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CONTENT

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EVALUATION OF WINDOW GLASSES TRANSMISSION AND SUNLIGHT GUIDING SYSTEM IN A SOLAR-BASED VERTICAL GREENHOUSE

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ABSTRACT

Vertical farming is believed to be a solution to the potential global food shortage in the future. However, it also receives many doubts about using excessive energy to support its artificial lighting system. In this paper, a solar-based vertical greenhouse is investigated based on a baseline greenhouse configuration. Ray tracing method is utilized to simulate the solar energy delivered to crop surfaces through window glasses transmission and artificially from a sunlight guiding system. Simulated results demonstrated that elevating the floor height and introducing a sunlight guiding system can improve the sunlight amount without using artificial lighting.

1.Introduction

On the global level, urbanization has been accelerated by population growth in the past decades. Unprecedentedly, over half of the world's population lives in urban areas. According to the UN's Dept. of Economic and Social Affairs prediction, the world`s population will surpass the 9 billion mark by 2050 (United_Nations, 2004). This trend has threatened the production, processing, and delivery of food. Sustainable solutions are expected to supply fresh, local food for the urban population. Researchers believe that closed production systems in cities can be the cure, namely plant factories, vertical farms and rooftop greenhouses (Kozai, Ohyama, & Chun, 2006). Vertical Farming (VF) is attracting public attention because it can achieve remarkable food production with very limited land area usage. Skyscrapers are equipped with artificial lighting infrastructure, environmental adjusting facilities, nutritional controlling appliances to realize fast growth and high yield of crops (Beacham, Vickers, & Monaghan, 2019; Ng & Foo, 2020; Kozai, 2013; Specht et al., 2013). Nevertheless, although these sorts of equipment benefit the production rate, they also bring problems to the system. One critical problem of VF is that it needs large energy consumption, especially on the artificial lighting system (Germany_Aerospace_Center, 2012). According to a feasible study of vertical farm conducted by Germany Aerospace Center, to construct a 37-floor vertical farm building will cost 139,454 Euros, however, the total cost of above-mentioned equipment for this building is 145,215 Euros, even higher than the building itself, 59% of these special expenses are utilized on lighting equipment such as LED panels and heat exchanger systems to cool down the LEDs. Besides, in daily usage, the artificial lighting system also requires 20% of the total energy consumption, contributes to the second-biggest consumer of electric power in operating a VF. Reducing the dependence on artificial light is

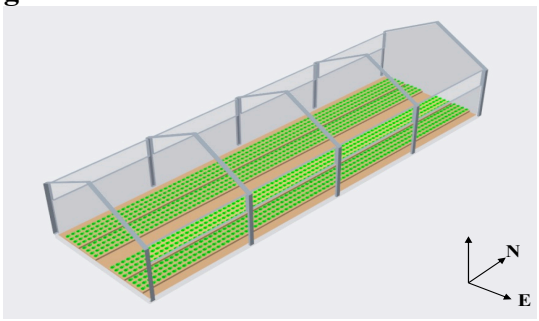
significant to push VF towards large-scale applications. In this context, guiding more sunlight might be a promising solution to cut energy consumption.

Generally, windows and sunlight guiding system are two paths for daylight transmission into a VF. The purpose of this research, therefore, is to evaluate these two methods and their effects of guiding daylight into vertical farms based on a comparison study to a baseline greenhouse.

2. Material and methods

The typical agriculture building utilizing the maximum of solar energy is the greenhouse. In the urban areas, the rooftop greenhouse is also a solution to the food production problem (Specht et al., 2013). To study how the sunlight enters the indoor area, a single floor rooftop greenhouse is built as a baseline as shown in Fig. 1. Total solar energy during the shortest day of the year is evaluated and recorded, the date and place are 22nd December in Singapore. Then, vertical multi-floor greenhouses are designed to evaluate whether each floor can still absorb enough sunlight for crop cultivation. Two different floor heights are set to evaluate the amount of window glasses transmission, the normal floor height is 3 meter and an elevated height is 6 meter, results are compared to evaluation whether elevating the height can effectively improve the sunlight amount. Additionally, a sunlight guiding system is built to study the amount of solar energy introduced by sunlight collectors (Ullah & Shin, 2014; Yalçın & Ertürk, 2020) though the fully glazed greenhouse may not need a sunlight guide system during the sunny day. Results are compared to window transmission. Note that crop growth depends on PAR illumination and plants might also get stressed under high PAR illumination reducing the crop growth. Thus, ensuring growth everyday does not necessarily ensures maximum crop growth.

2.1. Modeling of greenhouses and vertical greenhouses



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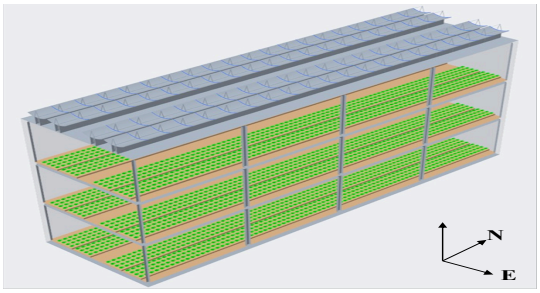

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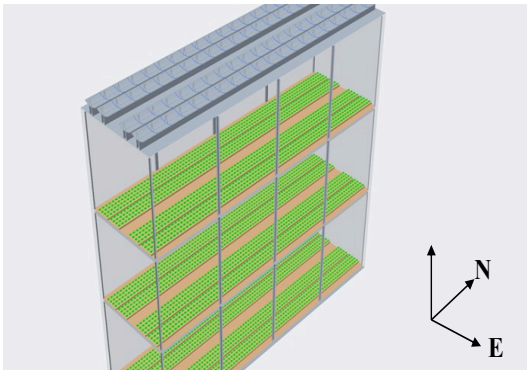


(c)

Figure 1. Layouts of the: (a) baseline greenhouse, (b) vertical multi-floor greenhouse and (c) vertical greenhouse heightened (elevated floor height)

Fig. 1 (a) exhibits the layout of a rooftop baseline greenhouse. All the walls are built by glasses so that sunlight can easily reach the crop surfaces. The rooftop position ensures the ideal location with no shade and shadow from other buildings in cities. Thus, results based on this model will be the baseline for the upcoming evaluations.

Figs. 1 (b) and (c) show the structure of two three-floor vertical greenhouses with a floor height of 3 meters and 6 meters, respectively. Since cost estimate from references has demonstrated that VF building is more affordable than its equipment, (Germany_Aerospace_Center, 2012) the 6-meter solution will be considered and assumed as an affordable scenario, which is supposed to add less cost than a sunlight guiding system. For these three buildings, two larger façades are placed toward east and west to absorb more sunlight energy. Layout parameters are listed in Table 1.

Table 1. Layouts of the baseline greenhouse, vertical greenhouse and vertical greenhouse heightened

Name of building	Length [m]	Width [m]	Floor height [m]	Number of floors	Crops Area (m^2)	Collectors Area (m^2)
Baseline Greenhouse	20.2	6	3	1	80	0
Vertical Greenhouse	20.2	6	3	3	240	80
Vertical Greenhouse Heightened	20.2	6	6	3	240	80

2.2. Modeling of sunlight guiding system

The sunlight guiding system is supposed to collect abundant sunlight on the building roof, and then transfer the energy into indoor areas as a supplemental approach. To achieve a high concentration of sunlight, a parabolic trough is designed as shown in Fig. 2(a). Aluminum foil coated on the surface can help to realize the sunlight reflect rate of 95% (Venegas-Reyes, Jaramillo, Castrejón-García, Aguilar, & Sosa-Montemayor, 2012).

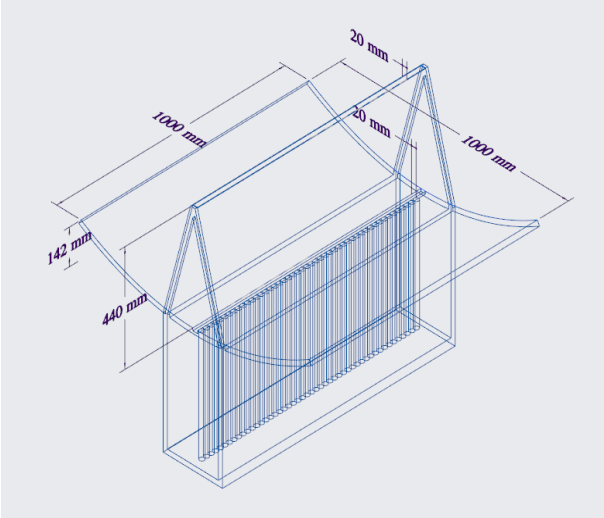
The rectangular aperture height and the rectangular aperture width are both set as 1000 mm so that the vertical projection area is 1 m^2. The parabolic trough in the middle and the convex parabolic reflector on the top match one another to ensure that the sunlight collected by the parabolic trough can be guided into the optic fibers inlet in the bottom, as shown in Fig. 2(a). The optical fibers inlets are in a linear array so that they can receive the maximum solar energy and transfer it to the crops. The proper arrange of reflectors to collimate the sunlight is designed by:

