16
	E	3.57	3.71	4.51	4.85	7.28
	W	6.91	13.22	8.88	4.60	7.17
	P	2.73	2.02	0.85	4.14	7.86
	M	6.59	3.20	4.16	1.22	6.69

*All values are the mean ± standard deviation of three replicates.

a, b, c, d (↓) Different letters within a column are significantly different ($p<0,05$).

It was reported that three CIE LAB units color differences (ΔE^{*}_{ab}) are generally considered as an estimate of the differences perceived by the human eye (Martinez et al., 2001). This means that pre and deep fat fried chicken nuggets were significantly ($p<0.05$) different from control group (Table 6). Loewe (1993) indicated that the chemical browning reactions between reducing sugars and protein sources, the absorption of frying oil, density of the fried product, the temperature and frying time lead to color development during frying process. Additionally, Sahin (2000) reported that color development in fried products is also

influenced by starch gelatinization and protein denaturation. In this study, color differences during pre-frying of nuggets with pectin were significantly less than the other treatment groups. Loewe (1990) indicated that the ability of gums to bind moisture prevents dehydration and inhibits the Maillard browning reaction. In general the color changes are confirmed by an increase in parameter a* and the reduction of L* during conventional deep frying (Innawong et al., 2006, Dogan et al., 2005).

3.5. Texture analysis

The effect of gluten-free wheat flour, cellulose, egg powder, whey powder and pectin on hardness and fracturability values of chicken nuggets was shown in figure 3. Results showed that control group had the highest hardness values ($p<0.05$). The lowest ($p<0.05$) hardness values were obtained in the nuggets manufactured with gluten-free wheat flour. This result may be related with its lowest pick-up value of gluten-free wheat flour compared to other tested ingredients ($p<0.05$). (Suderman (1983)) reported that thicker coatings are very smooth and lack in texture, however, a thinner and less viscous coating from these same ingredients may allow air bubbles to appear on the surface which results in more appealing texture.

3.6. Sensory analysis

Sensory evaluation of chicken nuggets revealed that use of gluten-free wheat flour, cellulose, egg powder, whey powder and pectin had no significant effect on sensory properties of coated samples compared to the control group. Sensory analysis results indicated that there was no significant differences among treatment groups regarding color and color intensity, textural attributes (ease of fracture, firmness, coating crunchiness and greasiness), flavor and off-flavor, odor and the overall acceptability (data is not presented). Regarding overall acceptability, all treatment groups received high overall acceptability scores ranging 5-7 from panelists.

4. Conclusions

Present study showed that combination of whey powder and gluten-free wheat flour could be effectively incorporated in the preparation of gluten-free chicken nugget without any significant adverse effect on the quality characteristics of the product. The oil uptake and batter pick-up value in combination of whey powder and gluten-free wheat flour were found be similar with control group. In addition, the chicken nuggets produced with a combination of whey powder and gluten-free wheat received the similar overall acceptability with the control group. It is suggested that the combination of gluten-free wheat flour and whey protein can be a suitable alternative to produce good-quality gluten-free coated chicken products.

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CHARACTERISTICS AND QUALITY OF VIRGIN COCONUT OIL AS INFLUENCED BY MATURITY STAGES

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ABSTRACT

Characteristics and quality of Virgin Coconut Oil (VCO) extracted from coconut with three different maturity stages including immature coconut (IMC), mature coconut (MC) and overlay mature coconut (OMC) were comparatively studied. The highest recovery (95.64%) was found in VCO from OMC ($p<0.05$), followed by those from MC (84.40%) and IMC (61.06%), respectively. All VCO samples had water-like appearance and contained medium chain fatty acid (MCFA), especially lauric acid (C12:0) as a major fatty acid, (49.74-51.18 g/100g). Myristic acid (C14:0) in the range of 18.70-19.84 g/100g was present in all VCO. Quality parameters of all VCO samples complied with Asian Pacific Coconut Community (APCC) standards. All VCO samples had low lipid hydrolysis and oxidation, indicating that maturity stages had no influence on oil oxidative stability. Thus, maturity stages played an essential role in recovery, but showed no impact on fatty acid composition and physicochemical properties of resulting VCO.

1. Introduction

Coconut oil is one of important oils used extensively in many traditional foods of Asian and Pacific regions (Onsaard et al., 2005). Coconut oil is different from other vegetable oils due to high content of medium chain fatty acids (MCFAs), particularly lauric acid. MCFAs are burned up immediately after consumption and therefore the body uses it instantly to make energy, instead of storing it as body fat (Enig, 1996). Lauric acid is converted into a very valuable compound known as monolaurin, which has antiviral and antibacterial properties (DebMandal & Mandal, 2011). It is therefore assumed that consumption of coconut oil may help to protect the body from infections. Coconut oil is generally extracted from wet coconut kernel (meat) by mechanical or natural means, with or without the use of heat. Chemical refining, bleaching or de-odourising processes are not required. As a consequence, the nature of oil is not altered and is known as virgin coconut oil (VCO) (Villarino et al., 2007). VCO is rapidly gaining immense importance due to various health benefits, high degree of saturation and good stability. VCO is the purest form of coconut oil with natural distinctive coconut taste and smell. It is solidified at low temperature and becomes colourless like water when liquefied (Marina et al., 2009).

Coconut milk, a milky white oil-in-water emulsion, has been used as a starting material for VCO production. The emulsion in coconut milk

was naturally stabilised by coconut proteins: globulins and albumins, as well as phospholipids (Raghavendra & Raghavarao, 2011). To obtain VCO, destabilisation of coconut milk emulsion using different extraction methods has been implemented (Raghavendra & Raghavarao, 2010). Amongst all processes, the enzyme-assisted separation process has been known to be effective and less time consuming. Additionally, high yield could be obtained from coconut milk with the aid of protease like Alcalase (Man et al., 1996). The efficiency of enzyme in extraction of oil is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Rahayu et al., 2008).

Yield is generally one of prime parameters in VCO extraction. Properties of VCO are also essential and must meet the specification set by the organisation with authority. Both yield and quality of VCO could be affected by several factors, both intrinsic and extrinsic ones. Maturity stage of coconut could be another factor influencing oil level in coconut kernel associated with oil yield as well as properties of VCO obtained. The objective of this study was to comparatively investigate the recovery, fatty acid composition and properties of VCO from coconut milk with three different maturity stages.

2. Materials and methods

2.1. Chemicals

Potassium hydroxide and ρ-anisidine were purchased from Sigma (St. Louis. MO, USA). Sodium thiosulfate, isooctane and ferrous chloride were obtained from Merck (Darmstadt, Germany). Methanol, ethanol, acetic acid, chloroform, petroleum ether, hydrochloric acid, sulphuric acid, n-hexane and cyclohexane were procured from Lab-Scan (Bangkok, Thailand). Alcalase (2.4L) with the activity of 2.4 AU/g and a density of 1.17 g/mL was purchased from Brenntag (Toronto, Canada).

2.2. Preparation of coconut milk with different maturity stages

Coconuts from three different maturity stages including immature coconut (IMC) (9-10 months old from pollination), mature coconut (MC) (11-12 months old from pollination) and overlay mature coconut (OMC) (14-15 months old from pollination) were purchased from a plantation site in Yaring district, Pattani province, Thailand and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. Coconuts were subjected to deshelling, paring and removal of water. Coconut kernel was removed manually and grated using a rotary wedge cutter machine. Coconut milk was then prepared from grated kernel without water addition using a hydraulic press machine (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand). Coconut milk from IMC, MC and OMC had oil contents of 17.28%, 30.18% and 46.2%, respectively, as determined by the Bligh and Dyer method (Bligh & Dyer, 1959).

2.3. Separation of virgin coconut oil (VCO) using Alcalase

Coconut milk was adjusted to pH 8 using 1 M NaOH and added with Alcalase at a level of 0.5% (v/v). The hydrolysis was performed at 60 °C for 90 min in a shaking water bath (W350, Memmert, Schwabach, Germany). After the designated time, the mixtures were centrifuged at 4900xg for 30 min at room temperature (26 – 28 ºC) using a centrifuge (Beckman coulter, Allegra™ centrifuge, CA, USA). VCO was collected from the upper oil phase. All VCO samples from coconuts with different maturity stages were calculated for the recovery and subjected to analyses.

2.4. Oil recovery

The Bligh and Dyer method (Bligh & Dyer, 1959) was employed to determine total oil content of coconut milk. The weight of oil separated after Alcalase aided process (VCO) was determined. Oil recovery was calculated

using the following equation (Mansor et al., 2012):

$$\text{Oil recovery (\%)} = (A / B) \times 100 \quad (1)$$

where A: Weight of VCO;
B: Weight of total oil in coconut milk.

2.5. Characteristics and quality of VCO

2.5.1. Determination of fatty acid profile

Fatty acid profile of VCO samples was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared according to the method of AOAC (AOAC, 2000). The prepared FAMEs were injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionisation detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m ´ 0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 ºC and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC /min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

2.5.2. Fourier transform infrared (FTIR) spectra analysis

FTIR analysis of VCO samples was performed in a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Prior to analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run. For spectra analysis, VCO sample (200µl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra, in the range of 4000–400 cm^{-1} (mid-IR region) with automatic signal gain, were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 ºC. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co., Ettlingen, Germany).

2.5.3. Colour determination

Colour of VCO samples were measured using a colourimeter (HunterLab, Model ColorFlex, VA, USA). The colour was reported in L^*, a^*, b^* values representing lightness, redness/greenness and yellowness/ blueness, respectively. Total difference in colour (ΔE*) and the difference in chroma (ΔC*) were also calculated using following equations:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

where, ΔL*, Δa* and Δb* are the differences between the corresponding colour parameter of the sample and that of white standard (L*= 93.55, a* = 0.84, b* = 0.37).

$$\Delta C^* = C^*_{sample} - C^*_{standard} \quad (3)$$

where, $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

2.5.4. Determination of moisture content

VCO samples were analysed for moisture content according to the method of AOAC (AOAC, 2000).

2.5.5. Measurement of viscosity

Viscosity of VCO samples was determined using a controlled-stress rheometer (Rheostress RS1, Thermo Hakke, Germany) with parallel plates (35 mm diameter). The samples were sheared at a constant shear rate of 100 s^{-1} over temperatures ranging from 25 to 80 °C and their viscosity was then measured.

2.5.6. Measurement of refractive index (RI), relative density and specific gravity

RI of VCO samples was determined using a refractometer (Model AR3D-AR64, Abbe, Hamburg, Germany). Measurements were performed at 40 ºC. The relative density and specific gravity of all VCO samples were measured according to the AOAC method (AOAC, 2000). Measurements were performed at 30 ºC.

2.5.7. Measurement of iodine value (IV), saponification value (SV), peroxide value (PV) and ρ-anisidine value (AnV)

IV, SV, PV and AnV of all VCO samples were analysed according to the method of AOCS (AOCS, 2009).

2.5.8. Measurement of acid value and free fatty acid (FFA)

Acid value of all VCO samples was determined by the method of AOCS (AOCS, 2009) and FFA was determined by the following equation, using the conversion factor of 2.81 for lauric acid.

$$\text{Acid value} = \% \text{ FFA} \times 2.81 \quad (4)$$

2.5.9. Thermal analysis by DSC

Thermal properties of VCO samples were determined using a differential scanning calorimeter (PerkinElmer, Model DSC-7, Norwalk, CT, USA). The DSC instrument was calibrated with indium (m.p. 156.6°C, ΔHf = 28.45 J/g). Samples (10–12 mg) were weighed into aluminum pans and were hermetically sealed. Prior to analysis of samples, the baseline was obtained from an empty hermetically sealed aluminum pan. Samples were subjected to the following temperature program: 80 °C for 5 min, cooled at 5°C/min to –80°C and held for 5 min. The same sample was then heated from –80 to 80°C at the same rate. Melting and crystallisation temperatures and enthalpy of samples were determined.

2.5.1. Cloud and pour points

Cloud and pour points of all VCO samples were determined according to ASTM D 2500 and ASTM D 97 standard method, respectively. Cloud and pour point analyser (Walter Herzog GmbH, Lauda-Königshofen, Germany) was used. The samples (50 mL) were cooled to -40^0C with continuous agitation. The temperature of first crystallisation, at which a thermometer in the sample was no longer visible, was considered as 'cloud point'. Pour point was defined as the temperature, at which oil sample was solidified and is not able to flow.

2.5.11. Flash point

Flash point of VCO samples were determined according to the ASTM D 93 standard method using a flash point tester (Pensky-Martens Model HFP 386, Walter Herzog GmbH, Lauda-Königshofen, Germany). The samples (100 mL) were heated using an electric heater. The temperature, at which the first flash appeared on the surface of sample, was recorded.

2.5.12. Sensory characteristics

Thirty non-trained panellists (aged between 25 and 40 years) were used for sensory evaluation. They were the students and staffs from the Department of Food Technology, who were acquainted with VCO. VCO samples from three different maturity stages were coded with 3-digit random numbers. VCO samples and one blind control MC sample with a volume of 20 mL were served at room temperature under the fluorescent daylight-type illumination. The panellists were asked to evaluate the difference from the blind control for colour, odour, viscosity, flavour and overall of VCO samples using 10-point scale (0, no difference; 9, extreme difference) (Meilgaard et al., 2006). Between samples, the panellists were asked to rinse their mouth with warm water.

2.6. Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Mean comparison was carried out by the Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1960). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Oil recovery

Varying oil recovery was observed, depending on maturity stages. The highest oil recovery (95.64%) was found when coconut milk from OMC was used ($p<0.05$), followed by

MC (84.45%) and IMC (61.06%). The result suggested that the emulsion of coconut milk from OMC was less stable than others. Therefore, Alcalase might be able to hydrolyse the proteins stabilising oil droplets in MC milk emulsion more effectively. Coconut milk is naturally stabilised by coconut proteins (McGlone et al., 1986). During germination, proteins in kernel were plausibly hydrolysed, in which free amino acids could be used for embryo. Man et al. (1996) reported that Alcalase played a significant role in destabilisation of coconut milk emulsion, thereby liberating free oil. On the other hand, low oil recovery (61.06%) was obtained when IMC milk was used. Proteins in coconut kernel at this stage might be more resistant, due to the high complexation. Therefore, the proteins could stabilise oil droplets in coconut milk effectively and thus oil released was lower. The result suggested that maturity stage of coconut fruit was an important factor governing the oil released from coconut milk emulsion when Alcalase was employed.

3.2. Characteristics and quality of VCO

3.2.1 Fatty acid compositions

Fatty acid profiles of VCO separated from coconut milk with three different maturity stages are shown in Table 1. Regardless of maturity stages, all VCO samples dominantly contained medium chain fatty acids (MCFA) especially lauric acid (49.74 – 51.18%). The second major fatty acid was myristic acid with 18.70 – 19.84%. The VCO from OMC had the highest lauric acid, whilst that from MC showed the highest myristic acid ($p<0.05$). The most abundant MCFA present in coconut oil is lauric acid, which is responsible for health benefits (Enig, 1996). MCFAs have 6 to 12 carbons, including caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids (Papamandjaris et al., 1998). Palmitic acid ranged from 8.16 to 8.87 g/100g. Caprylic acid and capric acid constituted 7.20 – 7.45 and 6.11 – 6.52 g/100g, respectively. Oleic acid was found in the range of 3.73-4.58 g/100g and the lowest content was found in VCO from MC milk ($p<0.05$). Stearic acid was found at low level, but VCO from MC showed the highest content, compared to others. In general, there was no marked difference in fatty acid profiles between VCO extracted from coconut milk having different maturity stages. Furthermore, the fatty acid composition was comparable to those guided by APCC standards. Due to the high level of saturated fatty acids, VCO could be solidified at low temperature with ease.

3.2.2. Fourier transform infrared (FTIR) spectra

FTIR spectra at wavenumber ranging from 4000 to 500 cm^{-1} of VCO prepared from coconut milk with three different maturity stages are shown in Fig. 1. Infrared mid region of the spectrum has been used for characterisation of vegetable oils and the absorption bands are associated with the vibration of particular functional groups (Guillén & Cabo, 1997). For all VCO samples, the major peak was found at wavenumber of 1742 cm^{-1}. Generally, the carbonyl absorption of the triglyceride ester linkage was observed at 1741 – 1743 cm^{-1} (Chaijan et al., 2006). Sánchez-Alonso et al. (2012) reported that the ester bond of hake lipids was found in the 1743–1740 cm^{-1} region. The result indicated that triglyceride containing ester bonds constituted in all oil samples. Major peaks were also observed at a wavenumber range of 2922 cm^{-1} and 2853 cm^{-1} indicating asymmetrical and symmetrical stretching of $–CH_2$, respectively. Furthermore, peaks at the wavenumbers of 1462 ($–CH_2$ bending), 1418 (cis =C–H bending), 1375 ($–CH_3$ bending), 1228 (–C–O stretch), 1153 (–C–O stretch; $–CH_2$ bending), 1109 (–C–O stretch), 963 (trans-CH=CH– bending out of plane), and 722 cm^{-1} (cis-CH=CH– bending) (Guillén & Cabo, 1997; Lerma-García et al., 2010) were also observed in all samples, irrespective of maturity stages. Overall, the similar spectra were observed in all VCO samples, which was in agreement with similar fatty acid profiles of the oils (Table 1).

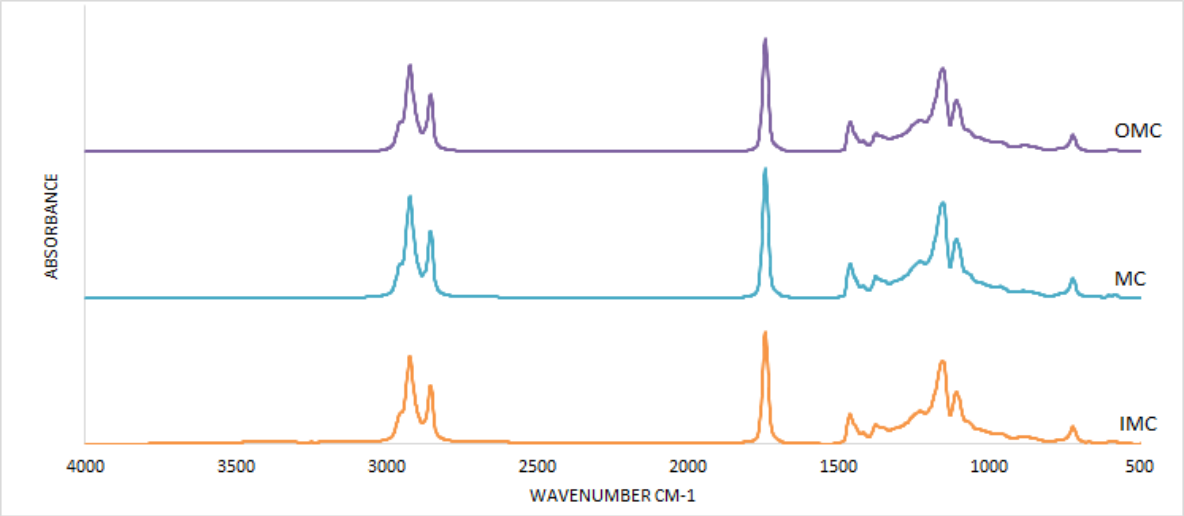

Figure 1. Fourier transform infrared (FTIR) spectra of virgin coconut oil with three different maturity stages. IMC: Immature coconut; MC: mature coconut; OMC: Overlay mature coconut.

Table 1. Fatty acid profile of virgin coconut oil with three different stages.

Fatty acids (g/100 g oil)	**IMC**	**MC**	**OMC**	**APCC Standards**
Caproic acid (C6:0)	0.32±0.03b	0.41±0.05a	0.31±0.02b	0.10 – 0.95
Caprylic acid (C8:0)	7.30±0.37a	7.45±0.41a	7.20±0.34a	4 – 10
Capric acid (C10:0)	6.37±0.11a	6.11±0.07b	6.52±0.10a	4 – 8
Lauric acid (C12:0)	50.32±0.23b	49.74±0.27c	51.18±0.10a	45 – 56
Myristic acid (C14:0)	18.98±0.16b	19.84±0.15a	18.70±0.16b	16 – 21
Palmitic acid (C16:0)	8.47±0.06b	8.87±0.07a	8.16±0.09c	7.5 – 10.2
Stearic acid (C18:0)	2.62±0.01b	3.08±0.02a	2.56±0.03c	2 – 4
Cis-9-Octadecanoic acid (C18:1 n-9)	4.58±0.03a	3.73±0.03b	4.58±0.06a	4.5 - 10
Cis-9,12-Octadecadienoic acid (C18:2 n-6)	0.93±0.00a	0.65±0.00c	0.70±0.01b	0.7 – 2.5
Arachidic acid (C20:0)	0.05±0.00a	0.05±0.00a	0.05±0.00a	-
Unidentified peak	0.07±0.00a	0.06±0.01a	0.03±0.00b	-

Values are mean ± standard deviation (n=3). Different lowercase letters in the same row indicate significant difference between means ($p<0.05$).
IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut

The peak near 3006 cm^{-1} was associated to the stretching vibration of C=C bonds. These bonds are mostly presented in unsaturated fatty acids (Henna Lu & Tan, 2009). However, the peak near 3006 cm^{-1} was absent in all VCO samples, indicating the low degree of unsaturation. This was in agreement with fatty acid profile (Table 1). From the spectra, there was no peaks at 3470 cm^{-1} and 1711 cm^{-1} detected in all samples, representing hydroperoxides and FFAs, respectively (Van de Voort et al., 1994; Guillén & Cabo, 1997). The result indicated that coconut milk from different maturity stages had no impact on oxidative stability of the resulting VCO.

3.2.3. Colour

The colours of VCO extracted from coconut milk with different maturity stages expressed as *L**, *a** and *b** values, are shown in Table 2. All

VCOs were clear like water and showed high *L**-value with the distinct coconut aroma. VCO separated from OMC milk showed the lowest *L** value ($p<0.05$), whereas the highest *a** and *b** values were found for the oil of IMC ($p<0.05$). Since VCO was extracted from a fresh coconut kernel, negligible pigments associated with deterioration were contaminated into the separated oil. The lower ΔE* and ΔC* were observed for the VCO of OMC ($p<0.05$). This was plausibly due to the differences in indigenous pigments found in kernel from coconut with different maturity stages. VCO is generally colourless and has natural pleasant coconut smell. The refining, bleaching, and deodourising (RBD) process may cause yellow colour of coconut oil (Marina et al., 2009). In the present study, no further refining process was implemented. As a result, colour and smell of VCO had natural characteristics.

3.2.4. Moisture content

Moisture content of all VCO samples was negligible (~0.1%) (Table 3). As per APCC standard, VCO must have moisture in the limited range (max 0.1%) (APCC, 2003). High moisture content indicated that VCO could be prone to hydrolysis process, leading to the enhanced rancidity (Raghavendra & Raghavarao, 2010). In the present study, all VCO samples had very low moisture content, thereby assuring their long shelf-life. Furthermore, oil fraction could be separated from aqueous phase effectively after Alcalase aided process. This was evidenced by low moisture content of separated VCO.

Table 2. Colour of virgin coconut oil with three different maturity stages.

Sample	*L**	*a**	*b**	ΔE*	ΔC*
IMC	97.13±0.20a	-1.68±0.03a	1.78±0.08a	4.15±0.26a	2.25±0.11a
MC	97.53±0.07a	-1.51±0.02b	1.26±0.02b	4.19±0.08a	1.56±0.04b
OMC	96.86±0.04b	-1.54±0.02b	0.96±0.04c	3.20±0.07b	1.29±0.05c

Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference between means ($p<0.05$). IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut

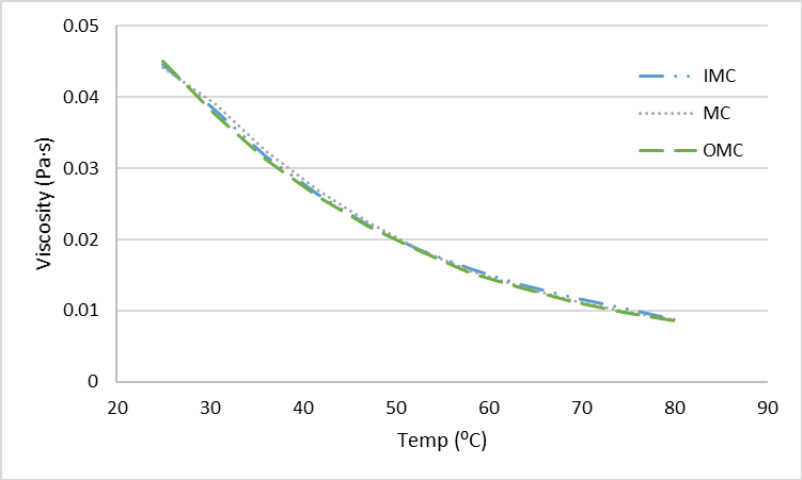

Figure 2. Viscosity of virgin coconut oil with three different maturity stages as a function of temperatures.
IMC: Immature coconut; MC: mature coconut; OMC: Overlay mature coconut.

Table 3. Properties of virgin coconut oil with three different maturity stages.

Sr. No.	APCC Parameters	IMC	MC	OMC	APCC Standards
1.	Moisture (%)	0.1±0.03a	0.1±0.01a	0.1±0.01a	Max 0.1
2.	Refractive index at 40°C	1.4486±0.00a	1.4486±0.00a	1.4486±0.00a	1.4480 – 1.4492
3.	Relative density	0.921±0.00a	0.921±0.01a	0.921±0.00a	0.915 – 0.920
4.	Specific gravity at 30 deg./30 deg. C	0.921±0.00a	0.921±0.01a	0.921±0.00a	0.915 – 0.920
5.	Iodine Value (g I_2/100g oil)	5.54±0.25a	4.70±0.10b	5.39±0.04a	4.1 – 11
6.	Saponification Value (mg KOH/g oil)	258.42±0.77a	256.76±0.75c	257.29±0.63b	250 – 260
7.	Free fatty acid (%)	0.06±0.03a	0.06±0.02a	0.06±0.02a	Max 0.2
8.	Peroxide Value (meq O_2/kg)	1.66±0.57a	1.66±0.58a	1.66±0.57a	Max 3
9.	ρ-anisidine	0.57±0.02a	0.57±0.02a	0.57±0.03a	-

The mean ± standard deviation, n=3. Different lowercase letters in the same row indicate significant difference between means ($p<0.05$). IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut

3.2.5. Viscosity

Viscosity of all VCO samples as a function of temperatures ranging from 25 to 80 °C is shown in Fig. 2.

All VCO samples showed a non-linear decrease in viscosity with increasing temperature. VCO with different maturity stages showed the similar viscosity over the temperatures tested. Heat applied to oil has been recognized to reduce intermolecular interactions by thermal molecular movement (Santos et al., 2005). Coconut oil mainly consists of medium chain fatty acid (MCFA) and hence its viscosity is lower as compared to oils with long chain fatty acid (Akhtar et al., 2009). Additionally, fatty acids with more double bonds do not have a rigid and fixed structure. The loosely packed structure is associated with more fluid-like nature (Abramovic & Klofutar, 1998). Although all VCO samples had slight difference in fatty acid composition, similar viscosity was noticeable. The results suggested that there was no marked difference in viscosity of VCO samples with three different maturity stages.

3.2.6. Refractive index (RI), relative density and specific gravity

RI values of different VCO samples are shown in Table 3. All samples had the same RI (1.4486), which was within the standard range (1.4480 – 1.4492) of VCO guided by APCC (APCC, 2003). Refractive Index (RI) is related with the degree of unsaturation in lipid. RI increases proportionally with increasing number of double bonds. It is also used to indicate impurity of the oil since each substance has a different RI (Kamariah et al., 2008).

Both relative density and specific gravity were similar between all VCO samples obtained from coconut milk with various maturity stages. The values were 0.921, which were close to the standard range (0.915 - 0.920) following the guidance of APCC (APCC, 2003). Specific gravity is the ratio of the density of a substance to that of a reference substance at the same temperature. The density varies according to the temperature of oil as reported by Kamariah et al. (2008). The result indicated that VCO from coconut milk having different maturity stages more likely had similar compositions, especially lipids or fatty acids. This was confirmed by

similar fatty acid compositions between samples (Table 1).

3.2.7. Iodine value (IV)

All VCO samples had IV with the range of 4.70-5.54 g I_2/100g oil (Table 3), which were in accordance with APCC standard (APCC, 2003). IV is used to measure the degree of unsaturation in fatty acids. The lowest IV was observed for the oil of MC ($p<0.05$), which was correlated with lower contents of oleic acid (C18:1) and linoleic acid (C18:2) (Table 1). The low IV found in all VCO samples indicated that the VCO samples were highly saturated, which ensured their stability against oxidative rancidity. Additionally, they could be solidified at low temperature, e.g. refrigerated temperature.

3.2.8. Saponification value (SV)

The relative high SV were found for all VCO samples, ranging from 256.76 to 258.42 mg KOH/ g (Table 3). High SV of all VCO samples indicated that VCOs contained high amount of short chain or medium chain fatty acids (Marina et al., 2009). This coincided with the high contents of fatty acids with chain length of C8-C16 (Table 1). Amongst all VCO samples, slight differences in SV were observed. This was probably due to some differences in fatty acid composition. The values were within the standard range (250 – 260 mg KOH/ g) guided by APCC standard (APCC, 2003). Saponification value (SV) is an index of the average molecular weight of triglycerides present in sample. Saponification is the process of breakdown of a fat sample into glycerol and fatty acids by treatment with strong base (KOH). SV is inversely proportional to the molecular weight of triglycerides (Opoku-Boahen et al., 2012). The long chain fatty acid on glycerol backbone gives a low SV, however short chain counterpart renders a high SV (Marina et al., 2009).

3.2.9. Peroxide value (PV)

Low PV (1.66 meq O_2/kg) was found in all VCO samples (Table 3). PV of VCO samples were within the standard range (max 3 meq O_2/kg) guided by to APCC standard (APCC, 2003). PV is used to determine the primary oxidation products of oil (Choe & Min, 2006). Low PV in VCO indicated that low lipid oxidation took place in all samples. This was more likely due to the low content of unsaturated fatty acids confirmed by iodine value. Conversely, saturated fatty acids were prevalent (Table 1). This made VCO more stable to oxidation. When comparing PV between samples, the similar PV was observed, suggesting the similar oxidative stability of VCO from coconut milk with different maturity stages. This was in agreement with FTIR spectra, in which no peak representing hydroperoxide was detected (Fig. 1).

3.2.10. Anisidine value (AnV)

Low AnV was observed in all VCO samples (0.57) (Table 3). There were no differences in AnV among the samples ($p>0.05$). According to Rossell (1983), oils with an AnV below 10 were considered as good quality. AnV is used to determine the secondary oxidation products of oil. The primary oxidation products i.e. peroxides are not stable and decomposed to the secondary products such as aldehydes, which are responsible for rancid odour and taste. An increased AnV indicates an increase in the amount of the non-volatile oxidation product (Choe & Min, 2006). Low AnV of all samples was in agreement with low PV, regardless of maturity stages. The results reconfirmed high oxidative stability of all VCO samples.

3.2.11. Free fatty acid (FFA)

FFA contents of VCOs separated from coconut milk with various maturity stages are shown in Table 3. All VCO samples had the low FFA content (0.06%). The result indicated that ester bonds of triglycerides were cleaved to low degree. This was in agreement with FTIR spectra, in which no peak at 1711 cm^{-1} representing the free fatty acid was noticeable (Fig. 1). FFAs are responsible for unacceptable taste and aroma in oils and fats. It was reported that VCO produced by enzymatic process

contained low FFA with a good smell and long lifetime (Rahayu et al., 2008). Marina et al. (2009) found that FFAs were high in coconut oils having high moisture content. There was no difference in FFA content amongst VCOs obtained from coconut with different maturity stages. FFA contents of all VCO samples were below APCC standard (APCC, 2003), indicating their good initial quality.

3.2.12. Thermal behaviour

Melting and crystallisation temperatures of all VCO samples are shown in Table 4.

Table 4. Melting and crystallisation temperatures and enthalpy of virgin coconut oil with three different maturity stages.

Samples	Melting (Endotherm)		Crystallisation (Exotherm)	
	Temperature(°C)	Enthalpy (J/g)	Temperature(°C)	Enthalpy (J/g)
IMC	23.30±0.37b	112.16±1.05b	-1.89±0.07b	-104.29±1.68a
MC	24.87±0.40a	117.96±2.49a	-1.13±0.26a	-110.73±0.75b
OMC	23.94±0.49ab	114.17±0.91b	-1.74±0.18b	-105.78±0.37a

Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference ($p<0.05$). IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut

Table 5. Cloud, pour and flash points of virgin coconut oil with three different maturity stages.

Samples	Cloud point (°C)	Pour point (°C)	Flash Point (°C)
IMC	18.5±0.70a	15.5±0.70a	232±2.82a
MC	16.0±1.41a	13.5±2.12a	227±0.70a
OMC	18.0±1.41a	15.0±0.00a	227±0.70a

Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference between means ($p<0.05$).IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut

Table 6. Difference from blind control test scores of virgin coconut oil with three different maturity stages.

Samples	Colour	Odour	Viscosity	Flavour	Overall
IMC	0.32±0.07a	0.94±0.40a	0.47±0.13a	0.61±0.29a	0.85±0.29a
MC	0.33±0.19a	0.99±0.32a	0.56±0.15a	0.51±0.31a	0.86±0.31a
OMC	0.36±0.21a	0.97±0.49a	0.55±0.24a	0.51±0.19a	0.96±0.56a

Values are mean ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference ($p<0.05$). IMC: immature coconut, MC: mature coconut and OMC: overlay mature coconut

Endothermic peaks, representing melting point, were observed for VCO from MC, OMC and IMC at 24.87, 23.94 and 23.30 °C, respectively. Exothermic peaks of VCO from MC, OMC and IMC were found at -1.13, -1.74 and -1.89 °C, respectively. Generally, the highly saturated oil melts at higher temperatures than the unsaturated oil. The former oil also undergoes crystallisation at higher temperature. VCO sample from MC had lower degree of unsaturation than others as indicated by lower IV (Table 3). Thus, it was expected to melt and crystallise at higher temperature when compared to OMC and IMC samples. Melting and crystallisation, two commonly used physical events to characterise thermal behaviour of VCO samples, require the intake or release of thermal enthalpy, respectively. The melting and crystallisation enthalpies of all VCO samples are shown in Table 4. VCO samples from MC showed the highest melting enthalpy at 117.96 J/g ($p<0.05$), followed by OMC (114.17 J/g) and IMC (112.16 J/g), respectively. Crystallisation enthalpy of MC was -110.73 J/g, and OMC (-

105.78 J/g) and IMC (-104.29 J/g). Generally, the oil samples with high degrees of saturation showed slightly higher enthalpy than those with a high degree of unsaturation (Tan and Che Man, 2000). VCO sample from MC had lower degree of unsaturation. As a result, higher enthalpy was required for melting process and less energy was released during crystallisation process. The obtained results were in accordance with those reported by Tan and Che Man (2000) for coconut oil, in which melting and crystallisation temperatures were observed at 22.45 and -0.70 ^{0}C. Melting and crystallising enthalpies were 120.6 J/g and -106.5 J/g, respectively. DSC results indicated that the differences in the thermal behaviour of VCO samples with three different maturity stages were more likely associated with different chemical compositions.

3.2.13. Cloud and pour points

Cloud and pour points of VCO samples with three different maturity stages are shown in Table 5. In general, there was no difference in both cloud and pour points amongst all samples ($p>0.05$). Cloud and pour points of VCO samples in the present study were higher than those reported by Akhtar, et al. (2009), in which coconut oil had the higher cloud and pour points (13.1 and 12.7 °C) than other oils. This was plausibly owing to the differences in process, variety, and fatty acid compositions. The cloud point is the temperature at which a cloud is formed in the oil due to the beginning of crystallisation and pour point is the temperature at which the oil is solidified enough to resist the flow (Akhtar et al., 2009; Bello et al., 2015). The chain length of fatty acids affects the cloud and pour points. With the shorter chain length, oil has higher cloud and pour points (Akhtar et al., 2009). VCO contained high amounts of short chain or medium chain fatty acids (Marina et al., 2009). The results suggested that high cloud and pour point of VCO samples were more likely due to the presence of short or medium chain fatty acids.

3.2.14. Flash point

Flash point was observed at very high temperature for all VCO samples as shown in Table 5. However, there was no difference amongst the samples ($p>0.05$). Flash point specifies the temperature, at which a flash appears at any point on the surface of the sample. The oil with low flash point can ignite when exposed to heat easily (Bello et al., 2015). The flash points for all VCO samples were very high. This made VCO suitable for deep frying and cooking.

3.2.15. Sensory characteristics

There were no differences in colour, odour, viscosity, flavour and overall scores amongst different VCO samples ($p>0.05$) (Table 6). All VCO samples were transparent and clear like water. All samples had natural odour and flavour without fermentation smell. It can be inferred that there were no differences in sensory properties of VCO samples with different maturity stages.

4. Conclusions

VCO separated from three different maturity stages of coconut had similar fatty acid composition and other properties. VCO had high contents of MCFAs, especially lauric acid. However, VCO separated using Alcalase showed the highest recovery when coconut milk from overlay mature coconut was used as starting material. The maturity stages had the influence on oil recovery, in which the overlay mature coconut rendered the highest yield. Nevertheless, the maturity stages had no influence on properties, oxidative stability and sensory characteristics of resulting VCO.

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EFFECTS OF CARBON SOURCES, NITROGEN SOURCES AND SALTS ON GROWTH OF *SACCHAROMYCES BOULARDII*

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ABSTRACT
To investigative the effect of carbon sources, nitrogen sources and salts on growth of *Saccharomyces boulardii*, the optical density (OD) in the medium was measured at 560nm by the single factor text. The results of this study indicated that *S. boulardii* grew better in medium consisted of 4% glucose, 0.8% soybean peptone, beef paste or yeast extract, 0.6% bovine serum or malted milk, and the addition of Na_2HPO_4 had a promotion on growth of *S. boulardii*..

1. Introduction

In recent years, the use of functional foods containing probiotics for health promotion and disease prevention has increased significantly. According to the definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO Working Group, 2002). Among the most studied probiotics are strains of *Saccharomyces boulardii*. *S. boulardii* is a strain of probiotic yeast isolated from litchi fruit in Indonesia, its optimum temperature is 37 ^{0}C, both in vitro and in vivo, which belongs to the subspecies of *Saccharomyches cerevisiae* (Martins, et al.,2013; Buts, *et al.*, 2006, Edwards, *et al.*,2007). *S. boulardii* has the unique biological activity, it can inhibit the growth of some pathogenic microorganisms.

From the beginning of the 1980s, studies have evaluated the profits of *S. boulardii* for the host and determined its mechanisms of action (Czerucka, *et al.*,2007). Currently *S. boulardii* is widely applied in the prevention and treatment of several of diarrhea and inflammatory bowel disease (Pothoulakis, *et al.*, 2009). This yeast has been used in many countries as lyophilized product for the treatment of diarrhea in children and adult (Fietto, *et al.*,2004, E lm, *et al.*,2010). Pharmacokinetic studies have shown that, after continuous oral, *S. boulardii* can exist in the colon stability about 3 days, and after the cessation of the drug, it will be excreted in feces, not be colonized in the intestine (Czerucka, *et al.*,2002; Blehaut, *et al.*,1989).

Moreover, the use of bacteria as probiotics may spread its drug resistance genes to pathogens; however, using yeast as probiotics there is no such a problem, it is a safe and micro-ecological regulating agent (Hennequin, *et al.*,2000; Perapoch, *et al.*,2000). As one kind of probiotics, *S. boulardii* has the advantage of natural, non-toxic side effects, safe and reliable, no residue, ect. *S. boulardii* belongs to fungi preparation, no degradation by antibiotics, which can be used in conjunction with antibiotics, and it can effectively prevent the use of antibiotics with dysbacteriosis (Akil, *et al.*,2006). *S. boulardii* can also stimulate the immune system, as the application of feed additives has been recognized by many

countries, including China, and thus it will have a broad prospect of application in livestock industry (Giang, *et al.*,2012; Oeztuerk, *et al.*,2005).

S. boulardii as probiotics, to ensure its biological effect, the intestine must achieve a sufficient number of viable cell count. Therefore, this paper studied the effect of some medium components on growth of S. boulardii, and initially identified the culture conditions which contributed to the growth of the cells (Du, *et al.*,2012).

2. Materials and methods

2.1. Microorganism

The probiotic strains employed in the present study was *Saccharomyches boulardii*, which were obtained from School of Food and Biological Engineering, Shaanxi University of Science & Technology. All chemicals used were of analytical grade.

The amount of *S. boulardii* strains was dissolved in sterile water, added to the configured YPD medium and cultivated 36h under the temperature of 37℃.The activated yeast suspension was added to the YPD medium with the amount of 2%, and activated twice under the same conditions.

2.2. Culture conditions

YPD was prepared, consisting of yeast powder 1%, glucose 2% and peptone 2%.

2.3. Growth measurement

The growth of strains was monitored by measuring the optical density at 560nm (OD_{560}) through a spectrophotometer (SP-756PC, Shanghai Spectrum Instruments Co., Ltd., Shanghai. China).

3. Results and discussion

3.1. Effect of carbon sources on growth of *S. boulardii*

The carbon sources were added to the tubes according to the concentration gradient, and there was 10ml broth whose composition was 1% yeast extract and 2% peptone in each tube, then it was sterilized after cooling, inoculated with 2% *S. boulardii* that cultured 36h under the temperature of 37℃ in the shaker, and measured the OD value at 560nm.The results showed as Figure 1.

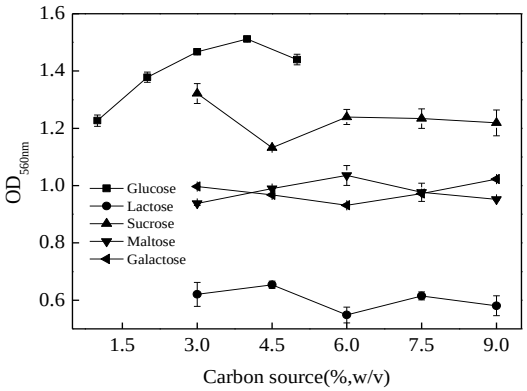

Figure 1. Effect of carbon sources on growth of *S. boulardii*

Figure 1 showed that with the increasing concentration of glucose and maltose, the OD value increased at first and then decreased, and the optimum concentration on growth of *S. boulardii* were 4%, 6%, respectively. However, when the carbon source was sucrose or galactose, the OD value decreased and then increased, and the optimal concentration were 3%, 9%, respectively. While the concentration of glucose, sucrose, maltose, galactose were 4%, 3%, 6%, 9%, the OD value reached 1.512, 1.322, 1.036, 1.023, respectively. Therefore, glucose was the optimal carbon source for the growth of *S. boulardii*, and sucrose, maltose, galactose were followed, whereas the effect of lactose on growth of *S. boulardii* was the worst. The possible reason was that the ability of the yeast to degrade or ferment lactose was poor. So the next experiment selected glucose as carbon source.

3.2. Effect of nitrogen sources on growth of *S. boulardii*

3.2.1. Effect of organic nitrogen sources on growth of S. boulardii

Basal medium was composed of 2% peptone and 2% glucose, and used soy peptone, tryptone, beef extract, yeast extract, bovine serum, casein

hydrolysate, malted milk, urea instead of original yeast extract, respectively. These nitrogen sources were added to the tube in the concentration gradient, and there was 10ml broth in each tube. Then it was sterilized after cooling, inoculated with 2% *S. boulardii* that cultured 36h under the temperature of 37℃ in the shaker, and measured the OD value at 560nm. The results showed as Figure 2.

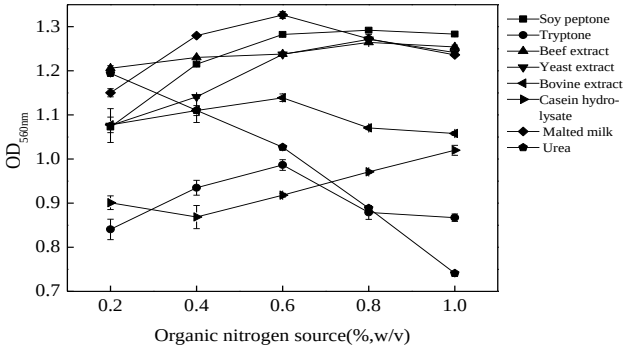

Figure 2. Effect of organic nitrogen sources on growth of *S. boulardii*

As Figure 2 showed, with increasing concentration, the OD value of *S. boulardii* increased and then decreased, when the organic nitrogen sources were soy peptone, tryptone, beef extract, yeast extract, bovine extract, malted milk, respectively, and the optimal concentration of soy peptone, beef extract and yeast extract on growth of *S. boulardii* were 0.8%, which of bovine extract and malted milk on the growth of *S. boulardii* were 0.6%. While the concentration of casein hydrolysate increasing, the OD value reduced and then raised, and the optimal concentration was 1%. However, the OD value reduced rapidly with urea as the organic nitrogen source, which demonstrated that urea significantly inhibited the growth of *S. Boulardii.*

Except urea, when the other 7 organic nitrogen source were at the optimal concentration, the OD value were 1.292, 0.987, 1.265, 1.272, 1.139, 1.020, 1.327, respectively, which indicated that compared with casein hydrolysate and tryptone, soy peptone, beef extract, yeast extract, bovine serum, malted milk had a greater influence on the growth of *S. boulardii.*

3.2.2. Effect of inorganic nitrogen sources on growth of S. boulardii

Basal medium was composed of 2% peptone and 2% glucose, and used inorganic nitrogen sources of $(NH_4)_2SO_4$, diammonium hydrogen citrate, ammonium citrate, NH_4NO_3 instead of the original yeast extract, respectively. These nitrogen sources were added to the tube in the concentration gradient, and there was 10ml medium in each tube. Then it was sterilized after cooling, inoculated with 2% *S. boulardii* that cultured 36h under the temperature of 37℃ in the shaker, and measured the OD value at 560nm. The results showed as Figure 3.

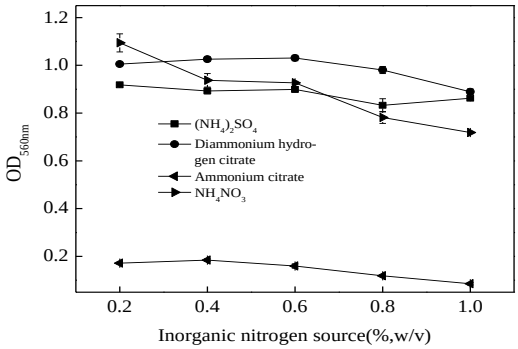

Figure 3. Effect of inorganic nitrogen sources on growth of *S. boulardii*

According to the results shown in Figure 3, with the concentration of ammonium citrate increasing, the OD value increased slowly from 0.172 at ammonium citrate 0.2% to 0.185 at ammonium citrate 0.4%, and then decreased slowly from 0.185 at ammonium citrate 0.4% to 0.086 at ammonium citrate 1%. As the concentration of NH_4NO_3 increasing, the OD value reduced from 1.094 at NH_4NO_3 0.2% to 0.719 at NH_4NO_3 1%. While the inorganic nitrogen sources were $(NH_4)_2SO_4$, ammounium hydrogen citrate, respectively, the changes of the OD value were not noticeable. Figure 3 indicated that compared with other 3 inorganic nitrogen sources, the addition of ammonium citrate had an inhibition on growth of *S. boulardii*. The yeast can hardly grow in the presence of ammonium citrate.

3.3. Effect of salts on growth of *S. boulardii*

YPD broth was used as basal medium, and supplemented with salts, which were K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, $MgSO_4$, $CaCl_2$, sodium citrate, sodium glutamate, respectively. These salts were added to the tube in the concentration gradient, and there was 10ml medium in each tube. Then it was sterilized after cooling, inoculated with 2% *S. boulardii* that cultured 36h under the temperature of 37℃ in the shaker, and measured the OD value at 560nm.The results showed as Figure 4 and Figure 5.

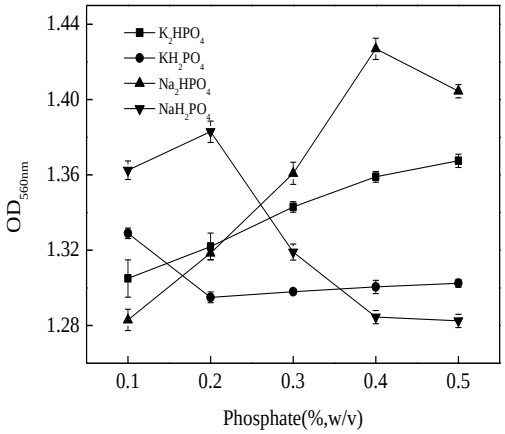

Figure 4. Effect of phosphates on growth of *S. boulardii*

As shown in Figure 4, the OD value increased rapidly and then decreased with the concentration of NaH_2PO_4 and Na_2HPO_4 increasing. And the optimal concentration of NaH_2PO_4 and Na_2HPO_4 were 0.2%, 0.4%, respectively. The OD value increased from 1.305 at K_2HPO_4 0.1% to 1.368 at K_2HPO_4 0.5% with the concentration increasing. The OD value decreased at first and then tended to be flat along with the consistence of KH_2PO_4 increasing, and the optimal concentration was 0.1%.

As Figure 5 revealed that, the OD value rapidly reduced from 1.280 to 1.054 with the concentration of $CaCl_2$ increasing, and it decreased from1.214 to 0.677 as the concentration of sodium citrate increasing. While the changes of the OD value were not significant with the concentration of $MgSO_4$, sodium glutamate increasing.

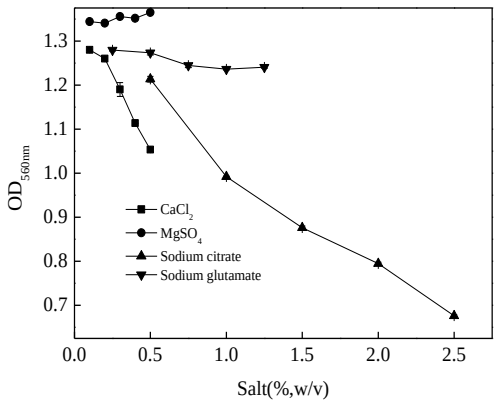

Figure.5. Effect of other salts on growth of *S. boulardii*

Figure 4 and Figure 5 indicated that compared with other salts, *S. boulardii* grew better in the presence of phosphates, and almost determined the addition of phosphates had a promotion on growth of *S. boulardii*, in which the effect of NaH_2PO_4 was most obvious. When the concentration of NaH_2PO_4 was 0.4%, the OD value reached 1.368. Sodium glutamate and $MgSO_4$ also had a promoting effect on the growth of *S. boulardii*, but its effect was not obvious. However, $CaCl_2$ and sodium citrate significantly inhibited the growth of *S. boulardii.*

4. Conclusions

The glucose was the optimum carbon source compared with others, and the optimal concentration of glucose was 4%. When the nitrogen source was 0.8% soybean peptone, 0.8% beef paste, 0.8% yeast extract, 0.6% bovine serum, 0.6% malted milk, respectively, it could significantly promote the growth of *S. boulardii.* Moreover, the addition of phosphates played an important role in promoting growth of *S. boulardii*, especially NaH_2PO_4, and its optimal concentration was 0.4%.

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THE EFFECTS CLO_2 & O_3 WITH MAP ON MICROBIAL LOAD REDUCTION OF MUNG BEAN SPROUTS

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Modified atmosphere packaging and microbial load.

ABSTRACT

Many of the agricultural products which are highly-damped and nutrient-rich become microbially spoiled after a short while and are subsequently omitted from the human food chain. Therefore, employing different techniques of food preservation with the intention of increasing their shelf life and reducing their waste is extremely important. Accordingly, the present study aimed to explore the effect of antiseptic compounds including Sodium Hypochlorite and Ozone packed with modified atmosphere in which CO_2 density was 1.5 % and 2.5% and conventional air at three different times (3,6 and 9 days) on the microbial spoilage of Mung. The results revealed that Sodium Hypochlorite plays a more effective role on the reduction of microbial load in comparison with Ozone. It was also found that packaging with modified atmosphere is effective on the reduction of mesophilic aerobic bacteria but the amount of mold and yeast increased due to the high levels of perspiration and water ellipsis in the packets.

1.Introduction

In recent years, the fruit production and vegetable packaging industry has improved considerably (annually about 10%). Many factors contribute to this rapid growth, one of which is an increase of the demands of consumers because of fruits and vegetables' good impacts on human health and their comfort of production and consumption (Rico *et al.*, 2007).

Over the past decade, the use of seed sprouts in many countries has increased due to their relatively easy production and nutritional value which has increased the epidemic of infectious diseases caused by eating contaminated products. For example, alfalfa and mung sprouts often are involved in outbreaks of disease due to the Salmonella and E. coli O157: H7 in North America and Canada (Jorge F *et al.*, 2013).

Mung bean sprouts were responsible for the outbreaks of salmonellosis in Ontario of Canada in the year 2005 when more than 600 cases were reported (Canada Health, 2012). High nutritional value of this new product acts as an excellent source for micro-organisms growth due to nutrients. In addition, the seed buds sprout under conditions of heat and humidity which makes an ideal place for the accumulation and replication of bacteria.

In the sprout industry, reducing the risk of diseases arising from them through the use of chemical disinfectants to decline microbial load of sprouts is preferred because they are more economically viable than other technologies.

One of the disinfectants which is used for this purpose is sodium hypochlorite. The effect of this material on reducing the production of spoilage and pathogenic bacteria during post-harvest processing has been fully studied. Sodium hypochlorite is one of the most frequently used disinfectants in the fresh fruits

and vegetables packaging industry. In Brazil, the most common method for cleaning and disinfecting vegetables is putting the vegetables in a sodium hypochlorite solution with the concentration of 200 mg/L for 15 min. (Gill *et al.*, 1996).

Ozone is a strong oxidizing compound which has recently been the focus of many studies which intended to utilize it as a suitable disinfectant for the packaging of fresh fruit and vegetables. These studies have revealed that this material has an inhibitory effect on a wide range of microbes (Kim *et al.*, 1999). Todays, ozone is widely used for water and nutrient disinfecting (Guzel cesium *et al.*, 2009).

Although treatment with chemical disinfectants is effective in preventing foodborne pathogens from foods, some cells may still remain alive or may only get damaged by the chemical material. These cells can revitalize and grow in food during the storage phase. Therefore, employing other maintenance methods such as MAP after treatment with chemical substances may make it possible to keep a controlled amount of food pathogens in foods which remain after treatment with chemical substances (Jayas & Jeyamkondan, 2002). Success in modified atmosphere packaging method requires knowledge about the correct gas mixture inside the package and the use of suitable coating materials. While the appropriate choice of gas condition causes an increase in the postharvest life of the product, inappropriate conditions such as low oxygen or high carbon dioxide concentration cause an anaerobic respiration in the product and lead to the reduction of its durability (Mortazavi *et al.*, 2006).

In this study, the use of ozone water and sodium hypochlorite with modified atmosphere packaging and their probable impact on the reduction of micro flora and external quality of sprouts are examined.

2.Materials and methods

2.1. Preparation sample

Mung bean (from Dezful area) and polyethylene polyamide plastic with 80 micron thickness were purchased from the market for packaging. After cleaning and re-cleaning the prepared seeds, they were washed with the usual water and were again rinsed with water containing 12ppm chlorine (CLO_2). Then, the washed seeds were soaked after being transferred into stainless steel containers or glazed with water containing chlorine 4ppm for 15 hours at 22-25 ° C . At the end of this soaking phase, the puffy seeds were transferred to the special baskets and were kept for 5 hours to drain out the excess water. The mentioned baskets were moved to the germination set after dewatering stage.

At this stage, the amount of humidity, temperature, and light have particular importance. Temperature is 25-27 ° C, humidity is 85% and germination period is 3 to 5 days.

2.2. Washing and disinfecting of sprouted mung bean

Sprouted mung beans were divided into three parts. One part was washed with water and the other part was disinfected with sodium hypochlorite and last part was disinfected with ozone water.

2.2.1.Disinfecting mung bean sprouts with sodium hypochlorite

Mung bean sprouts were placed for 10 minutes in a solution with the concentration of 150ppm of sodium hypochlorite and then were rinsed with water and water was drained out.

2.2.2.Disinfecting mung bean sprouts with ozone water

Ozone production was done with ozone generator machine (OZONICA SERIES) that was connected to an oxygen generating machine for feeding with pure oxygen, and ozone was produced with 1.5 ppm concentration. The amount of ozone was adjusted based on the oxygen input into ozone generating machine and its ppm amount was measured with portable devices using a colorimetric assay kit DPD-No.4. Produced ozone gas was entered in a stainless steel chamber containing 50 ml of water and was distributed by stirring like fine

bubbles into the water chamber. Baskets containing the sprout were immersed into it for 10 minutes and were placed in a solution of 1% sodium thiosulfate to neutralize the residual ozone and were then placed on the sanitary conditions to dry.

2.3.Packaging

Sprouts washed with water and disinfected with sodium hypochlorite and ozone water were shed separately into containers (disposable) of polyethylene and were packaged in three modes: normal air, gas concentration 1.5% and 2.5% CO_2 with MAP packaging machine (Table1) and were stored at 4 ° C for 6, 3 and 9 days, respectively.

2.4.Statistical analysis

The statistical analysis was done through SPSS, version 19 (Stanford, California, USA). The results were Calculated based on Bon Ferroni at alpha level 0.05 %.

Table 1. Treatments applied in this research

Timar name	Cod	Timar name	Cod	Timar name	Cod
Without disinfection & Normal air	I	disinfection with sodium hypochlorite & Normal air	IV	disinfection with ozone & Normal air	VII
Without disinfection & 1.5% CO_2	II	disinfection with sodium hypochlorite & 1.5% CO_2	V	disinfection with ozone & 1.5% CO_2	VIII
Without disinfection& 2.5% CO_2	III	disinfection with sodium hypochlorite & 2.5% CO_2	VI	disinfection with ozone & 2.5% CO_2	IX

2.5.Microbial testing

Before opening the packages, gas composition of the package was determined using a gas analyzer. Then 10 g of mung bean sprouts were weighed and mixed well with 90 ml of buffered peptone and ether in a blender for 2 min and buffered peptone and ether ten dilutions to 10^{-7} were prepared. The cultivation environments of PCA, YGC, VRB and MRS agar were utilized for cultivation of aerobic mesophilic bacteria, molds and yeasts, coliform bacteria, and lactic acid bacteria each of which were incubated respectively at 30 °C for 24-48 h, at 25 °C for 3-5 days, at 37 °C for 24 h and at 30 °C for 24-48 hours (Bahreini *et al.*, 2011a).

2.5.1.Investigating the sensory and physical properties of mung bean sprouts

The sensory and physical properties of mung bean sprouts were evaluated based on visual observations and taste test by five persons. Based on the amount of created changes that were observable, the properties were graded from zero to 5. The physical properties under study included: discoloration, colorlessness and transparency, being slimy and wilting and sensory properties based on flavor and taste changes (Bareini *et al.*, 2011a).

3. Results and discussion

3.1. The Effect of Ozone Water and Sodium Hypochlorite on the Microbial Load of Mung Bean Sprouts

In the Table 2, the results of the influence of ozone water at 1.5 ppm concentration and sodium hypochlorite at 150 ppm concentration on the normal flora of mung bean sprout have been presented. Population of bacteria has diminished in disinfected packages compared to packages that were not disinfected. Disinfection with sodium hypochlorite has been shown to have a greater reduction in the number of mesophilic aerobic bacteria, coliform and lactic acid bacteria in comparison to disinfection with

ozone (Figure 1). The lowest amount of mesophilic aerobic and lactic acid bacteria in disinfected packages was observed on the third day of the experiment. The whole population of coliforms was made negative through disinfection (Figure 1).

Table 2. Average number of bacteria

Bacteria	Time (day)	I	II	III	IV	V	VI	VII	VIII	IX
Aerobic mesophilic	3	a8.46A	ab8.33A	b8.24A	cf7.55a	cd7.41A	d7.33A	e7.76A	e7.75A	ef7.64A
	6	a8.61AB	b8.46A	b8.37A	c7.74B	d7.63B	e7.5B	f7.96B	c7.83A	c7.76B
	9	a8.76B	a8.46A	acde8.33A	b7.84C	b7.75B	b7.67C	bc7.93AB	bd7.96B	be7.87C
Lactic acid	3	a8.52A	a8.38A	a8.42A	b7.73A	c7.45A	c7.34A	b7.85A	b7.68A	c7.41A
	6	a8.09B	ab7.83B	abe7047B	bcf7.18B	cdg6.76B	d6.33B	efh7.41B	bgh7.06B	di6.2AB
	9	a7.6C	ab7.31C	ab6.91C	b6.8C	c5.91C	d4.57C	ab6.9C	c5.93C	c5.33B
Mold and yeast	3	a4.51A	ae4.67A	b5.17A	c3.72A	d4.18A	a4.52A	ad4.36A	ae4.59A	be4.89A
	6	af5.03B	bgh5.51B	bc5.86AB	d4.1B	e4.66B	bgh5.57A	ae4.84B	fg5.3B	ch5.76B
	9	adgh5.72C	Abefh6.0C	ac6.47B	d4.45C	degh5.19C	adgh5.31A	bfg4.93B	bcg6.0C	ch6.35C
coliform	3	a2.28A	ab2.31A	b2.43A	A0c	A0c	A0c	A0c	A0c	A0c
	6	a2.59B	a2.53A	b2.69B	A0c	A0c	A0c	A0c	A0c	A0c
	9	a2.76B	b2.88B	b2.94C	A0c	A0c	A0c	A0c	A0c	A0c

* Latin small letters (a through i) show significant differences between averages of bacteria in a variety conditions of disinfection and packaging in each unit of time (in a row).
* Latin capital letters (A through C) show significant difference between the averages of bacteria at period of time in each variety of disinfection and packaging (in a column)

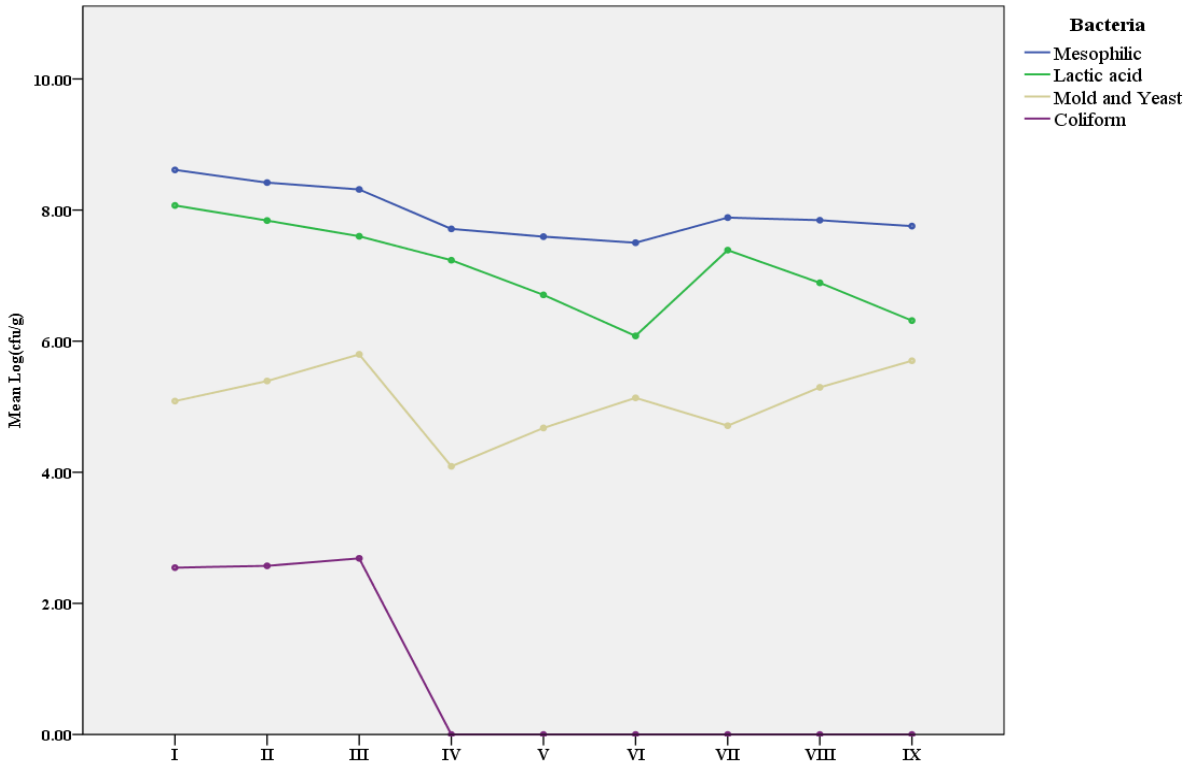

Figure 1. The changes of average number of bacteria

3.2. Effect of CO_2 Gas on the Microbial Load of Mung Bean Sprouts

As shown in Table 2, CO_2 gas was effective in reducing the aerobic mesophilic microbes. In packages containing 2.5% CO_2 gas, more reduction was observed in the mesophilic aerobic bacteria population compared to packets with less gas content and with no gas. The lactic acid bacteria population in packages containing 2.5% CO_2 has more reduction than packages containing 1.5% c_2 gas and packages with no gas. Conversely, molds and yeasts in packages containing 2.5% CO_2 have greater increase than packages containing 1.5% CO_2 gas and with no gas. Evaluation of population of coliforms was not possible due to the effect of disinfection on them. According to Fig.1. it was observed that the simultaneous effect of disinfection and packaging with CO_2 gas caused more reduction in mesophilic aerobic and lactic acid bacteria than the packages that were not disinfected containing gas and also, disinfected packages without gas.

The influence of disinfectants has been accepted on reducing the microbial load of packaged vegetables. It has also been proven that in order to improve the quality and to increase the half-life of packaged fresh products, disinfection stage should be applied. Washing vegetables with water alone cannot have a significant impact on the reduction of contamination load. According to studies conducted by different researchers, it was proved that rinsing with water alone could reduce the population of pathogenic bacteria from the surface of vegetables approximately up 0.2 to 1.1 logarithm cycle (Sapers & Jones 2006) (Velazquez *et al.*, 2009). Studies have demonstrated that mung bean sprouts washed with chlorine at room temperature have generally better logarithmic reduction in the number of bacteria with increasing amount of chlorine and peroxy acetic acid (PAA) to (Shan Yu Neo *et al.*, 2013).

There are several reports on the effects of ozone on microbial quality of lettuce, Bahraini & et al (2014) reported that five ozone water concentrations (0.6, 0.8, 1.2, 1.6 and 2 ppm) could reduce aerobic mesophilic bacteria population, mold and yeast, coliform bacteria and lactic acid bacteria respectively up to 1.54, 0.94, 1.94 and 1.35 logarithmic cycle.

In JIN & LEE's (2007) study, the effect of chloride dioxide with the MAP modified atmosphere packaging in prevention of Listeria monocytogenes and Salmonella Typhimirium on mung bean sprouts during storage in a cool environment was worked on. Mung bean sprouts were packaged in 4 different ways (air, vacuum, N_2 gas, CO_2 gas) and washed with a solution 100ppt ClO_2 for 5 min and held at 5 ± 2 c. Mung bean sprouts mesophilic population was about 8.4 log10 CFU/g, and this level was not reduced significantly by washing with ClO_2. When samples were packed in a vacuum, N2 gas, and CO_2 gas with performance of CIO2, mesophilic population decreased significantly during storage. These results indicate that the combination of CIO_2 and MAP, such as packaging with CO_2 may be helpful to prevent microbial contamination and storage quality of mung bean sprouts during storage.

In this study, it was found that lactic acid bacteria decreased with increasing CO_2 gas. The effect of MAP on lactic acid bacteria may vary depending on the type of product packaged. Increasing concentrations of carbon dioxide and oxygen depletion which are commonly used in MAP, are suitable for the growth of lactic acid bacteria. This can accelerate the spoilage of susceptible products to lactic acid bacteria such as lettuce, chicory leaves and carrots. MAP impact is minimal on yeast, while fungi are aerobic microorganisms and carbon dioxide at low concentrations such as 10% could inhibit their growth, although it has not lethal effects on fungi (Littlefild NA *et al.*, 1996), (Molin G, 2000), (Nguyen & Carlin F, 1994).

Also, in this study, packages containing CO_2 gas due to excessive sweating caused an increase in humidity of environment and created an appropriate condition for yeast growth and alteration in the flavor. The number of yeast in the packages containing 2.5% CO_2 gas was higher than those containing 1.5% CO_2 gas and those without gas. Conversely, the number of

lactic acid bacteria in packages containing 2.5% CO_2 gas was observed to be less than 1.5% CO_2 gas and without gas.

The reason of simultaneous reduction in lactic acid bacteria and increase in yeast may be attributed to the competitive environment which was created. Considering the fact that the lactic acid bacteria was sensitive and required the condition of complete growth and due to the resistance of yeasts and the suitable conditions which was provided for their growth the population of yeasts increased and lactic acid bacteria population decreased.

4.Conclusions

According to the results of this study, ozone water and sodium hypochlorite solution are both effective in reducing microbial load of mung bean sprouts. The effect of disinfection with sodium hypochloritem, however, was more than that of ozone water in reducing the mesophilic aerobic bacteria and molds and yeasts. According to the Table 2, it can be concluded that CO_2 gas is effective in reduction of microbial load. Amount of yeast increased due to more sweating of packages containing CO_2 gas and caused the spoilage of product. Since mung bean sprout is active, alive, and is breathing, it is recommended that besides disinfecting of mung bean sprouts, active packaging be applied so that the durability and quality of the product would increase.

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EXPERIMENTAL STUDY OF GROMWELL EXTRACTION ON ACTIVITIES OF SERUM- ENZYMES AND ANTI-OXIDATION PROTECTION OF HEPATIC TISSUE IN ENDURANCE-TRAINED RATS

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ABSTRACT

Through the observation of biochemical indicator with hepatic tissue and blood serum in once exhausting with endurance-trained Rats, discussing the biological mechanism of Gromwell extract impacting to delay sports fatigue. Method: Choose 24 rats, and divide them into 3 groups--Exercise groups (group A), exhausted exercise group (group B), Gromwell with exhausted exercise group (group C). Three groups all have been trained 6 weeks. At last time, Group A was not trained as usually and adopted samples immediately, Group B and C were trained by exhausted exercise and then adopted samples. Result: After the exhaustive exercise, the activities of serum- enzymes with Group B can improve greatly ($P<0.05$ or $P<0.01$), the anti-oxidation ability of serum with Group B can lower greatly ($P<0.05$). compared with the Group B, The activities of serum-enzymes with Group C can lower greatly($P<0.05$), The anti-oxidation ability of serum and hepatic tissue with Group C can improve greatly($P<0.05$). Conclusion: Gromwell extract supplement can maintain the of ability anti-oxidation in high- intensity and long-time exercises, decrease oxidative stress injury of liver, prolong the exhaustive time of endurance-trained Rats.

1. Introduction

The liver is one of the largest organs of the body, and it is also the centre of the body's metabolism. In short, the liver is not just a drug, poison metabolism detoxification organ, but also sugar, protein, fat synthesis, decomposition, metabolism of the plant. In addition, the liver is also involved in bilirubin metabolism, urea synthesis, bile acid synthesis and transport, storage and metabolism of vitamins, hormone regulation and cytokine production, and so on. The maintenance of normal liver function is the guarantee of the health of the body. Modern Chinese medicine research shows that the exercise fatigue and recovery are intimately associated with the regulation of five organs, which lies in the liver regulating function disorder (Du *et al.*, 2008; and Dun *et al.*, 2013). Regulation and protection of liver function by targeted supplement of exogenous nutritional supplements or drug active ingredients is one of the effective ways to delay the exercise fatigue and promote recovery after exercise (Chi *et al.*, 2014; Zhang *et al.*, 2011; Xu *et al.*, 2015).

Modern sports medicine research indicates that the much more excessive free radicals which produced by high intensity exercise can damage the body cells and the organization of the system. The free radicals can Cause the membrane lipid peroxidation and also leading to protein denaturation and mitochondrial dysfunction, and so on. It caused cytoplasmic

Ca^{2+} accumulation, cell metabolism obstacle, impaired mitochondrial respiratory chain, and then leads to decreased ability of muscle contraction and fatigue (Zhang *et al.*, 2011). If the exercise fatigue has not eliminated in time, it will lead to a decline in the body's function, so that the decline in exercise capacity, and even lead to excessive fatigue, a serious impact on the health of the body (Zhang *et al.*, 2012; Tang *et al.*,2004). Previous studies showed that high intensity exercise training can result in liver cell damage, the reasons for the injuries. One is produced in the process of movement of physical damage, one is higher intensity exercise training produces free radicals caused by excessive oxidative damage.

Exercise can affect the liver, cause liver function changes, high intensity exercise will cause damage to the liver, and liver area pain after strenuous exercise is a clear manifestation. For oxidative damage, exogenous free radical scavenging agent can be used to reduce the free radical concentration in vivo, in order to reduce the degree of oxidative damage of liver cells, and maintain the normal structure and function of the liver.Gromwell was originally recorded in the book of “Shen Nong’s Herbal Classic ". It’s cold character, sweet and salty, with cooling, circulating blood and detoxification functions. It is applied to treat blood heat, Shing poisoning, boils, haematuria, blood lymph, blood dysentery, burns, malignant sore, and so on(Han *et al.*,2008).

Modern pharmacological research show that the main composition of Gromwell is the compounds of naphthoquinones and polysaccharide, which is good antibacterial, antitumor, antiviral, anti-inflammatory, anti-allergy, protecting liver, reducing enzyme etc. Gromwell is a very high medicinal value of plants (Song *et al.*,2013). This experiment through the established high intensity treadmill animal model, and investigate the biological mechanism of delaying exercise-induced fatigue of Gromwell. It can provide experimental basis for the applied of Gromwell as Sports supplement.

2. Materials and methods

2.1. Experimental Materials

Experimental drug was Xinjiang Gromwell Arn ebiaeuchroma (Royal) Johnston], purchased in Xinjiang Sen Rong Pharmaceutical.

The product specifications of Gromwell we re extracted by 10:1, provided by the Xi'an Nati ve Plant Engineering Technology company. Th e extraction process reference on the way with alcohol-water extraction of literature (Tan *et al.* ,2013). The main effective components of alco hol extraction are naphthoquinone compounds, and the effective components of water extractio n are mainly lithospermum.

2.2. Experimental Subjects

Sprague-Dawley (SD) male healthy rats 24, weight 180~220g, provided by the experimental animal centre of Xi'an Jiao Tong University School of medicine. National standard rodent animal feed, free diet, feeding temperature (23±5) ℃, the relative humidity of 40%~70%, lights along with the natural change, the sub cage adaptive feeding 7 d after the experiment.

2.3. Medication Method

Gromwell suspended in 0.5% sodium carboxymethyl cellulose solution, stiring well, the amount is 800mg/Kg (Mai, *et al.*,2007), and the dose is 2ml, intragastric to Gromwell with exhausted exercise group. The time is 8 o'clock in the morning. The control group is intragastriced by the same amount of 0.5% sodium carboxymethyl cellulose solution. The total of experiment is six weeks.

2.4. Training programs

The rats were randomly divided into three groups: Exercise group (group A), exhausted exercise group (group B), Gromwell with exhausted exercise group (group C), 8 rats in each group. Three groups of rats were 6 weeks of treadmill training. The training program refers to the tread mill training model of Benford (Bedford *et al.*,1979) (see Table 1).

The training lasts for 6 weeks and 6 days in a week. It is training after the exercise of 5min at 15m/min speed every day.

Table 1. Experimental animal motion schene

week	(speed)m•min^{-1}	(slope) %	(exercise time)min•d^{-1}	(exercise intensity)%
first week	15	0	20	
second week	15.2	5	20	58.4 ±1.7
third week	15.2	5	30	58.4 ±1.7
four week	26.8	5	20	74.3 ±2.9
five week	26.8	5	30	74.3 ±2.9
six week	26.8	10	20	81.0 ±3.5

2.5. Sampling and preparation

At last day of the 6 weeks, the rats of A group were not trained, killed in a quiet state and then Sample handling and index determination. The rats of B and C group were exercising until exhausted, exhaustive judgment standard refers to papa (Mao *et al.*,2008), killed immediately and then Sample handling and index determination.

Serum preparation: drawing blood from the rats, and take the blood into 37℃ water bathing for 30 min, and then centrifuged (3500rpm, 15min, 0-4℃), the serum was extracted and placed in 4℃ refrigerator to be tested.

Liver homogenates preparation: The Liver homogenates proportion is 10%, which contain the 0.9% NaCl solution. It is prepared with w(g)/V(ML)by the ratio of 1/9.It take the amount of liver tissue (0.2g-1g) and crushing grinding for preparation of 10% tissue homogenate, and then centrifuged (3500rpm, 15min, 0-4℃), the serum was extracted and placed in 4℃ refrigerator to be tested.

2.6. Test index and method

Superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px), Total antioxidant capacity (T-AOC), Malondialdehyde (MDA).The total indexes were tested by Nanjing Jian cheng kit and the testing method strictly according to kit instructions.

2.7. Statistical analysis and treatment

The experimental data were processed by SPSS12.0 software, and the experimental results were expressed as mean plus or minus standard deviation ($\bar{X}$±SD), and the test was performed between the groups. Significant differences were selected for $P<0.05$ and $P<0.01$ levels.

3. Results and discussions

3.1. The effects of Gromwell Extraction on antioxidant index in serum after exhaustive exercise in endurance training rats

Table 2 data show that the activities of SOD,GSH-PX,T-AOC in the serum of B group were significantly lower than that in group A($P<0.05$), the content of MDA in the serum of B group were significantly higher than that in group A($P<0.05$). Table 2 data also show that the activities of T-AOC in the serum of C group were significantly higher than that in group A($P<0.05$), the content of MDA in the serum of C group were significantly higher than that in group A($P<0.05$), the activities of SOD,GSH-PX, T-AOC in the serum of C group was significantly higher than that in group B($P<0.05$ or $P<0.01$), the content of MDA in the serum of C group were significantly lower than that in group B($P<0.05$).