$$F_1 = F_2 \tag{2.2.1}$$

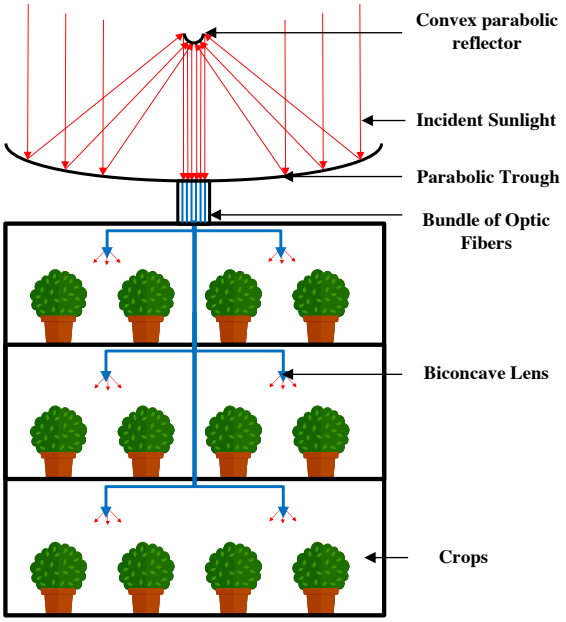
In this equation, F_1 and F_2 represent the focal point of the parabolic trough and the concave reflector, respectively. For the parabolic trough, the focal length is set to be 440 mm. Every light to the inlet surface of the optical fibers is defined by

$$H_{PR} = W_r \tag{2.2.2}$$

The H_{PR} represents the rectangular aperture height of the parabolic reflector and W_r means the width of the receiver of optic fiber inlet. In this case, W_r is 20 mm (Roland Winston, 2004).The sunlight captured by one collector will be transfer to 1 m^2 crop area on each floor as shown in Fig 2(b). A Biconcave lens is introduced at the end of each bundle of optic fibers to diffuse the sunlight and to achieve a uniform distribution on crop surface. The transfer efficiency is set to be 60% according to literature (Liang, Monteiro, Teixeira, Monteiro, & Collares-Pereira, 1998).



(a)



(b)

Figure 2. Layouts of (a) a single sunlight collector with projected area of 1 m^2;(b) Sunlight guiding system

2.3. Solar load method

To calculate the solar energy, the ray tracing method and solar load method is introduced. The Theoretical Maximum Method is applied using ANSYS Fluent. The equation for direct normal irradiation is introduced based on NREL (National Renewable Energy Laboratory)'s Solar Position and Intensity Code (Solpos):

$$Edn = S_{etrn}S_{unprime} \tag{2.3.1}$$

The S_{etrn} represents the direct normal solar irradiance from the top of the atmosphere. $S_{unprime}$ represents the correction number which can account for factors reducing solar load through the atmosphere.

The evaluation of the diffuse load in this solar model is calculated by methods from the 2001 ASHRAE Fundamental Handbook (ASHRAE, 2001). On a vertical surface, the definition for diffuse solar irradiation is represented as:

$$Ed = CYEdn \tag{2.3.2}$$

In which the C represents a constant according to Tab. 7 from Chapter 30 of the 2001 ASHRAE Handbook of Fundamentals, Y is the ratio of sky diffuse radiation on a vertical surface to that on a horizontal surface, which is count as a function of incident angle). *Edn* means the direct normal irradiation on a clear day.

Besides the vertical surfaces, diffuse solar irradiation for other surfaces is defined as:

$$Ed = CEdn\frac{(1+cos\,\varepsilon)}{2} \tag{2.3.3}$$

The tilt angle of the surface from the horizontal plane is defined as ε in this equation represents.

For coated glazing, spectral transmissivity and reflectivity (assume gray approximation with no dependence on wavelength) are computed based on the normal angle of incidence (Elizabeth U Finlayson, 1993).

Transmissivity is defined as:

$$T(\theta,\lambda) = T(0,\lambda)Tref(\theta,\lambda) \tag{2.3.4}$$

In which:

$$Tref(\theta) = a0 + a1\,cos(\theta) + a2\,cos(\theta^2) + a3\,cos(\theta^3) + a4\,cos(\theta^4) \quad (2.3.5)$$

Reflectivity is defined as:

$$R(\theta, \lambda) = R(0, \lambda)[1 - Rref(\theta)] + Rref(\theta) \quad (2.3.6)$$

In which:

$$Rref(\theta) = b0 + b1\,cos(\theta) + b2\,cos(\theta^2) + b3\,cos(\theta^3) + b4\,cos(\theta^4) - Tref(\theta) \quad (2.3.7)$$

The normal transmissivity and reflectivity are defined as 0.8 and 0.1 respectively for the window glasses both in the greenhouse and vertical farms (Farkas et al., 2001; Graamans, Baeza, van den Dobbelsteen, Tsafaras, & Stanghellini, 2018).

3. Result and discussion

3.1. Grid independence result

Three meshes with different elements number are built for each building. The solar heat flux on the unshaded top surfaces is recorded and converted into Daily Light Integral (DLI) value. Experiment results from literature carried out in Singapore are introduced to validate the simulation (Song, Tan, & Tan, 2018).

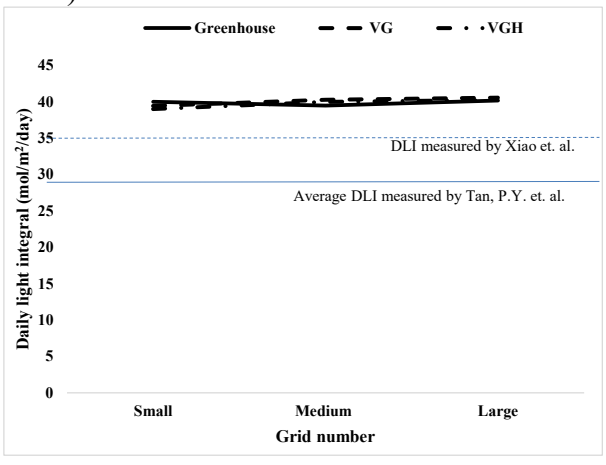


Figure 3. Grid independence result on layouts for baseline greenhouse, VG, VGH.

The grid independence results with ray tracing approach (ANSYS Fluent) in Fig. 3 shows that the increase in grid number from coarse, fine to finer does not obviously affect the DLI result. The values of daily maximum solar flux are converted to photosynthetically active radiation (PAR) based on a coefficient of 2.1 with reference to literature. (Robert W. Langhans, 1997) Calculated values of unshaded DLI are around 40 mol/m^2/day, which is slightly higher than values from the measurement. This is possible because the measurement can take the shade of cloudy weather, atmosphere thickness, and shadow from other buildings into account, whereas simulations in this study are aimed to evaluate the ideal situation which does not consider these factors.

3.2. Comparison of solar energy for different solutions.

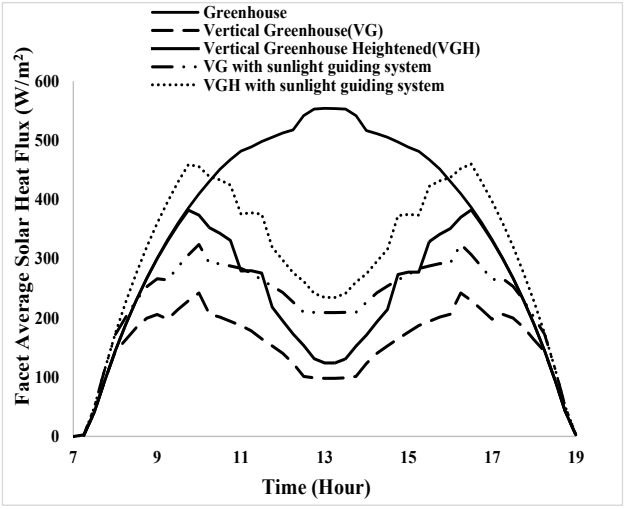


Figure 4. Solar energy during the day

Fig. 4 exhibits the simulation results of facet average solar heat flux. It can be observed that before 8:00 and after 18:00, the solar energy results in the greenhouse and vertical greenhouse are the same. This is because the solar zenith angles are low so the sunlight can enter the indoor area with no difference. However, when the solar zenith angle is high in the middle hours of the day, indoor areas of a multi-floor building cannot receive as much

solar energy as the baseline greenhouse can. The sunlight collectors contribute more obviously to introduce additional sunlight energy.

3.3. Comparison of DLI in different solutions.

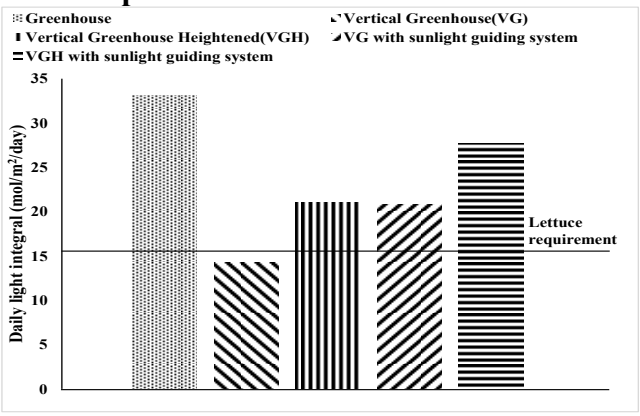


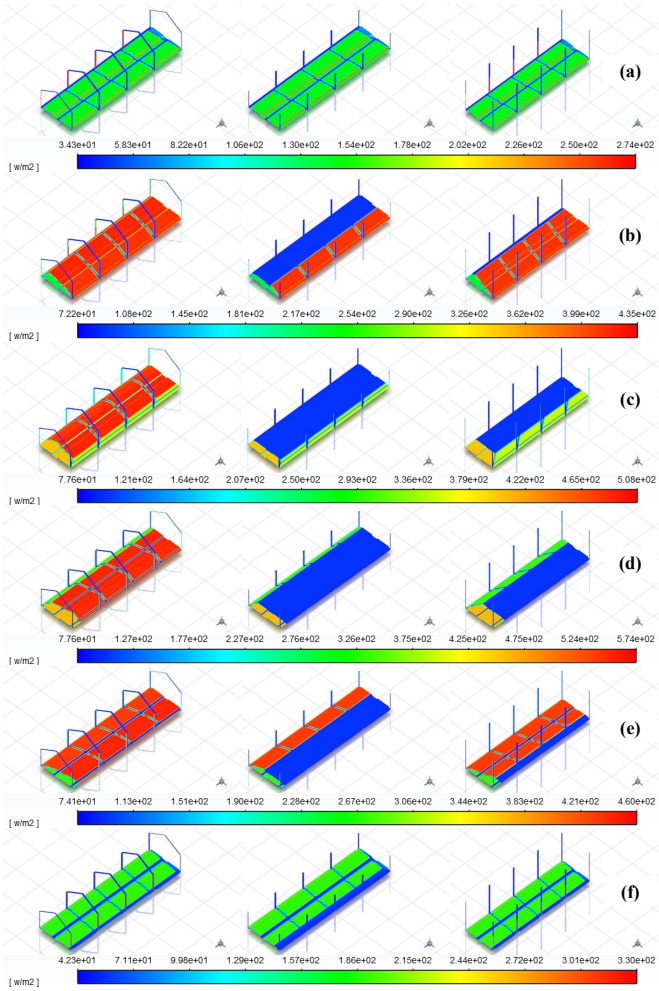
Figure 5. Comparison of DLI results for different layout solutions