Table 2. Comparison of the antioxidant index in serum with each group rats after exhaustive exercise

group	SOD (U• mg prot^{-1})	GSH-Px (U• mg prot^{-1})	T-AOC (U• mg prot^{-1})	MDA (nmol• mg prot^{-1})
Exercise group(A group)	267.56±15.94	478.63±21.54	5.80±0.40	3.63±0.32
exhaustive exercise group(B group)	203.92±21.02^{a}	327.76±25.67^{a}	3.49±0.51^{a}	6.07±0.58^{a}
Gromwell with exhaustive exercise group (C group)	252.34±20.65^{b}	430.49±27.46^{b}	7.56±0.73^{abb}	5.04±0.5^{ab}

Note: compare with the group A, ^{a} represent P<0.05, compare with the group B, ^{b}represent P<0.05.

Table 3. Comparison of the antioxidant index in hepatic tissue with each group rats after exhaustive exercise

group	SOD (U·mg prot^{-1})	GSH-Px (U·mg prot^{-1})	T-AOC (U·mg prot^{-1})	MDA (nmo·mg prot$^{-1)}$
Exercise group(A group)	319.84±25.67	113.31±10.07	2.04±0.23	2.56±0.24
exhaustive exercise group(B group)	227.31±28.63^{a}	76.15±11.83^{a}	1.08±0.18^{a}	5.19±0.54^{a}
Gromwell with exhaustive exercise group (C group)	282.88±27.34^{b}	106.46±10.83^{b}	1.88±0.18^{b}	3.67±0.32^{b}

Note: compare with the group A, ^{a} represent P <0.05, compare with the group B; ^{b} represent P <0.05.

Table 4. Comparison of the exhaustive time of Treadmill exercise with B group and C group

group	exhaustive time(min)	Time delay percentage (%)
exhaustive exercise group(B group)	75.89±18.37	
Gromwell with exhaustive exercise group (C group)	90.65±20.18$^{\#}$	19.45%

Note: The C group compare with the B group $^{\#}$ represent P<0.05.

3.2. The Effects of Gromwell Extraction on the exhaustive time of Treadmill exercise in endurance training rats

Table 4 shows that the exhaustive exercise time of supplementing Gromwell rats (C group) was significantly higher than exhaustive exercise rats(B group). The mean exhaustive time prolonged 19.45%, with statistical significance (P < 0.05).

3.3. Analysis and Discussion

3.3.1. The effects of Gromwell extraction on antioxidant index in serum and hepatic tissue in endurance training rats

Modern research indicates that the high intensity and long-time exercise can induce the body to produce a large number of endogenous free radicals, which can attack the cell membrane and enzyme protein, and induce oxidative denaturation, and then change the membrane permeability and fluidity, and further affect the integrity of the structure and function of tissue and cells, reducing the body movement ability (Mena *et al.*, 1996; Tiidus, *et al.*, 1996). However, free radicals are also present in the body to resist free radical damage, in which SOD is one of the antioxidant enzymes at the forefront of free radical defence. All the cells in the mammal have SOD, whose function is to produce H_2O_2, and then convert to water continuously by other antioxidant enzymes, which can achieve the purpose of removing free radicals. Long endurance exercise causes a large amount of free radical accumulation in all tissues and organs in the body, especially the excessive accumulation of SOD, so that the vitality of the organ tissue is significantly decreased. GSH-Px is a kind of free radical scavenging free radical and inhibiting free radical reaction in the body, which can remove the lipid peroxide and H_2O_2, and reduce the damage of organic hydrogen peroxide to the body. CAT is present in the intracellular catalase, which can catalyse the decomposition of H_2O_2, and participate in the production of H_2O_2 and many important enzymes in organic peroxide. Therefore, SOD, GSH-Px, CAT are considered to be the most

important three kinds of enzymes in the free radical scavenging system, and their activity values can be estimated to a large extent, to evaluate the functional status of the free radical scavenging enzyme system. MDA is lipid peroxidation is one of the main products, biological membrane lipid unsaturated fatty acids most easily by free radical attack and oxidation, so the content of MDA in is to reflect the number of free radicals in the tissues and cells of a commonly used indicator. The antioxidant enzyme activity is an important indicator reflecting the free radical scavenging ability, MDA content is an important indicator to reflect the level of lipid peroxidation and the degree of cell damage (Xiong *et al.*,2010).

A large number of studies have concluded that the right amount of endurance training can improve the body's ability to resist free radical damage and delay the occurrence of the defence mechanism. However, long-term overload training can increase the free radicals in the body, which may reduce the body's antioxidant capacity, resulting in damage to the body tissue and cell membrane structure or cause fatigue. High intensity exercise can damage the body's antioxidant substances, thereby reducing the body's antioxidant levels. The formation of MDA in the reaction of oxygen free radicals and lipid molecules can make the protein molecules cross - linking, which makes the biofilm become hard and easily broken. The ability of skeletal muscle to resist oxygen free radicals in the human body is poor, so it is highly sensitive to the role of superoxide, which is mainly due to the high basal metabolic rate of skeletal muscle in exercise. In the long time and high intensity exercise, the body is in a state of strong oxidative stress, the production and elimination of free radicals in vivo imbalance, increasing the number of free radicals, the oxidation of the body tissue. In the process of intensive endurance training, the body metabolism, the growth of the material oxidation, oxygen free radical generation is more obvious. Oxygen free radicals generated during the oxidation of substances can be combined with unsaturated fatty acids in the cell membrane to form MDA, and continuously release into blood and enter into the tissue cell and affect the activity of cells.

Table 3 and 4 showed that the activities of SOD, GSH-PX,T-AOC in serum and hepatic tissue of exercise group were significantly lower than that in group A($P<0.05$), the content of MDA was significantly higher than that in group A($P<0.05$).It showed that the free radical has accumulated in the body. The antioxidant capacity of liver and the whole body have decreased in the large intensity exhaustive exercise. The experimental results also showed that the activities of SOD,GSH-PX, T-AOC in serum and hepatic tissue of supplementing Gromwell rats(C group) were significantly higher than that in group B($P<0.05$), the content of MDA in serum and hepatic tissue of C group were significantly lower than that in group B($P<0.05$).It indicated that the supplementing Gromwell extraction can reduce the oxidative damage on the body in long time and high intensity endurance exercise, and protect the activities of antioxidant enzyme, and prevent free radical accumulated, and in order to protect the complete structure and function with liver and other tissues or organs. The recently research indicated that (Assimopoulou *et al.*,2004; Gong *et al.*,2011) the Gromwell rich in naphthoquinone compounds, which can has a good protective affection on CCl4 and alcoholic liver injury. The antioxidant mechanism of Gromwell extraction may be related to Gromwell ruching in naphthoquinone compounds. Gromwell is one of naphthoquinone compounds, and especially have 5, 8 phenolic hydroxyl on the structure of nucleus. The hydroquinone structure is unstable, extremely easy to outward transfer electrons and protons, and terminate free radical chain reaction in unsaturated and fatty acid. It has strong antioxidant capacity, can effectively delay the accumulation of free radicals in high intensity exercise, also can protect the activity of antioxidant enzymes and improve the tolerance and adaptability with exercise intensity.

3.3.2. The effects and mechanism of Gromwell extraction on the exhaustive time in endurance training rats

Exercise fatigue is a complicated physiological phenomenon and the reasons of opinion are various. Experts and scholars proposed many assumptions to explain the fatigue phenomenon and its causes, and has successfully explain the phenomenon of fatigue with certain aspects. But due to the complexity of metabolic fatigue process and the regulation mechanism. At present, various theories have one-sided or certain limitation. It's hard to explain the fatigue process mechanism in general (Roger *et al.,* 2008). The modern sports medicine research confirmed that the free radicals, clearance rate and production rate are not balance, and the cells of body are under oxidative stress, and then leads to cell damage, further the muscles are incapable to work and exercise-induced fatigue (Deng *et al.,*2013). Therefore, Nutritional or pharmaceutical active ingredients supplement can control free radical excessive accumulation and delaying or alleviate sport fatigue, and also is the effective way to exercise fatigue.

The experimental results showed that the exhaustive exercise time of supplementing Gromwell rats was significantly higher than exhaustive exercise rats. The mean exhaustive time prolonged 19.45%. It indicated that supplementing Gromwell can improve the exercise ability of rats and delay the generation of sports fatigue. The main mechanism is related to Gromwell rich in naphthalene quinines, benzoquinones, alkaloids, phenols, phenolic acids, triterpene acids, sterols, flavonoids and polysaccharides, and so on a variety of ingredients, also concerned with multiple pharmacological effect of anti-inflammatory analgesic, antioxidant, immune adjustment. Supplementing Gromwell can improve the level of oxidative stress adaptability in exercise, and protect the activity of antioxidant enzymes, and protect the integrity of the cell membrane and subcellular structure and function, and then delay the exercise fatigue. Moreover, shik on in has strong antioxidant activity, and has a positive affection to maintain liver function and promote the repair of liver injury, so as to ensure the movement of energy metabolism, substance metabolism and immune regulation function, in order to improve the body's exercise ability and prolong exercise time.

4. Conclusions

Traditional Chinese medicine is the quintessence of our country, inherit and carry forward the Chinese traditional culture, the development of traditional Chinese medicine and the use of the sport to, solid foundation and bright prospects. Now with the rapid development of competitive sports, competition has become increasingly fierce, people in the introduction and adoption of advanced training methods and techniques at the same time, consider the use of traditional Chinese medicine agent to fully tap the human potential, is possible and feasible. Gromwell is a treasure of Chinese herbal medicine resources, has rich medicinal function. This experiment shows that long term supplementation of Gromwell can effectively maintain the activity of antioxidant enzymes in high intensity exercise especially can reduce liver damage within deduced by oxidative stress, maintain the normal operation of the liver function, can significantly delay the treadmill exhaustive time of exercise training rats. Gromwell is a natural wild drug and its extraction can reduce and delay the exercise fatigue, Gromwell can be developed and utilized by sport supplements.

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STUDY ON ORDERING MODEL OF FOOD COLD CHAIN LOGISTICS WITH MULTI-ECHELON INVENTORY

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ABSTRACT
The echelon inventory levels of food cold chain logistics can be infinite in theory, but in the actual supply chain, the fact is that, the fewer the levels are, the better the situation is. Therefore, to provide convenience for study and description of the problem of multi-echelon inventory control model, the paper takes the three-echelon inventory composed of the cold storages at the place of production, the central place and marketing places as the study object to conduct overall optimization and control for the cold chain logistics, involving discussion of cold storage capacity scale and the optimal purchasing quantity of the multi-echelon cold chain logistics and distribution of purchasing quantity to the cold storages in the production place.

1.Introduction

With the rapid development of logistics industry, the cold chain logistics has changed from single-echelon inventory system to multi-echelon inventory system, including the place of production, central place and marketing place (Wang et al., 2009). The multi-echelon inventory system is not only connected with but also different from the single-echelon inventory system as it is formed and developed from the single-echelon inventory system, so some researches and discussions on the single-echelon inventory system are also applicable to the multi-echelon inventory system. However, there are also some problems that were not solved for the single-echelon inventory system, for example, for the single-echelon inventory system, the optimization is always performed for partial system by viewing form a certain node or link other than the optimization of the whole cold chain logistics industry. In order to realize the effect control on the chain logistics whole, the multi-echelon control method shall be taken to synchronize the operation of all relevant nodes and execute centralized management and decision for all of the cold chain inventory system, so as to obtain more economic benefits and higher profitability of the cold chain logistics industry (Tian and Taudes, 2015). In the paper, the discussion is focused on the configuration for cold storage capacity system of the multi-echelon cold chain logistics and the allocation and integration of multi-echelon cold inventory system.

2. Materials and methods

Multi-echelon inventory system is a complete system involving all inventory nodes based on the supply-demand relationship. Theoretically, the levels of multi-echelon inventory can be the infinite supply chain forming a net model, but practically, the more supply chains levels is not the better, and the fewer levels is the better for the convenience of research and the balance between the cold chain logistics theory and the practices.

Therefore, the multi-echelon inventory control model in this paper is defined as the classic three-echelon inventory control model that is enough to clearly state the operation matters of cold chain logistics system (Shen and

Liu, 2013). We divide the cold chain logistics inventory system model into three grades: the grade I is the called cold storage in the place of production that is used for refrigeration and preprocess for fresh food; the grade II is the cold storage center with the function of deep processing, rapid refrigeration and storage based on the grade I; and the grade III is the cold storage at the marketing place that is used for the delivery of the finished food. The cold chain multi-echelon inventory system structure is shown in Figure 1.

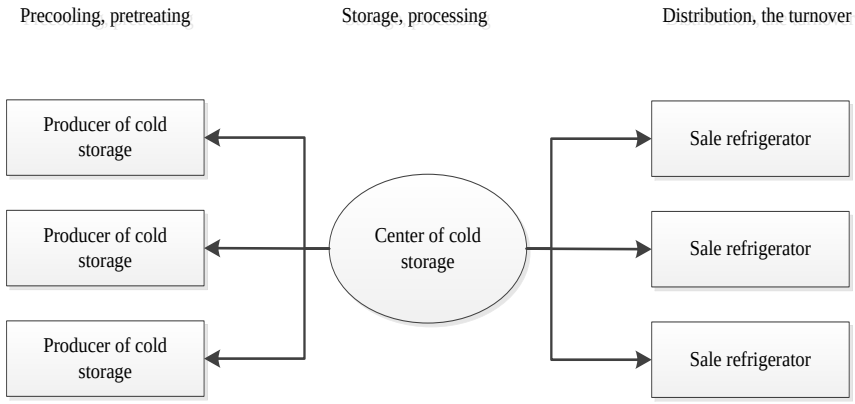

Figure 1: Food cold chain logistics multi-level inventory system

Clark and Scarf has started to study on the multi-echelon inventory at 1970s and presented the definition of "echelon inventory": echelon inventory of supply chain, the existing inventory of a certain inventory node, and the inventory transferred to under the transformation to the following nodes. Thus the inventory of multi-echelon inventory is mainly constituted by echelon inventory and point inventory: the echelon inventory of a certain node n refers to the storage volume of all nodes from the upstream to the downstream of the inventory nodes, wherein the inventory of the node n is the local inventory or the node inventory (Chen, 2014)..

Assuming the food cold chain logistics system is composed by n nodes (n is the number of nodes), and the food cold chain is set as the start point and to the end point the nodes are successively marked as $n = N, N-1, \cdots, 2, 1$. Nodes are the inventory points of the cold chain logistics system, the terminal demand is presented at the node 1, and the demand of node N is supplied by external manufactures. In addition, all relationship among the inventory nodes occurs within the system, namely, the node $N-1$ is supplied by node N, and $N-2$ by $N-1$, etc. The specific supply-demand relationship is shown in Figure 2. Let the storage volume of node i at the time node t is expressed by , and echelon inventory of node i at the time node t is expressed by $Q_i(t)$, then,

$$Q_i(t) = \sum_{k=1}^{i} Q_i(t), \quad i = 1, 2, \cdots, N \tag{1}$$

Similarly, the storage cost of multi-echelon inventory is also constituted by the costs of echelon inventory and point inventory. Assuming h_i is the echelon inventory cost of node i, the inventory cost h_i of node i can be expressed as:

$$h_i^* = h_i - h_{i+1}, \quad i = 1, 2, \cdots, N \tag{2}$$

The phase $N+1$, representing the supply by external manufactures, is set as $h_{N+1} = 0$. Providing the charges for maintaining the inventory is expressed as the whole process of the supply chain system from the beginning at upstream to the end at the downstream:

Thus:

$$h_i > h_{i+1}, \quad i = 1, 2, \cdots, N \tag{3}$$

The charge for the whole supply chain involving all echelon inventories calculated based on the Eq. (2) is:

$$h_i = \sum_{k=i}^{N} h_i^*, \quad i = 1, 2, \cdots, N \tag{4}$$

Figure 2: Multilevel inventory system

3. Results and discussions

3.1. Optical scale for cold storage with determined demand

The model research in this paper is manly mainly based on EOQ model. Based on the existing EOQ model, we can obtain the optimal ordering quantity under the determined demand, so that the costs for storage and purchasing can be minimized based on this premise. However, EOQ model also has disadvantages: for example, the investment for the fixed assets related to the inventory is neglected. The cost for building food cold storage system is much higher than that for normal cold storage, so the investment for the fixed assets related to the cold storage must be taken into consideration. Therefore, to minimize the charges for storage, purchase and investment, we should determine the optimal scale of cold storage and further the optimal purchasing quantity according to the scale. According to elementary mathematic principle, if $a = b$, the sum of $a + b$ is the lowest, namely, investment cost=storage charges+ purchasing charges. The cold storage scale is optimal when the food cold storage inventory cost is lowest. Based on the above analysis, we divided the model into the three steps as follows:

Step 1: firstly, calculate the purchasing quantity Q_j^* and the corresponding total cost C_i based on the existing EOQ model.

Step 2: then find out the optimal scale range of the cold storage that can satisfy the condition investment cost=storage charges+ purchasing charges.

Step 3: at last, determine the optimal scale of the cold storage on the premise of the optimal scale scope. According to the above steps, the calculation process of the model is as follows:

$$C_i = \frac{b_j r Q}{2} + \frac{SD}{rD} \tag{5}$$

Wherein: C_i is the storage charges and purchasing charges, b_j is the annual storage charges, r is the percentage of purchasing quantity to the total storage volume, S is the cost of purchasing each time, and D is the total purchasing quantity of a year.

If the sum of storage cost and the purchasing cost is lowest, then,

$$\frac{\partial C_i}{\partial Q} = b_j r - \frac{SD}{2Q^2} = 0 \tag{6}$$

Solve the equation,

$$Q_j^* = \sqrt{\frac{2SD}{b_j r^2}} \tag{7}$$

$$C_i = \sqrt{2SDb_j} \tag{8}$$

In the above calculation, only the storage cost and purchasing cost are involved in the lowest cost but the investment cost is not included. In order to minimize the total cost of the cold storage, the investment cost need to be involved in the model, and the details are as below:

$$C_{Total} = C_i + C_I^* = \sqrt{2SDb_j} + \frac{C_I}{\frac{1-(1+i)^{-n}}{i}} \tag{9}$$

Providing the investment cost scale for cold storage is relatively high, and the payback period is long, the investment cost hereby is calculated by its present value and its will be shared to each year based on the compound interest factors of the present value to show the time value of money, wherein, C_I^* is the cost shared to each year, and C_I is the investment cost. Considering C_I^* is a piecewise function that cannot be derived directly, and the sum of $a + b$ is smallest only in case of $a = b$ according to the primary mathematic principle, the total cost of cold storage is lowest when $C_I = C_I^*$, and under this condition, the storage scale will result in an optimal capacity. On the premise of the above condition and the calculated C_i and C_I^* under different scales, the scale scope on the condition

of $C_I^* = C_i$ is (Q_1, Q_2), or the optimal scale scope of the cold storage is Q^*. If $Q_j^* \in (Q_1, Q_2)$, then $Q_j^* = Q^*$; if $Q_j^* < Q_1$, then $Q^* = Q_1$; and if $Q_j^* > Q_1$, then $Q^* = Q_2$.

3.2. Optical scale for cold storage with undetermined demand

As for the undetermined demand that cannot be obtained immediately, the following matters shall be worked out: if the cold storage volume is excessively large that exceeds the actual demand, there will be lots of unused capacity, resulting in failure for utmost use of the storage space and increase of the storage cost. Once the cold storage is constructed, it is difficult or even impossible to change the volume of the inventory system. Thus, the exceeding inventory space cannot be taken for use and its value will be not achieved, which will cause irretrievable loss. However, if the scale of cold storage is too small and cannot meet the final determined demand, there will be less marketing opportunity and customer resources, causing increase of opportunity cost, moreover, the company may have to pay more to rent cold storage to meet the actual demand. In order to avoid those problems, the newsvendor model will be used in this paper to build a model:

Assuming the use ratio of the cold inventory capacity is subject to the normal distribution of $N(\mu, \delta^2)$, then the density function is:

$$p(x) = \begin{cases} 0, x < 0 \\ \dfrac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}}, x \geq 0 \end{cases} \quad (10)$$

Wherein, $\mu, \sigma > 0$ and it is a constant, then the corresponding distribution function is:

$$F(x) = \frac{1}{\sqrt{2\pi}\sigma} \int_0^x e^{-\frac{(x-\mu)^2}{2\sigma^2}}, \quad (11)$$

Assuming the enterprise has its own inventory capacity of X tons, and purchasing quantity of Y tons, and the replenishment quantity of the enterprise is random, how many is the inventory capacity that will lead to the lowest inventory cost? The analysis on the question is as follows:

If $X > Y$, or the purchasing quantity of the enterprise is less than its own inventory capacity, the inventory cost is the sum of the inventory operation charges and the idle cost, namely,

$$C = YC_1 + (X - Y)C_2 \quad (12)$$

Wherein, C is the total inventory charges, C_1 is the use charges of the inventory capacity owned by the enterprise, and C_2 is the ideal cost of the inventory capacity owned by the enterprise.

If $X < Y$, or the purchasing quantity of the enterprise is larger than its own inventory capacity, the inventory cost is the sum of the operation charges and the rental fees, namely,

$$C = XC_1 + (Y - X)C_3 \quad (13)$$

Wherein, C_3 is the charge for unit capacity of the leased cold storage, so the expected cost of the enterprise is:

$$C(X) = \int_x^\infty \left[XC_1 + (Y - X)C_3\right] \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx + \int_0^X \left[YC_1 + (X - Y)C_2\right] \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx \quad (14)$$

$$\frac{dC(x)}{dx} = (C_1 - C_3) \int_0^\infty \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx + C_2 \int_0^x \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx \quad (15)$$

$$\int_0^x \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx = 1 - \int_x^\infty \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx \quad (16)$$

Distribution probability shown by its own inventory at the least cost can be calculated as:

$$F(x) = \frac{C_3 - C_1}{C_2 + C_3 - C_1} \quad (17)$$

According to the calculated quantity demanded to be purchased, a conclusion can be drawn that the model, i.e. a model to optimize the cold storage, has the same solution process with the description above. However, investment cost in the inventory has never been increased in the resolution process; therefore, to achieve the total cost minimization, the investment cost must be increased, and then solution can be carried out according to the above-mentioned computing method.

3.3. Model description

Let us suppose there are cold storage $A, B, C, \cdots, N$ in the place of production in multi-echelon inventory system, and the

corresponding inventory quantities will be $Q_1, Q_2, Q_3, \cdots, Q_n$ respectively. Purchasing charges and storage charges shall be focused on according to the inventory model of an independent cold storage in the place of production. An assumption is made that the corresponding purchasing charges of the cold storage in the places of production in the above-mentioned multi-echelon inventory system is $K_1, K_2, K_3, \cdots, K_n$, and the corresponding storage changes will be $h_1, h_2, h_3, \cdots, h_n$. And let's suppose that the purchase demanded of the inventory in the central place is D, and how to distribute the purchased demanded D to the cold storage in the places of production to make the lowest cost of the inventory system is our first problem to be resolved at present.

Let Q_j be the purchasing quantity of a certain node j in the food cold storage, and the inventory cost will be:

$$T_j(Q,D) = \frac{Q_j h_j}{2} + \frac{K_j D}{Q_j} \tag{18}$$

Wherein, $T_j(Q,D)$ is the inventory cost of node j, K_j the purchasing cost of node j, h_j the storage cost of node j, and D_j the purchase demanded distributed to joint j. Therefore, the echelon inventory cost of node j can be calculated to be:

$$T_j(Q,D) = \sum_{i=1}^{i=j}\left(\frac{Q_j h_j}{2} + \frac{K_j D}{Q_j}\right) \tag{19}$$

Therefore, the total cost for a N-echelon inventory within a continuous period is:

$$T(Q,D) = \sum_{j=1}^{N}\left(T_j(Q,D)\right) \tag{20}$$

Therefore, the corresponding mathematical model is:

$$MinT(Q,D)$$
$$s.t.\begin{cases}\sum_{j=1}^{N} D_j = D \\ Q_j \le Q_0\end{cases} \tag{21}$$

3.4. Algorithm design

As the model above is a nonlinear programming model, it is relatively difficult to be calculated. Therefore, a more optimized solution method is given in the paper, namely gradual decomposition method. The solution method is to divide all inventory nodes into two echelon inventories and distribute all purchases demanded to the two echelon inventories to minimize the total cost of the inventory, and then carry out gradual decomposition for the two echelon inventories above respectively until the optimum purchasing quantity for the inventory of each node which meets the actual demands is calculated, as shown in Figure 3.

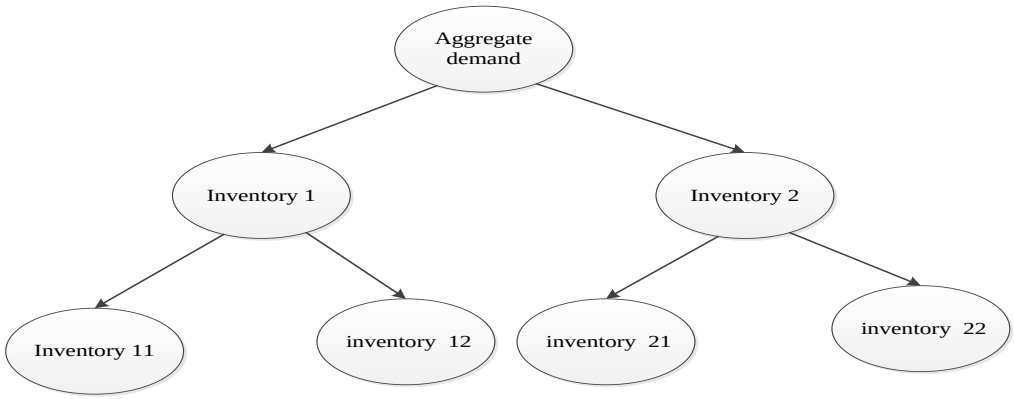

Figure 3: A decompose step by step method

$y=F\left(f_1(x_1), f_2(x_2),\cdots, f_n(x_n)\right)$, The principle of the algorithm originates from the theorem: let there be multivariate compound function: $y=F\left(f_1(x_1), f_2(x_2),\cdots, f_n(x_n)\right)$, if x_i^* is the minimal value of $f_i(x)$, x_i^* will be the minimal value of y. The calculation process is detailed below:

Firstly, decompose the total purchases demanded D into D_1 and $D-D_1$, and then the corresponding purchasing quantities of echelon inventory 1 and 2 can be determined respectively according to the purchases demanded distributed to them and their own cost functions. According to the inventory model described above, functions of Q_j^*, Q_2^*, C_1 and C_2 as well as the total cost can be obtained, as shown below:

$$Q_1^*=\sqrt{\frac{2h_1D_1}{k_1}} \tag{22}$$

$$Q_2^*=\sqrt{\frac{2h_2\left(D-D_1\right)}{k_2}} \tag{23}$$

$$C_1=\sqrt{2k_1h_1L} \tag{24}$$

$$C_2=\sqrt{2k_2h_2\left(D-D_1\right)} \tag{25}$$

Get derivation according to the total cost and let the derivation be equal to 0 to get the optimal quantities distributed to echelon inventory 1 and 2.

$$\frac{\partial C_{Total}}{\partial D_1}=\sqrt{\frac{k_1h_1}{2D_1}}-\sqrt{\frac{k_2h_2}{2\left(D-D_1\right)}}=0 \tag{26}$$

The equation above can be solved:

$$\begin{aligned} D_1&=\frac{k_1h_1D}{k_1h_1+k_2h_2}\\ D_2&=\frac{k_2h_2D}{k_1h_1+k_2h_2} \end{aligned} \tag{27}$$

If $Q_j^*>Q_{0j}$, it will be obtained that $Q_j^*=Q_{0j}$. Other purchases demanded will be distributed to other echelon inventories. And then calculation is conducted according to the above-mentioned scheme to respectively calculate the purchasing quantity D_1 and D_2 of echelon inventory 1 and 2 until the optimal purchasing quantity of each cold storage which meets the actual inventory demands is calculated.

3.5. Basic data

Due to the situation of food cold chain logistics with multi-echelon inventory, it's very difficult to collect all true data. Therefore, in the paper, examples are adopted to carry out calculation. Let us assume that there are three cold storages at the marketing place, one cold storage at the central place and four cold storages at the place of production in a food cold chain logistics system. The average purchasing quantity, purchasing standard deviation and purchasing cost of the cold storages at the marketing place are shown in Table 1.

Table 1. The optimal size of the calculation table sale cold storage 1

Number	Most distribution probability	Annual demand	The best better	The lowest operating costs
1	0.2315	7021	36	30236
2	0.2854	7362	39.2	30124
3	0.3625	7125	39.1	29026
4	0.3654	7141	40.2	28144
5	0.4125	7512	41.3	28652
6	0.4521	7162	41.5	28365
7	0.4123	7142	41.6	27145
8	0.5124	7210	44.2	26302
9	0.5212	7125	44.3	26119

Table 2. The optimal size of the calculation table sale cold storage 2

Number	Most distribution probability	Annual demand	The best better	The lowest operating costs
1	0.2512	7012	38.2	30251
2	0.2852	7059	39.3	32546
3	0.2632	7112	35.2	29215
4	0.3521	7184	35.9	29415
5	0.4152	7232	40.2	28365
6	0.4512	7265	41.3	28474
7	0.5236	7140	41.5	28695
8	0.5269	7254	44.2	27523
9	0.5481	7136	44.6	26359

3.6. Calculation results

The cold storages at the marketing place are to face end customers, so its purchasing quantities are determined according to the quantity demanded by the consumer market. However, market demands are distributed randomly, so the

scale of the cold storages in the marketing place is calculated according to any demand model to look for the optimal purchasing scale. The calculation results are shown in Table 2, 3 respectively.

Table 3. The optimal size of the calculation table sale cold storage 3

Number	Most distribution probability	Annual demand	The best better	The lowest operating costs
1	0.2512	9012	41.2	30251
2	0.2852	9059	42.6	32546
3	0.2632	9112	43.6	39215
4	0.3521	9184	44.2	38695
5	0.4152	9252	44.2	37523
6	0.4512	9255	44.2	26359
7	0.5236	9140	44.6	32123

The cold storages at the marketing place are to directly face sales market and the inventory turnover speed is relatively fast. Let us suppose that r is 0.6, we can see from Table 2 that the cold storage 1 at the marketing place has the minimum production and operation fee and annual present value of investment which are the most similar with those when the scale of the cold storage is 50～80 tons. Therefore, as for the cold storage 1 at the marketing place, the optimal scale range is (50, 80), and the corresponding optimal purchasing quantity of the optimal scale is 39.3 tons, not exceeding the lower limit of the scale range; therefore, we adopt the lower limit of the optimal scale range as the optimal scale of the cold storage 1 at the marketing place, namely, the optimal scale being 50 tons. If aiming for maximizing the cold storage profit, we set the storage price of the cold storage as RMB5/t, so the optimal purchasing quantity of the cold storage 1 at the marketing place is 36 tons from Equation (8).

The cold storages at the marketing place are to directly face sales market and the inventory turnover speed is relatively fast. Let us suppose that r is 0.6, we can see from Table 3 that the cold storage 2 at the marketing place has the minimum operation cost and annual present value of investment which are the nearest to those when the scale of the cold storage is 80～100 tons. Therefore, the cold storage 2 at the marketing place has the minimum operation cost and annual present value of investment which are the most similar with those when the scale of the cold storage is 80～100 tons. At this time, we find that the corresponding optimal purchasing quantity of the optimal scale in the range is 48 tons, not exceeding the lower limit of the scale range; therefore, we adopt the lower limit of the optimal scale range as the optimal scale of the cold storage 2 at the marketing place, namely, the optimal scale being 80 tons. If aiming for maximizing the cold storage profit, we set the storage price of the cold storage as RMB5/t, so the optimal purchasing quantity of the cold storage 2 at the marketing place is 42.3 tons from Equation 3.8.

The cold storages at the marketing place are to directly face sales market and the inventory turnover speed is relatively fast. Let us suppose that r is 0.6, we can see from Table 3 that the cold storage 3 at the marketing place has the minimum operation cost and annual present value of investment which are the most similar with those when the scale of the cold storage is 80～100 tons.

At this time, we find that the corresponding optimal purchasing quantity of the optimal scale in the range is 43.4 tons, not exceeding the lower limit of the scale range; therefore, we adopt the lower limit of the optimal scale range as the optimal scale of the cold storage 3 at the marketing place, namely, the optimal scale being 80 tons. If aiming for maximizing the cold storage profit, we set the storage price of the cold storage as RMB5/t, so the optimal purchasing quantity of the cold storage 3 at the marketing place is 37.9 tons from Equation (8).

4. Conclusions

The echelon inventory levels of food cold chain logistics can be infinite in theory, but in the actual supply chain, the fact is that, the fewer the levels are, the better the situation is. Therefore, to provide convenience for study and description of the problem of multi-echelon inventory control model, the paper takes the three-echelon inventory composed of the cold storages at the

place of production, the central place and marketing places as the study object to conduct overall optimization and control for the cold chain logistics, involving discussion of cold storage capacity scale and the optimal purchasing quantity of the multi-echelon cold chain logistics and distribution of purchasing quantity to the cold storages in the production place.

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THE APPLICATION OF GREY MATTER-ELEMENT METHOD IN DAIRY PRODUCT COLD CHAIN LOGISTICS EVALUATION

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ABSTRACT
In recent years, food safety accidents, especially the transportation accidents of dairy product cold chain logistics have occurred frequently. Dairy product cold chain logistics plays an important role in food cold chain. Because there are many processes in dairy product cold chain logistics, and its reliability is influenced by the effect dovetailed by those processes, the evaluation system of dairy product cold chain logistics is constructed according to its process characteristics in this paper, using grey matter-element method to establish the evaluation model of dairy product cold chain logistics, dividing its evaluation results into four grey clustering, which provides a scientific, reasonable and feasible method for the evaluation of dairy product cold chain logistics.

1. Introduction

Along with the improvement of social level and living standard, people's demand for dairy products is growing. Consumption idea changes from simple type to multiple one. Therefore, the development of dairy industry needs the support and guarantee of the cold chain logistics. Cold chain logistics is a system engineering that cold storage items (such as meat, fast food, dairy products, ice cream, etc.) are kept in the low-temperature environment (0℃ ~ 4℃) in the whole process of production, storage, transportation, reprocessing and sales so as to ensure the quality and performance of the goods. It is a supply chain system that low temperature environment is required as the core(David,2006). It develops with the rapid development of science and technology progress and the development of refrigeration technology. It is a low temperature logistics process with freezing technology as the basis and refrigeration technology as the means.

The study abroad about food cold chain mostly concentrated on cold storage technology and technique. There were also some studies about the management of food supply chain, for example, Goldring. Zack discussed the measures of maintaining the normal operation of cold chain logistics system in <Maintaining the cold chain>, and Neil Shister pointed the importance of management and technology in cold chain transport in <Managing The Global Cold Chain>, and so on (Rediers et al., 2009). Domestic researches have also gradually been expanded in recent years. The researches mainly focus on the basic theory and the status analysis of cold chain logistics, for example, Ruifu Zhang has studied the temperature demand in cold chain logistics and the effect the temperature has on cold chain logistics in <The research on cold chain logistics system based on temperature > , Yuehua Zhang represented the hazard analysis of fresh food and the theory of critical control point in < The technology innovation of fresh goods cold chain logistics under HACCP monitoring>.

Dairy product cold chain logistics is regarded as the research object in this paper.

According to the processes of dairy product cold chain logistics, the evaluation index system is constructed. AHP method is used to determine the weight of each second-level evaluation index, so as to set up the grey matter-element evaluation model.

2. Materials and methods

The evaluation system of dairy product cold chain logistics

2.1. The processes of food cold chain logistics

Food cold chain (cold chain) is a special supply chain system, which means from collecting zone purchase or after fishing, in order to ensure food quality and safety, reduce loss, and prevent pollution, in the process of production and processing, storage, transportation, sales, and terminal consumers, the perishable food must be kept in the necessary low temperature environment. Food cold chain logistics system is composed by four parts, that is, refrigeration processing, refrigeration storage, refrigeration transport, refrigeration sales.

(1) Refrigeration processing

Refrigeration processing includes not only the processing of all kinds of frozen food and dairy products at low temperature, and the processing process at low temperature (Zonaeshna, 2005), but also the precooling of fruits and vegetables, and the cooling of meat and poultry, fish and eggs. In this process, the key points are the cold chain equipment such as refrigerating installation and cooling device.

(2) Refrigeration storage

In order to ensure the storage and processing process of refrigerated food at a low temperature environment so as to guarantee the food delicious, the refrigeration installation equipment such as all kinds of cold storage and refrigerators are mainly used in this process to carry on the cold storage of the refrigerated food, including the CA storage of fruits and vegetables.

(3) Refrigeration transport

In order to ensure the food long-distance transportation and short-distance delivery process in low temperature condition (Fei, 1992; Hui, 2003), a variety of means of transportation at low temperature are used in this process. In this link, the transport needs to be in high requirement, especially for the long distance transportation. Only the transport with good performance could not only keep the required low temperature, but also maintain the stability of the temperature (Zhang and Du, 1994).

(4) Refrigeration sales

Manufacturers and sellers are involved in this link. It is a necessary step of sales and storage for cold-chain food. It is the key to make food cold chain complete. Refrigerated display cabinets and other equipment are widely used in all kinds of chain supermarkets. At the same time, it has gradually become the main sale mode of food cold chain (Gimenez, 2006).

2.2. The influence of each process on dairy product cold chain logistics

Analyze the process of dairy product cold chain logistics and the influence each process has given to the economy, performance and feasibility of dairy product cold chain logistics; determine the influencing factors of dairy product cold chain logistics evaluation. The specific details are shown in table 1.

Table 1. The influencing factors of dairy product cold chain logistics evaluation

The main processes	The influencing factors
Collecting and treating process A	raw milk collection, raw milk grading, preservative treatment, raw milk packaging, collecting zone refrigeration
Transport process B	long-distance transport, loading and unloading handling;
processing flow C	Insurance processing, dairy product storage;
delivery process D	Loading, short-distance transport and delivery
Sales process E	Storage, marketing

3. Results and discussions

3.1. Grey matter element analysis method

Matter-element analysis method is a kind of evaluating method of solving contradictions. It is to use correlation function to analyze the problems, transforming the incompatible problems into compatible problems, which is suitable to solve the fuzzy problems (Jiang and Liu, 1996; Zhang, 2000). The grey theory is to solve the social problems that are vague, unclear, with incomplete information. Combined the gray theory with the matter-element analysis method, it is a new attempt for the evaluation research of dairy product cold chain logistics.

3.2. Determine the grey number explicit values

Here we adopt grey element expression, that is, using the ordered triple of object, object characteristics, and grey number explicit value to describe objects, written as $\tilde{\otimes}R$ .and N indicates the object, c indicates object characteristics, $\tilde{\otimes}$ indicates the grey number explicit value corresponding to object characteristics c , therefore, grey element expression could be written as:

$$\tilde{\otimes}R=\begin{bmatrix} & N \\ c & \tilde{\otimes} \end{bmatrix}.$$

The evaluation of dairy product cold chain logistics is researched in this paper, therefore, N indicates dairy product cold chain logistics, c indicates each process of dairy product cold chain logistics.

3.2.1. Determine the grey clustering level

According to the analysis of dairy product cold chain logistics processes, combined with the experts' opinion, the evaluation results of dairy product cold chain logistics are divided into four levels, that is, excellent, good, general, and poor. The specific classification is shown in table 2.

Table 2. The evaluation level table of dairy product cold chain logistics (using a 10-point scale)

The evaluation level of dairy product cold chain logistics	Scale (no unit)
excellent	8—10
good	6—8
general	4—6
poor	1—4

3.2.2.Determine the whitenization weight function

There are three kinds of expressions of the whitenization weight function, that is:

1.The upper type

the grey number is $\otimes\in[0,d_1,+\infty)$ .its whitenization weight function is:

$$f_1(d_{ji})=\begin{cases} 0, & d_{ji}\in(-\infty,d_1) \\ \dfrac{d_{ji}}{d_1}, & d_{ji}\in[0,d_1] \\ 1, & d_{ji}\in[d_1,+\infty) \end{cases} \quad (1)$$

2. The middle type

the grey number is $\otimes\in[0,d_1,2d_1]$ .its whitenization weight function is:

$$f_2(d_{ji})=\begin{cases} \dfrac{d_{ji}}{d_1}, & d_{ji}\in[0,d_1] \\ 2-\dfrac{d_{ji}}{d_1}, & d_{ji}\in[d_1,2d_1] \\ 0, & d_{ji}\notin[0,2d_1] \end{cases} \quad (2)$$

3. The lower type

the grey number is $\otimes\in[0,d_1,d_2]$. its whitenization weight function is:

$$f_3(d_{ji})=\begin{cases} 1, & d_{ji}\in[0,d_1] \\ \dfrac{d_2-d_{ji}}{d_2-d_1}, & d_{ji}\in[d_1,d_2] \\ 0, & d_{ji}\notin[0,d_2] \end{cases} \quad (3)$$

The selection of d_1,d_2 in formula (1)(2)(3) is usually carried on according to the related standards or the previous experience, or could be valued according to the minimum, average, maximum of the sample matrix to determine

the threshold value of lower limit, middle and upper limit.

3.2.3 Determine the grey evaluation coefficient.

Using the evaluation the experts gave to each process of dairy product cold chain logistics, we could get D_{ji} .and $D_{ji}^{(A)}$ indicates the evaluation matrix the expert i gave to the j th influencing factor of the main process A.

Integrated $D_{ji}^{(A)}$ and $f_k(d_{ji})$, we could get the grey evaluation coefficient the influencing factor j to the main process A which belongs to the k th grey clustering is

$$\tilde{\otimes}_{ji}^{(A)} = \sum_{i=1}^{n} f_k(d_{ji}) \tag{4}$$

3.3. The grey matter elements of each process

$\tilde{\otimes}_{ji}$ $(j = 1,2,\cdots,m; i = 1,2,\cdots,n)$ is the corresponding grey number explicit value of n influencing factors under j th evaluation level, therefore we could get n -dimension grey element of j th evaluation level.

$$\tilde{\otimes}R_{jn} = \begin{bmatrix} & M_j \\ c_1 & \tilde{\otimes}_{j1} \\ c_2 & \tilde{\otimes}_{j2} \\ \vdots & \vdots \\ c_n & \tilde{\otimes}_{jn} \end{bmatrix}. \tag{5}$$

M_j indicates the j th evaluation level, c_j indicates the i th main process under the j th evaluation level, $\tilde{\otimes}_{ji}$ $(j = 1,2,\cdots,m; i = 1,2,\cdots,n)$ indicates the corresponding grey number explicit value.

For the convenience of calculation and analysis, gather the n -dimension grey element of m evaluation levels, write n -dimension composite grey element of m evaluation levels, use $\tilde{\otimes}R_{mn}$ to express, namely:

$$\tilde{\otimes}R_{mn} = \begin{bmatrix} & M_1 & M_2 & \cdots & M_m \\ c_1 & \tilde{\otimes}_{11} & \tilde{\otimes}_{21} & \cdots & \tilde{\otimes}_{m1} \\ c_2 & \tilde{\otimes}_{12} & \tilde{\otimes}_{22} & \cdots & \tilde{\otimes}_{m2} \\ \vdots & \vdots & \vdots & & \vdots \\ c_n & \tilde{\otimes}_{1n} & \tilde{\otimes}_{2n} & \cdots & \tilde{\otimes}_{mn} \end{bmatrix} \tag{8}$$

m indicates the number of evaluation levels, n indicates the number of influencing factors.

3.4. Use the relative optimal principle to construct n -dimension grey element of the ideal risk set.

Find out the optimal value from m evaluation levels, make up an ideal risk set so as to construct the n -dimension grey element of the ideal risk set.

$$\tilde{\otimes}R_0 = \begin{bmatrix} & M_0 \\ c_1 & \tilde{\otimes}_{01} \\ c_2 & \tilde{\otimes}_{02} \\ \vdots & \vdots \\ c_n & \tilde{\otimes}_{0n} \end{bmatrix}. \tag{7}$$

Normally, the optimal value is determined according to the minimum value, the medium value and the maximum value of grey number explicit value of each main factor, namely, there are 3 types:

(1)The smaller the optimal type: $\tilde{\otimes}R_{0i} = \tilde{\otimes}R_{1i} \wedge \tilde{\otimes}R_{2i} \wedge \cdots \wedge \tilde{\otimes}R_{mi}$;

(2)The moderate type: $\tilde{\otimes}R_{0i} = u_{ji}$;

(3)The bigger the optimal type: $\tilde{\otimes}R_{0i} = \tilde{\otimes}R_{1i} \vee \tilde{\otimes}R_{2i} \vee \cdots \vee \tilde{\otimes}R_{mi}$.

And $i = 1,2,\cdots,n$. $\wedge$ is min- operation, $\vee$ is max- operation.

3.5. Correlation degree analysis

3.5.1. Data processing

Use the interval data processing to carry on the dimensionless processing to the original data. There are three kinds of concrete forms, that is:

(1)The smaller the optimal type:

$$\tilde{\otimes}'_{ji} = \frac{\max\tilde{\otimes}_{ji} - \tilde{\otimes}_{ji}}{\max\tilde{\otimes}_{ji} - \min\tilde{\otimes}_{ji}}; \tag{9}$$

(2)The moderate type:

$$\tilde{\otimes}'_{ji} = \frac{\min(\tilde{\otimes}_{ji} - u_{ji})}{\max(\tilde{\otimes}_{ji} - u_{ji})}; \quad (10)$$

(3)The bigger the optimal type:

$$\tilde{\otimes}'_{ji} = \frac{\tilde{\otimes}_{ji} - \min\tilde{\otimes}_{ji}}{\max\tilde{\otimes}_{ji} - \min\tilde{\otimes}_{ji}}. \quad (11)$$

And $j = 1,2,\cdots,m$. $i = 1,2,\cdots,n$.

3.5.2. Correlation analysis

The n -dimension correlation coefficient composite grey element $\tilde{\otimes}R_{\xi}$ of m evaluation levels is as follows:

$$\tilde{\otimes}R_{\xi} = \begin{bmatrix} & M_1 & M_2 & \cdots & M_m \\ c_1 & \tilde{\otimes}\xi_{11} & \tilde{\otimes}\xi_{21} & \cdots & \tilde{\otimes}\xi_{m1} \\ c_2 & \tilde{\otimes}\xi_{12} & \tilde{\otimes}\xi_{22} & \cdots & \tilde{\otimes}\xi_{m2} \\ \vdots & \vdots & \vdots & & \vdots \\ c_n & \tilde{\otimes}\xi_{1n} & \tilde{\otimes}\xi_{2n} & \cdots & \tilde{\otimes}\xi_{mn} \end{bmatrix}. \quad (12)$$

$\tilde{\otimes}\xi_{ji}$ indicates the correlation coefficient explicit value of i th main process under the j th evaluation level after the standardization transformation, $j = 1,2,\cdots,m; i = 1,2,\cdots,n$ .and

$$\tilde{\otimes}\xi_{ji} = \frac{\Delta\min + \rho\Delta\max}{\Delta_{ji} + \rho\Delta\max} \quad (13)$$

The Δ_{ji} in formula (5) is the absolute value of grey number explicit value after the i th main process under the j th evaluation level is carried on the data standardization and ideal risk set data standardization, that is, $\Delta_{ji} = \left|\tilde{\otimes}'_{0i} - \tilde{\otimes}'_{ji}\right|$. $\Delta\max$ indicates the maximum value of absolute error Δ_{ji} , $\Delta\min$ indicates the minimum value of absolute error Δ_{ji} , ρ indicates the resolution coefficient, normally, $\Delta\min = 0$. $\rho = 0.5$.

3.5.3. Calculate the correlation degree

The analytic hierarchy process (ahp) is used to determine the weight of each evaluation index ω_k, ω_{kn} in dairy product cold chain logistics, and ω_k indicates the weight of the main process, ω_{kn} indicates the weight of n th influencing factor under k th main process.

Carry on the weighted calculation, we could get:

$$\tilde{\otimes}A_k = \omega_k \cdot \tilde{\otimes}R_{\xi} \quad (14)$$

$\tilde{\otimes}A_k$ indicates the overall grey correlation degree the main process of dairy product cold chain logistics to the evaluation level k .

Finally, according to the maximum membership degree principle, the evaluation level of dairy product cold chain logistics could be determined.

3.6. The simulation calculation of Deyi dairy product cold chain logistics evaluation in Weifang area

Referring to table 2 and the formula (1)(2)(3), we could determine the four grey clustering function expressions of Deyi dairy product cold chain logistics in Weifang area are as follows:

$$f_1(d) = \begin{cases} \frac{d}{8}, & 0 \le d \le 8 \\ 1, & d > 8 \\ 0, & d < 0 \end{cases}.$$

$$f_2(d) = \begin{cases} \frac{d}{8}, & 0 \le d \le 8 \\ 2 - \frac{d}{8}, & 8 < d \le 16 \\ 0, & d > 16, d < 0 \end{cases}. \quad (15)$$

$$f_3(d) = \begin{cases} \frac{d}{6}, & 0 \le d \le 6 \\ 2 - \frac{d}{6}, & 6 < d \le 12 \\ 0, & d > 12, d < 0 \end{cases}.$$

$$f_4(d) = \begin{cases} 1, & 0 \le d \le 4 \\ 2 - \frac{d}{4}, & 4 < d \le 8 \\ 0, & d > 8, d < 0 \end{cases}.$$

Four experts are hired to score and evaluate Deyi dairy product cold chain logistics in Weifang area, the results are shown in table 3.

Table 3. The scores the experts give to Deyi dairy product cold chain logistics in Weifang area

The influencing factors	Expert 1	Expert 2	Expert 3	Expert 4
raw milk collection	7.3	6.8	6.5	6.8
raw milk grading	8.2	7.8	8.3	7.5
preservative treatment	8.1	8.5	8.8	7.6
raw milk packaging	5.5	5.2	5.3	6.0
collecting zone refrigeration	6.2	6.8	6.6	6.5
long-distance transport	4.8	4.3	4.8	4.2
loading and unloading handling	4.5	5.6	4.8	6.0
Insurance processing	6.0	6.2	6.1	5.9
dairy product storage	5.1	6.2	6.8	4.2
Loading	6.2	5.4	6.2	6.8
short-distance transport	4.3	5.8	5.8	4.2
delivery	5.2	5.5	4.8	3.8
Storage	6.6	5.6	5.2	4.4
marketing	5.8	6.8	7.8	5.4

Therefore, we could get

$$D^{(A)}=\begin{pmatrix}7.3 & 6.8 & 6.5 & 6.8\\ 8.2 & 7.8 & 8.3 & 7.5\\ 8.1 & 8.5 & 8.8 & 7.6\\ 5.5 & 5.2 & 5.3 & 6.0\\ 6.2 & 6.8 & 6.6 & 6.5\end{pmatrix}.$$

$$D^{(B)}=\begin{pmatrix}4.8 & 4.3 & 4.8 & 4.2\\ 4.5 & 5.6 & 4.8 & 6.0\end{pmatrix}.$$

$$D^{(C)}=\begin{pmatrix}6.0 & 6.2 & 6.1 & 5.9\\ 5.1 & 6.2 & 6.8 & 4.2\end{pmatrix}. \quad (16)$$

$$D^{(D)}=\begin{pmatrix}6.2 & 5.4 & 6.2 & 6.8\\ 4.3 & 5.8 & 5.8 & 4.2\\ 5.2 & 5.5 & 4.8 & 3.8\end{pmatrix}.$$

$$D^{(E)}=\begin{pmatrix}6.6 & 5.6 & 5.2 & 4.4\\ 5.8 & 6.8 & 7.8 & 5.4\end{pmatrix}.$$

From the formula (4) and $D^{(A)}$, the 4 grey clustering evaluation coefficients of the first influencing factor under the main process A could be calculated separately as follows:

$$\tilde{\otimes}_{111}=\sum_{l=1}^{4} f_1(d_{1l})=f_1(7.3)+f_1(6.8)+f_1(6.5)+f_1(6.8)=3.4$$

$$\tilde{\otimes}_{211}=\sum_{l=1}^{4} f_2(d_{1l})=f_2(7.3)+f_2(6.8)+f_2(6.5)+f_2(6.8)=3.4$$

$$\tilde{\otimes}_{311}=\sum_{l=1}^{4} f_3(d_{1l})=f_3(7.3)+f_3(6.8)+f_3(6.5)+f_3(6.8)=3.5$$

$$\tilde{\otimes}_{411}=\sum_{l=1}^{4} f_4(d_{1l})=f_4(7.3)+f_4(6.8)+f_4(6.5)+f_4(6.8)=1.2$$

(17)

In the same way, the 4 grey clustering evaluation coefficients of the 2nd, 3rd, 4th, 5th influencing factor under the main process A could be calculated as follows:

$$\tilde{\otimes}_{112}=3.9\,.\,\tilde{\otimes}_{212}=3.7\,.\,\tilde{\otimes}_{312}=2.7\,.\,\tilde{\otimes}_{412}=0.2;$$

$$\tilde{\otimes}_{113}=3.95\,.\,\tilde{\otimes}_{213}=3.8\,.\,\tilde{\otimes}_{313}=2.5\,.\,\tilde{\otimes}_{413}=0.1;$$

$$\tilde{\otimes}_{114}=2.75\,.\,\tilde{\otimes}_{214}=2.75\,.\,\tilde{\otimes}_{314}=3.7\,.\,\tilde{\otimes}_{414}=2.5;$$

$$\tilde{\otimes}_{115}=3.3\,.\,\tilde{\otimes}_{215}=3.3\,.\,\tilde{\otimes}_{315}=3.65\,.\,\tilde{\otimes}_{415}=1.5\,.$$

(18)

Therefore, we could get the grey evaluation coefficient matrix $\tilde{\otimes}R$ of the main process A as follows:

$$\tilde{\otimes}R_A = \begin{pmatrix} 3.4 & 3.4 & 3.5 & 1.2 \\ 3.9 & 3.7 & 2.7 & 0.2 \\ 3.95 & 3.8 & 2.5 & 0.1 \\ 2.75 & 2.75 & 3.7 & 2.5 \\ 3.3 & 3.3 & 3.65 & 1.5 \end{pmatrix}. \quad (19)$$

Using the bigger the optimal principle, the optimal solution of the main process A could be determined as follows:

$$\tilde{\otimes}R_0^{(A)} = \begin{bmatrix} & M_0 \\ c_1 & 3.5 \\ c_2 & 3.9 \\ c_3 & 3.95 \\ c_4 & 3.7 \\ c_5 & 3.65 \end{bmatrix}. \quad (20)$$

Use the bigger the optimal criterion to carry on the standardized processing to $\tilde{\otimes}R_A$, use the formula (5) to calculate the correlation grey matter element of the main process A, that is,

$$\tilde{\otimes}R_\xi^{(A)} = \begin{pmatrix} 0.95 & 0.95 & 1 & 0.33 \\ 1 & 0.98 & 0.61 & 0.33 \\ 1 & 0.99 & 0.44 & 0.33 \\ 0.57 & 0.57 & 1 & 0.44 \\ 0.83 & 0.83 & 1 & 1.33 \end{pmatrix}. \quad (21)$$

The analytic hierarchy process (AHP) is used to calculate the evaluation index weight of Deyi dairy product cold chain logistics in Weifang area. The results are shown in table 4.

Table 4. The evaluation index weight of Deyi dairy product cold chain logistics in Weifang area

The main processes	weight	The influencing factors	weight
Collecting and treating process A	0.30	raw milk collection	0.22
		raw milk grading	0.15
		preservative treatment	0.20
		raw milk packaging	0.15
		collecting zone refrigeration	0.28
Transport process B	0.24	long-distance transport	0.60
		loading and unloading handling	0.40
processing flow C	0.10	Insurance processing	0.45
		dairy product storage	0.55
delivery process D	0.26	Loading	0.32
		short-distance transport	0.46
		delivery	0.22
Sales process E	0.10	Storage	0.56
		marketing	0.44

So

$\omega = (0.30, 0.24, 0.10, 0.26, 0.10)$.

$\omega_A = (0.22, 0.15, 0.20, 0.15, 0.28)$.

$\omega_B = (0.60, 0.40)$.

$\omega_C = (0.45, 0.55)$.

$\omega_D = (0.32, 0.46, 0.22)$.

$\omega_E = (0.56, 0.44)$.

According to the formula (6), we could calculate

$$\tilde{\otimes}A = (0.22, 0.15, 0.20, 0.15, 0.28)) \begin{pmatrix} 0.95 & 0.95 & 1 & 0.33 \\ 1 & 0.98 & 0.61 & 0.33 \\ 1 & 0.99 & 0.44 & 0.33 \\ 0.57 & 0.57 & 1 & 0.44 \\ 0.83 & 0.83 & 1 & 1.33 \end{pmatrix} = (0.88, 0.87, 0.83, 0.35) \quad (22)$$

.

In the same way, we could calculate that

$$\tilde{\otimes}B = (0.35, 0.55, 0.48, 1);$$

$$\tilde{\otimes}C = (0.56, 0.76, 0.63, 0.58);$$

$$\tilde{\otimes}D = (0.42, 0.82, 0.62, 0.55); \quad (23)$$

$$\tilde{\otimes}E = (0.52, 0.72, 0.52, 0.60).$$

Finally, we could get the correlation coefficient of Deyi dairy product cold chain logistics evaluation in Weifang area as follows:

$$\tilde{\otimes}P = (0.565, 0.706, 0.640, 0.606). \quad (24)$$

According to the maximum membership degree principle, the evaluation result of Deyi dairy product cold chain logistics evaluation in Weifang area is good.

4. Conclusions

Dairy industry has long industrial chain, and many links. And its range is wide. The loss rate of current dairy product logistics is high, and its safety and reliability is low, which needs to be changed to improve the reliability of the whole system effectively. Dairy product cold chain logistics involves each link of the whole supply chain, That any process goes wrong would influence the safety and quality of dairy products. In this paper, combined with each process of dairy product cold chain logistics, dairy product cold chain logistics is evaluated, establishing a fair, scientific and reasonable evaluation model.

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RESEARCH ON CURRENT SITUATION OF NUTRITION FOOD SUPPLEMENT IN CHINA

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ABSTRACT
This research focuses on the current situation of sports health food. It has made comprehensive analysis of the current situation of sports health food approved by Ministry of Health and SFDA as well as the sales and operation situation of sports health food on the market based on the comprehensive and systematic analysis of formation and development of our traditional health care theory; drawn lessons from the specific measures and methods of registration examination and approval management of sports health food at abroad, proposed comments and suggestions for the existing problems and development trend in the future of our sports health food and expected to provide scientific basis for the healthy and orderly development of our sports health food industry and provide important reference for further establishment and improvement of our standard system of laws and regulations for sports health food.

1. Introduction

Sports health food is an emerging industry born gradually from the continuous development of diet therapy theory and practice. Sports health food has the function of maintaining health and strengthening health by acting on healthy people; has the function of removing the sub-health state of body, controlling the transformation to disease state or recovering to health state by acting on sub-health people; has the function of assisting treatment, which can not only relieve the side effects of drugs but also increase the sensitivity of drugs by acting on the people with disease (Li et al, 2010). Therefore, sports health food has popular, specialized and legalized features. At the beginning of reform and opening up, sports health food was in a mess situation without legal basis (Palacio and Nuin, 2009). After unremitting efforts of numerous people with lofty ideas for nearly 30 years, the sports health food has developed from existed as a form of food under the premise of not promoting its health care function and not naming sports health good by the government to the situation that clauses about sports health food management have been listed in Law of the People's Republic of China on Food Hygiene issued on Oct. 30th 1995 (Qi and Tian, 2011); and then to the situation that State Food and Drug Administration (hereinafter as SFDA) started to perform the registration examination and approval function officially in Oct. 2003 and related laws, regulations and standard system related to sports health food also made great progress, at present, it has developed into a sunrise industry with extensive development prospect (Lan and Xue, 2013). Till the end of 2005, there are totally 8,684 sports health products approved by the country. But till today, there are few researches on the theory and practice of sports health food and there is no report for the research on current situation of sports health products approved by the country (Hangan, 2015). The comparative research on related laws and regulations of sports health

products both at home and abroad needs to be strengthened in a further way. The theoretical system for research on sports health food development and marketing and management model has not been established (Qi and Tian, 2011). The enterprises working on research and development as well as operation of sports health food are still in a state of blind project and simple copy and the phenomenon of low-level repetition is very serious. The market supervision and management of sports health food as well as personalized sales mode have not been formed yet (Lan and Xue, 2013). The establishment of quality standard system which is extracted with advanced production technology and usually used for the animal and plant extracts of sports health food has not gained the attention from related departments, which has greatly restricted the further development of sports health food industry and produced great negative effect on the reputation of our sports health food in international market (De et al., 2013).

Table 1. In each of the total number of health food approval TAB

Year	Domestic health food	Imported health food
1996	591	3
1997	954	125
1998	602	62
1999	1136	45
2000	696	85
2001	956	62
2002	902	46
2003	545	48
2004	1520	95
2005	994	28

Table 2. Has been approved by the health food function distribution statistical table

Health care function	The total number of approval	The percentage	2003	2004	2005
Enhance immunity	2485	32.25	150	484	312
Alleviate physical fatigue	1452	16.35	86	235	163
Auxiliary fall hematic fat	1263	14.25	95	203	45
Nutrient supplements	658	10.32	50	135	48
anti-aging	448	7.54	45	98	36
purge	356	5.23	62	78	59
Auxiliary fall blood sugar	365	3.65	52	14	25
beauty	302	3.45	30	25	5
Improve sleep	236	3.02	41	54	12
lose weight	245	2.65	21	56	36
Liver injury protection	268	2.16	15	85	20
hypoxia	131	2.54	10	25	14
improve the memory	161	1.54	25	12	25
Qing pharynx and larynx	121	1.56	6	23	45
Improve anemia	532	1.24	9	36	55
Resistance to radiation	123	1.06	4	24	23

For this, this research focuses on the current situation of sports health food. It has made comprehensive analysis of the current situation of sports health food approved by Ministry of Health and SFDA as well as the sales and operation situation of sports health food on the market based on the comprehensive and systematic analysis of formation and

development of our traditional health care theory; drawn lessons from the specific measures and methods of registration examination and approval management of sports health food at abroad (Giannaki, 2014).

Table 3. The SFDA in 2004 approved by the health food products to the statistics of traditional Chinese medicine as the main raw material

The main raw material	Frequency of usage	New formula	Traditional Chinese medicine and animal and plant extract formula	Combination formula
Chinese wolfberry	200	146	24	33
American ginseng	165	123	15	26
ginseng	136	102	29	25
Poria cocos	118	92	46	42
angelica	95	66	41	21
hawthorn	88	68	15	19
yam	85	54	14	17
Cassia seed	75	58	16	26
Kudzu root	74	49	16	25
Ginkgo biloba	72	42	5	23
Zizyphus jujube benevolence	70	70	8	10
Huang jing	65	56	9	2
Fruit of Chinese magnoliavine	69	34	12	5
Aloe vera	54	35	10	9
pearl	58	39	6	14
Red flowers	55	75	8	5
chrysanthemum	56	58	9	3
Rhodiola rosea	23	69	8	2
single	53	48	5	8
notoginseng	54	25	7	17
acanthopanax	36	26	9	16
Eucommia ulmoides	39	54	12	2
cinnamon	54	48	3	3
licorice	45	62	6	25
Fructus ligustri lucidi	65	36	5	2
Longan meat	23	32	2	2
Barley rice	26	12	3	1
Radix paeoniae alba	28	15	1	2
Large-headed atractylodes	12	25	5	3
Son Tu silk	16	24	4	5
lily	14	28	6	5

Proposed comments and suggestions for the existing problems and development trend in the future of our sports health food and expected to provide scientific basis for the healthy and orderly development of our sports health food industry and provide important reference for further establishment and improvement of our standard system of laws and regulations for sports health food (Burkhart, 2010).

2. Materials and methods

Since the official release of "measures for the administration of sports health food" on Jun. 1st 1996, there are totally 8684 sports health products approved by the nation till end of 2005 since the nation performed approval system for foods with health care function, in which there are 8109 national sports health foods made in China and 575 foreign sports health foods. There are 5535 sports health foods made in China

approved by the Ministry of Health and 490 imported sports health foods (Burkhart, 2010) (Goston, 2010); there are 2574 sports health foods made in China approved by SFDA and 85 imported sports health goods. This research takes technical review database of sports health food in SFDA sports health food review center as basis (checking the original file for products without complete technical material approved by Ministry of Health in early years), refers to sports health food announcement of Ministry of Health and SFDA, makes comprehensive and systematic statistical analysis of the total products number, functional distribution, main ingredients, using frequency of efficacy components, distribution of dosage form as well as regional distribution of reporting units etc. of sports health food approved by Ministry of Health and SFDA and looks forward to provide basis for establishing and improving the laws and regulations for registration and approval of sports health food and provides importance reference to the scientific development of sports health food etc (De et al. 2013).

2.1. Data source and statistical analysis method

It is mainly based on material of sports health food approval certificate database approved by sports health food technology evaluation system in SFDA sports health food evaluation center, which includes all materials from the No. 001 (96) approved health and food index on Jun. 1st 1996 to No. G20050994 national health and food index approved by SFDA (Giannaki, 2014); including all the content of 8684 sports health food approval certificate databases as well as the content of sports health food notice released by Ministry of Health and SFDA, original files of partial products also included(Gong and Spear, 1988).

Distribution of health care function: the distribution of health care has made statistical analysis by taking all the products approved by Ministry of Health and SFDA as base and made following adjustments for the inconsistent names of health care functions before and after the implementation of technical specifications for inspection and evaluation of sports health food (2003 version) (Burton et al. 2006). For items without obvious changes of evaluation indexes and methods, take the name of new function as standard and the old functions are included (such as immune regulation, anti-fatigue, regulating blood pressure, regulating blood lipid and improving memory etc.) (Gong and Spear, 1988). New functions of removing acne, dispelling chiliasm, improving skin moisture and improving skin oil have been listed into beauty function; make statistics for functions not in the new program based on former functional names (such as anti-mutation and anti-aging etc.); ¼ make separate statistics for items with two functions, the calculated percentage is the percentage of health care function to the approved product (Ewert and Yoshino, 2008); ½ take all the functions included in nutrients supplementation as a big functional group to make statistics.

2.2. Using frequency of main raw materials and efficacy components

(1) Using frequency of main raw materials of sports health foods which takes the items of food and drug and items used for sports health food as basic formula: first make statistics for the using frequency of items listed in the attachments 1,2 of No. 2002851 health supervision in the approved 7762 sports health foods (922 nutrients supplementations are not included) from the notice about further standardize the management of raw materials of sports health food realized by Ministry of Health, and then select 33 items with using frequency above 30 times and finally make comprehensive analysis of formula and/or main raw materials (Guo et al. 2014).

(2) The using frequency of main raw materials of sports health foods taking traditional Chinese medicine and/or food extract and non-traditional Chinese medicine as the basic formula: make statistical analysis of sports health foods which take traditional Chinese medicine and/or food extract and non-traditional Chinese medicine animals (including insects) as main raw materials and need to add suitable addictives due to the process needs.

(3) Using frequency of efficacy components and/or marking components: make statistical analysis of efficacy components of sports health food listed in technical specifications for inspection and evaluation of sports health food issued by Ministry of Health (2003 version) and efficacy components listed in health indicators inspection specification (Schmitt, 2007).

2.3. Regional distribution of products and reporting units

Because the database material before 2003 is not complete and the data recording making technology transfer after approval is limited, in addition, blind reporting phenomenon is serious and many products reporting units were trying to report for approval before the publish of measures for administration of sports health food registration on Jul. 1st 2005, so it is difficult to reflect the real situation. Therefore, this research only makes statistics for the sports health products approved in 2004 and its reporting units.

3. Results and discussions

3.1. Statistical analysis results of total sports health foods approved annually

It can be seen from table 1 and Figure 1 that for the annually approved sports health foods made in China, 1996 is with the least number (59), followed by 2003 (545), while the number of sports health foods approved in 1997 is about 20 times of that in 1996 (949 in total); 2004 is with the most quantity (1503) (Burton et al. 2006). 1997 is with the most imported sports health foods (101), followed by 1999 (98), with a decreasing trend in the following and in 2005, there are only 27 approved. In addition, the product approval number approved by Ministry of Health in 1996 is not standardized, the years are with both two digits and four digits, for example, No. xxx (96) approved health and food index, No. xxx (1996) approved health and food index; the import sports health food approval indexes of 1996, 1997, 1998 and 1999 are also in a mess, which not only include No. xxx health and food index (year), but also No. xxx food and health index (year)(Gong and Spear, 1988).

3.2. Distribution statistical results of health care function (as shown in table 2 and Figure 2)

It can be learnt from table 2 and Figure 2 that the function of strengthening the immunity function account for the most among all the functions of approved sports health foods (2789 in total, 32.12%), followed by the function of relieving fatigue (1405 in total, 16.18%) and then followed by the function of assisting in reducing the blood lipid (1241 in total, 14.29%). Products with functions of strengthening immunity, relieving fatigue and assisting in reducing the blood lipid account for 62.59% of the total approved sports health foods. Among all the sports health foods approved by Ministry of Health in 1999, products with two or over two functions account for over 40%, in which there are five functions at most for the declared products; among all the sports health foods approved after 2000, the number of products with two functions accounts for 20.27% of the total approved products. As a big functional group, 922 nutrients supplementations have been approved, which account for 10.62% of the approved product functions, in which there are 332 products with calcium supplement function, accounting for 36.01% of the total nutrients supplementations.

3.3. Using frequency of main raw materials, efficacy components and/or marking components

Using frequency of main raw materials of sports health foods which takes the items of food and drug and/or items used for sports health food as basic formula, it can be seen from table 3 that the top three sports health foods taking item as both food and drug based on health supervision [attachment of file 2002151] are meddler (1040 times), tune (506 times) and hawthorn (430 times); among sports health foods taking goods in health supervision [attachment 2 of file 2002851] as the main raw materials of sports health food, the American ginseng is with the most using frequency (594 times), followed by astragals meme panaceas (515 times) and ginseng (494 times). There are 106 protects taking pan ax as main raw material, which

accounts for 18.01% of the total sports health foods with ginseng.

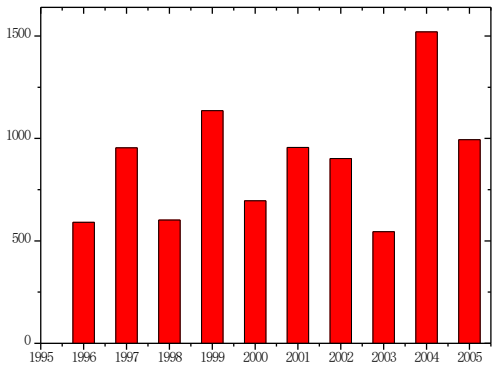

Figure 1. Domestic health food for 1995-2005

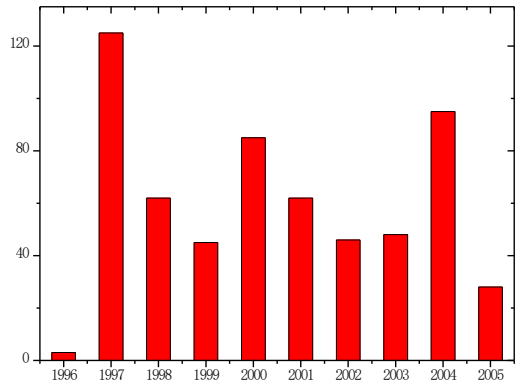

Figure 2. Imported health food for 1995-2005

There are totally 102 products with single ginseng as main raw material, which accounts for 20.65% of the total sports health foods with ginseng. The using frequency of pearl lists No. 5 (218 times), but the proportion of sports health food taking single pearl as main raw material is more (14.53%). Among 1638 sports health food approved by SFDA in 2004 (81 approved in 2003 included), there are 34 products with single ginseng as main raw material, which accounts for 20.99%of the total sports health foods with American ginseng. The using frequency of pearl is low (57 times) and there are nearly 50% sports health foods are added with addictive of biological activity due to the need of health care function (Hangan, 2015).

The using frequency of main raw materials of sports health foods taking traditional Chinese medicine and/or food extract and non-traditional Chinese medicine as the basic formula (as shown in table 3): it can be seen from table 3 that the using frequencies of plant extracts and non-traditional Chinese medicine ranked top three are propels/propels freeze-dried powder with the most using frequency (191 times), followed by grape seed/grape seed extract (116 times) and extract of ginkgo balboa (flavones) (95 times). It needs to pay attention that sports health foods made of above raw material by adding some auxiliaries take bigger proportion in total raw material using, which are 42.93%, 46.55% and 35.79% respectively (Stewart et al. 2007). The using frequency of soy is flavones ranked at No. 4 (92 times), but there are a lot of sports heath products taking single product as raw material, accounting for 47.83%.

Appearance frequency of efficacy components and/or marking components (as shown in table 3): It can be seen from table 3 that the total spooning is with the most appearance frequency of efficacy components and/or marking components (733 times), followed by total flavones (603 times), crude polysaccharide (596 times), ginseng spooning (221 times) and pro cyaniding (75 times). Among 1638 sports health foods approved by SFDA in 2004 (including 81 approved in 2003), total spooning is the most (375 times), followed by total flavones (350 times), two efficacy components of total spooning and total flavones account for 44.26% of the total approved number. The using frequencies of pro cyaniding and ginseng saponins are also very high, which are 58 times and 56 times respectively; both the using frequency and proportion have exceeded 50% of the total using frequency (as shown in table 3).

3.4. Statistical results for regional distribution of products and reporting units

It can be seen from table 3 that, the sports health foods approved by SFDA in 2004 mainly distribute in Beijing, Zhejiang, Guangdong, Shandong, Shanghai and Jiangxi. There are totally 909 products in these six provinces and

cities, which account for 55.49% of the total approved number, in which Beijing has the most, with 289 products, accounting for 1.64% of the total approved number; there are 163 and 157 products in Zhejiang and Guangdong respectively. There are totally 22 products in western less developed areas, such as Xinjiang, Xizang, Ningxia and Qinghai, which accounts for only 1.34% of the approved products. Among 1638 products approved in 2004, there are totally 1161 reporting units involved, in which there are 237 units reported by two or over two units. Beijing is with the most reporting units for sports health food, 254 in total, accounting for 21.88%, in which there are 54 joint declarations between outside reporting units and Beijing report units. There are only 56 reporting units in Shandong while there are 113 products in total and each reporting unit possesses two products on average.

4. Conclusions

This research has made comprehensive and systematic analysis of the current situation of our approved sports health food, current situation of products which are already on the market after approval, the market sales situation of famous enterprises as well as the sales trend of products of famous brands based on the purpose of this subject and compared comprehensively the differences in supervision and management of sports health food between China and Japan, America, EU, Australia and other countries and regions. The conclusions are as following:

1. There are a lot of approved products (8684), but the phenomenon of simply copy and low-level repetition is very serious and the general technology content is not high; the products function is too limited and the regional distribution is uneven.

2. The sales modes are diversified and the market potentiality is great. The self-protection awareness of consumers is weak and the advertisement effect is obvious. Personalized health management sales model has not attained enough attention and it lacks specific guidance from professional personnel in the sales team of sports health food.

3. Compared with developed countries, the supervision and management of our sports health food is with unique features and related laws and regulations for sports health products are relatively complete. However, because there are serious limitations in relevant management rules or standards of sports health food, the technical evaluation standards of sports health food, label standard, quality standard of raw material extracts, technical guiding principle of products development and research etc have not been established, which greatly restricts the sustainable development of sports health food industry.

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COMPREHENSIVE EVALUATION RESEARCH OF FOOD SAFETY BASED ON GREY CLUSTERING

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ABSTRACT

The food safety issue is the big matter that is related to human life, and it is the chief national policy of each country government. This paper revolves around food safety issues, and firstly confirms the evaluation system of taking food quantity safety, food quality safety and food sustainable safety as main indexes. By using Analytic Hierarchy Process (AHP), and according to previous data, the weight of each index is calculated. Through national food safety standard, the grey clustering of comprehensive evaluation of food safety is confirmed. And the whitenization weight function of comprehensive evaluation model of food safety is established. Finally, calculate the whitenization weight clustering coefficient and clustering vector of comprehensive evaluation model of food safety, and then get the evaluation results. The evaluation method and evaluation process are scientific and reasonable, combining subjective evaluation and objective calculation. The evaluation results accord with the actual situation, and provide theoretical support to enact food safety policies.

1. Introduction

How to evaluate food safety level, especially the food safety level of an industry evens an area, is a difficult issue in current food safety field in our country. Food safety evaluation work involves a very wide variety of aspects. A large number of researchers develop all kinds of evaluation and research jobs in this field. Especially the achievements of comprehensive evaluation aspects emerge in an endless stream (Ma et al., 2007). Basic index establishment is the most basic job for single evaluation or comprehensive evaluation. Compared with comprehensive evaluation research, basic index and exponent research develop slowly all the time (Chen, 2010). By now, the supervision departments in our country still utilize the traditional fraction defective index. And because many comprehensive evaluation exponents the theoretical circles proposed can't support the effective supervision demand, and always stop at the thesis research level, they are not applied by actual supervision departments (Li, 2004). Among all kinds of food safety indexes, fraction defective index is a basic index that is adapted in the widest scopes, whose index meaning is clearest, and whose operation is simplest. This index is wildly used in the food safety supervision from center to local levels in our country.

Food safety evaluation has important significance towards food safety work. Currently the experts at home and abroad carry on a relatively deep research on this aspect. Because food safety evaluation is a kind of research that is related to a very wide aspect, and can't be divided from the researches of food safety main body action recognition, food safety risk warning and food safety information release (Li et al., 2012; Sperber, 1998).