Fig. 5 shows the DLI result of different layout solutions. The baseline greenhouse receives a DLI of 33.12 mol/m^2/day. Compared to the unshaded value without glass walls of DLI around 40 mol/m^2/day in the grid independence test, the lower DLI inside the greenhouse is caused by the reflection and absorption effect of the glass walls. However, this value can meet the requirement of various crops as presented in Table 2. The 3-meter vertical greenhouse receives an average DLI of only 14.23 mol/m^2/day, which cannot meet the lettuce`s DLI requirement of 17. Elevate the floor height or introduce the sunlight by the collector can increase the DLI up to 21.01 and 20.85, respectively. Implement both floor heightened and sunlight collector methods simultaneously can raise the DLI value to 37.32, which is even higher than that of the baseline greenhouse.

Table 2. Crops requirements of DLI from reference

Crops	DLI requirement (mol/m^2/day)	Reference source
Lettuce	12-17	(Song et al., 2018) (NASA, 2018)
Tomato	27-30	(Song et al., 2018) (NASA, 2018)
Onion	17	(NASA, 2018)
Rice	33	(NASA, 2018)
Cucumber	5.5-30	(Song et al., 2018)

3.4. Effects of elevating the floor height

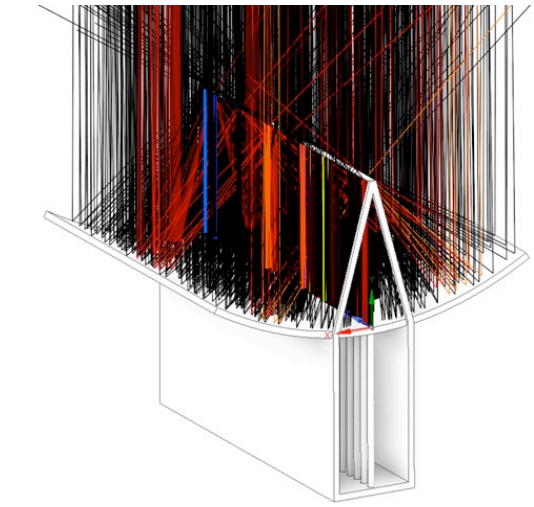


Baseline VG
VGH

Figure 6. Solar heat flux distribution at different time during the day (a) 8:00, (b) 10:00, (c) 12:00, (d) 14:00, (e) 16:00, (f) 18:00

Fig. 6 shows the solar heat flux distribution at different hours during the day. It helps to explain the improvement caused by elevating the floor height. When the solar zenith angle is not high at times such as 10:00 and 16:00 as shown in Figs. 6 (b) (e), heightened floor allows more sunlight to pass the glass wall to enter the building. Crops can, therefore, extend the sunlight duration and grow better.

3.5. Effect of the sunlight guiding system



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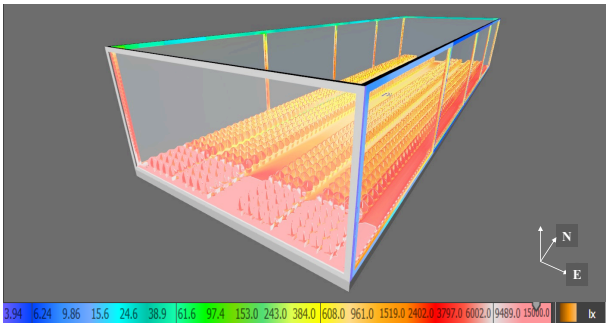

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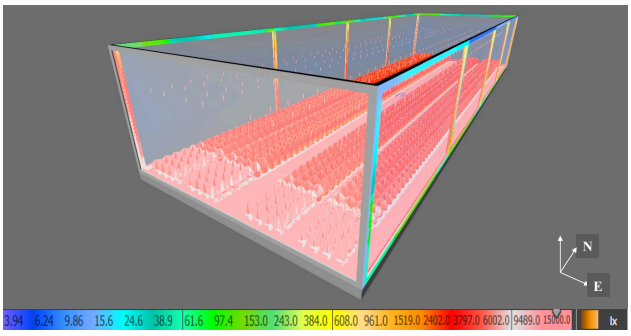

(c)

Figure 7. (a) Ray tracing of sunlight on the collector (b) Solar distribution on the vertical greenhouse without sunlight guiding system (c) Solar distribution on the vertical greenhouse with sunlight guiding system

Fig. 7 (a) visualized how the incident sunlight rays are collected and guided into the optical fibers. Figs. 7 (b) and (c) exhibit the lighting distribution of a vertical house with and without sunlight guiding system at 13:00 hour, it can be observed that with a dense array of diffuse lens, the sunlight collected by the system can be equality distributed on each of the crop surfaces and obviously elevated the illuminance on crop surfaces. In Fig. 7(c), a boundary can still be observed, dividing the area into the north part with an illuminance of about 3000 lux and the south part with an illuminance over 10000 lux, which means compared to the sunlight from window, the sunlight from the guiding system is less abundant. Three reasons might contribute to this difference. Firstly, at collecting stage, the sunlight guiding system collects the sunlight on only one floor on the roof but distributes the rays to multiple floors under the roof. In present study, one collector with the projection area of 1 m^2 can feeds three different 1 m^2 crops areas on three floors, therefore it is a ratio of 1/3 in area. It is reasonable to infer that when the crops floors increase, the energy guided from the roof will be further distributed and insufficient, this is probably the reason that the sunlight guiding system can only be a supplementary approach to the LEDs rather than working independently in many other solutions. In comparison, the window transmission naturally does not have this one-to-many problem. Considering that most building roof has the same area of each floors, it is necessary to increase the collecting area of this system for further optimization, for instance, to expand the roof of buildings or introduce façades to collect more solar energy (Tablada et al., 2017). Secondly, the different transmission efficiencies of sunlight guiding system and glass window are accountable for this variation. The sunlight guiding system is comprised of various components in collecting, transporting, and distributing stages, each of them has a loss rate. In the collecting stage, the

rays reflect twice with a loss of 5% at each time. In the transporting stage, the optical fiber bundles have a transmittance of 87% at a length of 3 m and 72% at a length of 8 m(Kandilli & Ulgen, 2009). In this study, even the lower design of vertical greenhouse has an overall height of 9 m, this means the optical fiber will lose or absorb probably 28% of the energy when transporting the sunlight. In the distributing stage, lens efficiency will further decrease the sunlight energy carried to the crop surface, the overall efficiency of this study is 0.6. In comparison, the glass window has a transmissivity of 0.8. How to expose the crops in a vertical farm to the maximum of sunlight in a less wastage way is a question to be answered for further optimization.

4. Conclusions

Both proposed solutions, increasing the floor height and implementing a sunlight guiding system, can introduce additional solar energy to elevate the DLI and therefore help crops grow better. Artificial-light-free solutions for growing lettuce can be achieved in these cases based on the simulations. Elevating the floor height is an economic choice because the spending on construction and maintaining of sunlight guiding system can be saved. Additionally, compared to three stage of sunlight collecting, transporting and distributing in the sunlight guiding system, the window transmission has only one stage, optimizing the window layout and improving the window transmissivity might be future solutions to introduce more sunlight and reduce the dependence on LEDs. The sunlight guiding system also has its own benefits. It can bring more flexibility and improved efficiency in using the land area, and the sunlight introduced can be more evenly organized. However, expanding the collecting area and improving the efficiency of the three-stage system are necessary to cut down the usage of artificial light. For instance, façade integrating approaches has been studied to provide more choices in sunlight collecting (Tablada, Kosorić, Huang, Lau, & Shabunko, 2020), fluorescence coatings are utilized to improve the sunlight distribution efficiency (Yalçın & Ertürk, 2020). This study is thus far limited to a simulation study and our further study will focus on these optimization directions with validations to confirm the artificial-light-free solutions for growing lettuce. The solar modelling of the sunlight collector could also be validated by experimental data monitored from a real or scaled greenhouse since this sunlight transmittance system could be very complicated where such a complex optical system will not be easily calculated using several simple algorithms.