Therefore, besides reading and analyzing the research references of food safety evaluation, other present research situations about food safety risk analysis, food safety main body action recognition, food safety risk warning and so on, should also be analyzed and summarized. Foreign researches on food safety evaluation mainly focus on two aspects, and they are food safety connotation and influencing factor analysis and food safety risk analysis. Currently domestic researches on food safety connotation and influencing factor analysis aspect mainly focus on two aspects, and they are basic research of food safety evaluation connotation, and present food safety situation analysis. Especially on the aspect of basic researches of food safety connotation, more academic achievements are acquired.

As for the unceasingly outbreak of food safety events and the actual situation of our national supervision main body, the evaluation method of food safety status and the establishment of food safety index should not be over complicated, and the related variables should not be too many (Ban et al., 2003). Few variables and simply operated evaluation methods will be more easily popularized and applied in a larger scope.

2. Materials and methods

2.1. Establishment of food safety evaluation index system

2.1.1. Selected principles of food safety evaluation index

The premise of comprehensively evaluating food safety is the selection of food safety index, and it should follow the basic principles of establishing comprehensive evaluation index (Banati, 2003). This paper is based upon the needs that supervision departments carrying on unqualified sequence evaluation and value evaluation (risk level) towards participating objects, constructing reliable, objective, convenient, and identified food safety index, and adheres to the principles of comprehensiveness, objectivity, relativity, comparability, clarity and simplicity; at the same time, according to the following four principles to select the final food evaluation index:

(1) Material determination principle

The object of food safety evaluation should lock up the material in the food(Boon, et al,2008). Key material element that is directly related to food safety should be chosen to construct the excellent and reliable evaluation index.

(2) Source determination principle

According to data mining theory, the establishing process of food safety exponent is a process of data mining. The difference of different initial data will lead to the big difference of data mining and outputting. Therefore, the initial data source that can be tested is the basis that can guarantee the evaluating information quality (Dong et al., 2010; Jiang et al., 2005).

(3) Limited standard principle

In the research of current food safety evaluation, most established evaluation index doesn't take the objective limited standard as basis to design the evaluation index, but take the experts' subjective judgement as basis to design the evaluation index. By using the subjective fuzzy mathematics evaluation method to develop the comprehensive evaluation of food safety, can't ensure the evaluation information is reliable, because its designed membership index relies on the experts' subjective judgement (Liu and Zhou, 2004; Liu and Xie, 2011). By comparing the actual value in the food testing project with the limited standard, to design the evaluation index, not only has the reliable basis that has been tested, but also has the authorized guarantee provided by official standard.

(4) Comprehensive information principle

In order to support the effective supervision, the food safety index is required to provide more comprehensive information (Zheng, 2005). According to different participating objects, through food safety index to evaluate comprehensively, multi-angle and multilevel food safety evaluation information can be provided. The evaluation of specific testing

project of food safety status, and the evaluation of different food classification with type average of testing project, and different districts and overall evaluation can be acquired (Sheng et al., 2012).

2.1.2. Constructing food safety evaluation index system

According to the selecting principle of food safety evaluation index, the index system of food safety evaluation is established, as table 1 indicates.

Table 1. Food safety comprehensive evaluation index system

target layer	standard layer(risk source)	Index layer
food safety comprehensive evaluation P	food quantity safety exponent A	(1) heat intake per capita per day A1 (2) grain reserve ratio A2 (3) low income stratum food safety guarantee levelA3 (4) grain self-sufficiency ratioA4; (5) grain occupancy per capita per yearA5; (6) total grain output A6;
	food quality safety exponent B	(7) the proportion of high quality protein occupying total protein B_1; (8) ratio of fat and heat energyB_2; (9) heat ratio provided by animal foodB_3; (10) pesticide residue sampling pass rateB_4; (11) veterinary drug residue sampling pass rateB_5; (12) food hygiene monitor overall pass rateB_6;
	food sustainable safety exponent C	(13) percentage of forest coverC_1; (14) water resources quantity per capitaC_2; (15) area of soil and water lossC_3; (16) cultivated land per capitaC_4.

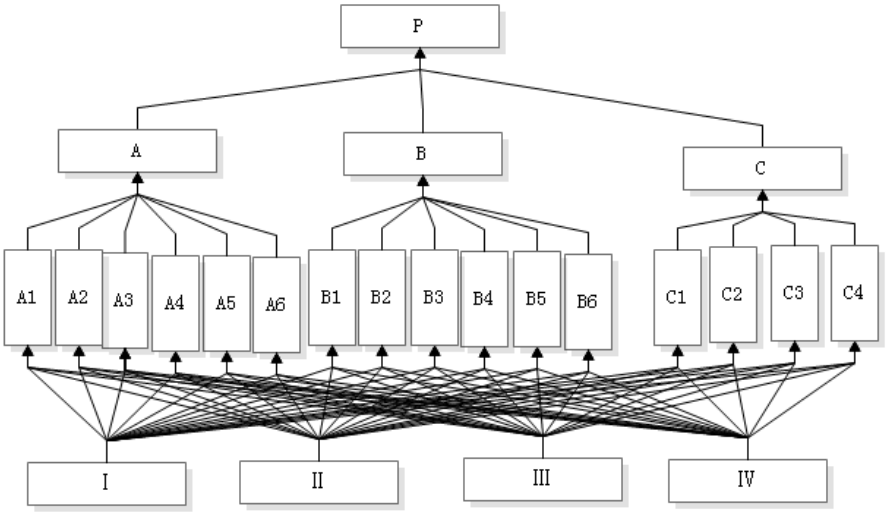

Figure 1. Food safety evaluation hierarchical chart

2.2. Establishing food safety evaluation model of grey clustering

2.2.1. Calculate and determine the weight coefficient of each food safety evaluation index

In the grey clustering evaluation model of food safety, aiming at constructing food safety index system, using the analytic hierarchy process (AHP) to determine and calculate the weight of each evaluation index (Yin and Li, 2009):

Firstly, construct the hierarchical chart of food safety evaluation. See figure1: target layer

is P; standard layer factor gather includes (A, B, C); index layer includes (A_1, A_2, A_3, A_4, A_5, A_6, B_1, B_2, B_3, B_4, B_5, B_6, C_1, C_2, C_3, C_4,); decision making layer;

Then, using expert judgment method to construct paired comparison matrix of each index level of food safety evaluation, and using MATLAB programming to calculate the weight of each evaluation index, and ensure that the calculation results all pass the consistency check. About the weight of food safety evaluation index, see table 2.

2.2.2. Determine the grey clustering of food safety evaluation

According to the results of food safety evaluation, combining with some experts' suggestions, food safety evaluation results can be divided into four grades, grade I, II, III and IV. There are four corresponding evaluation grey classes, and the grey class k=1, 2, 3, 4. The corresponding description of each food safety evaluation grade, as table 3 indicates.

Table 2. Weight of food safety comprehensive evaluation index

index	P_1	P_2	P_3	**weight** W_i
	0.63	0.19	0.18	
A_1	0.04			0.025
A_2	0.26			0.164
A_3	0.45			0.284
A_4	0.15			0.095
A_5	0.03			0.019
A_6	0.07			0.044
B_1		0.16		0.030
B_2		0.16		0.030
B_3		0.16		0.030
B_4		0.42		0.080
B_5		0.21		0.040
B_6		0.09		0.017
C_1			0.40	0.072
C_2			0.28	0.050
C_3			0.11	0.020
C_4			0.21	0.038

Table 3. Classification standard of food safety evaluation grade

grade	classification standard
I	Grain reserve surplus; meet self sufficiency;per capita share is high; high-quality protein proportion is big; no drug residue; rich water resources; large coverage of forest and green land;
II	Grain reserve in abundance;self sufficient basically;per capita share is relatively high;little drug residue;water resources can meet self need; larger coverage of forest and green land;
III	Grain reserve sufficient basically;per capita share is relatively low; vegetable and meat with little drug residue; lack of water resources relatively; less coverage of forest and green land;
IV	Grain reserve not sufficient;can not meet the self-sufficiency;per capita share is very low;much drug residue; lack of water resources; low coverage of forest and green land;

According to the data of food safety exponent and national standard of food safety, combined with requirement of food safety evaluation, by using Delphi method, the grey numbers of four grey classes of food safety evaluation clustering index are acquired, as table 4 indicates (Dai, 2009).

Table 4. Grey numbers of four grey classes of food safety evaluation clustering index

index	**weight**	Ⅰ	Ⅱ	Ⅲ	Ⅳ
A_1	0.025	$6\leq A_1<12$	$12\leq A_1<17$	$17\leq A_1<24$	$24\leq A_1<30$
A_2	0.164	$40\leq A_2<50$	$50\leq A_2<60$	$60\leq A_2<70$	$70\leq A_2<80$
A_3	0.284	$24\leq A_3<29$	$29\leq A_3<36$	$36\leq A_3<44$	$44\leq A_3<52$
A_4	0.095	$8\leq A_4<12$	$12\leq A_4<16$	$16\leq A_4<24$	$24\leq A_4<32$
A_5	0.019	$20\leq A_5<25$	$25\leq A_5<30$	$30\leq A_5<38$	$38\leq A_5<44$
A_6	0.044	$15\leq A_6<20$	$20\leq A_6<24$	$24\leq A_6<30$	$30\leq A_6<40$
B_1	0.030	$18\leq B_1<22$	$22\leq B_1<27$	$27\leq B_1<34$	$34\leq B_1<41$

B_2	0.030	$30\leq B_2<40$	$40\leq B_2<50$	$50\leq B_2<75$	$75\leq B_2<85$
B_3	0.030	$21\leq B_3<32$	$32\leq B_3<38$	$38\leq B_3<44$	$44\leq B_3<52$
B_4	0.080	$14\leq B_4<22$	$22\leq B_4<27$	$27\leq B_4<34$	$34\leq B_4<40$
B_5	0.040	$0\leq B_5<7$	$7\leq B_5<15$	$15\leq B_5<22$	$22\leq B_5<28$
B_6	0.017	$12\leq B_6<22$	$22\leq B_6<28$	$28\leq B_6<34$	$34\leq B_6<42$
C_1	0.072	$13\leq C_1<17$	$17\leq C_1<22$	$22\leq C_1<26$	$26\leq C_1<33$
C_2	0.050	$24\leq C_2<30$	$30\leq C_2<35$	$35\leq C_2<41$	$41\leq C_2<50$
C_3	0.020	$6\leq C_3<9$	$9\leq C_3<13$	$13\leq C_3<18$	$18\leq C_3<24$
C_4	0.038	$10\leq C_4<15$	$15\leq C_4<20$	$20\leq C_4<25$	$25\leq C_4<30$

2.2.3. Establishing whitenization weight function of food safety evaluation model, and calculate its function value

On the basis of analyzing different clustering objects, grey clustering evaluation method is applied to calculate the whitenization weight function of the related different index objects, and then synthesize all index weight to calculate the clustering grey numbers thus conclude and study all the indexes according to different grey classes and grey numbers. The clustering style of clustering object belongs to which kind of grey class has big relations with the influencing of this calculated index towards the clustering objects. In this paper the clustering objects are the three risk sources that food safety includes. The clustering index is each food safety evaluation index that the three risk resources correspond. Here the grey evaluation method, based on endpoint triangle whitenization weight function, is applied.

$$f_i^k(x_i)=\begin{cases} 0, & x_i\notin[a_{k-1},a_{k+2}] \\ \dfrac{x_i-a_{k-1}}{\lambda_k-a_{k-1}}, & x_i\in[a_{k-1},\lambda_k] \\ \dfrac{a_{k+2}-x_i}{a_{k+2}-\lambda_k}, & x_i\in[\lambda_k,a_{k+2}] \end{cases} \quad (1)$$

In this formula, x_i is the score value of index i. k indicates its evaluation grey.

As for the food safety evaluation index, the experts' score value x_i is substituted in the formula (1), calculating and getting the four grey trigonometric weight function $f_i^k(x_i)$ of each food safety evaluation index. In the formula $\lambda_k=\dfrac{a_k+a_{k+1}}{2}$, $a_0=a_1-5$, $a_6=a_5+5$.

2.2.4. Calculate the whitenization weight clustering coefficient and clustering vector of food safety evaluation model

$$\sigma_i^k=\sum_{i=1}^{m} f_i^k(x_i)W_i \quad (2)$$

Formula (2) is the grey variable weight comprehensive clustering coefficient of grey k. In the formula, x_i is the evaluation value of food safety evaluation index; $f_i^k(x_i)$ is index i subclass k whitennization weight function; W_i is the comprehensive clustering weights of index i.

By using formula (2), applying software MATLAB, calculate comprehensive clustering coefficient of each grey class, and get the clustering coefficient set of clustering objects $(\sigma_i^1,\sigma_i^2,\sigma_i^3,\sigma_i^4)$. According to the maximum membership principle, determine the clustering grade of clustering objects.

3. Results and discussions

Simulating calculation of food safety evaluation

According to the grey numbers of four grey classes of food safety evaluation clustering index in the above verse, combined with the five food safety experts' grading to the sixteen secondary class index in the evaluation model, the grey scorecard of food safety evaluation of weifang district is acquired, as table 5 indicates.

Table 5. Grey scorecard of food safety evaluation of weifang district

index	expert 1	expert 2	expert 3	expert 4	expert 5	average x_{ij}
A_1	18	16	21	15	23	19
A_2	68	44	72	48	64	59
A_3	39	37	24	28	33	32
A_4	26	28	16	18	19	21
A_5	38	44	40	36	42	40
A_6	32	21	38	25	30	29
B_1	36	32	27	24	28	29
B_2	65	48	58	42	54	53
B_3	46	42	28	30	34	36
B_4	14	16	11	16	18	21
B_5	18	22	14	8	12	15
B_6	14	25	23	16	18	19
C_1	12	13	11	11	15	12
C_2	52	48	56	46	54	51
C_3	9	12	7	8	10	9
C_4	18	26	20	19	21	21

The average value x_{ij} of the five food safety experts' scores in table 5, is substituted in the formula (1), and get the four grey whitenization weight function values those are corresponded by sixteen secondary class evaluation indexes, and then get the whitenization weight function value of food safety evaluation in weifang district, as table 6 indicates specifically (Chen, 2003).

Table 6. Whitenization weight function value of food safety evaluation index in weifang district

index	average x_{ij}	$f_i^1(x_{ij})$	$f_i^2(x_{ij})$	$f_i^3(x_{ij})$	$f_i^4(x_{ij})$
A_1	19	0.0000	0.5236	0.8235	0.2000
A_2	59	0.0667	0.7333	0.6000	0.0000
A_3	32	0.4211	0.9412	0.2727	0.0000
A_4	21	0.0000	0.3000	0.9167	0.4167
A_5	40	0.0000	0.0000	0.4000	0.9091
A_6	29	0.0000	0.1250	0.8462	0.4545
B_1	29	0.0000	0.5236	0.8235	0.1905
B_2	53	0.0000	0.7333	0.5778	0.1000
B_3	36	0.1739	0.8889	0.6667	0.0000
B_4	21	0.6667	0.6667	0.0000	0.0000
B_5	15	0.0000	0.6364	0.6957	0.0000
B_6	19	0.8182	0.5385	0.0000	0.0000
C_1	12	0.5714	0.0000	0.0000	0.0000
C_2	51	0.0000	0.0000	0.0000	0.4211
C_3	9	0.7273	0.6000	0.0000	0.0000
C_4	21	0.0000	0.5333	0.8000	0.1333

By formula (2), using MATLAB software, the comprehensive clustering coefficient σ_i^k of four grey of food safety evaluation in weifang district can be calculated, as table 7 indicates specifically.

As table 7 indicates, $\sigma^1 = 0.0581$ $\sigma^2 = 0.0233$, $\sigma^3 = 0.1108$, $\sigma^4 = 0.1101$, so the possibility size of the four grey clustering of food safety evaluation in weifang district is $\sigma^3 > \sigma^4 > \sigma^1 > \sigma^2$. According to the maximum membership principle, the overall evaluation level of food safety in weifang district is class III.

Table 7. Comprehensive clustering coefficient of food safety evaluation in weifang district

grey	clustering index			total
	A	B	C	
I	0.0686	0.0094	0.0728	0.0581
II	0.0254	0.0064	0.0339	0.0233
III	0.0743	0.1297	0.2185	0.1108
IV	0.1326	0.0480	0.0968	0.1101

4. Conclusions

Food safety evaluation is a hot topic. The research of food safety evaluation is related to a very wide variety of aspects. This paper meets the requirements of the supervision departments of simple index(Chen,2002), reliable information, subjective standard and sensitive exponent and so on. On the basis of food safety limited standard, the research of food safety evaluation is developed. On the precondition of comprehensively considering all kinds of influencing factors of food safety, three first class indexes, and sixteen secondary class indexes are selected, and the grey clustering evaluation model of food safety evaluation is established. This model is reasonable and feasible.

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THE COMPARISON OF PROTEIN EXTRACTION METHODS OF ACTINIDIA ARGUTA BASED ON TWO-DIMENSIONAL ELECTROPHORESIS

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ABSTRACT

Protein extraction from fruit is a key step to achieve high-resolution protein separation in two-dimensional electrophoresis. Three routine cellular total protein extraction methods were compared. Compare the total protein extraction efficiency for fruits of three protein extraction methods which were used in two-dimensional gel electrophoresis. *Actinidia arguta* treated with homogenization, TCA method and phenol extraction method to extract total protein of the fruit, measured the protein content using a spectrophotometer. According to the complexity of the operation, the time of the operation, the cost, dissolution rate and protein content, use analytic hierarchy process to choose the best method to extract total protein from the fruit. The results showed that the homogenate extraction method is more suitable to extract total protein from *Actinidia arguta* for two-dimensional electrophoresis.

1.Introduction

Proteomics refers to the study of protein features at the level of high throughput, including expressions, post-translational modifications, interaction and so on. Technologies involved include protein extraction, isolation, identification and bioinformatics analysis, and protein extraction is an important process.(Barraclough et al., 2004). The fruit tissue contains an extremely low amount of protein content, sufficient protease and a large quantity of interfering substance, which thus brings many difficulties to the subsequent analysis. (Bolgingh, 2000; Bradford, 1976) Therefore, it is of great necessity for the study on proteomics to select the appropriate method for the extraction of fruit protein.

Actinidia arguta (Sieb. et Zucc.) Planch. ex Miq is a perennial deciduous vine. as an *Actinidia*, it is the most widely distributed wild fruit tree in China (Carpentieret al., 2005). *Actinidia arguta* is also distributed in Russia, North America, Japan, Korea and other Asian countries. Its fruit contains a variety of nutrients, like sugar, protein, minerals, amino acids, vitamins, especially a relatively high content of Vc. Its green fruit has hairless skin, refreshing fragrance and a soft and juicy taste, which also promotes the production of body fluid and nourishes the lung, and holds certain treatment effect on high blood pressure, angina and high cholesterol (Chan et al., 2007). The fruits and the seeds can be used as medicines, and they have physiological functions for the treatment of diuresis, quenching thirst, anti-oxidation, anti-tumor, and alleviation of dysphoria with smothery sensation, improvement of immunity of the organism, anti-viral, liver protection, and prevention of cardiovascular and cerebrovascular disease. Its main ingredients are actinidine, cistanche base, saponin, flavonoid glycoside and other

effective ingredients (Damerval et al., 1986, Tian et al., 2011)

TCA extraction method is the classical method of extracting fruit protein, which originates from Damerval's extraction of wheat seedling protein (Deytieux et al., 2007). The largest drawbacks of this method are that incomplete precipitation will cause the loss of protein, and that the precipitated protein hardly dissolves in the lysis buffer. Also, the purity of the acquired protein is not high, with considerable impurities included (Hurkman and Tanaka, 1986). All of the demerits bring about difficulties to the subsequent research. Thus, its application is limited. Sarry et al., 2004, adopts this method to obtain a higher rate of grape skin protein.

Phenol protocol comes from the extraction of Hurkman (Rodrigues et al. 2009). For the proteins of plant cell membrane, which is now widely used in the extraction of fruit proteins, (Saaty et al., 2000) Deytieux et al., 2007 have modified this method to obtain a favorable purity of grape skin protein. Negri et al. 2008 and other researchers use this method to construct an extraction method for the cell wall proteins of grape fruit, with a higher yield of protein.

The homogenation method is also known as the urea-thiourea method. The lysis buffer acquired from the solubilized protein precipitation is used to directly extract the protein. The extracted protein impurities in this method are in a small amount, but the protein yield is lower than that of the previous methods. The resulting precipitation needs to be washed repeatedly to remove the lysates. (Barraclough et al., 2004). Apply this method with acetone precipitation to extract the protein in the fruits of apple, avocado, lemon and peach, with a high protein yield and purity.

This study compares the extraction efficiency of the total protein of this fruit in the three protein extraction methods that are currently used in the two-dimensional gel electrophoresis. *Actinidia arguta* is treated by liquid nitrogen, and the total proteins are respectively extracted by homogenation, TCA method and phenol protocol, and they are dissolved in the lysis buffer. The protein content is measured by a spectrophotometer. Considering the complexity of the extraction operation, operation time, cost, the difficulty degree of dissolution and protein content, the relatively best method of extracting the total protein is identified.

2. Materials and methods

2.1. Source of sample

Sample fruits are collected from the same tree in Qianshan mountain located in northeast china, then stored at 4±0.5 °C with 80-90% relative humidity, which physiological indexes of sample, such as table 1.

Table 1. Physiological indicators of *Actinidia arguta*

	Firmness (kg/cm^2)	Soluble solid content (%)
Values	2.44±0.14	9.72±0.11

Note: n≥100, P< 0.05

2.2. TCA method

The TCA method is slightly improved with reference to the method of Sarry et al [9]. 4g pulp is weighted and ground into powder in a mortar with liquid nitrogen. Then, four times of the volume of 10% ice-cold TCA (containing 0.07% β -mercaptoethanol) is added. The homogenization buffer is overnight cultured at minus 20℃ with gauze filter. The filtrate is centrifuged for 30 minutes at 20000×g(12000r) and collected for precipitation. The precipitation is washed three times with cold acetone (containing 0.07% β -mercaptoethanol), dried at 4 ℃, and then dissolved in the lysis buffer for alternative uses.

2.3. Phenol protocol

Protein extraction is performed using the phenol protocol with some modifications based on the achievements of Saravanan and Rose (Wang et al., 2006). 4g pulp is weighted and ground into powder in a mortar with liquid nitrogen. Then, 4ml of the homogenation buffer (it contains 20 mol/L Tris-Hcl (pH7.5), 1.5 mol/L sucrose, 10 mol/L EDTA , 1 mmol/L

PMSF, without 1 mmol/L DTT and 1% Triton X-100) and the grinding continues. The homogenation buffer is centrifuged for 30 mins at 20000×g (12000r). An equal volume of Tris-saturated phenol with a pH of 7.8 is added in the supernatant, fully shaken, mixed, and then centrifuged for 30 mins at 10000×g (6000r) at 4 ℃. The upper layer is phenol, the white substance in the middle is impurities, and the lower layer is water. Phenol is recycled. Five times of the volume of the pre-cooled methanol containing 0.1mol/L ammonium acetate is added, fully mixed, and overnight cultured at -20℃ to precipitate the proteins. The precipitation is washed three times with pre-cooled methanol containing 0.1mol/L ammonium acetate and acetone respectively, then dried at 4℃, and dissolved in 1ml pyrolysis buffer for alternative uses.

2.4. Homogenation

4g pulp is weighted and ground into powder in a pre-cooled mortar with liquid nitrogen. Then, 20ml of the homogenization buffer (it contains 20mmol/L Tris-Hcl (pH7.5), 250mmol/L sucrose, 10mmol/L EDTA, 1mmol/L PMSF, without 1mmol/L DTT and 1% Triton X-100) is added and the grinding continues. The homogenate is centrifuged for 40 minutes at 20000×g (12000r) at 4 ℃. The supernatant is transferred to a new centrifuge tube, and an equal volume of 20% TCA solution is added to make the final concentration of TCA solution reach 10%, with 3 hours' standing at 4℃. After 40 minutes of centrifugation at 20000×g (12000r) at 4℃, the precipitation is collected, rinsed three times with cold acetone, dried at 4℃and stored at -20℃ for alternative use (White et al., 2005)

2.5. Protein dissolution

The protein powder is subpackaged into the 100mg/2ml tubes, and 1ml of lysis buffer solution (now 0.01g DTT) is added. The mixer is whirled to speed up the protein dissolution. Since the vortex produces heat, every 5 mins of revolution requires 4 to 5 mins of ice bath, in order to avoid protein denaturation caused by overheating. If there are undissolved proteins left, ultrasonic waves are applied under the condition of ice-bath to help the solubilization at the rate of 2 mins per time. Protein dissolution is observed. Then this solution is the sample protein solution.

2.6. Determine the Protein Content

Coomassie brilliant blue method is employed to determine the protein content in the sample (Wang et al. 2010)

2.7. Establish the Analytic Hierarchy Process Model

In the 1970s, T. L. Saaty (Saaty, 2000) and other researchers have proposed a practical method that is able to effectively address this kind of problem, namely the Analytic Hierarchy Process (AHP), which is an analytical method with the combination of qualitativeness and quantitativeness, systematization and hierarchicalization. When AHP is applied, five factors are considered—protein content, the complexity degree of the operation, and the difficulty level of dissolution, operation time and cost. The model is constructed to determine the relative optimal extraction method.

3. Results and discussions

3.1. Effects of different extraction methods on the dissolution level of protein

The protein dissolution conditions in the three extraction methods are illustrated in the following table2.

Table 2. The protein dissolution

Method	Complexity of dissolution	Ultrasonic aided	Dissolution effect
TCA	Difficult	So many times	Failed dissolved
Phenol protocol	Middle	Few time	Fully dissolved
Homogenation	Easy	Many times	Fully dissolved

3.2 Effects of different extraction methods on the protein content

Bovine serum albumin is set as the standard substance. The Coomassie brilliant blue method is adopted to measure the protein. The regression equation

$Y=0.016X+0.337(R^2=0.998)$ is built, and the standard curve is plotted in Figure 1.

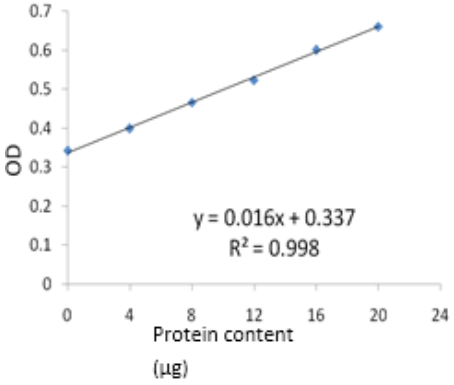

Figure 1. Protein standard curve

The extraction results of the protein content in the three methods are illustrated as Figure 2. The protein content extracted by TCA method is relatively small; the extracted protein content in the homogenation method is relatively high; the phenol protocol has extracted the most protein content, indicating a high protein content obtained by the phenol protocol.

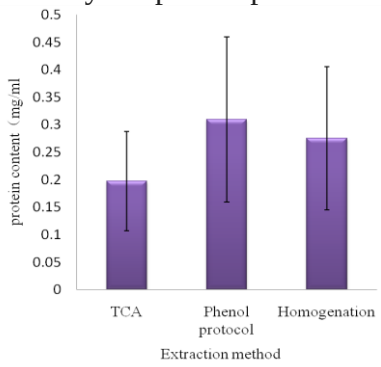

Figure 2. Total protein content

3.3. Determine the relative optimal extraction method by AHP

3.3.1. Determine the hierarchical structure

Some factors need to be considered when dealing with the issue of decision-making. In case of AHP, five factors are considered—protein content, the complexity degree of the operation, the difficulty level of dissolution, operation time and cost. The model is constructed to determine the relative optimal extraction method（Figure 3）.

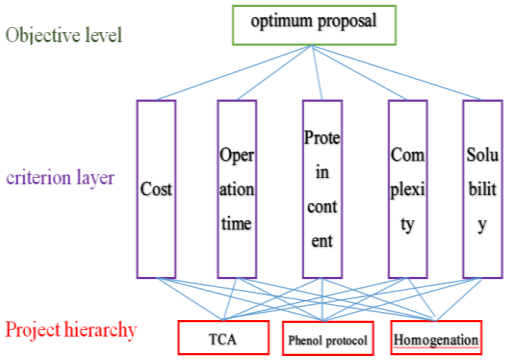

Figure 3. Choose the optimal extraction method of hierarchy

3.3.2. Construct pairwise comparison matrix among all levels of factors

Through comparison, the weights of various factors in the second layer relative to the first layer as well as the weights of various plans to each element are determined. These weights are usually qualitative in the human thinking process. When comparing the impacts of two factors with possibly different properties--C_i and C_j on the factor in the last layer O, the 1-9 ratio scale proposed by Saaty et al. is applied, which ranges from 1, 2, ..., 9 and its opposite numbers are 1,1 / 2 , ..., 1/9 (see Table 3).

Table 3. The meaning of scale a_{ij}

Scale	Meaning
1	The impact of C_i and C_j is the same
3	The impact of C_i is slightly stronger than that of C_j
5	The impact of C_i is stronger than that of C_j
7	The impact of C_i is evidently stronger than that of C_j
9	The impact of C_i is absolutely stronger than that of C_j
2,4,6,8	The impact ratio of C_i and C_j lies between the above adjacent scales
1,1/2,…1/9	The impact ratio of C_i and C_j is the opposite number of the abovementioned a_{ij}

The weights of the third layer to the second layer as well as the weights of the second layer to the first layer are used to determine the weight of the third layer to the first layer.

Assume that the impacts of the n factors C_1, C_2,…, C_n in a certain layer on one factor O in the last layer are to be compared. Two factors, C_i and C_j, are taken out each time. a_{ij} is used to represent the ratio of the impacts of C_i and C_j on O. All the comparison results could use the pairwise comparison matrix.

$$A=(a_{ij})_{n\times n}\ ,\ a_{ij}>0\ ,\ a_{ji}=\frac{1}{a_{ij}} \qquad (1)$$

Based on the characteristics of a_{ij} in the above formula, A is known as the straight reciprocal matrix.

The 1-9 ratio scale proposed by Saaty et al compares the impacts of any two factors among the five factors on the factor in the last layer. In the matrix, a_{ij} represents the ratio of the impacts of each two factors on O. According to the specific circumstances of the experiment, the pairwise comparison matrix obtained by paired comparison method is:

$$A=\begin{bmatrix} 1 & 1/2 & 4 & 3 & 3 \\ 2 & 1 & 7 & 5 & 5 \\ 1/4 & 1/7 & 1 & 1/2 & 1/3 \\ 1/2 & 1/5 & 2 & 1 & 1 \\ 1/3 & 1/5 & 3 & 1 & 1 \end{bmatrix} \qquad (2)$$

3.3.3. Consistency Test

Pairwise comparison matrix is not usually the consistent matrix. However, in order to use its feature vector that corresponds to the characteristic root λ as the weight vector of the compared factor, its degree of inconsistency should be within the tolerance range. Thus the consistency index is introduced, namely,

$$CI=\frac{\lambda-\mathrm{n}}{n-1} \qquad (3)$$

In order to determine the tolerance range of the inconsistency range, it is required to identify the standards of the consistency index CI that measures A. The random consistency index, RI, is introduced, and the values are as follows:

Table 4. The values of random consistency index

N	1	2	3	4	5	6	7	8	9	10	11
RI	0	0	0.58	0.90	1.12	1.24	1.32	1.41	1.45	1.49	1.51

In the table 4, when n=1, 2, RI=0, because the positive reciprocal matrixes of the 1st and 2nd order are always the consistent matrix.

For the $n\geq 3$ pairwise comparison matrix, the ratio of the consistency index CI of A to the random consistency index RI at the same order is called the consistency ratio CR. When

$$CR=\frac{CI}{RI}<0.1 \qquad (4)$$

It is believed that the inconsistency degree of A lies within the tolerance range, and its feature vector can be used as weight vector. The

selection of 0,1 in the formula is with certain subjective reliability.

The above formula and Table 7 are used to test *A*, which is called the consistency test. When the test is failed, pairwise comparison needs to be re-examined, or the existing *A* needs to be modified.

Based on the A in the above formula,

$\lambda = 5.703$

Normalize the eigenvector,

$$w = (0.263, 0.475, 0.055, 0.099, 0.110)^T \quad (5)$$

So,

$$CI = \frac{5.073 - 5}{5 - 1} = 0.018 \quad (6)$$

According to the values of random consistency index *RI* in Table 7,

$$RI = 1.12$$

So,

$$CR = \frac{0.018}{1.12} = 0.016 < 0.1 \quad (7)$$

Consistency test is passed, and the above w can be used as the weight vector.

3.3.4. Calculate the combined weight vector and conduct the combined consistency test

The weight vector of the second layer to the first layer is now obtained and it is referred to as

$$\mathrm{w}^{(2)} = \left(w_5^{(2)}, \ldots, w_5^{(2)}\right)^T \quad (8)$$

The same method is employed to construct the pairwise comparison matrix of each factor in the third layer to the second layer. Set them as

$$B_1 = \begin{bmatrix} 1 & 2 & 5 \\ 1/2 & 1 & 2 \\ 1/5 & 2 & 1 \end{bmatrix} \quad B_2 = \begin{bmatrix} 1 & 1/3 & 1/8 \\ 3 & 1 & 1/3 \\ 8 & 3 & 1 \end{bmatrix}$$

$$B_3 = \begin{bmatrix} 1 & 1 & 3 \\ 1 & 1 & 3 \\ 1/3 & 1/3 & 1 \end{bmatrix} \quad (9)$$

$$B_4 = \begin{bmatrix} 1 & 3 & 4 \\ 1/3 & 1 & 1 \\ 1/4 & 1 & 1 \end{bmatrix} \quad B_5 = \begin{bmatrix} 1 & 1 & 1/4 \\ 1 & 1 & 1/4 \\ 4 & 4 & 1 \end{bmatrix}$$

The element $b_{ij}^{(k)}$ in B_k(k=1, …, 5) is the relative properness of the extraction method in the third layer to the superiority of each consideration factor in the second layer.

The weight vector $w_k^{(3)}$, the maximum characteristic root λ_k and consistency index CI_k are calculated by the pairwise comparison matrix B_k in the third layer. The results are shown in Table 5.

Table 5. The results of the third layer of the question choosing the best extraction method

k	1	2	3	4	5
$w_k^{(3)}$	0.595	0.082	0.429	0.633	0.166
	0.277	0.236	0.429	0.193	0.166
	0.129	0.682	0.142	0.175	0.668
λ_k	3.005	3.002	3	3.009	3
CI_k	0.003	0.001	0	0.005	0

It is obviously indicated that, since the random consistency index *RI*=0.58 when n=3, all the above CI_k s pass the consistency test.

The weight vector of various consideration factors relative to the optimum extraction method is $\mathrm{w}^{(2)}$, and the weight vector of each consideration factor is $w_k^{(3)}$ (k=1, …, 5). Both of them are used to calculate the weight vector of each consideration factor, which is called the combination weight vector, denoted by $\mathrm{w}^{(3)}$. The combination weight of the phenol protocol in the target should be their respective pairwise sum of the products, namely

$$0.595 \times 0.263 + 0.082 \times 0.475 + 0.429 \times 0.055 + 0.633 \times 0.099 + 0.166 \times 0.110 = 0.300 \quad (10)$$

In the same way, the combination weights of the TCA method and the homogenation to

the relative optimum extraction method are calculated as 0.246 and 0.456, so the combination weight vector is

$$w^{(3)} = (0.300, 0.246, 0.456)^T \quad (11)$$

Thus, the combination weight of the homogenation method is the largest. With an overall consideration of the five factors, the results show that the homogenation method is more suitable for the two-dimensional electrophoresis extraction of the total protein of Actinidia arguta.

4. Conclusions

Total proteins in the fruit are extracted to be applied in the two-dimensional electrophoresis.(Table 6) From the perspective of five aspects including the complexity of the operation, the condition of protein dissolution, protein content, extraction time and cost, the AHP model is constructed for analysis. In the end, it is concluded that the homogenation method is suitable for the total protein extraction of two-dimensional electrophoresis *Actinidia arguta*.

Table 6. Comparison of three protein extraction methods

Methods	Advantages	Disadvantages
TCA	A large number of soluble protein interferents are removed, prompting the protein precipitation.	Incomplete protein precipitation with low content; precipitated protein is insoluble in the lysis buffer
Phenol protocol	A large number of interferents are effectively removed, prompting the precipitation of protein denaturation. The resulting protein is of high content and high yield.	Cumbersome operation procedure
Homogenation	Small amount of protein impurities	Lower protein yield. Precipitated protein needs to remove the lysate repeatedly

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BODY EFFECT AND FOOD NUTRITION REGULATION RESEARCH ON ATHLETES' AFTER TRAINING

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ABSTRACT

This article observed the training of athletes nutritional recovery, a more comprehensive biochemical marker tests. The relevant summarize the work together to find practical and effective method of assessing performance athletes and nutritional supplements means. In order to find athletes explore ways before the body function and nutritional recovery, the grand prix of the 32 track and field athletes over a period of 4 weeks and biochemical indicators of nutritional supplements test comparison. Through this training period, the restoration of nutrition experimental observation results showed that the functional state of athletes is better. Generally results suggested that reasonable arrangements for exercise stress and nutrition programs to improve the athletes' function situation, as well as to create a good athletic performance plays. A crucial role in this experiment may explain nutrient solution to increase testosterone levels in the blood. Male athletes have a certain effect combined with years of research and practice with the team, initially developed with hemoglobin (Hb), blood urea nitrogen (BUN), creatine kinase (CK), serum testosterone (T) and cortisol (C) and other biochemical indicators to assess the ability of the athletes' function standard.

1.Introduction

A series of physiological and biochemical changes during the timing of the body human motion, which is a reflection of its objective to bear the exercise load. The body reflects the ability of exercise training stress(Pasiakos, 2015; MacKenzie-Shalders, 2015). Motion stimulation can cause changes in many aspects of the human cardiovascular, endocrine, immune, skeletal muscle, oxygen transport and utilization, energy metabolism and substance metabolism and so on(Manore, 2015; Pasiakos, 2016; Howe, 2016; Laakso, 2015). If the exercise load appropriate and timely measures to restore the body there will be adaptations good; training load is too small, to improve exercise capacity is not obvious; the training load is too large, not only can not enhance athletic ability, but there will be overtraining(Escalante, 2016). Thus, in the practice of sports training, scientific and rational use of medical laboratory technology and biochemical indicators to assess the functional state of athletes, training load control, judgment sports fatigue, rational nutrition supplements and athletic performance prediction has special significance. Our research services in the long term with the team which discovered the coaches monitor athletes functional status and take the necessary measures as nutrition an important part of scientific training(MacKenzie-Shalder, 2016; Sundgot-Borgen, 2013).

From a practical perspective, self-control is very important for everyone, through rational self-control in favor of individuals to achieve their goals in the sport is very important(Halson, 2014; Joy, 2013; Pasiakos, 2015; MacKenzie-Shalders, 2015). This study will focus on the

study athletes adjustment process through events and experiences, through the athlete's self-description, to understand what factors had an impact on the athletes play, and how these factors impact on athletic performance. Varsity athletes in this study were selected Sports Institute, they can be seen as sports experts, they were able to grow from PE student athletes to participate in various competitions, both successes and failures, their game experience or experience is unique. At the same time their awareness of sport and race there may be some difference with ordinary students, they should be self-regulation is typical of the experience. Athletes take an active advocate of self-control, self-control to adjust the strength and direction, and thus the performance of sports training and catalytic role (Moore, 2014; Pasiakos, 2016).

This article is in the training of athletes do nutritional recovery was observed, and a more comprehensive biochemical marker tests. The relevant summarize the work together to find practical and effective method of assessing performance athletes and nutritional supplements means. In order to find and explore ways athletes before the body function and nutritional recovery during the grand prix of the 32 track and field athletes over a period of 4 weeks and biochemical indicators of nutritional supplements test comparison. Through this training period before the restoration of nutrition experimental observation results show that the functional state of athletes is better, generally better results, suggesting that reasonable arrangements for exercise stress and nutrition programs to improve the athletes' function situation, as well as to create a good athletic performance plays.

2.Materials and methods

2.1. Test subject

Track and field team 32 athletes (16 men each). The average age of male athletes was 22.5 years, the average life of 9.4 years of training, the average height of 1.84m, the average weight of 84.0 kg; the average age of female athletes was 20.3 years, mean age 7.2 years of training, the average height of 1.75 m, the average weight of 68.3 kg. The sportsmen and women were randomly divided into experimental and control groups (n = 8). Subjects are more than two athletes in good health. Test times are in the morning 8:00 ~ 9:00.

2.2. Indicators and test equipment

Test Index hemoglobin (Hb), hematocrit (HCT), red blood cell count (RBC), blood urea nitrogen (BUN), creatine kinase (CK), serum testosterone (T), cortisol (C), immunoglobulin A, M, C. Equipment for the F - 270 automatic hematology analyzer (Nissan), Reflotron blood analyzer (German production), radioimmunoassay -γ cytometer (Xi'an yield).

In the period before the training, we give the experimental group received nutrition is: Men are: 1) Road safety solution formula for the Millennium rare, Codonopsis, ginseng, day 6 (60 ml); 2) strong recovery granules, recipe oligosaccharides, L- glutamine, vitamins, taurine, gynostemma, 40 g per day; 3) creatine powder, five days before 20 g per day, after a 5 g per day; 4) active sugar recipe 1 , 6 diphosphate, taurine, citric acid, amino acids, vitamins, four per day. Women are: 1) Changbai Faerie King oral solution formulation of Rhodiola, Epimedium, meat Cong Rong, Ginseng and Ganoderma lucidum, 4 (40 ml) per day; men with other 2), 3), 4). Taking time is four weeks.

2.3. Technical route and key technology

Feasibility study mainly related research about self-loss and self-control are more easy to collect data; Secondly, this study does not involve complex large number of experiments, the researchers take the questionnaire, a number of high-level athletes questionnaire interviews: third, the questionnaire appropriately modified according to the characteristics of athletes; fourth, loss of self-control to avoid the behavior, the author mainly through the athlete's subjective memories, and dynamic investigations in thought before the race, study its controlled through their competition results loss of influence.

This paper has a certain degree of innovation, in the following aspects: First in innovative research ideas; It has been more concerned about

the loss caused by the effect of self-control, more emphasis on the promotion of the study of the effect of self-control, and in theory practice has a certain value. Secondly, the study on innovation paradigm; existing research on suppression of self-control, not related to the use of dual-task paradigm of self-control, but this study while using dialogue, in the form of interviews, psychological motivations real spy athlete and a true reflection game scene, combined with the highest number of keyword research appears athlete interviews. At the same time, the use of the experimental method of questionnaires and interviews drawn from the analysis strategies empirical research. Finally, in the past researchers have often focused on how to compensate the loss after self-generated, the present study is focused on the investigation of the loss of self-athletes reasons, and proposed prevention strategies. Key technical route was shown in Figure 1.

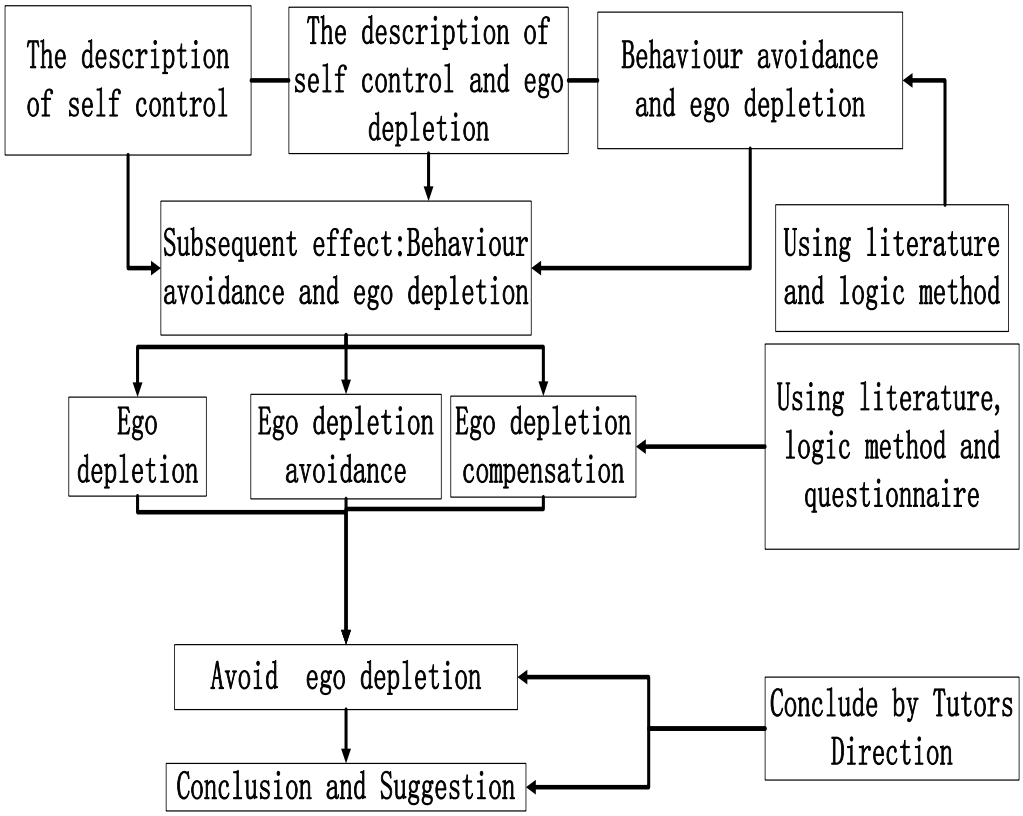

Figure 1. Key technical route

3.Results and discussions

3.1. Effectiveness of physical effects

In the practice of the present study, uninterrupted and timely feedback control, to some extent, the effect of the student body has played a positive role can be seen from Table 1, by teaching and training more than five months, the students steady weight level rise and its fluctuation is only 1.78%, corresponding to 91.07% never% body fat at the beginning of the semester, end of the period fell to 31.97%. Increase in lean body mass, suggesting that improved muscle quality food, and this corresponding to the grip level and back strength levels corresponding increase in the cardiovascular indicators, morning pulse level gradually falling blood pressure levels are relatively stable (CV only 92.53%), also shows the regulation of teaching training process performance in cardiovascular the validity of the change in blood urea blood biochemical parameters. Five months were within the range of normal monitoring levels of volatility, no

signs of fatigue appear, it is apparent that the participants of the training cycle of teaching and training the body to adapt the overall level is good, with effectiveness.

Table 1. The average level of different body effect and nutrition regulation indexes in different periods of students of PE major when they are in training ($\bar{x}$ ±S).

	SEP.	OCT.	NOV.	DEC.	JAN.	Average	CV%
A	175.2 ±3.12	172.2 ±5.21	174.3 ±3.33	174.2 ±2.12	174.4 ±1.12	174.8 ±3.12	0.50
B	68.21 ±6.12	69.11 ±5.22	68.88 ±4.22	69.51 ±3.45	70.21 ±8.12	69.21 ±6.12	1.55
C	43.39 ±6.55	43.78 ±5.20	43.71 ±2.11	41.96 ±7.52	43.27 ±3.54	43.22 ±4.55	7.33
D	40.59 ±1.19	42.49 ±3.75	44.79 ±1.27	41.70 ±2.53	42.92 ±3.49	42.50 ±2.35	5.22
E	105.82 ±15.09	149.40 ±5.52	141.68 ±13.59	119.02 ±13.10	157.01 ±3.25	128.55 ±10.20	11.55
F	18.90 ±0.77	19.59 ±2.52	15.47 ±0.76	11.38 ±2.44	11.49 ±0.73	15.37 ±1.44	14.32
G	68.88 ±11.32	62.07 ±8.22	58.55 ±6.55	58.33 ±5.38	59.50 ±7.85	51.65 ±4.58	7.56
H	110.22 ±4.73	103.84 ±3.51	102.16 ±8.33	102.76 ±5.85	106.77 ±4.55	105.22 ±5.58	2.11
I	69.58 ±2.84	66.42 ±0.38	68.78 ±0.27	68.76 ±2.65	66.90 ±3.89	68.09 ±2.01	3.22
J	4699.38 ±374.08	4961.18 ±225.27	4757.78 ±41.91	4992.44 ±114.48	5013.52 ±456.67	4884.94 ±242.48	6.55
K	133.04 ±15.49	146.51 ±3.73	140.76 ±33.66	149.92 ±0.16	131.56 ±27.12	140.36 ±16.03	5.23
L	146.34 ±40.00	147.37 ±21.57	131.68 ±45.53	137.99 ±9.09	135.19 ±13.19	139.72 ±28.87	9.20
M	14.32 ±0.88	14.58 ±0.66	14.88 ±0.68	14.66 ±0.55	15.11 ±0.88	15.01 ±0.72	3.74
N	22.85 ±5.49	26.77 ±5.79	28.78 ±5.41	28.88 ±5.88	30.22 ±5.57	26.58 ±3.95	14.88

Note: A:Height(cm) B:Weight(kg) C:LH(kg) D:RH(kg) E:Back(kg) F:Fat (%) G:Morning Pulse(c.p.m) H:SBP(mmHg) I:DBP(mmHg) J:Vital capacity(ml) K:Acoustic response L:Light response M:HGB(g%) N:BU(mg%)

Therefore, physical education and training must follow the laws of the body's physiological function, scientific and reasonable arrangement of training load, in order to improve the effect of teaching and training, and to athletes to develop. Because professional sports teaching and training is different from the professional sports team training, there is a diversity of teaching and training programs, and other physical and functional requirements of comprehensive features, we need to have arrangements for the teaching and training of macro-control, teaching the effectiveness of training objective evaluation. therefore, the need of our understanding and knowledge of physical education and training process, students physical function variation and characteristics, to understand and grasp the situation of load level at all stages of the body to adapt to the physical education and training, as well as dietary reasonable arrangement. for this

purpose, we conducted a follow-up study over five months. now on physical education and training, the effectiveness of the athlete's body effect and nutritious meals, and certain physical characteristics like effect question.

3.2. Athletes' function state diagnosis method

We focus on the domestic sports reference in physiological and biochemical research scholar athlete performance assessment and diagnosis of fatigue, combined with years of experience in athletics technology services, we developed a track and field athlete physical function indicators for monitoring and diagnosis of the state of the reference range. The use of hemoglobin, red blood cell count oxygen transport capacity of three indicators reflect the focus of athletes and training status, hemoglobin man peacetime training should be maintained at 14 ~ 17 g / dl, the woman should be 12 ~ 16 g / dl, before It should be close to or reach their own best level; blood urea nitrogen and creatine kinase reflect the degree of adaptation to the training load of the body and fatigue recovery.

BUN recovery value should be lower than men's training 7 mmol/L, the woman should be below 6 mmol / L, CK restore the value of man should be less than 300 IU / L, the woman should be less than 200 IU/L, race BUN before the optimum range of 5 ~ 7 mmol/L, CK value of 150 IU/L or less. Serum testosterone and cortisol is a manifestation of muscle synthesis and catabolism of endocrine level, peacetime training should be in control of the men T of not less than 380 ng/dl, Women T is not lower than 30 ng/dl, before man T should be 500 ~ 800 ng/dl, woman T should be 45 ~ 80 ng/dl, normal cortisol indicators should generally be less than 25 ug/dl, before the corresponding values in the individual's optimal level; IgA, IgG, IgM immune markers, reflected athletes during training and fatigue immune function of the body, the normal range for men and women athletes are IgA: 0.5 ~ 1.9 g/L, IgM: 0.85 ~ 1.85 g/L, IgG: 8 ~ 16 g/L. In practice, the training of scientific research, we found that athletes' function indicators have reached a desired level, their best race condition occurs, the performance of athletic performance better. If the function of state is not good, it should be timely adjustment Steering coaches load and recovery with targeted nutrition, to avoid fatigue, to ensure the normal training and competition.

3.3. Analysis of test results

Experimental test results before and after the two men's athlete of the indicators (Table 2, Table 3).

Table 2. Hemachrome level of male athletes before and after experiment

Group	Exp	HB/g.dl^{-1}	PCV/%	RBC/×1.012.L^{-1}	BUN/mmol.L^{-1}	CK/IU. L^{-1}
Control (n=8)	Before	16.22±0.53	49.99±2.35	4.95±0.3.8	5.87±0.33	166.5±18.8
	After	15.44±0.38	48.32±1.02	4.96±0.44	5.77±0.69	235.5±18.1
Exp (n=8)	Before	15.22±0.25	46.75±0.55	4.78±0.15	8.14±0.48	455.5±95.2
	After	14.33±0.48	44.35±1.23	4.25±0.33	7.58±0.53	386.4±90.1

Table 3. Testosterone level of male athletes before and after experiment

Group	Exp	Testosterone/ ng.dl^{-1}	Cortisol/ ng.dl^{-1}	IgG A/g.L^{-1}	IgG M/g.L^{-1}	IgG Gg.L^{-1}
Control (n=8)	Before	485.4±30.3	19.8±2.3	1.11±0.11	1.25±0.13	8.65±1.18
	After	454.4±15.8	11.3±1.5	0.96±0.09	0.97±0.06	5.90±0.55
Exp (n=8)	Before	385.2±71.2	16.7±1.8	1.88±0.05	0.48±0.08	10.88±0.33
	After	14.33±0.48	19.3±2.2	1.25±0.13	0.88±0.13	9.02±1.55

As can be seen from Table 3 and Table 2, the index had no significant difference (P> 0.05) in the control group before and after the experiment; the experimental group before and after the blood-testis experimental values are significantly different (P <0.05). This shows that this nutritional rehabilitation program to improve man's endocrine function, promote androgen secretion have better results. We believe that "Daoan fluid" may be a major factor in blood testosterone levels in athletes affected. Athletes blood test before and after the experimental group CK value dropped 53IU / L, after contrast control experiments improved 70.5 IU / L, there was a significant difference (P <0.05) difference between the two groups, indicating that the recovery measures for enhancing athletes anti-load capacity, speed up recovery after exercise has a role. After taking the experimental group athletes (before 5 days) BUN and CK values were 7 .51 mmol / L and 387.5 IU / L, reflecting the strength of the athletes before training is still large, the body has not been fully restored, which participate race course negative.

Experimental group hemoglobin of 15 g / dl, blood urea nitrogen of 6.82 mmol / L, creatine kinase (CK) fell to 254 IU/L, serum testosterone increased from 273.7 462.6ng / dl, cortisol was 12.2 ug / dl . Although CK value of the athlete is still high, but other indicators of performance is quite satisfactory, it is a good player in the game (won 3) results. Another example is hemoglobin after the experiment was 13 .4 g / dl, blood urea nitrogen was 8.20mmol / L, serum testosterone was 694.9 ng / dl, cortisol was 6.2 ug / dl, CK value of 513 IU / L. Although serum testosterone and cortisol index value is good, but the game poor performance athletes, coaches can also be considered a first lap run, you can not run the second lap. We believe that before the body of his state of fatigue (CK and BUN values were higher), low hemoglobin values, endurance athletes will be affected. Female athletes before and after the experiment two groups each index test results (Table 4, Table 5).

Table 4. Hemachrome level of female athletes before and after experiment

Group	Exp	HB/g.dl^{-1}	PCV/%	RBC /×1.012.L^{-1}	BUN /mmol.L^{-1}	CK/IU. L^{-1}
Control (n=8)	Before	12.22±0.43	42.90±0.95	3.95±1.38	6.47±0.33	118.8±18.4
	After	13.44±0.48	42.92±2.02	4.36±2.44	6.77±0.19	158.4±6.70
Exp (n=8)	Before	13.72±0.25	46.75±0.75	4.78±0.15	6.84±0.28	150.2±20.55
	After	13.33±0.48	43.34±1.23	4.25±0.33	6.18±0.31	155.8±18.33

Table 5. Testosterone level of female athletes before and after experiment

Group	Exp	Testosterone /ng.dl^{-1}	Cortisol / ng.dl^{-1}	IgG A/g.L^{-1}	IgG M/g.L^{-1}	IgG G/g.L^{-1}
Control (n=8)	Before	35.41±9.03	12.8±2.32	1.11±0.31	0.92±0.13	9.44±0.55
	After	28.15±1.58	10.3±2.10	0.86±0.29	0.87±0.06	6.88±0.22
Exp (n=8)	Before	33.21±7.20	13.7±1.81	1.88±0.15	0.78±0.18	12.0±0.65
	After	33.33±0.48	12.3±1.92	1.25±0.23	0.98±0.13	12.7±0.82

As shown in Table 4 and Table 5, there was no significant difference (P> 0.05) before and after the experiment two groups of indicators. After the experiment is only two groups of serum testosterone 29.95 ng / dl and 33 .60 ng / dl, it seems the use of traditional Chinese medicine and nutrition methods to improve blood testosterone female athletes still need further

study. For example, in the experimental group Xu ×, hemoglobin after the experiment was 14.6 g / dl, CK was 174 IU / L, serum testosterone from 20.4 ng / dl becomes 22 .2 ng / dl, cortisol was 10.6 ug / dl, since the blood testosterone has been low, the lower the intensity of training, poor race results. Others, such as after the king ××, experimental hemoglobin of 13.2g / dl, CK was 136 IU / L, testosterone is 46 ng / dl, cortisol is 15 ug / dl, since the indicators ideal, normal game play.

(1) Through this training period before the restoration of nutrition experimental observations indicate the functional state of the athlete better competition results in general good, and prompt and reasonable arrangements for exercise stress and nutrition programs to improve the athletes' function situation, as well as to create a good performance it plays a vital role.

(2) The experiment may explain male athletes after taking to improve blood testosterone levels and promote the body's recovery has some effect, which is consistent with the findings. But still we need to expand the test sample with the cycle, further observation.

(3) With hemoglobin, creatine kinase, serum testosterone, cortisol, blood urea nitrogen and other indicators assess the functional state of track and field athletes have stronger practicability and pertinence. We must combine characteristics of the project and practical training, conduct a comprehensive analysis.

3.3. Athletes self-control analysis

Mental ability to control their players is one of the most important factors in the course of the game can play out their own level. The most important include, mood, motivation, attention, volition. Emotions can affect the athlete's self-control, self-control, or individual success out of control, will lead to behavioral or impulse control, laissez-faire behavior, the behavior will produce different results of different emotions. There is a close relationship between mental fatigue and exercise motivation of athletes, athletes no more motivation, the more mental fatigue, and a tendency to avoid the more obvious.

Emotional needs are met substantially as metastasis. When people's needs are met and it will produce a happy mood. Then altercation with his teammates is not caused by the understanding; the results of the questionnaire and the issues under investigation, during the game likely to influence the mood of players mainly from two aspects, one is the referee errors, and opponent's foul dispute, the ability to handle several aspects of this problem, are key determinants of athletes emotional control.

Table 6 sportsmen and women can be found in emotional control or there is a big gap, which encountered unjust verdict can stay awake, the proportion of male athletes representing 80% of the sample; the proportion of female athletes accounted for only 40% of the sample; think he will not in the game overwrought male athletes accounted for 90% of the sample proportion, sample proportion of women athletes accounted for 60%; obviously easier to treat male athletes unfair referees and smooth game mentality. However, female athletes are more likely to accept criticism and to correct 70% of the sample; there are few athletes could safely disposed of in the event of attack, female athletes are more susceptible to emotional teammate. Sportsmen and women athletes, only about 40% can be resourceful, express tactical thinking, emotional control instructions athletes also need to continue to improve on a great level.

Table 6. Emotion Control

Emotion	male	ratio	Female	ratio
Stay calm when being attacked	12	30%	3	30%
Stay calm when judge is unfair	15	80%	4	50%

Forget troubles when on the field	13	40%	3	30%
Accept criticism and correct it	7	55%	7	70%
Uneasy to be affected	17	35%	2	30%
Adaptable	9	45%	4	40%

Traditional forms of motivation revolves was around reward and punishment. Term behaviorism is in terms of "positive reinforcement" or "negative reinforcement" motivation. Competition is to promote the game of internal power athletes, including strength and point to two aspects of the strength of an athlete in order to achieve a goal efforts, point refers to the choice of the direction of the target. Thus, athletes race motivation whether correct relations with the athletes to participate in the power level of the game, they will have to pay their hard work and the ability to obtain the corresponding results important problem.

Table 7. Motivation

Motivation	male	ratio	Female	ratio
Knowing the relation between gives and takes	15	50%	6	65%
I must win	16	85%	4	40%
To get material wealth	14	60%	7	80%
To get the experi-ence	8	25%	8	50%
Upset when lose the game	16	85%	7	60%
To get respect and honor	21	100%	9	90%

In the process of the athletes survey, many people want to participate in the competition to win, some people think that they win is all the game, it was afraid of losing, not afford to lose; but how correct athlete competition motivation is to improve the athlete self key controls, but also to reduce the loss of self-important way athletes, because often want the greater the disappointment will be greater, the athlete, the greater the psychological damage caused. As can be seen from the survey results, male and female athletes in control of the game motivation is a big gap on the lose, I have to win," the motivation point of view, 85 percent of male athletes want to be sure to win; only 40 percent of female athletes this indicator relatively strong; and to participate in the competition for the experience of the game is to get the motivation point of view, 25% of men agree with this view, but more than half of female athletes experience the game as is. From athlete "You play to get the honor, won the respect of " motivation level, all athletes want to get the respect of others by winning the race, in which male athletes 100%; 90% female athletes.

4.Conclusions

Under the conditions of collective meals, by means of sports medicine, to carry out comprehensive medical supervision, throughout the implementation of the regulation, to ensure that athletes nutrition and dietary health effects are positive and effective. However, due to professional sports teaching and training is different from professional sports sports training team, Athletes' Physical large individual differences, coupled with the diversity of teaching and training courses, to a certain extent, led to a teaching training period (five months), and significantly less athlete effects. This study should continue in-depth, in order for large period of one academic year teaching and training, and three to four year of teaching and

training throughout the study, and thus a more comprehensive exploration reveals PE teaching and training, the body effect and dietary regulation law athletes and methods to facilitate and enhance the effectiveness of training and sports teaching quality to provide a scientific basis.

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ANALYSIS OF PHYSIOLOGICAL ACTIVITY OF KELP IN ELIMINATING EXERCISE INDUCED PRESSURE

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ABSTRACT

The purpose of this study was to study the effect of kelp in eliminating exercise induced pressure and its physiological activity. Kelp water and laminarin which were prepared taking kelp as the raw material were taken as the experimental samples. One hundred and thirty-five healthy male mice were selected as research subjects and randomly divided into three groups for functional experiments concerning exercise induced pressure elimination. Hypoxia tolerance test, exhaustive swimming test and detection experiment on blood lactate dehydrogenase activity, blood lactic acid content, blood urea nitrogen content and hepatic/muscle glycogen content were carried out to investigate the effect of kelp in eliminating exercise-induced pressure and its activity. Compared to the control group, the mice in the experimental dose groups showed significantly increased duration of hypoxia endurance and exhaustive swimming time, higher blood lactate dehydrogenase activity, lower content of blood lactic acid and blood urea nitrogen, and increased hepatic/muscle glycogen content. The anti-hypoxia, exhaustive swimming and relevant qualitative change detection experiments suggested that, kelp could eliminate exercise-induced pressure.

1.Introduction

Fatigue (Mikovits and Ruscetti, 2012; Buchwald et al., 2015) is a phenomenon which appears when complicated biochemical and physiological changes occur in human body. Exercise-induced fatigue, also called physical fatigue, refers to the fatigue occurred when human body function cannot be maintained at a certain level or organs cannot maintain scheduled exercise intensity during the physiological process of body (Sun et al., 2011). With the rapid development of modern society, the living state of human is under constant changes, which makes fatigue an ordinary phenomenon nowadays. The occurrence of physical fatigue can affect the exertion of human body functions, resulting in a sub-health status (Guo et al., 2010; Li et al., 2010), and severely influence the working efficiency and living quality of human. Long-term fatigue can damage body functions and induce a series of functional dysfunctions and diseases. Therefore, anti-fatigue has been a social need. Exercise-induced fatigue has become a key research direction in the medical field and physical exercise field currently.

To rapidly relieve exercise induced fatigue and avoid the influence of side effects of drugs, some functional natural food with medicinal values (Kumar et al., 2012; Zheng et al., 2013) has been gradually applied in food and health care product industry. Marine plants and animals are important resources for developing marine drugs and health care products. Kelp as an important economic alga (Chen, 2012) has been extensively used in multiple food processing and health care products to relieve

exercise induced fatigue because of its more than 60 ingredients such as algin, amino acid and organic iodine (Steeples et al., 2016; Shimamoto et al., 2011) which have functions of antioxidation (Sang et al., 2016) and iodine deficiency prevention. Taking kelp as the raw material, we prepared kelp water and laminarin (Yu and Chao, 2013). The effect of kelp in relieving exercise-induced fatigue was investigated by carrying out the functional experiments such as anti-hypoxia and exhaustive swimming as well as the detection of relevant indexes, which aimed to provide a theoretical basis for the development of kelp as a kind of functional food and health care product.

2.Materials and methods

2.1. General materials

The main material used in this study was fresh kelp purchased from the market in Xuhui District in Shanghai. One hundred and thirty five healthy clean male mice with an average weight of 28 ~ 30 g and an average age of 7 ~ 8 weeks were bought from Shanghai Slac Laboratory Animal Co., Ltd., Shanghai, China. They were kept at 24 °C with 40% ~ 60% humidity. Ordinary fodders and tap water were used to raise the mice.

2.2. Main instruments and reagents

Main instruments included high-speed refrigerated centrifuge, ion chromatograph, homogenizer, fully automatic biochemical analyser, constant temperature incubator, electronic scale and 721spectrophotometer. Main reagents included blood lactate dehydrogenase, blood urea nitrogen reagent, whole blood lactate detection agent and normal saline.

2.3. Experimental method

2.3.1. The preparation of kelp water extractive and laminarin

Fresh kelp was washed by tap water and then distilled water and drained off. After the kelp was completely drained off, 500 g of it was taken, cut off, and added with distilled water. Then the kelp was mashed using a homogenizer and leached in 100 °C shaking bath for four and half hours. After it cooled, the kelp water extractive was obtained by separating the supernate using centrifuge. After that, the extractive was concentrated by rotary evaporation. In addition, absolute ethyl alcohol was added to adjust the concentration to 75%. After several minutes of stirring, the solution was filtered to obtain sediment. After the sediment was dissolved with distilled water, absolute ethyl alcohol was added again to adjust the concentration to 75%. After several minutes of stirring, polysaccharide precipitated. After six times of redissolution and sedimentation and the removal of ethyl alcohol, laminarin was obtained.

2.3.2. The grouping and feeding of experimental mice

One hundred and thirty-five healthy clean male mice weighed from 28 to 30 g were randomly divided into seven groups, i.e., three kelp water extractive groups (low, medium and high dose) (15 in each group), three laminarin groups (low, medium and high dose) (15 in each group) and one negative control group. The mice were gavaged every day, for one month. During the experiment, the mice could freely eat fodder and drink tap water. The dietary status, growth condition and daily behaviors and activities of the mice were observed. The mice in the high, medium and low dose groups were gavaged according to the weight, i.e., 0.1 g/kg, 0.5 g/kg and 1.5 g/kg, while the mice in the negative control group were gavaged with distilled water in the same volume.

2.3.3. Anti-hypoxia experiment and exhaustive swimming experiment

The mice in different groups were gavaged strictly following the specified dose. One hour after the last time of gavage, the mice were put into sealed bottles loaded with 20 g of lime. The bottlenecks were smeared with Vaseline to strengthen sealing. The time from sealing to

respiration cease, i.e., hypoxia endurance time, was recorded.

One hour after the last time of gavage, exhaustive swimming experiment was performed. The root of each mouse tail was installed with a lead wire whose weight was 5% that of the weight of the mouse. Then the mice were put into an incubator in a size of 50 × 50 × 40 cm (water depth: 40 cm; water temperature: 25 ~ 30 °C). The swimming behaviors of the mice were observed. The duration of loaded swimming (from the beginning to not emerging from water in 10 s) was recorded.

2.3.4. The detection of blood lactate dehydrogenase activity

After died of exhaustive swimming, the mice were taken out of water. The whole blood was collected from the eyeballs of the mice. The content of blood lactate dehydrogenase was detected using fully automatic biochemical analyser and blood lactate dehydrogenase (Brunauer, 2016) detection reagent.

2.3.5. Detection of the content of blood lactic acid, blood urea nitrogen and hepatic/muscle glycogen

After the mice died of exhaustion, they were taken out immediately. The blood was collected from the tail tip of each mouse. The whole blood was collected using blood lactic acid test paper. The content of blood lactic acid in each mouse was detected using a fully automatic biochemical analyser and reagents (Ba *et al.*, 2009). After the detection of blood lactic acid content, fresh livers were removed from the mice, washed by normal saline, and dried by filter paper. 100 mg of liver sample was weighed using an electronic balance. Then the content of hepatic/muscle glycogen was detected following the detection method specified on the hepatic/muscle glycogen detection reagent kit. Blood was collected from the orbit of each mouse. After the blood was kept in a static state for 3 hours, serum was separated from the blood using a centrifugal machine. Then the content of serum urea nitrogen was detected according to the method specified on the serum urea nitrogen detection reagent kit.

2.4. Statistical method

All the data were expressed as mean ± standard deviation (SD) and statistically processed by SPSS ver. 18.0. $p < 0.05$ meant there was a difference and $p < 0.01$ meant the difference was significant.

3.Results and discussions

3.1. The results of hypoxia endurance and exhaustive swimming time

The hypoxia endurance experiment and exhaustive swimming experiment could make corresponding evaluation of kelp in relieving exercise induced fatigue, and the detailed results are shown in table 1, figure 2 and 3. It could be noticed from the table and figures that, the mice which were gavaged with kelp water extractive and laminarin could tolerant hypoxia and exhaustive swimming longer compared to the mice in the control group, and the difference between the medium and high dose groups and the control group had statistical significance. It indicated that, kelp water extractive and laminarin could prolong hypoxia endurance time and exhaustive swimming time.

Table 1. Hypoxia endurance and exhaustive swimming time of mice gavaged with kelp water extractive and laminarin

	Kelp water extractive		Laminarin	
	Hypoxia endurance time (min)	Exhaustive swimming time (min)	Hypoxia endurance time (min)	Exhaustive swimming time (min)
High-dose group	35.26±6.78	23.35±3.68	34.63±3.26	25.35±3.36
Medium-dose group	37.23±5.21	26.50±4.22	36.11±2.45	27.52±4.23
Low-dose group	31.24±1.56	21.28±3.95	32.85±1.24	23.25±3.75
Control group	28.12±2.55	15.22±3.28	29.12±2.65	18.22±3.23

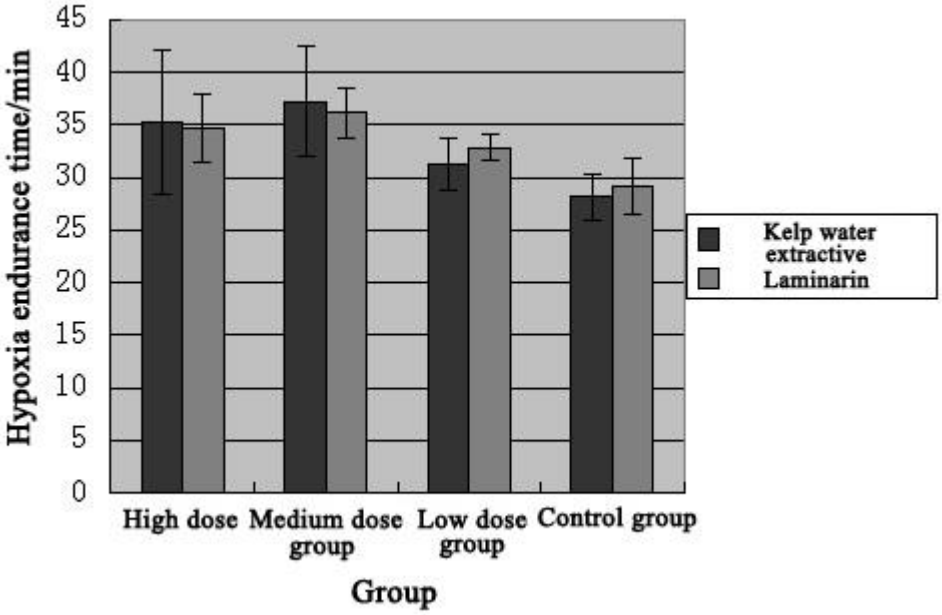

Figure 1. The result of hypoxia endurance time of the mice

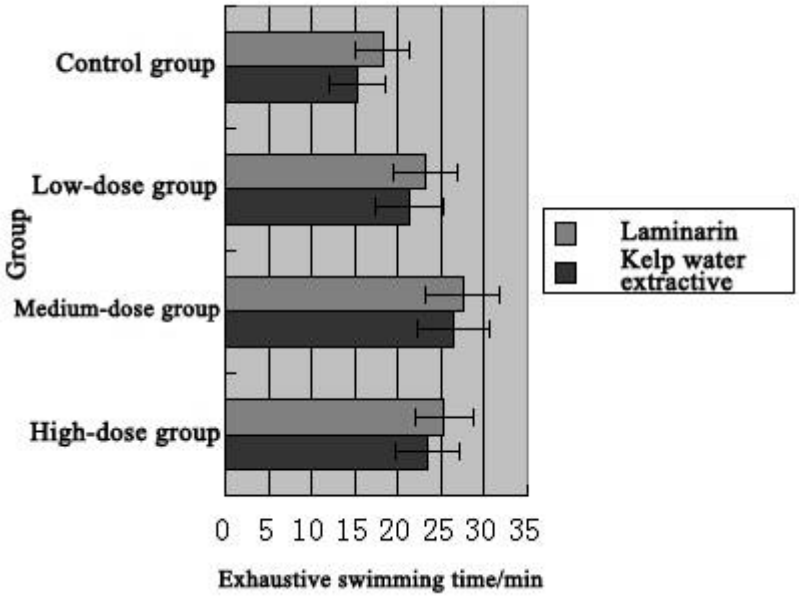

Figure 2. Exhaustive swimming duration of mice

3.2. Effects of kelp on blood lactate dehydrogenase activity

It could be noticed from experimental measurement that, the activity of blood lactic dehydrogenase of the mice gavaged with kelp water extractive and laminarin was higher than that of the control group, though the difference had no statistical significance. It indicated that, kelp water extractive and laminarin could strengthen blood lactate dehydrogenase activity of the mice after exercise, but the effect was not remarkable. The detection results are shown in figure 3.

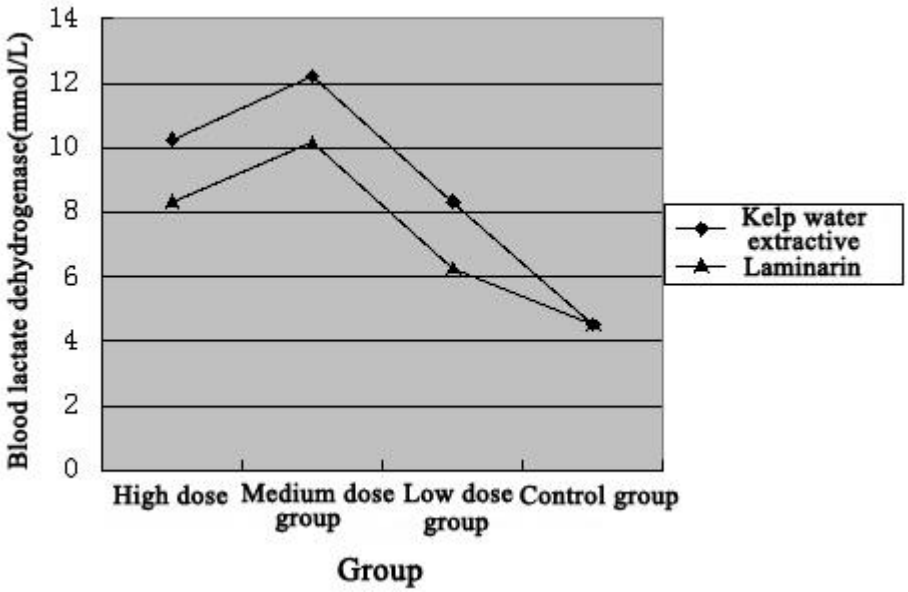

Figure 3. The detection result of blood lactate dehydrogenase activity

3.3. The content changes of blood lactic acid, blood urea nitrogen and hepatic/muscle glycogen

The detection of the blood lactic acid, blood urea nitrogen and hepatic/muscle glycogen content suggested that, the mice in the experimental dose groups had lower content of blood lactic acid and blood urea nitrogen and higher content of hepatic/muscle glycogen compared to the control group, and the difference between the medium and high-dose groups and the control group was notably significant, indicating kelp water extractive and laminarin could decrease the content of blood lactic acid and blood urea nitrogen and improve the reserve of hepatic/muscle glycogen. The experimental results are shown in table 2.

Table 2. The detection results of various biochemical indexes of the mice in groups

	High-dose group		Medium-dose group		Low-dose group		Control group
	Kelp water extractive	Laminarin	Kelp water extractive	Laminarin	Kelp water extractive	Laminarin	
Blood lactic acid (mmol/L)	3.66±1.78	14.12±0.89	6.13±1.93	13.23±1.98	7.82±3.36	10.48±3.21	9.50±0.95
Blood urea nitrogen (mmol/L)	6.55±0.26	8.98±0.85	4.75±0.62	9.68±1.36	6.52±1.25	7.26±0.29	8.12±0.63
Hepatic glycogen (mg/g)	10.11±0.36	7.65±0.88	9.21±0.22	8.91±1.75	8.69±0.69	9.58±0.85	4.96±0.09
Muscle glycogen (mg/g)	1.03±0.03	1.22±0.50	1.22±0.50	1.33±0.12	1.35±0.02	1.28±0.01	1.06±0.03

4.Conclusions

Exercise-induced fatigue can result in the decline of exercise capacity. Exercise tolerance and hypoxia response are the most direct markers for reflecting the fatigue of human body. In this study, an anti-hypoxia experiment and an exhaustive swimming experiment which could objectively display exercise capacity were carried out on the mice gavaged with kelp water extractive and laminarin.