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ELABORATION AND CHARACTERIZATION OF GLUTEN-FREE PIZZA AND COOKIE DOUGHS WITH BANANA WASTE FLOUR: ALTERNATIVES TO CELIACS

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ABSTRACT

The present study aims to evaluate the nutritional composition and acceptability of gluten-free pizza and cookie dough produced with banana (Musa ssp.) waste (bract)to improve their nutritional quality and reduce banana farming waste production. Bract flour was analysed for centesimal composition, water activity, minerals (Fe, Na and K) and color. For pizza dough elaboration, rice, sweet tapioca and bract flours were used. For cookieselaboration, cassava starch and bract flours were used to replace wheat flour. The centesimal composition and the phenolic compounds content were determined, and the sensory analyses were performed for both formulations. The results showed that bract flour presentsa high dietary fiber and minerals content and a lowcaloric value. Sensory analysis revealed the formulations acceptance and an even better evaluation for the cookies. The results suggested that banana bract flour may be included in gluten-free bakery products to improve their nutritional quality.

1.Introduction

Wheat products manufacture occurs predominantly due to thegluten proteins technological characteristics, whose hydration provides viscoelastic properties to the batter that incorporate gases during fermentation (Sciarini et al. 2010). On the other hand, some individuals are gluten intolerant (Fritz & Chen, 2017). In this context, the unique treatment for celiac disease (CD), a chronic immune-mediated enteropathy triggered by genetically predisposed individuals' exposure to dietary gluten, is agluten-free (GF) diet (Farage et al. 2018; Verma et al. 2017).

As the diet for celiacs is considered repetitive and restrictive, the food industry has invested in improving the gluten-free foods production to please consumers making the new products sensorially similar to those containing gluten. International studies show that 1% of the world population is celiac andaccording to the Brazilian Federation of Celiac Associations (Fenacelbra), around 2 million people in Brazil are affected by the disease (PRATESI and GANDOLFI, 2005). According to Farias (2009), the foods with the highest demand for transformation by the industry to be included in the celiac diet are bread, chocolate, pasta in general (spaghetti

and gnocchi), pizza and sausages (sausageand mortadella). Despite this, there is a lack of raw materials for their preparation.

When compared to conventional products, GF products generally present higher fat and sugar content, as well as lower protein and dietary fiber amounts (El Khoury et al. 2018; Fry et al. 2018). Due to their lower structural quality, these products are often less attractive in terms of appearance, taste, aroma and texture (El Khoury et al. 2018). Techniques, such as the use of dietary fibersare applied to improve their properties, affecting the dough and starch cohesion properties (Aprodu & Banu, 2015; Gao et al. 2018).

There is a trend among consumers for healthier foods and a demand for sustainable production by the agro-industrial waste recovery and reuse (Vialta et al. 2010). Some studies have revealed the relevant nutrition potential fromsuch waste including vitamins, minerals, fiber and bioactive compounds (Gawlik-Dziki, 2012).

According to Shah et al. (2005), banana cultivation (Musa spp.) generates approximately 220 tons of waste per hectare. The banana inflorescence is formed by male flowers with their respective bracts (Sheng et al. 2010). Since it has no market value in Brazil, it is discarded as banana waste (Sheng et al. 2011). The bract is dietary-fiber-rich and presentskey antioxidant properties due to the presence of bioactive compounds (Padam et al. 2012). According to Basumatary & Nath (2018), banana bract presents an excellent phenolic compound content playing a significant role in free radical inhibition processes (Fawole et al. 2012).

Thus, the gluten-free pizza and cookie doughs formulating addedwith banana bract flour may facilitate the consumption of a potentially functional ingredient; adding value to this waste, in addition to improving the nutritional quality of the products without significantly affecting the sensory attributes, hence the need to evaluate this effect and the acceptance level.

2.Materials and methods

2.1.Banana bract flour, pizza and cookie doughs elaboration

Banana (Musa spp.) bracts were obtained from the Federal University of Grande Dourados (UFGD – Campus II) organic vegetable garden, Dourados -MS, Brazil. Harvesting took place in the mornings, between August 2017 and March 2018.

The preparations were done at the UEMS Food Laboratory - Naviraí Campus. The flour was elaborated according to methodology described by Borges & Mendonça (2019). The bracts were removed from the inflorescence, washed in tap water, and then cut into standardized sizes with an approximate 1 cm thickness. Afterward, they were weighed and immersed in a 200 $mg.L^{-1}$ sodium hypochlorite solution for 15 minutes. After that, they were bleached and immersed in a 0.5% citric acid solution for 1 minute, placed in aluminum containers (30 cm x 40 cm) and oven-dried at 65 ºC for 18 hours. The milling was performed in a blade mill, obtaining 60-mesh size flour. The flour was stored in dark glass bottlespreviously sanitized and stored at 4 °C, until its use.

The other essential ingredients were purchased from local markets in the municipality of Naviraí-MS. The ingredients and their concentrations were defined by preliminary sensory tests, maintaining the formulations main characteristics, as per Tables 1 and 2.

For the pizza dough preparation, baker's yeast was hydrated with sugar and warm water (50 ml). The other dry ingredients were added and homogenized with eggs and the remaining water. Olive oil and a pre-prepared mixture were added tothe other ingredients until asmooth-structured homogeneous dough was obtained. The doughs were cut into equal parts, placed on 23 cm diameter aluminum forms and baked in a gas oven for 20 minutes at 180 °C.

For the cookies preparation, pre-tests were carried out to determine the banana bract flour and cassava starch concentrations. The methodology by Silvia et al. (1998) was used,

with adaptations. The dry ingredients were homogenized with eggs and butter until smoothing. The dough was divided into portions, laminated at 5 mm thickness and cut into a 30 mm diameter. Uniform circumference discs were weighed before baking in an automatic electric oven for 15 minutes at 200 °C.

Table 1. Pizza formulations with variation of banana bract flour and mixed gluten free flour.

Ingredients	Pizza P1	Pizza P2	Pizza P3
Sugar (g)	5.00	5.00	5.00
Water (ml)	142.00	142.00	142.00
Extra Virgin Olive Oil (ml)	15.00	15.00	15.00
Banana Bract Flour (g)	30.00	30.00	50.00
Mixed GF Flour (g)	450.00	246.00	450.00
Biological yeast (g)	10.00	10.00	10.00
Eggs (g)	30.00	30.00	30.00
Salt (g)	3.50	3.50	3.50

Mixed GF flour composed of rice flour (50%) and sweet tapioca flour (50%).

Table 2. Cookie formulations with variation of banana bract flour and cassava starch.

Ingredients	Cookie C1	Cookie C2	Cookie C3
Brown Sugar(g)	75.00	75.00	75.00
Refined Sugar (g)	100.00	100.00	100.00
Baking Soda(g)	5.00	5.00	5.00
Cocoa Powder 50% (g)	10.00	10.00	10.00
Banana Bract Flour (g)	10.00	10.00	15.00
Cassava Starch Flour (g)	250.00	200.00	225.00
Butter w/ salt (g)	75.00	75.00	75.00
Eggs(g)	15.00	15.00	15.00

2.2.Banana bract flour characterization

Banana bract flour centesimal composition analysis was performed in triplicate, as follows: moisture by the desiccation mass loss gravimetric method in an oven at 105 ºC; ash by incineration method in muffle at 550 °C (AOAC, 2005); crude protein by Micro-Kjeldahl method (AOAC, 2011), using 5.75 as a nitrogen conversion factor into plant proteins (Brazil, 2001) and lipids by Bligh & Dyer (1959) method. Total dietary fiber and soluble and insoluble fractions were performed by theenzymatic method (AOAC, 2000), and water activity performed by hygrometer (Labuza, 1975).

Carbohydrates were determined by the difference of the values found for moisture, ash, protein, lipid and total dietary fiber in 100g of product (AOAC, 1995). The total caloric value was calculated using lipids (9.03 kcal/g), protein (4.27 kcal/g) and carbohydrates (3.82kcal/g)as parameters (Merrill & Watt, 1973).

Samples were digested in a microwave oven using nitric acid, and the macro minerals (Fe, Na, K) were determined by atomic absorption spectrophotometry and the results expressed as mg 100 g^{-1} sample and standard deviation (Aquino et al. (2014, Xu et al. 1988). Color analysis was evaluated by the CIELAB system in terms of L, a* and b* colorimetric coordinates (Gonnet, 1998). The average results were expressed as percentage ± standard deviation.

2.3.Pizza and cookie doughs characterization

Centesimal composition analyses were performed on the three formulations of both products using the methods previously described for moisture, ash, lipids, proteins and carbohydrates. Total phenolic compounds determination was performed by Follin-Ciocauteau method with modifications proposed by Asami et al. (2003), in triplicate. Extracts were prepared with a 70% ethanol (v/v) extraction solution. 200 µl aliquots of extract, 60 µl Follin-Ciocauteau reagent and 2 mL sodium carbonate solution (7% w/v) were taken. The readings were taken at a 720 nm wavelength. The results were expressed as mg gallic acid equivalent (GAE) / g dry matter.

2.4.Pizza and cookiedoughssensory analysis

The sensory analysis was performed with the UEMS Human Ethics Committee approval under opinion # 1,858,034/2016. The formulation tests were performed at the UEMS Food Laboratory / Naviraí Campus, with 100 randomly chosenuntrained (19-59 years old) (male and female) adult tasters.

The samples were presented in coded disposable plastic plates on trays with a glass of water and a napkin. The pizzas were prepared with industrialized tomato sauce, mozzarella cheese and oregano. For the evaluation, the structured nine-point hedonic scale was used (Dutcoski, 2013).

2.5. Statistical Analysis

The results were expressed as average ± standard deviation (SD) and the triplicate measurements of pizza and cookie doughs were submitted separately to the analysis of variance (ANOVA) and Tukey test at a 5% significance level ($p \leq 0.05$) using the STATISTICA 7.0 software (Statsoft, 2004).