Results demonstrated that, the maximum hypoxia endurance time and the exhaustive swimming time of the mice in the kelp water extractive group was 42.45 min and 30.72 min and the time in the laminarin group was 36.11 min and 27.45%, much higher than the control group, indicating kelp water extractive and laminarin could significantly prolong the hypoxia endurance time and loaded swimming time of the mice. The above finding suggested kelp could relieve exercise induced fatigue and improve exercise capacity.

Oxidation energy supply of sugar during exercise can be divided into two processes, i.e., anaerobic glycolysis (Clomburg and Gonzalez, 2012) and aerobic oxidation. During high-strength exercise, the anaerobic energy of sugar is released and a large amount of lactic acids generates. The increase of lactic acid concentration will induce fatigue after high-strength exercise. Lactic dehydrogenase existing in cell tissues is one of the important indexes for metabolic regulation during exercise. It is responsible for transforming the lactic acid accumulated in muscles into pyruvic acid and reducing its accumulation in muscles. Serum urea nitrogen, the product of protein metabolism, can reflect the adaptability, metabolic capability and fatigue degree of the body. The detection of the content of blood lactate dehydrogenase, blood lactic acid and blood urea nitrogen of the experimental groups and the control group indicated that, the highest content of blood lactate dehydrogenase among the experimental groups was 13.37 mmol/L, higher than that of the control group, and the lowest content of blood lactic acid and blood urea nitrogen was 1.88 mmol/L and 4.13 mmol/L respectively, much lower than the control group. It verified that, the physiological activity of kelp could relieve exercise induced fatigue and promote fatigue recovery.

Sugar is an important energy source of muscle tissue in human body. The more muscle glycogen reserve, the more sufficient the energy supply and the better the endurance. The reserve of hepatic glycogen is of great importance to main the blood glucose level during exercise. The reserve of hepatic glycogen and muscle glycogen can directly affect the exercise capacity of human body. Human body is less likely to get tired if the reserve is sufficient. The detection results of hepatic/muscle glycogen of the mice suggested that, the highest content of hepatic and muscle glycogen of the mice in the experimental groups was 10.66 mg/g and 1.72 mg/g respectively, much higher than that of the control group, indicating kelp water extractive and laminarin could effectively improve the reserve of hepatic and muscle glycogen and kelp could relieve exercise induced fatigue.

The kelp mentioned in this study is a kind of important brown alga containing rich sulfated polysaccharides such as sodium alginate, fucoidin and laminaran. As a natural food, kelp tastes good and has functions of resisting oxidation, regulating immunity and relieving fatigue. In this study, kelp water extractive and laminarin were prepared taking kelp as the raw material. Taking the prepared kelp water extractive and laminarin as the experimental sample and the mice as the research subjects, gavage and functional experiment such as anti-hypoxia experiment and exhaustive swimming were performed. The research results demonstrated that, kelp water extractive and laminarin could prolong the hypoxia endurance time and exhaustive swimming time, enhance the activity of blood lactate dehydrogenase, improve the reserve of hepatic and muscle glycogen, and reduce the generation of blood lactic acid and blood urea nitrogen. The above findings were enough to verify that kelp had a physiological activity of relieving exercise induced fatigue and

improving exercise capacity. This work lays a basis for the development of kelp as a kind of health food.

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PACKAGING DESIGN OF PRODUCTS OBTAINING DIETARY FIBERS THROUGH GANODERMA LUCIDUM FERMENTATION FROM THE GREEN PERSPECTIVE

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ABSTRACT
In order to provide references for the green packaging design of products acquiring dietary fibers through ganoderma lucidum fermentation, this study combined current problems in the food packaging design and put forward relevant green concepts of food packaging design based on the green packaging of green food. Taking obtaining dietary fibers from corn skin through ganoderma lucidum fermentation as an example, we developed a kind of green and pollution-free products. Under the guidance of green food concepts, the packaging design of products obtaining dietary fibers through ganoderma lucidum fermentation was researched. According to the analysis of current design procedures of food package, we found that several aspects should be considered in the package design of products obtaining dietary fibers through ganoderma lucidum fermentation. Package materials should be non-toxic and non-pollution; the package shape should be easy to grasp; the package color should be natural; package languages should be comprehensible; relevant components of the product should be noted on the package, etc.

1. Introduction

As an important kind of functional food nutrients, dietary fibers can effectively maintain blood glucose as well as prevent obesity and diabetes, etc. (Misra *et al.*, 2011). Ganoderma lucidum itself can nourish vitality, soothe the nerves, relieve cough and asthma as well as prolong life. Therefore, apart from above efficacies, the ganoderma lucidum mycelia produced by ganoderma lucidum fermentation can also relieve anemia, resist cancers and improve immunity (Kim *et al.*, 2014). Dietary fiber products obtained through ganoderma lucidum fermentation are based on green materials as well as green acquiring means, thus they belong to green products. The packaging of green products should also highlight the "green properties".

As the green concept deepens, the concept of green packaging comes into being (Rokka and Uusitalo, 2008). The green packaging of foods is an inevitable trend of development. Materials of the green packaging should be combined with specific foods and harmless and degradable materials should be selected. For example, materials such as plant fibers and edible materials, etc., can be used (Ahmed and Varshney, 2011). Apart from the selection of materials, designs of shapes, colors, images and words should be based on consumption habits of consumers. Resources saving, moderate consumption and sustainable development should all be considered (Ouyang, 2014).

Taking bagasse, wheat bran and bean dregs as raw materials, an experiment of obtaining fiber products through ganoderma lucidum

fermentation was carried out in this study to explain the green properties of fiber products. On the basis of the green packaging theory, the packaging of fiber products was designed.

According to the analysis of current food packaging, we found that the packaging design of products obtaining dietary fibers through ganoderma lucidum fermentation should consider every aspect of the products.

For example, packaging materials should be non-toxic and non-pollution; the package shape should be easy to grasp; the package color should be natural; package languages should be comprehensible; relevant components of the product should be noted on the package, etc.

2.Materials and methods

Experiment of obtaining dietary fibers through ganoderma lucidum fermentation

The taste of foods containing rich dietary fibers is not favored by most people (Sangnark and Noomhrm, 2004).

Therefore, products obtaining dietary fibers through ganoderma lucidum fermentation should guarantee the original nutrients as well as the flavor.

2.1. Experimental materials and equipment

Fresh bagasse and bean dregs containing 13% water content were purchased from the local market.

Fresh wheat bran was purchased from Shandong Yao Hui Commercial and Trade Co., Ltd. Ganoderma lucidum seeds were purchased from the Zhi Cheng local specialty sales department of Songjiang River of Fusong County of Jilin Province. UONI2010001 sterile inoculation box was purchased from Nanjing Unix Bio-Technology Co., Ltd. CLG full-automatic high pressure sterilization pot was purchased from Southeast Technology Co., Ltd. RT-100 jacketed pan was purchased from Zhucheng De Chuan Gong Mao Co., Ltd. R30 electric mixer was purchased from American Fluko Company.

ZR-360 full-automatic make-up machine was purchased from Dong Guna Zhan-Rong Electronics Mechanical Co., Ltd.

2.2. Experimental procedures

First of all, the bagasse was washed and then added with 1% lime supernate. After 24 h of soaking, the bagasse was dried in the sun. Then 60 g of bagasse, 20 g of wheat bran and 20 g of bean dregs were mixed well with 1 g of edible $CaSO_4$.

After that, the mixture was added with water and citric acid for 2 h of soaking.

The water content of culture medium was 60% and the potential of hydrogen (PH) was 5.2. Then the mixture was poured into a heat-resisting plastic bag and put into a sterilization box for 1 h at 120 °C. The relative humidity was set at 85% and the temperature was 25 °C.

Then ganoderma lucidum seeds were put into the aseptic environment for 40 days of fermentation.

After that, the temperature was set at 40 °C and the fermented culture solution with 50% of water content was put into a drying oven.

The obtained solid was smashed by a pulverizer and fine powders were sieved using an 80-mesh sifter.

Obtained powders were then transferred to a jacketed pan and added with 40 ml of cold water, an appropriate amount of tapioca and flour and well mixed. Then the mixture was steamed for 30 min.

The steamed material was then pressed into 3 mm of thickness by a make-up machine and sliced up. Slices were then put into a microwave oven for drying and puffing under 2500 MHz.

After that, the obtained materials were sieved using a 120-mesh sifter twice for selection of final puffed products.

3. Results and discussions

3.1.Influence of different temperatures on cellulose degradation

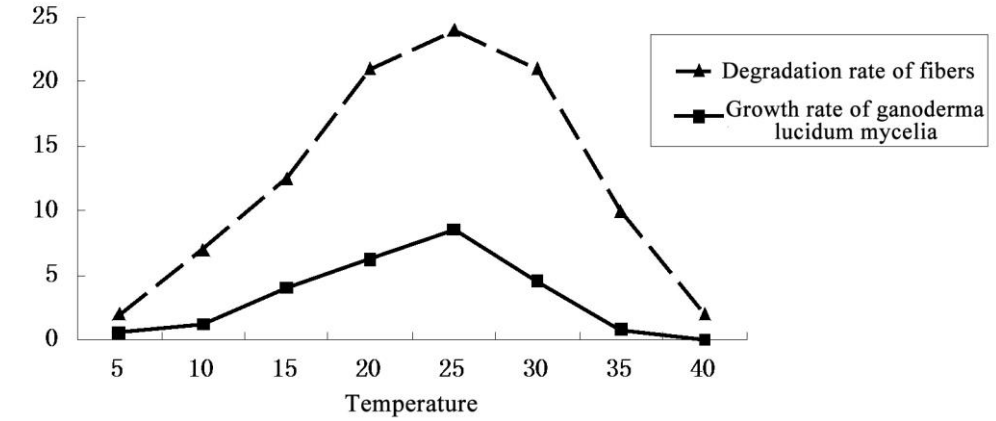

Figure 1. Influence of different temperatures on cellulose degradation and growth rate of ganoderma lucidum mycelia

As shown in figure 1, the degradation of mycelia on fibers is in direct proportion to its growth rate. The degradation rate reaches the maximum at 25 °C, thus the accumulation of non-starch polysaccharides also reaches the maximum. When the temperature is below 5 °C or over 30 °C, the growth of mycelia is restrained, thus the degradation is also affected. When the temperature is over 40 °C, mycelia begins to die. Thus the optimal temperature for degradation is at around 25 °C.

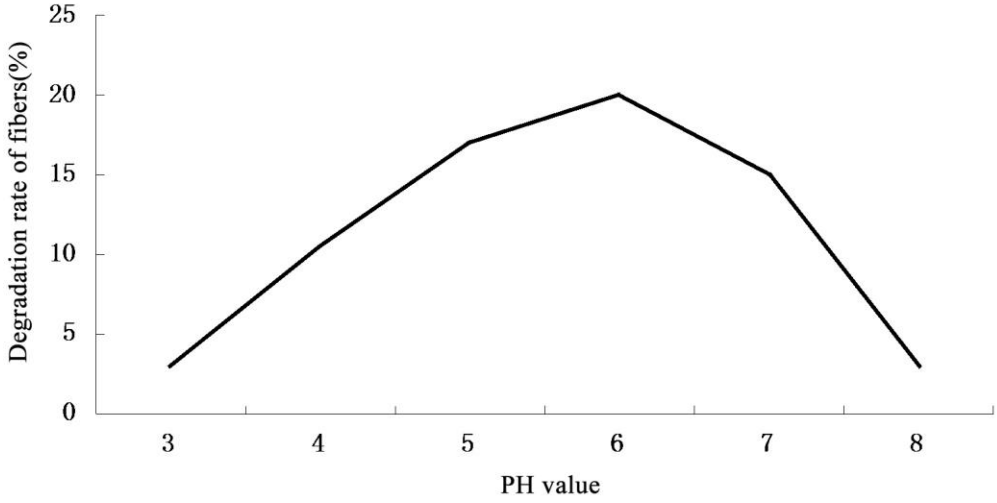

Figure 2. Degradation rates of cellulose under different PH values

3.2.Influence of different culture time on cellulose degradation

From the 1st day to the 7th day, mycelia grew rapidly and extended on the surface of culture medium. From the 7th day to the 25th day, mycelia began to extend to the inside of culture medium; soluble dietary fibers grew rapidly and the maximum growth rate reached 20%. From the 25th day to the 37th day, the vitality of cellulose decreased while mycelia regained vitality and the enzymatic activity of cellulose increased gradually. From the 37th day to the 50th day, the growth rate of soluble dietary fibers increased gradually. Therefore, there could be four phases, including planting phase, logarithmic phase, adjustment phase and growth phase.

3.3.Influence of different PH values on cellulose degradation

As shown in figure 2, the degradation rate of cellulose increases first and then decreases as PH value increases. When the PH value is 6, the degradation rate reaches the highest, thus the accumulated non-starch polysaccharides also reaches the maximum. Therefore, cellulose can be fully degraded at around PH 6.

3.4.Preparation of product formula

Puffed products made from 60% ganoderma lucidum mycelia powders, 30% tapioca and 10% flour were given a factor-level orthogonal experiment with powdered sugar and milk powder. Results are shown in table 1.

Table 1. Orthogonal factor levels

Serial number	Puffed products (kg)	Powdered sugar (kg）	Milk powder (kg）
1	55	24	21
2	60	22	18
3	65	20	15

Table 2. Results of orthogonal experiment

Serial number	Factor			Score (full mark=10)
	Puffed products	Powdered sugar	Milk powder	
1	1	1	1	7.6
2	1	2	2	8.2
3	1	3	3	8
4	2	1	2	9.2
5	2	2	3	8.6
6	2	3	1	7
7	3	1	3	7.4
8	3	2	1	6.8
9	3	3	2	7.2

According to table 1 and 2, we found that the 4th experiment had the highest score. Thus puffed product 6 kg, powdered sugar 2 kg and milk powder 18 kg were taken as the product formula. In conclusion, raw materials and producing procedures of products obtaining dietary fibers through ganoderma lucidum fermentation were green and non-pollution. Meanwhile, such kind of product had rich active nutrients. In every 100 g of the product, the water content ≤ 8%, protein ≥ 12%, 8% ≤ dietary fibers ≤ 10%, 350 mg ≤ calcium ≤ 450 mg; the total number of bacteria was fewer than 3000, the number of escherichia coli in every gram was fewer than 30 and there were no pathogenic bacteria.

3.5.Problems of food packaging

The food packaging nowadays shows problems like over packaging, unreasonable modeling composition, excessive hazardous substances and the lack of creativity of visual design (Sun and Jiao, 2011). Over packaging refers to that costs of the product packaging are far more than the value of the product itself, such as moon cake packaging. In order to raise the price, some merchants purposely expand the packaging volume and make the product delicately packed. Such kind of behavior can result in the waste of resources, accumulation of lots of rubbishes as well as environmental destruction. Unreasonable modeling composition reflects in big internal space and small product quantity. For example, most spaces are filled with plastic foams. Packaging materials commonly used in China include plastics, glass and papers, etc., which may contain chloroethylene, heavy metal and other hazardous substances. Therefore, such kind of packaging can cause negative influence on human health. Besides, the lack of creativity of visual design (He, 2015; Zhang, 2009) refers to that the packaging of some products imitates some high-end products to make visual similarity and thus to attract more customers. Such kind of products is lack of creativity.

3.6.Application of green packaging

3.6.1. Green materials of food packaging

Green materials of food packaging mainly reflect in follows aspects. The acquiring of materials should not damage the environment. Materials themselves should be non-toxic or low-toxic. Materials should be reproducible and recoverable and can be applied repeatedly. More degradable organic materials should be used (Dong *et al.,* 2014). The products obtaining dietary fibers through ganoderma lucidum fermentation belong to green products, thus their packaging should also use green materials. Commonly used materials at present include wood materials, organic materials, paper materials and edible materials, etc. (Wang *et al.,* 2008). Based on characteristics of products, bamboos are selected as the main packaging materials. Bamboos have fresh scent and can be easily degraded. Thus even if the products were thrown away, the environment would not be polluted. Moreover, bamboos have hard texture and can be stored for a long time after certain processing; they are difficult to be deformed and can be repeatedly used for several times.

3.6.2. Green shapes of the food packaging

Spaces between bamboo joints are hollow and no extra processing is needed. Thus the bamboo itself is a convenient packing box. Moreover, bamboos already have a shape and no modification is needed. As shown in figure 3, the packaging has no unnecessary spaces nor padding, which well follows the purpose of green packaging. In addition, the carry bag is made of recycled waste papers, as shown in figure 4.

Figure 3: Image of the bamboo shape

Figure 4: Image of the hand bag

Figure 5. Wash painting of ganoderma lucidum

3.7. Green visual elements on the food packaging

Food products can be vitalized by packaging colors. Purchase intentions of consumers can be affected by the identification of colors (Beltrán-González *et al.*, 2008). Colors of food packaging can be divided into active colors and passive colors. Active colors include red and yellow, etc., while blue and green belong to passive colors. Active colors can improve product appeal and passive colors give a feeling of harmony (Labrecque and Milne, 2011). The bamboo has a feeling of elegant and its green color is very eye-catching under an environment with massive active colors.

3.8. Words and images on the food packaging

In view of the green characteristics of dietary fiber products, the images and colors of the packaging should also follow the green concept. Moreover, the packaging should express a feeling that dietary fibers are good for body health, which should be the distinctive emblem from other normal food products (Sauracalixto, 1998). In addition, the image design should use theories of vision expression marketing as reference; colors should be pleasing and images should follow populace's aesthetic. For example, a wash painting of ganoderma lucidum can be added onto the packaging, which can not only indirectly inform the materials, but also emphasize Chinese culture (figure 5). In the meantime, introductions on the packaging should be concise and easy to understand; important information, such as date of production, expiration date, nutrients, etc., should be noted.

4.Conclusions

The packaging design of foods obtaining dietary fibers through ganoderma lucidum fermentation should emphasize the green concept of dietary fiber products. The functional orientation of these products should be explicit, thus to promote the continuous consumption (Ferguson *et al.*, 2001). The selection of packaging materials should not damage the environment and also aspects of economic benefits should be considered (Giacin, 2007). Budget analysis is needed before selection to guarantee relevant profits of companies. In addition, colors and shape designs of the packaging should be creative, instead of imitating others. Consumption psychology and habits of customers should also be followed (Puskás and Perényi, 2015).

The process of obtaining dietary fibers through ganoderma lucidum fermentation in this study is green and non-pollution. Compared with other dietary fiber products, the products in this study are richer in nutrients. Thus as long as the packaging is attractive, they can stand out from other products. The green packaging of food products not only reflects in green materials, shapes, colors and images, but also in uniqueness. Therefore, bamboos are selected as the packaging materials in this study.

In conclusion, the packaging of dietary fiber products should focus on green packaging and selected materials should be non-toxic and non-pollution. The packaging shape should be easy to grasp and there should not be much spaces left unused. Colors should give priority to natural colors and instructions should be easy to understand. Thus more benefits can be created on the basis of environmental protection.

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CONSTRUCTION OF HYPERSPECTRAL QUANTITATIVE ANALYSIS MODEL USED FOR DETECTING THE QUALITY OF CHILLED PORK

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ABSTRACT

With the progress of times and the development of technology, the hyperspectral imaging technology has been effectively applied to the detection of the quality of meat products. However, there are also some problems need to be solved. In this study, the chilled pork was taken as the research object, and the water content, potential of hydrogen (PH) value and total volatile basic nitrogen (TVB-N) content of chilled pork were taken as the evaluation indexes. According to various kinds of computational statistics theories and computer technology, a hyperspectral quantitative analysis model which was used for detection of the quality of chilled pork was constructed for further analysis. The main research contents are as follows: the Monte Carlo second detection method of abnormal samples based on detection of the quality of chilled pork was put forward and such method was found to be effective in the detection of abnormal chill pork samples; a kind of optimal spectrum pretreatment fast select method was put forward to compare the model performance of each chilled pork sample set, and the partial least squares regression (PLSR) model was found to be the optimal model of the water content, PH value and TVB-N content of chilled pork; finally, the hyperspectral model transmission was analyzed and the VSWS-PDS model transfer algorithm was put forward to provide technical support for the detection of the quality of chilled pork.

1.Introduction

Pork, which is rich in protein, calcium, phosphorus, iron and other nutrient contents, has become one of the main meat products favored by Chinese residents (Heeren and Smith, 2013; Yun *et al*., 2013). With the development of technology and the improvement of people's living standards, disadvantages of traditional methods for detecting the quality of meat products have showed up, such as time-consuming, inefficient, tedious steps, low accuracy and sample damage, etc. Therefore, these methods are no longer suitable for the detection of the quality of agricultural and animal products. In the meantime, the hyperspectral analysis technology is widely applied to various fields, especially the field of agricultural products, due to its advantages like fast, accurate, non-pollution and non-sample damage, etc. (Tabile *et al*., 2011; Lee *et al*., 2013). The hyperspectral analysis technology is a kind of photoelectric detecting technique based on optical properties. Its spectrum response range includes ultraviolet region, visible light region and near-infrared region and the spectral resolution is pretty high (Wu *et al*., 2011; Otsu *et al*., 2014).

At present, the detection of the quality of meat products mainly stays in sensory evaluation and physical-chemical analysis stage. Although physical-chemical analysis methods have high measurement precision,

they have tedious steps and can damage detection samples. Therefore, these two kinds of methods are not suitable for fast detection in meat products circulation. However, the hyperspectral imaging technology can simultaneously detect the internal and external quality of agricultural and animal products; moreover, its operation is simple and it has high resolution ratio, thus it is widely applied to nondestructive examination of agricultural and animal products. Based on this, the chilled pork which plays a dominant role in meat consumption of residents was taken as the research object in this study, and the nondestructive examination method of chilled pork quality using the hyperspectral imaging technology was further analyzed (Dong and Guo, 2015; Qiu *et al*., 2012; Lavanrra and Sanjeevi, 2013).

The constructed hyperspectral quantitative analysis model for the detection of the quality of chilled pork successfully provided a theoretical basis and technical support for the development and improvement of the hyperspectral imaging technology. Such model can be used to detect the quality of freshness of chilled pork in the circulation and sales links, which can further promote the production process of industrialization of the pork.

2.Materials and methods

Model construction of starred hotel's catering quality detection

2.1. Acquisition and preparation of samples

2.1.1. Acquisition and preparation of samples for detection of water content and PH values

The sampling time of chilled pork samples used for detection of water content was 10 days, 15 samples for each day. Samples were collected from the supermarket and packaged using preservative bags and delivered to the laboratory. Then samples were segmented for sample preparation and then sealed in hermetic bags and numbered. After that, samples were preserved in a biochemical incubator (0 °C- 4 °C). Besides, some pork tenderloin (about 20 g) was prepared for detection of the physical reference value of water content. It was also segmented and sealed in hermetic bags and numbered and then preserved in the biochemical incubator (0 °C- 4 °C) as well.

2.1.2. Acquisition and preparation of samples for detection of TVB-N content

Samples were cut into meat blocks in 8 cm * 6 cm * 1 cm and sealed in hermetic bags and numbered. Then they were preserved in the biochemical incubator (0 °C- 4 °C). Besides, we also prepared 90 En Shi Mountain (in Hunan Province of China) black pig samples and 90 local pig samples. The preparation of a large quantity of samples was beneficial to the detection of total volatile basic nitrogen (TVB-N) content.

2.2. Measurement of physical reference values

2.2.1. Statistical analysis of data of the water content of chilled pork

After examination and selection, the numbers of local pig samples and En Shi Mountain black pig samples were 79 and 77 respectively. The water content of local pig samples was 80.1% - 72.4%, while the water content of En Shi Mountain black pig samples was 71.4% - 77.3%. Details are shown in table 1.

Table 1. Data of water content of different chilled pork sample sets

Variety of pork	The number of samples	Maximum (%)	Minimum (%)	Mean value (%)	Standard deviation (%)
Local pig	79	80.1	72.4	76.3	1.0
En Shi Mountain black pig	77	77.3	71.4	74.2	1.2

2.2.2. Statistical analysis of PH values of chilled pork

After anomalous sample detecting of the PH values of chilled pork, the numbers of local pig samples and En Shi Mountain black pig samples were 79 and 82 respectively. The PH values of local pig samples were 5.2 ~ 6.4, while the PH values of En Shi Mountain black pig samples were 5.3 ~ 6.5. Details are shown in table 2.

Table 2. Statistical table of PH values of different kinds of chilled pork

Variety of pork	The number of samples	Maximum	Minimum	Mean value	Standard deviation
Local pig	79	6.4	5.2	5.5	0.2
En Shi Mountain black pig	82	6.5	5.3	5.6	0.3

2.2.3. Statistical analysis of TVB-N content of chilled pork

After anomalous sample detecting of the TVB-N content of chilled pork, the numbers of En Shi Mountain black pig samples and local pig samples were both 90. The TVB-N content range of En Shi Mountain black pig samples was 10.2 mg/100 g ~ 32.6 mg/100 g, while that of local pig samples was 10.1 mg/100 g~29.7 mg/100 g. The mean values of En Shi Mountain black pig samples and local pig samples were 17.9 and 19.0 respectively and the standard deviations were 5.2 and 6.7 respectively.

2.3. Monte Carlo second detection method of abnormal samples

2.3.1 Detection process

(1) The scatter diagram of statistics characteristic parameters of each sample was obtained and abnormal samples were determined preliminarily, i.e., the mean value or the standard deviation was far higher than the 2.5 times limit of the overall mean value.

(2) After the preliminarily determined abnormal samples in step 1 were eliminated from the original overall sample set, the remaining is the newly obtained overall sample set.

(3) The obtained new sample set was calculated using Monte Carlo abnormal samples detecting method, thus to further acquire the scatter diagram of statistics characteristic parameters of newly obtained samples.

(4) The obtained scatter diagrams were combined with the results of the first Monte Carlo detection as well as the distribution of current scatter diagram. An appropriate threshold value was selected for further determination of abnormal samples.

2.3.2 Detection results (table 3, 4 and 5)

Table 3. Abnormal sample detection results of the water content of local pig samples

Step	Abnormal samples	Model parameters after elimination of abnormal samples
		RMSECV
Monte Carlo method	8,43,46,32,22	0.279%
Significantly abnormal sample	8,43,46	0.275%
Monte Carlo second detection	8,43,46,45	0.263%
Second judgment of suspicious samples	8,43,46,45,32	0.257%
	8,43,46,45,32,22	0.274%

Table 4. Abnormal sample detection results of the PH values of local pig samples

Step	Abnormal samples	Model parameters after elimination of abnormal samples
		RMSECV
Monte Carlo method	8,28,1,14,50,9,66	7.88%
Significantly abnormal sample	8,28	9.54%
Monte Carlo second detection	8,28,1	8.92%
	8,28,1,14	8.94%
Second judgment of suspicious samples	8,28,1,50	8.72%
	8,28,1,50,9	8.50%
	8,28,1,50,9,66	7.83%

Table 5. Abnormal sample detection results of the TVB-N content of En Shi Mountain black pig samples

Step	Abnormal samples	Model parameters after elimination of abnormal samples
		RMSECV
Monte Carlo method	63,29,90,82,80,2,16,22,35	1.12
Significantly abnormal sample	63,29,90	1.31
Monte Carlo second detection	63,29,90,2,80,82,16	1.15
Second judgment of suspicious samples	63,29,94,2,80,82,16,22	1.12
	63,29,90,2,80,82,16,22,35	1.12

Table 3, 4 and 5 show that, Monte Carlo second detection could further determine that the partial suspicious samples obtained from the Monte Carlo method were abnormal samples, and it could discover abnormal samples that Monte Carlo method could not find out, which eliminated the effect of significantly abnormal samples on the sample set and other samples.

3.Results and discussions

Construction and analysis of hyperspectral quantitative analysis model used for detecting the quality of chilled pork

3.1. Spectral pretreatment method

(1)Centralization processing method: the mean value of data set was taken as the original point and coordinates of data set space were changed according to changes of the original point, thus to make data characteristics be clearer.

(2)Smoothness: the correction window with a certain width was selected and spectral values of the spectrum dot were averaged to obtain the estimated value of the dot.

(3)Multiplicative scatter correction: suppose there was a linear relation between every measured spectrum and ideal spectrum (only contains sample information), the approximate linear relation between the spectral value of each wavelength point of the measured spectrum and the spectral value of the ideal spectrum at that point was calculated, thus to correct the original spectrum.

3.2.Modeling of the hyperspectral quantitative analysis model used for detecting the quality of chilled pork

Quantitative modeling analysis of spectra is called quantitative correction. Commonly used multivariate calibration methods include multiple linear regression (MLR), principal component regression and partial least squares regression, etc., in which the MLR is a common method used in early spectrum quantitative analysis. According to the Lambert-Beer's law:

$$Y = XB + E \quad (1)$$

In the equation, Y refers to the concentration matrix of calibration set and X is the spectrum matrix; B refers to regression coefficient matrix and E refers to the concentration residual matrix. The least square solution of B is

$$B = \left(X^T X\right)^{-1} X^T Y \quad (2)$$

According to equation 1 and 2, the quantitative analysis model can be further constructed:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_j x_j + \ldots + b_n x_n + e \quad (3)$$

In equation 3, y refers to the concentration content of samples; x_j is the spectral value at wavelength j; b_j is the regression coefficient of x_j; e refers to the residual.

3.3 Model evaluation indexes

3.3.1 Determination coefficient

$$R^2 = \frac{\sum_{i=1}^{n}\left(\hat{y}_i - y_i\right)^2}{\sum_{i=1}^{n}\left(\hat{y}_i - y_m\right)^2} \quad (4)$$

In the equation, y_i refers to the measured value of the ith sample; $\hat{y}_i$ is the predicted value of the i^{th} sample; ym is the mean value of the measured values of all samples; n refers to the number of samples in the calibration set.

3.3.2 Root-mean-square error using cross validation

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{n}\left(\hat{y}_i - y_i\right)^2}{n-1}} \quad (5)$$

3.3.3 Predicted root-mean-square error

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{m}\left(\hat{y}_i - y_i\right)^2}{m-1}} \quad (6)$$

In the equation, m refers to the number of samples in the test set.

3.4. The optimal model PLSR of the chilled pork detection

Table 6 shows that, the water content model of the principle variety has good predicting performance. Before predicting the subordinate variety samples using principle variety water content model, the principle variety model should be maintained. After spectral pretreatment, the performance parameters of PLSR calibration model of the water content of chilled pork are shown in table 7.

Table 6. Performance parameters of the PLSR model of water content of local pig samples

Cross validation results			Predicting results of principle variety test set			Predicting results of subordinate variety test set		
R_{cv}^2	RMSECV%	RPD_{CV}	R_p^2	RMSEP%	RPD_p	R_p^2	RMSEP%	R_p^2
0.96	0.21	5.0	0.93	0.25	3.5	0.27	1.03	0.9

Table 7. Performance parameters of PLSR calibration model of the water content of chilled pork

Cross validation results			Predicting results of principle variety test set			Predicting results of subordinate variety test set		
R_{cv}^2	RMSECV%	RPD_{CV}	R_p^2	RMSEP%	RPD_p	R_p^2	RMSEP%	R_p^2
0.86	0.43	2.1	0.78	0.47	1.9	0.86	0.37	2.4

Table 7 shows that, the calibration model of the water content of chilled pork can have approximate quantitative calculation on variety samples.

3.5. Pass algorithm of the water content model and the PH value model of chilled pork

3.5.1. Pass algorithm of VSWS-PDS model (figure 1)

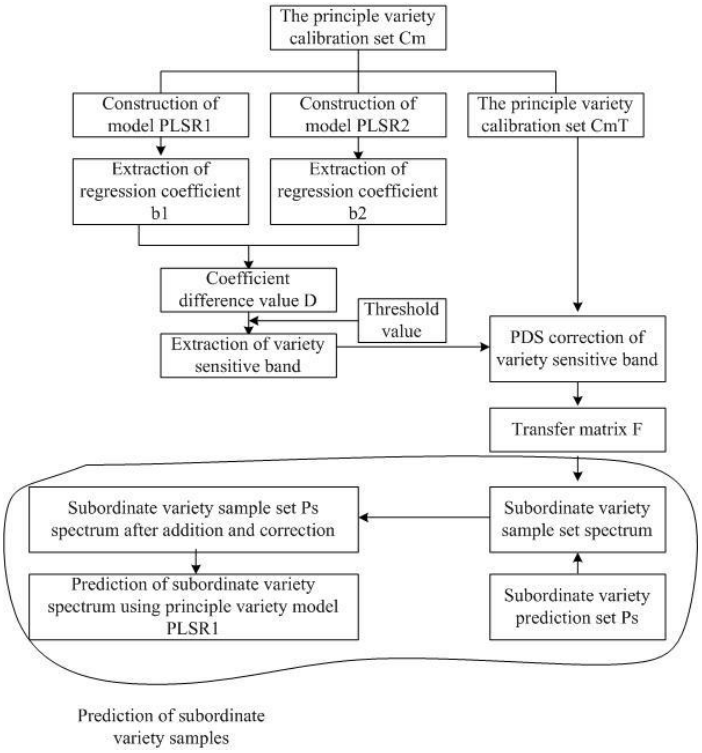

Figure 1. Flow chart of the pass algorithm of VSWS-PDS model

3.5.2. Hyperspectral model transfer

During the model transfer of water content of the chilled pork using VSWS-PDS algorithm, several representative subordinate variety samples were added to the principle variety calibration set to obtain the regression coefficients of updated model PLSR2. The obtained regression coefficients were compared with the regression coefficients of model PLSR1 to select variety sensitive band for subsequent spectral correction (Zhou *et al.*, 2012; Chen *et al*., 2013).

The process of spectral correction was influenced by three parameters, which were the number of samples q of the transfer set, the width of window k and the variant threshold value γ of regression coefficients. Figure 2 and figure 3 mainly show the effect of the variant threshold value γ on transfer results of the water content model VSWS-PDS and the hyperspectral model transfer results of PH values of chilled pork(Seuffert *et al*., 2012; Navarra, 2013).

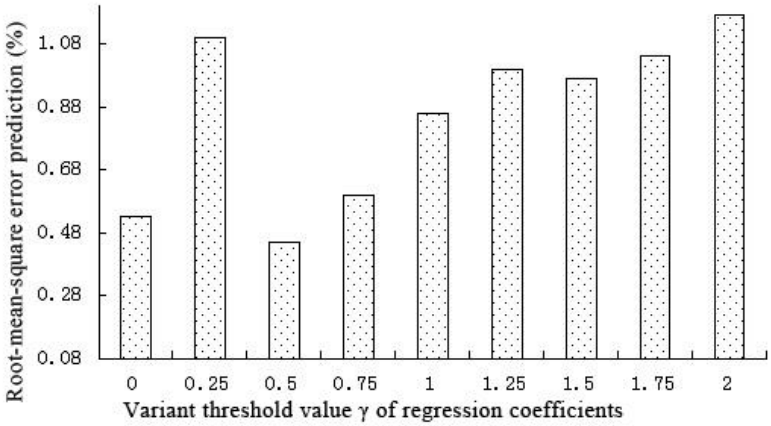

Figure 2. Effect of the variant threshold value γ on transfer results of the water content model VSWS-PDS of chilled pork

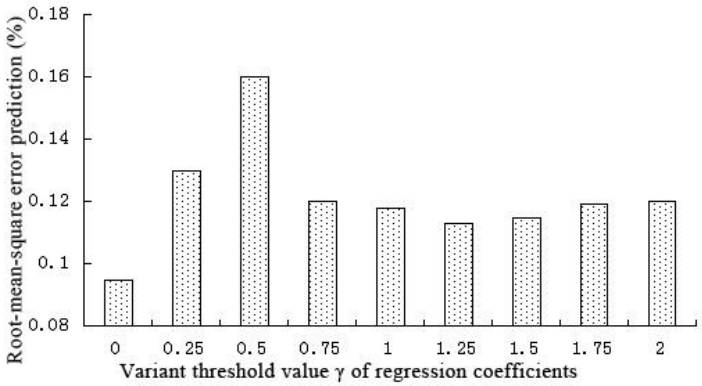

Figure 3. Effect of the variant threshold value γ on hyperspectral model transfer results of PH values of chilled pork

In order to verify the transfer effect of VSWS-PDS model transfer algorithm on hyperspectral quantitative analysis model of PH values of chilled pork, 50 remaining samples from the En Shi Mountain black pig samples were selected as the test set. When q=27, k=15 and γ=0.9, the transfer results of VSWS-PDS algorithm were R_p^2 =0.68, RMSEP=0.09 and RPD_p =1.5. Therefore, the VSWS-PDS algorithm significantly improved its prediction ability to subordinate variety samples.

4.Conclusions

This study mainly analyzed the construction of hyperspectral quantitative analysis model which was used for the detection of the quality of chilled pork as well as relevant contents. The chilled pork was taken as the research object and the water content, PH value and TVB-N content of chilled pork were taken as the evaluation indexes (Ye, *et al*., 2012). According to various kinds of computational statistics theories and computer technology, a hyperspectral quantitative analysis model which was used for detection of the quality of chilled pork was constructed for further analysis. The research contents included the acquisition and preparation of samples for detection of water content, PH values and TVB-N content of chilled pork; the Monte Carlo second detection method of abnormal samples was put forward (Özcomak *et al.*, 2013; Albert *et al.*, 2012)and the obtained results using such method were stable and

effective, which was significantly suitable for detection of agricultural and animal products (Silvia *et al.*, 2013); then reflection spectrum data in the hyperspectral images of pork samples were extracted (Yoshida *et al*., 2012; Zhang *et al.*, 2014) to construct a PLSR model of the TVB-N content of En Shi Mountain black pig samples, which was found to be the optimal model for detection of TVB-N content of En Shi Mountain black pig samples (Cheng and Sun, 2015); finally, a new VSWS-PDS algorithm was put forward and after VSWS-PDS model transfer, the prediction coefficients increased significantly, the predicted RMSEP reduced and the predicted bias ratio increased significantly (Schimleck, et al, 2011; Giannopoulos, *et al*., 2013), which effectively solved the influence of different varieties on prediction results of PH value model of chilled pork as well as significantly improved its prediction ability to subordinate variety samples (Cheng, et al, 2014; Cai, et al, 2011; Qiu *et al*., 2012).

In conclusion, the construction of hyperspectral quantitative analysis model which was used for detection of the quality of chilled pork significantly improves the applicability and reliability of spectrum detection models, as well as accelerates the promotion of hyperspectral non-destructive testing technology for detection of the pork quality, which has important scientific significance and good application prospect.

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CHARACTERIZATION BY CHEMICAL AND SENSORY ANALYSIS OF YOUNG AND AGED ROMANIAN PLUM DISTILLATE - ŢUICA

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ABSTRACT

The main chemical parameters and sensory characterization of aged plum distillate, from a mixture technological of varieties, have been determined in this study. Significant differences were obtained for the main concentration of the chemical parameters determined between crude and aged distillates. Also, some differences appear between crude and aged distillates from different aging methods. From the point of view of sensory qualities, rapid aging distillate has not obtained the maximum score as the aging classic distillate for 3 years.