3.Resultsanddiscussions

3.1.Banana bract flour characterization

The physicochemical and color analysis results are shown in Table 3. The result for moisture content is in accordance with the Brazilian Law (Brazil, 2005), which establishes a 15g-100g maximumlimit forproduct. The water activity results (0.454 ± 0.04) ensure greater product stability (Cecchi, 2003).

The ash content was higher than that obtained for green banana flour (2.27%) (Medeiros et al., 2010). Both ash and protein contents (9.98 ± 0.13%) were lower than those for dehydrated banana inflorescence (14.4 ± 0.64% and 14.5 ± 0.40%, respectively). On the other hand, lipids contentwas higher (6.27 ± 0.24%) when compared with the same study (4.04 ± 0.07%) (Fingolo et al., 2012).

The results for total dietary fiber (56.14 ± 2.41%) and insoluble fiber (50.32 ± 1.35%) were higher than those found by Scorsatto et al. (2017) in eggplant flour, 39.2% and 28.83% respectively. Bhaskar et al. (2012) obtained highertotal fiber, soluble and insoluble fraction results (65.6 ± 1.3; 7.3 ± 0.2% and 58.3 ± 1.0%, respectively) for dehydrated banana inflorescence.

Banana bract flour is classified as high fiber content (Brazil, 2012)and to achieve this classification it must contain 6g of fiber per 100g.Therefore, it is a potential product to achieve the dietary recommendation since the fiber intake by the population is below the recommended one (Grooms et al. 2013). Both the amount of carbohydrates and the caloric valuewere lowerthan those forwatermelon rind flour (65.95 ± 0.04% and 317.88 ± 0.04 Kcal, respectively) (Cristo et al. al., 2018).

Borges et al. (2009), analyzed green banana flouras having higher potassium content (1,180 mg 100 g – 1) and lower iron content (17.80 mg 100 g – 1) than the bract flour. For color,the L* value shows the luminosity and the higher this value, the lighter the sample thus, the flour tended to darken. Fora*, a positive value was obtained, which indicates an even greater darkening. According to Takatsui (2011), when a* and b* coordinates approach zero, the colors are neutral.

Table 3. Characterization and color analysis of banana bract flour

Composition	%±DP
Moisture	3.86±0.11
Water Activity (Aw)	0.454±0.04
Ash	10.46±0.02
Proteins	9.98±0.13
Lipids	6.27±0.24
Total Fiber	56.14±2.41
Soluble fiber	5.82±2.28
Insoluble fiber	50.32±1.35
Carbohydrates	12.42±0.53
VC (kcal/100g)	150.40±10.63
Fe	65.22 ± 0.95
Na	294.46 ± 3.97
K	322.04 ± 2.53
L*	33.66±0.11
a*	4.28±0.06
b*	1.98±0.11

Data were presented as mean ± standard deviation (SD). VC: Caloric value.
The theoretical calculation was performed for carbohydrates and caloric value (Kcal/100g).

3.2. Pizza and cookie doughs characterization

According to the Brazilian Institute of Geography and Statistics (IBGE, 2011), wheat-based pizza dough formulations contain an approximate 39.79%moisture content. Moisture contents are related to product crispness, an important sensory attribute that, is reduced at high moisture levels (Guimarães & Silva, 2009). When evaluating the results for doughs, all formulations were below this content level.

For the cookie formulations, a significant statistical difference in moisture content between C3 and the other formulations could be observed due to the higher bract flourcontent. According to the Brazilian Health Regulatory Agency (ANVISA), the maximum moisture content for cookies must be 15% w / w (BRAZIL, 2005). Thus, the cookies are in accordance with Brazilian law.

For pizza dough`s ash, protein and lipid content, the highest averages obtained for the P2 formulation were inversely proportional to the mixed flour concentration. One of the reasons was the other ingredients concentration in P2 formulation.

The three pizza dough formulations had a lower protein content compared to wheat-based pizza dough, with an 8.22% averageprotein content (IBGE, 2011). According to the Brazilian Food Composition Table (TACO, 2011), wheat flour has a 9.8g / 100g protein average content and, as already described, banana bract flour has an 9.98g / 100g average protein content (Table 4), making the amount of the latter necessary for the dough preparation lower than that of wheat flour. Rice flour and sweet flour were used as replacements for wheat flour, both presenting reduced 8.4g / 100g and 0.4g / 100g protein valueson a dry basis, respectively (TACO, 2011). These values may justify the results found for protein levels on formulations when compared to a wheat-based pizza formulation.

Pizza (P2) formulation obtained a lipid average similar to the findings from Monteiro (2013) on pizzas made with rice, yam and quinoa flours with 4.74% to 6.17% averages. However, all samples presented lower lipid contents than those reported by IBGE (2011) for wheat-based pizza (11.35%).

Carbohydrate contents showed a significant statistical difference between the three pizza samples. El-Beltagi et al. (2017) evaluated pizza doughs with partial wheat flour replacement by three different chickpea flour or carp fish powder concentrations, which present higher protein and carbohydrate contents. However, a lower ash contents was observed, mainly due to the high ash contents in banana bract flour.

For cookie formulations, C2 showed a lower ash value (1.55 ± 0.08), since it has a lower banana bract flouramount. C1 and C3 formulations achieved similar results to the study by Freitas et al. (2014), who evaluated gluten-free cookies added with pumpkin seed flour and baru seed flour obtaining 1.93g / 100g and 1.76g / 100g, respectively.

There was no statistical difference forthe cookies' protein, lipid and carbohydrate contents($P \geq 0.05$). Mariani et al. (2015) prepared gluten-free cookies from rice bran, rice flour and soy flour and obtained higher protein values (11.16 - 14.22g / 100g) and lower lipids (20.29 - 22.13g / 100g) and carbohydrates (37.27 - 48.20g / 100g) values. These results may be due to the energetic and non-protein characteristics of the ingredients used in the formulations.

From the results, the pizza dough with the lowest flour content showed the highest phenolic compoundscontent, mainly due to the highest concentration of products used, however, no statistical differences were seen in the pizza samples. For cookies, C3 formulation with the highest banana bract flour content presented the highest phenolic compoundscontent, which was statistically different ($P \leq 0.05$) from the other samples, confirming the considerable phenolic compounds contentin banana bract flour.

One of these products benefits was the good nutritional composition, obtaining a higher fiber and phenolic compounds content and a lower carbohydrate content.

Several studies have shown that the consumption of antioxidant substances in a daily dietproduces an effective protective action against the natural oxidative processes in the body, preventing diseases and delaying the body`s aging process. Also, adequate fiber intakeappears to reduce the risk of chronic diseases development such as coronary artery disease, stroke, hypertension, diabetes mellitus and some gastrointestinal disorders, among other health benefits.

Table 4. Characterization of pizza dough and cookie biscuits.

Sample	Moisture (g/100g)	Ash (g/100g)	Proteins (g/100g)	Total Lipids (g/100g)	Carbohydrates (g/100g)	Phenolics Compounds (mg EAG/100g
P1	28.63±0.31^{b}	2.49±0.08^{b}	0.62±0.01^{b}	3.73±0.09^{b}	64.53±0.23^{a}	72.08±0.20^{a}
P2	28.63±0.31^{b}	3.80±0.06^{a}	0.75±0.02^{a}	5.71±0.12^{a}	61.24±0.50^{b}	98.58±0.22^{a}
P3	28.49±0.31^{ab}	2.33±0.02^{c}	0.61±0.03^{b}	3.44±0.01^{c}	58.51±1.00^{c}	87.36±0.11^{a}
C1	7.31±0.15^{a}	1.70±0.05^{a}	0.43±0.23^{a}	34.16±15.31^{a}	56.40±15.48^{a}	195.21±0.41^{b}
C2	7.64±1.33^{a}	1.55±0.08^{b}	1.02±0.60^{a}	31.90±8.81^{a}	57.88±8.53^{a}	307.29±0.65^{b}
C3	2.78±1.51^{b}	1.80±0.02^{a}	0.89±0.15^{a}	38.04±9.80^{a}	56.50±8.91^{a}	471.21±0.20^{a}

Date were present as mean ± standard deviation (SD). The pizza dough formulations were analyzed separately from the cookie formulations. The averages in the same column. followed by different letters. differ statistically from each other by Tukey test at 5% probability.

[1]P1 Pizza Dough: 450g mixed GF flour and 30g bract flour; P2: 250g of mixed GF flour and 30g of bract flour; P3: 450g of mixed GF flour and 50g of bract flour.

[2] Cookie C1: 250g of cassava starch flour and 10g of bract flour; C2 200g of cassava starch flour and 10g of bract flour; C3 225g of cassava starch flour and 15g of bract flour.

Table 5. Averages of sensory acceptability and purchase intention of the pizza doughs of the cookie biscuits added banana flour

Sample		Color	Aroma	Texture	Flavour	Global Impression
Pizza Dough	P1	6.21±1.85[a]	6.88±1.68[a]	6.42±1.79[a]	6.68±1.69[a]	6.56±1.72[a]
	P2	5.76±1.72[a]	6.47±1.60[ab]	5.99±1.62[ab]	6.46±1.68[ab]	6.25±1.69[ab]
	P3	5.83±1.71[a]	6.00±1.84[b]	5.73±1.65[b]	5.96±1.73[b]	5.72±1.66[b]
Cookie	C1	6.88±1.63[a]	7.04±1.52[ab]	6.93±1.55[b]	6.69±1.69[b]	6.80±1.54[b]
	C2	7.09±1.58[a]	6.90±1.62[b]	7.06±1.36[ab]	6.84±1.77[b]	7.12±1.57[ab]
	C3	6.98±1.58[a]	7.49±1.33[a]	7.53±1.34[a]	7.78±1.32[a]	7.38±1.26[a]

Data were presented as mean ± standard deviation (SD). The pizza dough formulations were analyzed separately from the cookie formulations. The averages in the same column. followed by different letters. Differ statistically from each other by the Tukey test at 5% probability.

1 P1 Pizza Pasta: 450g mixed flour and 30g bract flour; P2: 250g of mixed flour and 30g of bract flour; P3: 450g of mixed flour and 50g of bract flour.

2 Cookie C1: 250g of manioc starch flour and 10g of bract flour; C2 200g of manioc starch flour and 10g of bract flour; C3 225g of manioc starch flour and 15g of bract flour.

3.3. Pizza and cookiedoughs sensory analysis

Sensory analysis is a critical factor for new products acceptance or rejection(Sivakumar et al. 2010). Changes in the modified foods sensory characteristics are expected, such as in the dough structure with the gluten removal (Padalino et al. 2016). The sensory analysis results are shown in Table 5.

Taste and texture are the determining factors in choosing pizza dough (Bingham et al. 2011). Gluten-free pizza dough formulations added with banana bract flour received between 5.57 (“neither liked nor disliked”) and 6.88 (“slightly liked”)on average for all hedonic attributes. Such results were similar to those obtained by Russo et al. (2012), who developed pizza doughs added with whole wheat flour and different flaxseed flour concentrations, with aroma, taste, texture and color attributesclassified as “slightly liked”. The exceptions occurred for the “flavor” attribute, where the formulation added with 5% flaxseed flour was classified as “moderately liked” and for the “texture” attribute, where the formulation added with 7.5% flaxseed flour was classified as “neither liked nor I disliked it”.

No significant statistical difference was observed in the “color” attribute among the pizza dough samples. Pizza dough P3 formulation added with 450g mixed flour and 50g banana bract flour was the least accepted among the tasters, receiving the lowest averages for the “aroma, texture, taste and overall impression” attributes. A significant difference ($P \leq 0.05$) was observed among the

P1 samples elaborated with the lowest banana bract flourconcentration, were the most accepted.

According to Lambert et al. (2006), the main cookie quality attributes are appearance, taste and texture. All cookieformulations were well-accepted by the tasters, with hedonic averages ranging from 6.69 ("liked slightly") to 7.78 ("liked moderately"). C3 formulation with the highest banana bract flour content was the highest ranked, except for the “color” attribute that showed no statistical difference among the formulations.

The cookies producedhad higher averages than those obtained by Queiroz et al. (2017) who developed gluten-free cookies with potato starch and coconut flour, with averages ranging from 6.0 (“like”) to 7.0 (“like”) for sensory attributes. The study by Giovanella et al. (2013), who developed gluten-free cookies added with quinoa flour and potato starch, obtained similar results in terms of taste, texture and overall impression.

4. Conclusions

In this study, further discussions were sought on the production of new, affordable, and easily accessible gluten-free products. The banana bract flour (Musa ssp.) analysisrevealed its considerable nutritional value and may be considered a significant nutritional supplement based on the dietary fiber content.

This study showed the feasibility of using a little known flour such as banana bract flour, as a wheat flour replacementon gluten-free foods production. It was seen that both pizza and cookie doughsadded with bract flour had a good response from tasters, with an overall acceptance above 65%, showing that it staste would not be a problem for products elaboration. It has shownitspotential as a raw material for new products, soit further testsshould be performed for it to enter the market in the future.

The gluten-free pizza and cookie doughs development added with banana bract flour has allowed obtaining more nutritious products with higher fiber and phenolic compounds and lower carbohydrate content. Sensory analysis showed that the formulations were accepted, with the best evaluation for cookie samples.

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OPTIMIZATION OF HOT-AIR AND MICROWAVE DRYING PROCESS PARAMETERS FOR EVALUATION OF PHENOLICS AND ANTIOXIDANT ACTIVITY IN SLICED WHITE BUTTON MUSHROOM (*Agaricus bisporus*) USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

This study was conducted to investigate hot-air and microwave dryings on phenolics and antioxidant activity of dried white button mushroom slices (*Agaricus bisporus*) using the response surface methodology. It was also aimed to determine optimized drying conditions. In hot-air drying, total phenolics content reduced with corresponding increase in drying temperature and slice thickness, whereas in microwave drying, the total phenolics content increased with microwave power. Although the effect of drying temperature on antioxidant activity is not significant, there is a decrease in antioxidant activity as the slice thickness of the samples increases. Microwave power had also a significant linear effect on antioxidant activity of dried samples ($p<0.01$). Both increases and decreases in individual phenolic compounds were detected with the change of drying conditions and sample thickness. A quadratic model was well fitted to all responses. As a result of numerical optimization, optimum conditions for hot-air drier and microwave oven were suggested as 50 °C and 600 Watt having mushroom slices of 2 mm thickness, respectively. Our results show that the quality of dried mushroom depends on the drying method and conditions. Also, microwave drying is suitable method for drying of mushroom slices within a shorter time compared with hot-air drying.

1.Introduction

Mushrooms have been widely consumed by humans for centuries in many countries for their characteristic delicate flavor and taste (Giri and Prasad, 2007; Celen *et al*., 2010). Besides, they are rich in carbohydrates, proteins, fibers, vitamins, minerals, unsaturated fatty acids. Mushrooms are recognized as an important source of biologically active compounds. So, they have several beneficial activities like antibacterial, antifungal, antioxidant, anti-tumor, antiallergic, antiatherogenic and anti-inflammatory (Manzi *et al*., 2001; Giri and Prasad, 2007; Vaz *et al*., 2010; Argyropoulos *et al*., 2011; Kalac, 2013). They are also an excellent source of biologically active compounds such as polyphenolics classified as free radical inhibitors (Choi *et al*., 2006; Heleno *et al*., 2010; Yahia *et al*., 2017). The main phenolics are known as gallic acid, pyrogallol, protocatechuic acid, naringin and myricetin in edible mushrooms such as *Agaricus bisporus, Pleurotus ostreatus, Flammulina velutipes, Pleurotus eryngii,* and *Lentinus edodes* (Kim *et al*., 2008).

It is reported that there are more than 38000 mushroom varieties in nature, but only 22 of them are produced and one of the most popular varieties is known as white button mushroom called *Agaricus bisporus*. This variety

contributes about 40 % of the total World production (Manzi *et al*., 2001; Walde *et al*., 2006; Giri and Prasad, 2007). According to Food and Agricultural Organization (FAO), world production of mushrooms exceeds ten million tons in 2017. The production of cultivated mushrooms in Turkey is around 40.000 tons in the year of 2017 (FAOSTAT, 2019).

Since mushrooms contain high level of moisture (ranging from 85.2 to 94.7 %) and activity of enzymes, they deteriorate rapidly after harvest (Giri and Prasad, 2007; Manzi *et al*., 1999; Manzi *et al*., 2004). For this reason, mushrooms are suggested to be consumed or preserved immediately (Giri and Prasad, 2007). Drying is one of the important preservation methods by reducing the moisture content to a level for safe storage (Giri and Prasad, 2007; Argyropoulos *et al*., 2011). Conventional hot air drying is a simple and practical method, but it requires more energy and long-time (Argyropoulos *et al*., 2011). Since microwave drying has some advantages such as increased drying rate, maintenance of nutritional value, color and original flavor, it has been proposed as a rapid and efficient drying alternative to conventional hot air drying (Maskan, 2001; Giri and Prasad, 2007; Askari *et al*., 2009; Valadez-Carmona *et al*., 2017).

To improve quality and yield of dried product, optimization study could be used in industrial process. Response surface methodology (RSM) is a statistical procedure widely used for effect of process parameters and determination of process optimization (Erbay and Icier, 2009; Šumić *et al*., 2017). Current study was carried out with the aim to use RSM for the optimization of drying parameters including both hot-air drying temperature/slice thickness and microwave power/slice thickness on white button mushroom (*Agaricus bisporus*).

In this research, it is aimed that effects of hot-air and microwave dryings on bioactive properties such as total phenolic content, antioxidant activity and individual phenolic compounds of mushrooms with 2-6 mm thickness. Different hot-air temperatures (50º, 60º and 70 ºC) and microwave powers (90, 345 and 600 W) were performed to compare bioactive properties. There are researchers about changes in total phenolics (Bhattacharya *et al*., 2014; Radzki *et al*., 2014; Šumić *et al*., 2017) but any research was found related with the variation of individual phenolics during drying of mushrooms. The objective of this study is also to determine optimum process conditions for drying of mushroom slices by using response surface methodology.

2. Material and methods

2.1. Material

Freshly harvested white button mushrooms *Agaricus bisporus* were provided from a private company in Bolu, Turkey. The samples immediately were washed and stored at +4º C until drying process. Mushroom slices were obtained by cutting mushrooms vertically using a hand operated food slicer. The thickness of mushroom slices had been used as 2, 4 and 6 mm. They were immediately weighed and dried at hot-air drier and microwave oven. The initial moisture content of the fresh mushrooms was 91.44 %. Drying experiments were carried out with two replicates. Analyses were performed in duplicate.