1.Introduction

The aging process of distilled beverages is a complex system based on the extraction of molecules from the wood and interaction with the liquid, the phenomenon of migration of wood constituents, as well as the formation and degradation of some compounds (Alcarde et al., 2014).

Several distillates from different origins (whisky, rum, cognac, armagnac, cachaça and brandy) are submitted to a long aging process in wooden barrels with the aim of improving their sensory quality quality (Ledauphin et al., 2010; Durán Guerrero et al., 2011; Rodriguez-Solana et al., 2012). During this stage, several chemical reactions such as hydrolysis and oxidation can occur in the distillate and simultaneously a great variety of compounds are also extracted from the wooden barrels, modifying the chemical profile.

A large number of studies have been published about these beverages and confirm this positive impact on their quality (Apostolopoulou et al, 2005; Caldeira et al., 2010; Plutowska et al., 2010; Shyr and Young, 2016). The chemical and sensory modifications are influenced by several factors such as the species of wood and the heat treatment, the use of new or already used barrels and their capacity, the temperature and humidity of the cellar, the aging time and the initial composition of the distillate distillate (Prida, and Puech, 2006; Gambuti et al., 2010, Rodriguez-Solana et al., 2012).

In Romania, plum distillate is a national beverage called *ţuică,* obtained by traditional alembic distillation. Distillates produced in this way are distinguished from other similar products and appreciated by consumers and is closely related with the history and tradition of a particular geographical region like Ţuica de Vâlcea, Ţuica de Piteşti, Ţuica de Bistriţa, Ţuica de Zalău.

Ţuica is obtained from the distillation of the fermented plum mash. Young plum distillates are colourless and in the aroma phase they are characterized by sensory attributes such as floral, fruity, herbaceous, ensilage and heads,

with astringent and alcoholic notes in taste .After the aging process, these'sharp' sensory characteristics are removed and the mature distillate shows different sensory qualities in colour (yellow-golden, orange-amber) and new attributes in flavour and aroma. However, and despite these positive changes, in the case of *ţuica*, this aging step is not required and for this reason only a few distilleries submit a small part of their distillate production to an aging process (Crowell and Guymon, 1973; Onishi et al., 1977; . Lehtonen and Eriksson, 1983; Spaho et al., 2013).

The main goal of this study was to compare, by chemical determinations, the characteristics of tuica aged in barrels of different capacity and different periods of aging time, as well as barrels with different length, also analysis of aging distillates by rapid method using wood shavings compared with the traditional method.

2.Materials and methods

2.1.Material used

To obtain plum distillate was used a technological mixture of of four varieties (Stanley, Anna Spath, Vânăt românesc, d' Agen) cultivated in Dragasani area, Valcea

Ţuica that was used in the aging process has been obtained by the traditional method of distillation in alembic copper. The concentration of in alcohol was 38% vol. Ensure that the alcoholic strength threshold was carried out using the aromatic waters (tails) in the second distillation.

The distillate was subjected to aging in oak barrels of capacities 50 liters. They were placed in a cellar where summer maximum temperature was 25° C and in winter by up to 15^{0} C.

2.2.Chemicals and standards

Acetaldehyde, ethyl acetate were supplied by Fluka (Switzerland); higher alcohols were purchased from Sigma-Aldrich (Switzerland). Ethanol (analytical grade) and methanol were supplied from Merck (Germany).

2.3.Analytical method

The contents of ethanol, methanol, higher alcohols esters (ethyl acetate) and aldehydes (acetaldehyde and furfural) were determined using a gas chromatography system, a VARIAN 450 gas chromatograph GC-FID detector (flame ionization detection) with a set of 275^{0}C temperature for both the column TG-WAXMS 60 m, ID 0.32mm, film, 0.25mm, injector temperature 150°C, column temperature: 35°C, 3 min stand, climb to 20°C / min., up to 70 to 150°C with 27°/ min., stand 2 minutes, climb 200^{0}C, stand 2 minutes, climb to 240°C with 20°C/min. and stands 6 min. He carrier gas (1.2ml / min). Injection volume is 1 µL. The identification was made by comparing the retention times of standards from the calibration curve.

2.4.Sensory analysis

The tasting panel was composed of a group of five professionals, all of them members of the official panel of ADAR and with great experience in the sensory analysis of assessing spirits, both aged and young, and wines, too. The judges evaluated the intensity of the descriptive parameters and the qualifying parameters (in visual, aroma, taste, aftertaste and general impression) for young and aged distillate from plum (*ţuica*). The tasters were asked to score each attribute using a structured scale (0, no perception; 1, very low; 2, low; 3, middle; 4, high; and 5, very high intensity). The panel also scored the overall quality of the *Ţuica* between 0 (without quality) and 20 (maximum quality).

3.Results and discussions

Results regarding the evolution of main compounds in young and aging plum distillate are presented in Table 1.

During the aging process, the plum distillate obvious is registering alcohol loses from 38.0% to 35.4% vol. According to Catão et al., during the aging process oscillations occur in the ethanol content of the distillate, a function of the temperature and humidity outside of the barrel, that is, the

higher the outdoor humidity, the greater the loss of alcohol. In conditions of low humidity, relative concentrations occur owing to loss of water through the pores of the barrel. Giménez Martínez et al. showed values between 38 and 40% (v/v) for the ethanol content in brandies (wine distillates), approximate value similar to that obtained from plum.

During the same period the pH value increases from 3.55 to 4.40. It also increases the extract content of 620-930 mg / L and that of the ash increases from 62.0 to 73.10 mg/L (Crăciunescu, 2014).

Table 1. Evolution of main compounds in young and aging plum distillate

Stages of analysis (years)	Alcoholic degree	mG/L distillate		mG/100 ml anhydrous alcohol					pH	Tasting note 1-20 points
		Extract	Ash	Total acidity	Acet-aldehyde	Ethyl acetate	Isobutyl alcohol	Furfurol		
Initial	38.0	620	62.0	35.0	5.5	60.5	138.12	0.10	3.55	7.9
1	37.7	640	62.2	38.1	5.8	64.9	260.05	0.75	3.55	15.5
1+½	37.4	722	64.4	38.6	7.2	67.8	170.2	1.45	3.78	17.9
2	36.6	788	68.6	51.7	7.9	72.6	195.3	2.10	3.85	18.5
2+½	35.9	820	71.15	55.3	8.8	79.4	202.6	2.89	4.10	19.0
3	35.4	930	73.10	78.2	11.1	80.3	223.1	3.55	4.4	20.0

At the same distillate as the acidity increases from 35.0 to 78.2 mg / 100 ml, a.a. This parameter increases significantly during the wood aging process, resulting from oxidation reactions of ethanol and from wood extraction (Caldeira et al., 2010). In Brazilian sugar cane spirits or 'cachaça', a similar alcoholic beverage to *ţuica*, the values of total acidity were lower, at 46.40 mg 100 mL^{-1} after 36 months of aging (Parazzi et al., 2008).

Acetaldehyde is a volatile compound formed during spontaneous or microbial mediated oxidation during the alcoholic fermentation of raw material. Its concentration in the final distillate is also influenced by the distillation system, the wood and the aging time (Rodríguez Madrera et al., 2003). The acetaldehyde concentration increased during aging to a lesser extent when the distillation of the raw material was carried out using a rectification column and when the wood species used was *Quercus alba* (American oak), as it has a lower porosity than *Quercus petraea* or *Quercus robur* (French oaks). At plum distillate is recorded evident increases the content of acetic aldehyde, increasing from 5.5 to 11.1 mg / 100 ml a.a.

Ethyl acetate is the most abundant acetate in the distillates derived from the secondary metabolism of the yeast during the alcoholic fermentation of marc plum. However, it is the product of acetic acid esterification and thus its concentration increases during the aging process (Onishi et al., 1977). A high content of ethyl acetate in the distillate, above its perception threshold of 180 g HL^{-1} a.a., has a negative impact on sensorial characteristics and is perceived as having a solvent character (Silva and Malcata, 1998). In the samples analysed, the content ranged from 60.5 to 80.3 mg / 100 ml a. a, contributing fruity and floral notes to the aroma of the distillate. Furfural increases about 4 times.

The amount of total higher alcohols in the samples analysed varied from 138.12 to 223.1 mg / 100 ml a.a. The concentration of amino acids, the yeast strain, the fermentation conditions (pH, temperature, time) and the distillation process are all important factors in terms of the concentration of higher alcohols in the final distillate. Higher alcohols comprise the group that is quantitatively more important in the distillates. These volatile compounds are positively involved in the sensory quality of the distillate, if they are not present in high concentrations. Snakkers et al. indicated an

increase in the content of higher alcohols in the cognac after aging as a result of the phenomenon of concentration by ethanol evaporation.

Appreciation organoleptic distillate received during various stages of aging appreciations increasingly better from 7.9 points which was offset raw distillate, after 3 years receives a maximum score of 20 points.

Over time, the concerns of more and more specialists are oriented towards finding a method of shortening the aging of distillates, regardless of their origin, from which it is expected the problems to reduce losses, the release storage facilities, of reducing the cost price but is required to maintain the same level of quality distillate outdated or obsolete quite close to those by the classical method.

A method of rapid aging of the distillate, developed for the first time in Romania (Popa,1985, 2002; Pomohaci, 2009), consists in the use of oak wood shavings activated at ambient temperature for 5 days in the oxidant solution of hydrogen peroxide H_2O_2 1% followed by 5 days with the electrolyte solution with a catalytic amount of $FeSO_4$-2 g / l, followed by washing with cold water and alcohol in wine.

By activating the wood shavings aims exhaustion its easily extractable components, which depreciates quality of distillate early aging. The dose of wood shavings used was 10 g / l and aims to increase the contact surface of distillate with oak wood that the presence of oxygen do to accelerate the hydrolysis reactions and oxidative shortening the period in which the sweet attributes taste of the distillate and its bouquet are made, the distillate can be quickly put into use.

The results are shown in Table 2. The dosage of wood shavings 10 g / l provides about 100-150 cm^2 / L. Distillates undergo such rapid aging were chemically analyzed after 30, 60, 90 and 120 days.

Table 2. Evolution of the main chemical components of distillate subjected to rapid aging with oak shavings

Stages of analysis (days)	Alcoholic degree	mG/L distillate		mG/100 ml anhydrous alcohol					Tasting note 1-20 points
		Extract	Ash	Total acidity	Acet-aldehyde	Ethyl acetate	Isobutyl alcohol	Furfurol	
Initial	38.0	620	62.0	35.0	5.5	60.5	138.12	0.10	7.9
30	38.0	630	60.2	28.2	6.8	68.9	170.2	0.65	15.8
60	38.0	660	69.4	58.6	9.0	82.8	19102	0.69	17.9
90	38.0	740	78.1	71.1	10.9	92.6	248.3	0.86	18.5
120	38.0	749	82.2	85.1	11.8	99.4	250.0	0.96	19.0

In distillate subject to rapid aging oak shavings, quantities of extract increases with increasing duration of aging. Initially having 214 mg / L extract, for after 120 days to reach 720 mg / L. Naturally increase the ash content of 29.1 mg / L 73.1 mg / L.

Significant increases recorded and volatile chemical components. Acidity increases from 36.4 mg / 100 ml a.a. to 125.0 mg / 100 ml, a.a., acetaldehyde from 6.8 mg / 100 ml, a.a. 12.9 mg / 100 ml a.a. The content of ethyl acetate doubles the amount of 76.9 mg / 100 ml, a.a. to 140.1 mg / 100 ml, a.a. Also strong gains isobutyl alcohol are increasing from 152.0 mg / 100 ml a.a. to 260.0 mg / 100 ml, a.a.

It realizes the complexity of the chemical composition of the distillate subjected to rapid aging is highlighted in significantly improving sensory aspects. As a result, if crude distillate 37% vol. Alcohol had an appreciation of 7.9 points at the end of the rapid obsolescence of 120 days, it was rewarded with 19 points by maximum points possible 20.

As a result, these analyzes were identified some similarities but also some differences that appear with aging classical and rapid distillate.

Processes acting on classic technology distillates aging in oak barrels are the same and rapid technology aging.

Shortening aging processes is achieved through acceleration of all chemical and biochemical processes of oxidation, hydrolysis and extraction of more components of oak wood, oxidation, esterification, deamination and decarboxylation of several chemical distillates raw or extracted from wood that is spend and during the long aging in oak barrels.

Mentioned acceleration is achieved by: increasing reports values for the contact surfaces between oak and distillate young; intensifying oxidative activation of oak more vigorous and increasing the proportion of oxidants intermediaries - catalytically active; ensuring high temperatures (20-25^{0}C) during the aging processes

The duration of distillate contact with the barrel stave oak or oak shavings activated has a decisive influence both on enrichment in fixed and volatile chemical components and shape of aging processes distillate pleasant characters. The aging processes duration is much longer distillates have significant proportions of fixed and volatile chemical components and their character immobilizing amplified being fuller, softer, with more attractive color, more enjoyable and richly fragrant.

Comparison of composition and taste characteristics of distillates rapid aging in the presence of oak shavings previously activated with the long obsolete distillates in oak barrels, show similar values, sometimes a little higher, distillates rapid aging. This can be explained by operating more vigorous aging factors.

Accordingly, in connection with the rapid aging can find that: changes in chemical composition are the same as the classic aging technology, standing out is some predominance of some of the volatile components; increase production by increasing the proportions of esters formed by deamination of components extracted from oak wood and favoring esterification processes and increasing the proportion of higher alcohols by oxidative decarboxylation processes deaminease and, more evident in the rapid aging technology.

4. Conclusions

During aging processes, along with amplification quality and major losses occur and alcoholic strength volume

Activation oak wood (similar to stave vessel) by different physico-chemical processes can ensure rapid enrichment of the distillate in valuable compounds, specific natural products aging.

Comparison compositional characteristics of distillates rapid aging, in the presence the oak shavings previously activated, with the distillates aging long in barrels of oak, showing similar values, sometimes a little higher, distillates rapid aging. From the point of view of sensory qualities, rapid aging distillate has not obtained the maximum score as the aging classic distillate for 3 years.

Continue for about 12 months maturation in oak leads to better harmonize and highlight the qualities that characterize distillates aging

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CYTOTOXICOLOGY AND ITS APPLICATION IN FOOD SAFETY

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ABSTRACT

Cytotoxicology is a branch of toxicology which studies the mechanism and effect of exogenous substances on the damage of life cells. It can be used in the evaluation of food safety toxicology and the toxic mechanism of harmful substances in food. Food processing and production in the detection of toxic and hazardous substances improved functional foods in the efficacy of active ingredients of research. There are several methods of cytotoxicology studied the different effects of exogenous substances on the cells, including cell activity and genetic effects, which resulting in changes of cell morphology and apoptosis detection. Many factors affect the safety of food, including pesticide residues, food additives, heavy metal materials, biological toxins. Cytotoxicology is a rapid, accurate and economic analysis method for food safety by studying the toxicity, toxic metabolism and toxic effect mechanism of harmful factors in food. It has the advantages of fast, accurate and economic and has a good effect in food safety field. In this paper, the application of cytotoxic technology in screening of the above-mentioned hazards is expounded.

1.Introduction

Food is the material basis for human survival and development(UHL, 2000; Klein, 2016; Dutta, 2009). Recently, food safety has become an important global problem in the unprecedented prosperity of human society(Espitia, 2012). With the improvement of people 's living standard, food safety testing has become an urgent problem(Magnuson, 2011; Allahghadri, 2010). The research of food safety in our country started late, but the rapid development of food industry has promoted the research of food toxicology. Cytotoxicology is a branch of food toxicology(Fabra, 2016). The experimental tendency of replacing acute toxicity and whole animal is very obvious. It is a study of exogenous harmful factors, including physical, chemical and biological factors on life cell damage, the mechanism of toxic effect a methodology for risk assessment. Through the cytotoxic method, as is shown in Figure 1, the cell environment can be simplified and the cell line can be purified(Karmaus, 2016). Under the condition of cell survival, the changes of the fine structure and function of target cells can be observed. Its mechanism and dose-effect relationship can be analyzed and evaluated. Now the application of cytotoxic technology in food safety is expounded (Chau, 2015; Jain, 2016).

The most widely use of cytotoxic method in the field of food are MTT colorimetric assay, DNA synthesis assay, comet assay, western

blot, flow cytometry, NRU staining and immunocytochemical staining(Maldonado, 2015; Abdalhai, 2015; Duarte, 2015). Animal experiments was used to complete toxicological safety assessment of toxicology, which is widely used evaluation methods. But with the accumulation of experimental data, it was found that not all animal experiments can accurately predict the harmful substances on the human body, which need to sacrifice many animals. So from the economic and scientific point of view, people use in vitro animal cells, human cells, tissue culture and other new research methods(Deng, 2015). The choice of vitro methods to replace animal experiments possible, Europe has long proposed detection of chemical animal safety, unless there is no reliable alternative methods that can be used animal experiments, which is not only the protection of animal rights needs, but also highlights the progress of science, social and economic development(Lin, 2015).

In the 20th century, the principle of 3R, which were replacement, reduction and refinement, were proposed to treat the living animals and reduce the suffering of animal deaths, used by animals scientifically, rationally and humanely. International organizations such as the Organization for Economic Cooperation and Development (OECD), the European Center for the Validation of Alternative Methods (ECVAM) and the United States Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) have promoted the development of animal substitution law. The OECD has established the Good Laboratory Practice (GLP), the guiding principle for the in vitro substitution experiment, which together facilitate as the establishment of experimental guidelines and the international integration of experimental conditions. Toxicology experimental animal replacement methods include computer simulation methods and vitro experimental methods. The current animal replacement method, including cell culture, organ culture and embryo culture three methods. The development of cell culture achievements of the rise and the subject of cytotoxicology. The definition of cytotoxicology is the study of exogenous substances on the role of life cell damage and its mechanism of a toxicology branch of the discipline. People have established a number of cell lines for cytotoxic experimental studies, such as the use of liver cell line detection toxicity of the source material. Cytotoxicology has been used in the field of food, medicine, plant tissue culture, this paper focuses on cytotoxicology and its research methods in the field of food applications.

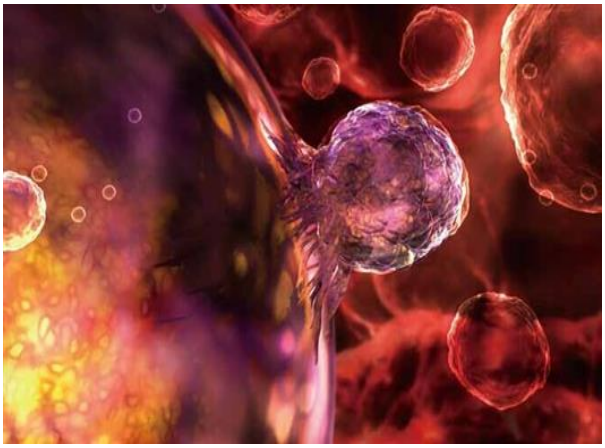

Figure 1. Microscopic cytotoxicology

2.Matherials and methods

2.1. Research of Cytotoxicology methods in food field

There are many research Cytotoxicology methods, the most mature in practice which is used by many researchers is the MTT method. For example, the study of lycopene polysaccharide, lycopene, curcuma oil on cancer cell inhibition(Lopez, 2015). This method can detect cell activity, but this is only one aspect of cytotoxicology, it requires a variety of methods to explore the different effects of foreign substances on the cells, including the genetic effects of cells, which resulting in changes of cell morphology and cell apoptosis detection. There are many research methods of cytotoxicology, but their research and development are not the same. In practice, not all methods have been widely used. This paper mainly introduces the current

situation of domestic and foreign laboratory personnel in the field of food cellular toxicology test method. Detection of the Cytotoxicology schematic was shown in Figure 2.

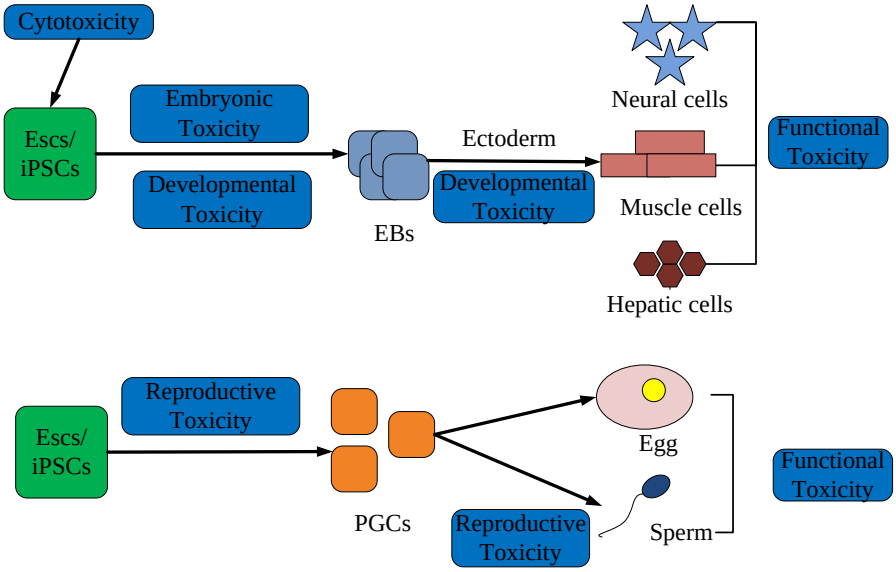

Figure 2. Detection of the Cytotoxicology schematic

2.1.1. MTT colorimetric experiments

MTT colorimetric assay is a method for cell survival and growth detecting. MTT is abbreviation of coloring agent thiazole blue, the chemical name is (2, 3, 4, 5-dimethylthiazol) and 2, 5-diphenyltetrazolium salt. MTT can be living cells mitochondria succinic acid, Dehydrogenase is reduced to an insoluble blue-violet crystalline form, which deposited in cells, whereas dead cells can not. The method is very mature, many researchers have used the test cell viability. Arsenic poisoning was caused by high arsenic concentration in drinking water. Bredfeldt used MTT and other vitro experiments to study the effects of carcinogenic trivalent arsenic and methylenic arsenic acid on human urethral epithelial cells. The results showed that the ratio of methylene arsenic acid arsenic (trivalent) toxicity is 20 times greater, and human urethral epithelial cells can be arsenic (trivalent) biotransformation into pentavalent and trivalent methylene metabolites.

For example, ochratoxin is produced in aspergillus and penicillium, it can contaminate crops such as rice, wheat, coffee, corn and so on. After ingestion of the animal, the mycotoxins in food will be harmful to human health. Liver, kidney will be infringed, severe teratogenic. Mwanza et al. tested the cytotoxic effect of OA on human and porcine lymphocytes by using the MTT assay. Although MTT colorimetric assay is widely used, it can not be used for the study of all substances. For example, some substances in flavonoids can not use this method to explore its effect on various cells. Peng et al. used cells in the absence of luteolin, flavonoids quercetin can also reduce the MTT, which will affect the role of these two flavonoids in cell growth measurement results.

2.1.2. Cell DNA synthesis assay

DNA is the basic material of cytogenetics, its structure has four kinds of bases, including thymidine (TdR) with its unique base. Therefore, by using 3H-TdR, DNA synthesis precursor incorporation of DNA synthesis and metabolism process measured the cell's radioactivity, which can reflect the cell DNA metabolism and cell proliferation. Cell DNA synthesis assays can be used to study the effects of exogenous substances on cellular DNA synthesis, thereby elucidating their toxic effects on cells. Conjugated linoleic acid cis-9 and trans-11CLA are physiological activity of a natural product, it can be anti-

atherosclerosis which improved immunity, anti-cancer effects. Other DNA synthesis using cell cytotoxicity test methods the cytotoxicity of cis-9 and trans-11CLA on breast cancer MCF-7 cells was studied. The results showed that cis-9 and trans-11CLA could block the cell cycle and inhibit the growth and proliferation of MCF-7 cells. Cyclosporine is a metabolically active metabolite produced by a fungus. It is an ideal immunosuppressive agent provided by Marionnet et al. They evaluated the immunosuppression of cyclosporin A in their study, DNA synthesis assay was used to evaluate the antiproliferative effect of cyclosporin A on normal human skin keratinocytes and HPV keratinocyte transfected cells. The DNA synthesis assay was used to measure the DNA synthesis of DNA synthesis quantit in 16-18 h.

2.1.3. Comet Electrophoresis (SCGE)

Endogenous Mg2+ and Ca2+ dependent endonucleases are activated during apoptosis, and there are three forms of DNA cleavage, namely cleavage of DNA strands between small bodies, cleavage of macromolecular DNA strands and DNA cleavage chain breakage. They can be used for food, medicine, cosmetics, aloe contains melatonin (MT), it is an indole hormone, which can antioxidant, anti-aging, contribute to the immune system, digestive system. Bagchi used electrophoresis the effect of melatonin on macrophage J774A cells which was studied by the method of cytotoxicology. In this study, macrophage J774A.1 cells induced oxidative stress and DNA damage induced by camphor balls. The results showed that melatonin on the damage by the mothballs macrophage J774A.1 cells whihc have a protective effect. Uhl et al. evaluated the toxic effects of genotoxic carcinogens on the Hep G2 hepatocellular carcinoma cell line by electrophoresis. They examined the toxic effects of 12 compounds, including yellow, which were used to detect the cytotoxic effects of carcinogens. (AFB1), sassafrasin, and some pyridine compounds. The results showed that 10 compounds resulted in a significant increase in DNA migration. The authors also compared their results with earlier experiments, indicating that the Hep G2/SCGE method is promising for predicting genotoxicity.

2.1.4. Western blotting

Western blot is a biological technique for the analysis of protein expression in cells. It is a method for the identification and quantification of proteins with specific antisera, by transferring non-labeled proteins separated by electrophoresis onto solid-phase carriers. This method can be used to study the toxic effects of exogenous substances on the genetic material in cells. Naringenin is a natural bitter substance with antibacterial, anti-inflammatory, anti-cancer, anti-atherosclerosis and other effects, Park and other use of westernblot and other cytotoxic test methods to study naringenin on cancer cells MCF 7 cells. The results showed that naringenin could inhibit the growth of MCF-7 cells in a dose-dependent manner. Naringenin could induce the endogenous activity of naringenin in a dose dependent manner. The activation of p38, SAPK/ JNK/, c-Jun signaling pathway, p38, SAPK/JNK/1/2, c-Jun can cause cell growth arrest in cytokines and oxidative stress. And cell apoptosis, while naringenin decreased the level of factor P-Erk1/2 which induced cell growth.

Flavonoids are rich in flavonoids and are often used as food additives and antibacterial agents. Flavonoids (RCMF) extracted from lacquer leaves can inhibit the growth and induce death of human osteosarcoma cells. Kook et al. induced apoptosis of human osteosarcoma cells which associated with p-53-mediated mitochondrial stress response and nuclear translocation of factor AIF and EndoG. AIF and EndoG were involved in the induction of cell apoptosis. Bcl-2, Bax and Cytochrome c, and RCMF can induce Bcl-2, Bax, and Cytochrome c release by induction of p53, and finally RCMF can induce human body osteosarcoma cell apoptosis.

2.1.5. Flow cytometry

Flow cytometry is a very useful tool in the food industry for the detection of food microbes. It can be used to detect microbial cells by simultaneously detecting membrane integrity and

membrane activity, membrane potential, respiration, intracellular pH and so on. In food microbes, it mainly used to determine the activity of microbial flora and cell count, spoilage and pathogen contamination testing, probiotics viability testing. Veal also in its literature describes the flow cytometry and fluorescence staining for the detection of microbial cells, including monitoring of water, food and beverage micro-organisms.

Flow cytometry can also be used to detect the effects of active ingredients in functional foods on human cells. Long-chain polyunsaturated fatty acids exist in many plants, which have a variety of physiological activity of the function, in food and more as a nutritional fortifier, mukaro and so on. The use of flow cytometry detection of food add omega-3 long chain saturated fatty acids in food, the human erythrocyte membrane lipid in the long-chain polyunsaturated fatty acids change is indirectly involved in the role of anti-inflammatory, that is helpful to the anti-inflammatory white blood cell volume and function change is related. In addition, it can also be used to detect harmful factors in food on the body cells of toxic effects, such as lag and so on. The use of flow cytometry and other toxicological experiments of cadmium induced primary epithelial lung cell death mechanism of action, the study pointed out that cadmium Induction of apoptosis is associated with p53 and bax, which did not involve oxidative metabolic pathways.

2.2. Experiment environment and condition

2.2.1. Experiment material

Subject: purchased from Beijing Songlan Pieces Factory, the product Eucommia ulmoides Euphorbia uluaridee soliver dry bark slices, using alcohol to mention. The extraction of ethanol, to alcohol-free flavor, continue to concentrate to about 1: 2, 2000ml, let cool, precipitate a add ethanol, and then centrifuged for 15 min (5000 r/min). The supernatant was further concentrated to 1000 ml and the relative density was 1.16 (60 oC). The blood volume was 4g / ml, bottled and autoclaved at 100 oC for 1h . Distilled water was used to make the desired concentration.

2.2.2. Instruments and reagents

Neutral red aqueous solution(LISHEN, BB 5060, (5 mg/ml), immobilization solution (1%, CaCl2 formalin aqueous solution) were used as the medium, (1% acetic acid a 50% ethanol solution), anatomical apparatus, biological microscope, slide, low temperature high speed centrifuge, low temperature refrigerator (80 oC), liquid tank, clean bench, constant temperature incubator, homogenizer, steam pressure cooker. Kunming grade mice male and female half 18-22g by the China Pharmaceutical and Biological Products Laboratory Animal Center provided. Salmonella typhimurium strains TA97, TA98, TA100, TA102 strains, tested strains identified to meet the requirements. Do ampicillin and ampicillin/tetracycline main plate, 4 oC preservation. Chinese Hamster Ovary Cell CHO, Chinese Hamster Lung Fibroblast Cell CHL, Mouse Lymphoma Cell L5178Y.

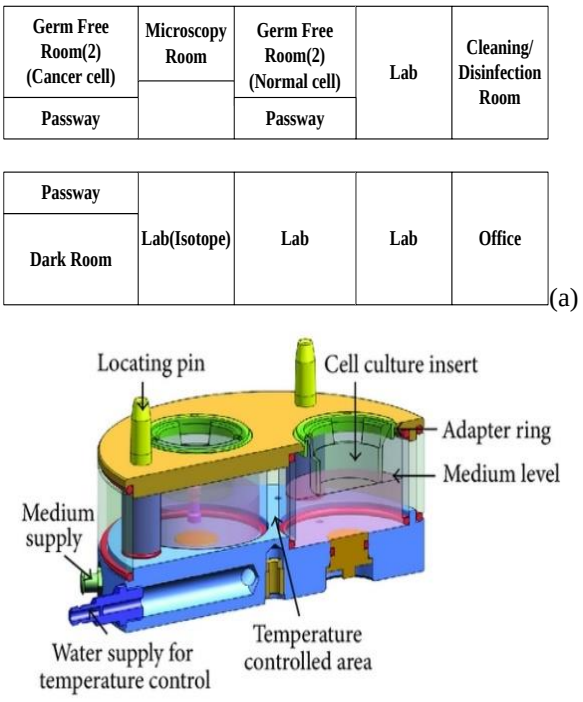

Figure 3. (a)Schematic diagram of cell culture laboratory design, (b) Physical map of cell culture laboratory design

3.Results and discussions

3.1. Cytotoxicity test

The results are shown in Table 1. When the concentration of Eucommia ulmoides was more than 125 mg/ml, the number of viable cells decreased drastically, the CHL of CHO cells was 109.38 mg/ml and the IC50 of CHO cells was 109.38mg / ml.

Table 1. The toxic effect of CHL and CHO cell

Cell Types	Dose mg/ml	n	OD540 value	Surviving Rate (×10-2)
CHL	0.98	12	1.40±0.04	98.30
	31.25	12	1.28±0.03	85.33
	62.5	12	1.15±0.02	71.68
	125.0	12	0.86±0.02	42.09
CHO	7.81	12	0.15±0.01	100.12
	31.25	12	0.14±0.00	94.00
	62.5	12	0.12±0.00	77.37
	125.0	12	0.10±0.00	40.07

When the cells are exposed to harmful factors, they can undergo morphological changes, poor adherence, decreased growth rate, cell degradation, impaired integrity, and even death. These changes can be detected by optical microscopy, electron microscopy and other methods directly observed. Cell density changed with time was shown in Figure 4.

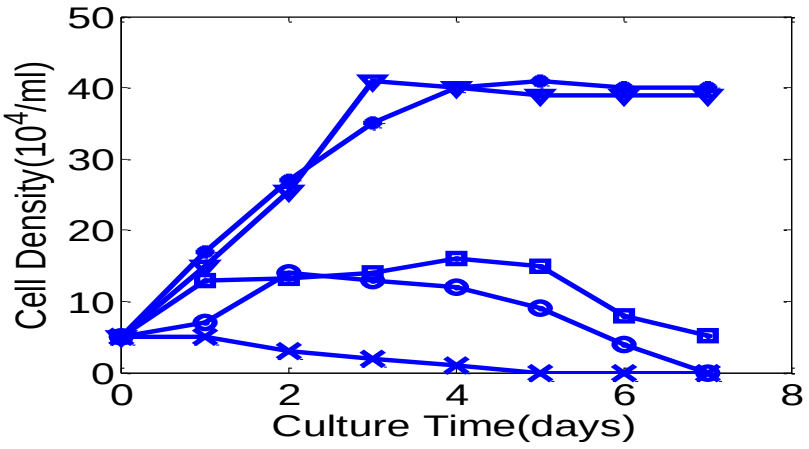

Figure 4. Cell density changed with time

3.2. Genotoxicity test

The micronucleus rate of the positive control group was higher than that of the negative control group (P <0.01), but the micronucleus rate of each group was not significantly different from the negative control group (P> 0.05). That is, Eucommia ulmoides bone marrow cell micronucleus test negative results were shown in Table 2

Table 2. The effect of bone marrow cell micronucleus rate

Dose (g/kg)		Sex	n	Micronucleus Rate (×10-3)
Du-zhong	0.00	male	5	0.16
	41.6		5	0.14
	83.3		5	0.20
	166.6		5	0.16
Du-zhong	0.0	female	5	0.22
	41.6		5	0.16
	83.3		5	0.20
	166.6		5	0.18

Compared with control group,*P<0.01

3.3. Tk gene mutation test

As shown in Table 3, the frequency of mutation (MF) in the MMS-positive and control groups was 2-fold higher than that in the solvent-control group, while the frequency of Eucommia ulmoides was not more than 2 times that of the solvent control group. No mutagenesis of gene in L5178Y mouse lymphoma cells was induced. Gene mutation results in different cell concentration was shown in Figure 5.

Table 3. The effect of tk genetic mutation

Group	Dose (ug/ml)	PE0	PE2	MF
H2O MMS Du-zhong	0.00	89.03	74.96	91.11
	10.00	86.64	54.77	523.7
	1250.00	64.87	50.62	98.98
	625.00	68.01	45.21	145.1
	312.50	77.01	48.32	128.3
	156.25	77.01	70.68	111.1

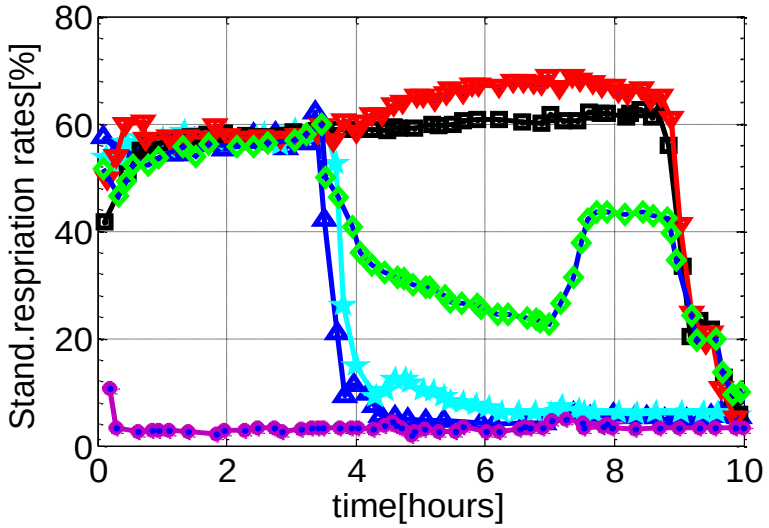

Figure 5. Gene mutation results in different cell concentration

3.4. Outlook for other monitoring methods

Common approaches to food research include neutral red uptake (NRU) cytotoxic assays and immunocytochemical staining techniques. The NRU experiment has been used in many studies to examine the effects of exogenous substances on cell viability and to study the efficacy of active ingredients in foods. The cytotoxic effects of harmful agents in food, and the toxic effects of food additives on the cells of organisms wait. For example, Jeong and other NRU, MTT and other cytotoxic experiments to study the water extract of camellia on neuronal cell PC12 protective effect. Handrich and so on using this method to detect the unknown mycotoxins on the body cells of toxic effects. Toxic effects of fungicide P40 on green monkey kidney cells were studied.

Immunocytochemical staining is a combination of serological methods and microscopic methods, which can be divided into immunofluorescence cytochemistry, immunoenzyme chemistry, immunoferritin technology, immunization gold-silver cytochemistry, affinity immunocytochemistry and radioimmunoassay. This technology can be used to study the morphological changes of cells and intracellular metabolites, which has been used in the field of food research, such as weaver using immunocytochemical staining to explore the carcinogen benzene on epithelial lung cancer cells the effects of oxymatrine. Bioactive component extracted from sophora flavescens root and other plants, on the proliferation and collagen synthesis of mouse skin fibroblast NIH3T3, which were studied by immunoenzymatic method and M-TT method.

4.Conclusions

Food safety has become a focus of modern life which get much public concern. Cytotoxicity is the application of in vitro cell culture methods and techniques to evaluate the harmful factors. It has the advantage of being simple, rapid, sensitive and accurate. It has been widely used in the study of the safety of many substances. In this paper, the cytotoxicity of food safety in the application of a brief overview. Traditional toxicological evaluation of food safety is an in vivo experiment using animals, which is time-consuming, laborious and costly. With the advocacy and implementation of the 4Rs (reduction, substitution, optimization and reliability) principle used in laboratory animals. Therefore, the research and application of cytotoxicology in the field of toxicology research has attracted more and more people's attention and attention in food toxicology safety evaluation. As a new research field, Cytotoxicology is one of the alternative methods in animal experiments, and the research in food field has a good prospect. Athletes physical health, physical fitness can form strong than short-term, long-term need for a reasonable and balanced diet. In order to allow the athletes come out of nutrition misunderstanding, we establish the correct concept of nutrition as soon as possible. It should be widely carried out in the athletes early groups, efficient sports nutrition knowledge and improve athletes overall understanding of nutrition science from the breadth depth. Players only through correct nutrition taught in various forms to understand the relevant knowledge of nutrition, and establish a positive, correct beliefs and attitudes, will it be possible to integrate the formation of the active feel good for their own healthy eating behavior. In this paper, the integrated use of expert interviews, literature review, questionnaires, mathematical statistics, methods and means, on the part of nutrition education and nutrition status of college athletes KAB investigation and analysis.

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