2.2. Drying

i) Hot-air drying

The sliced mushrooms were dried at hot-air temperature of 50º, 60º and 70 ºC with air flow rate of 1.5 m/s using a hot-air drier designed and fabricated in Eksis Industrial Drying Systems, Isparta, Turkey. Slices were spread in a single layer on the tray which was placed into the drier. The samples were dried till the moisture content was reduced to 8-10 %. Drying times were 5, 3.5 and 2.5 hours at applied temperatures, respectively.

ii) Microwave drying

Microwave drying of the sliced mushrooms was performed with microwave oven (Bosch-HMT84G421, P.R.C) with maximum output of 900 W at 2450 MHz. Sliced samples were dried at different microwave powers (90, 345 and 600W). Slices were placed in a single layer on the rotating table. The samples were dried till the

moisture content was reduced to 8-10 %. Slices (samples) were dried at applied microwave powers for 90, 35 and 15 minutes, respectively.

2.3. Modelling drying data (Response Surface Methodology)

The results were analyzed by response surface methodology (RSM) using the software Statease Inc. 9.1 (Minneapolis, ABD). The experimental design employed was a Central Composite Design for two independent variables each at three levels. The drying variables of temperature (ºC) and slice thickness (mm) for hot-air drying; microwave power (W) and slice thickness (mm) for microwave drying were studied using RSM. In this study, 12 experiments were created for each drying type according to a two-level factorial design with center and star points (Table 1).

Table 1. Levels of hot-air and microwave drying process variables

		Factor Levels		
Independent variable for hot-air drying (Units)	**Symbol**	**-1**	**0**	**+1**
Temperature (ºC)	β_1	50	60	70
Slice thickness (mm)	β_2	2	4	6
Independent variable for microwave drying (Units)	**Symbol**	**-1**	**0**	**+1**
Microwave power (Watt)	β1	90	345	600
Slice thickness (mm)	β2	2	4	6

Quadratic model proposed for response is shown in Equation 1:

$$Y=\beta_0+\sum_{i=1}^{n}\beta_i X_i+\sum_{i=1}^{n}\beta_{ii} X_I^2+\sum_{i<J}^{n}\beta_{ij} X_iX_j \quad \text{... Eq. (1)}$$

β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the cross product coefficient and x_i and x_j are independent variables.

Desired goals (maximization of total phenolics content and antioxidant activity) were used to perform optimization of variables and the response.

2.4. Methods

Moisture analysis

Moisture analysis was performed at 105 ºC (AOAC, 1990). Samples were analyzed in duplicate and average moisture content was recorded.

Chemicals

ABTS (3-ethylbenzothiazoline-6-sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2 carboxylic acid), pyrogallol, t-cinnamic acid, caffeic acid, p-coumaric acid and (+)-catechin were obtained from Sigma Aldrich (Sigma-Aldrich, Inc., Saint Louis, USA). Folin–Ciocalteu reagent, sodium carbonate, ethanol and the other HPLC grade solvents were purchased from Merck (Darmstadt, Germany).

Extraction

Extraction of samples was performed with methanol using the method of Thaipong *et al*. (2006), with some modifications. 3 grams of dried and milled mushrooms were mixed with 25 mL methanol and homogenized with ultra turrax (IKA, Germany). This solution was kept at 4 ºC for 12 h and then centrifuged at 9000 rpm for 25 min with a centrifuge (Nuve, Ankara, Turkey). The supernatant was transferred to a 25 mL volumetric flask, brought to volume with methanol and stored at – 20 ºC until analysis.

Analysis of total phenolics content

The amount of total phenolics was detected by using a modified Folin-Ciocalteu reagent colorimetric method (Shahidi *et al*., 2001). The absorbance of all samples was read at 720 nm using a UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan). The content of total phenolics in dried mushroom samples were determined using a standard curve prepared with (+)-catechin (10-150 mg/L) and expressed as (+)-catechin equivalents (mg/g dry matter).

Analysis of antioxidant activity (TEAC)

Trolox equivalent antioxidant capacity (TEAC) method was used to determine antioxidant activity of samples (Re *et al*., 1999).

15 µL of dried mushroom extract was added to 1 mL of diluted ABTS*+ solution; the mixture was mixed and the absorbance was recorded. The absence of ABTS*+ was determined by measuring the decrease of absorbance at 734 nm for 6 min. Results were analyzed by reference to the Trolox and expressed as micromolar Trolox equivalent antioxidant capacity (µM Trolox/100g dry matter).

Determination of individual phenolic compounds with HPLC

Phenolic compounds were separated on a Perkin Elmer C 18 column (5 µm, 250 x 4.6 mm i.d.) according to method described by Biswas *et al*. (2013). The column was operated at a temperature of 25 ºC. A Shimadzu HPLC pump and PDA detector (Prominence LC-20A, Shimadzu, Kyoto, Japan) were used. Phenolic compounds were detected at 280 nm (pyrogallol, (+)-catechin and t-cinnamic acid) and 320 nm (caffeic acid and p-coumaric acid) with a flow rate of 0.8 mL/min. Separations were carried out by varying the proportion of 2.5% (v/v) acetic acid in water (mobile phase A) and 70% methanol in water (mobile phase B). The solvent gradient elution program was as follows: 10% to 26% B (v/v) in 10 min, to 70% B at 20 min and to 90% B at 25 to 31 min, finally to 10% B at 39 to 45 min. The injection volume for all samples and standards was 10 µL. Identification of phenolics was carried out by comparison of the HPLC retention times with corresponding standards of pyrogallol (7.8 min), (+)-catechin (17.9 min), caffeic acid (21.7 min), p-coumaric acid (24.8 min) and t-cinnamic acid (30.4 min) (Figure 1). The concentrations were calculated, using the standard calibration curves of pyrogallol (30-150 mg/L), (+)-catechin (5-25 mg/L), caffeic acid (1-10 mg/L), p-coumaric acid (0.5-2.5 mg/L) and t-cinnamic acid (1-10 mg/L). The sample extracts were filtered through a 0.45 µm pore size syringe filter before HPLC analysis.

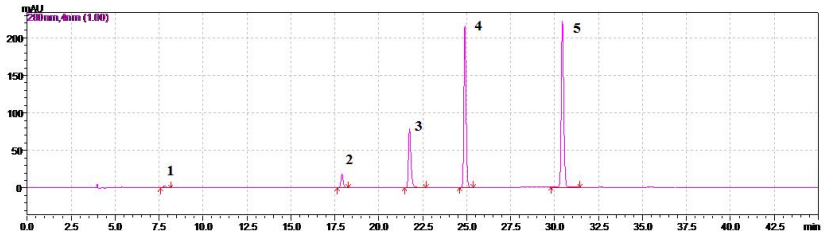

Figure 1. HPLC chromatogram of phenolic standards. Identified compounds 1, Pyrogallol; 2, (+)-catechin; 3, caffeic acid; 4, *p*-coumaric acid and 5, *t*-cinnamic acid

3.Results and discussions

3.1. Total phenolics content

Effect of hot-air drying conditions

Total phenolics content of fresh white button mushroom was 9.28 mg/g d.m. and this parameter ranged from 1.06 mg/g d.m. to 2.14 mg/g d.m. in samples after dried at hot-air (Table 2). The change in total phenolics was best described by the quadratic model ($p<0.01$; R^2: 0.967). Drying temperature and slice thickness had negative significant linear effects on total phenolics content of dried white button mushroom ($p<0.01$) (Table 3). Total phenolics content reduced with corresponding increase in drying temperature and slice thickness. Total phenolics content decreased to 1.61 mg/g d.m. from 2.14 mg/g d.m. when the slice thickness increased to 6 mm from 2 mm at 50 ºC (Figure 2). This data exhibits thinner mushroom samples showed higher total phenolics than thicker samples. Because, thicker samples dried longer than the thinner samples, so long drying time caused a higher loss of total phenolics. Table 2 also shows that the rise in temperature from 50° to 70 °C led to decline of total phenolics content of dried samples with the same slice thickness from 2.03 mg/g d.m. to 1.12 mg/g d.m.

Table 2. Central composite design with the observed responses for total phenolic content (TPC), antioxidant activity (AA) and content of individual phenolic compounds of hot-air and microwave dried white button mushrooms

	Run	Temp. (°C) / Mic. Power (W)	Slice thick. (mm)	TPC (mg/g d.m.)	AA (µmol Trolox/100 g d.m.)	Pyrogallol (mg/kg d.m.)	(+)-Catechin (mg/kg d.m.)	*t*-Cinnamic acid (mg/kg d.m.)	Caffeic acid (mg/kg d.m.)	*p*-Coumaric acid (mg/kg d.m.)
Hot-air	1	70 (+1)	2 (-1)	1.18	13.65	702.89	56.19	4.96	0.82	4.52
	2	70 (+1)	4 (0)	1.12	9.3	870.05	52.93	6.51	0.61	2.65
	3	60 (0)	4 (0)	1.4	9.3	647.73	79.58	12.82	0.51	3.21
	4	70 (+1)	6 (+1)	1.06	10.81	929.09	46.29	6.61	0.54	2.09
	5	50 (-1)	6 (+1)	1.61	11.76	843.48	57.26	7.71	0.33	4.29
	6	50 (-1)	2 (-1)	2.14	15.74	333.28	81.94	9.19	0.85	4.21
	7	60 (0)	2 (-1)	1.52	11.25	380.66	67.5	8.08	0.77	2.88
	8	60 (0)	4 (0)	1.26	9.87	635.04	79.93	12.15	0.64	2.74
	9	60 (0)	6 (+1)	1.3	11.08	963.13	67.53	12.29	0.68	3.1
	10	60 (0)	4 (0)	1.35	10.16	832.63	85.12	10.04	0.59	2.51
	11	60 (0)	4 (0)	1.28	10.39	714.3	88.05	12.55	0.43	2.86
	12	50 (-1)	4 (0)	2.03	10	534.47	65.15	7.29	0.41	3.2
Microwave	1	345 (0)	4 (0)	1.11	7.94	345.21	26.41	0.29	0.19	1.01
	2	600 (+1)	6 (+1)	1.96	12.71	361.44	21.34	0.39	0.16	0.67
	3	600 (+1)	4 (0)	2.01	10.71	375.65	19.27	0.42	0.15	0.79
	4	345 (0)	2 (-1)	1.39	12.18	312.11	39.17	0.41	0.24	2.02
	5	345 (0)	4 (0)	1.47	12.29	403.14	26.92	0.4	0.14	0.97
	6	600 (+1)	2 (-1)	2.83	12.75	267.97	38.41	0.5	0.24	1.33
	7	90 (-1)	2 (-1)	1.08	9.2	1359.3	35.3	5.15	0.13	1.19
	8	345 (0)	4 (0)	1.17	10.4	352.68	24.63	0.38	0.13	1.07
	9	90 (-1)	6 (+1)	1.21	8.41	992.43	31.21	10.74	0.14	0.51
	10	345 (0)	4 (0)	1.2	11.33	304.05	23.84	0.39	0.14	0.83
	11	345 (0)	6 (+1)	2.36	13.61	379.77	32.68	0.43	0.15	0.69
	12	90 (-1)	4 (0)	0.87	4.96	1040.24	32.52	5.58	0.09	0.81

Table 3. Analysis of variance of regression coefficients of the fitted quadratic model equations for the variations of the total phenolics content (TPC), antioxidant activity (AA) and content of individual phenolic compounds of hot-air and microwave dried white button mushrooms

	Coefficient	TPC	AA	Pyrogallol	(+)-Catechin	*t*-Cinnamic acid	Caffeic acid	*p*-Coumaric acid
Hot-air	β_0 (intercept)	1.35	9.58	701.52	80.33	11.57	0.55	2.71
	Linear							
	β_1 (temp.)	-0.4 **	-0.6233	+131.80 **	-8.16 *	-1.02	+0.063	-0.41
	β_2 (slice thick.)	-0.14 **	-1.165 *	+219.81 **	-5.76	+0.73	-0.15 *	-0.36
	Quadratic							
	β_{11}	+0.17 *	+0.7725	+12.56	-15.61 *	-4.03 **	-0.061	+0.44
	β_{22}	+0.003	+2.2875 *	-17.80	-7.14	-0.74	+0.15	+0.51
	Cross product							
	β_{12}	+0.1 *	+0.285	-71	+3.7	+0.78	+0.06	-0.63 *
	R^2	0.967	0.812	0.903	0.831	0.829	0.786	0.801
	Model (p>F value)	<0.01	<0.05	<0.01	<0.05	<0.05	<0.05	<0.05
Microwave	β_0 (intercept)	1.29	10.402	344.16	26.25	0.17	0.15	1.01
	Linear							
	β_1 (mic. power)	+0.606 **	+2.267 **	-397.82 **	-3.34 *	-3.37 **	+0.032 *	+0.047
	β_2 (slice thick.)	+0.038	+0.1	-34.29	-4.61 *	+0.91	-0.027 *	-0.45 **
	Quadratic							
	β_{11}	+0.05	-2.3913 *	+378.01 **	-1.96	+3.21 **	-0.029	-0.30 *
	β_{22}	+0.485	+2.6688 *	+16.01	+8.07 **	+0.63	+0.046 *	+0.26
	Cross product							
	β_{12}	-0.25	+0.1875	+115.09 *	-3.24	-1.41 *	-0.022	+0.005
	R^2	0.813	0.815	0.978	0.873	0.958	0.852	0.868
	Model (p>F value)	<0.05	<0.05	<0.01	<0.05	<0.01	<0.05	<0.05

** Significant at p<0.01; * Significant at p<0.05

The loss of total phenolics in this present study is in agreement with results obtained by other researchers who studied dried *Centella asiatica* (Niamnuy *et al*., 2013), sour cherry (Wojdylo *et al*., 2014) and chanterelle mushroom (Šumić et al., 2017). Wojdylo *et al*. (2014) indicated that phenolic compounds can be damaged by increasing temperature and a long exposing to high temperatures. In this study, combined effect of drying temperature and slice thickness also significantly reduced total phenolics of dried white button mushroom (p<0.05).

Effect of microwave drying conditions

Total phenolics content of white button mushroom dried at microwave showed significant decreases (0.87-2.83 mg/g d.m.) compared with fresh sample (9.28 mg/g d.m.). The change in total phenolics content of white button mushroom dried with microwave showed a good fit to a quadratic model (p<0.05; R^2: 0.813). Table 2 presents total phenolics of the samples dried at twelve drying conditions. Total phenolic was highest (2.83 mg/g d.m.) in the sample dried at a power of 600 W, with 2 mm thickness. The lowest total phenolics was found in the sample dried at 90 W, with 4 mm thickness (0.87 mg/g d.m.). Microwave power had a significant positive linear effect on total phenolics content of dried white button mushroom (p<0.01) (Table 3). On the other hand, the statistical analysis indicated that the

effect of slice thickness and interaction among parameters were insignificant ($p>0.05$).

Total phenolics significantly increased with the increase in the microwave power (Figure 2). Total phenolics increased to 2.83 mg/g d.m. from 1.08 mg/g d.m. when the microwave power increased to 600 W from 90 W at the same thickness value (Figure 2). This result is consistent with the other studies related with Thai red curry powder (Inchuen *et al*., 2010) and dried sage plants (Sellami *et al*., 2013). Another research about extraction of phenolic compounds in dried mushrooms by using oven and microwave, total phenolics increased with microwave power (Celebi Sezer *et al*., 2017). Al Juhaimi *et al*. (2018) also investigated the effect of microwave (360 W, 540 W and 720 W) oven roasting on oil yields, phenolic compounds, antioxidant activity and fatty acid composition of some apricot kernels. According to that study, total phenolics and antioxidant activities of kernels increased at 360 and 540 W, while those parameters decreased at 720 W.

In this research, the increment in total phenolics according to the intense of microwave can be explained by the effect of microwave treatment in releasing phenolic compounds. It is reported that plant cell wall polymers can deteriorate when intense heat formed from the microwaves leads a high vapor pressure and temperature in the tissue. So, cell wall phenolics or bond phenolics could be released and more phenolics could be extracted (Inchuen *et al*., 2010; Sellami *et al*., 2013).

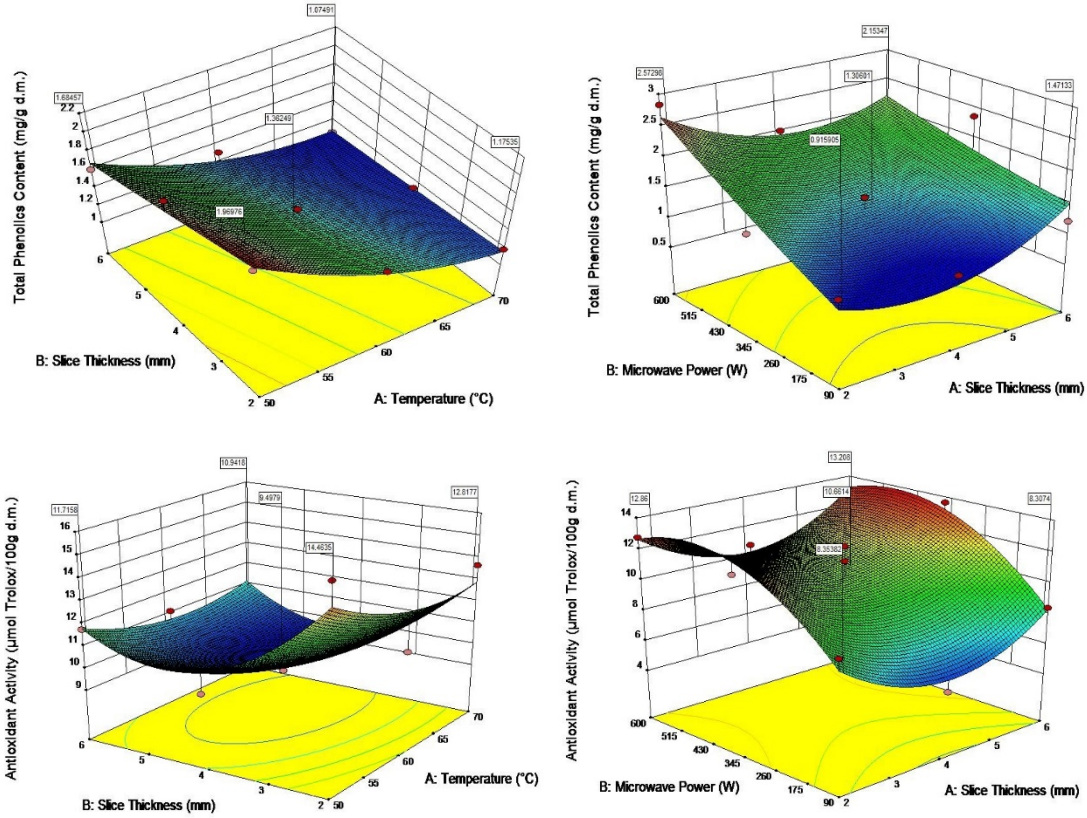

Figure 2. Response surface plot showing effect of different drying parameters on total phenolics content and antioxidant activity

3.2. Antioxidant activity

Effect of hot-air drying conditions

Hot-air drying process led to decrease in antioxidant activity of fresh white button mushrooms. The observed antioxidant activity of fresh sample (23.59 µmoL Trolox/100g d.m.) was found in the range of 9.3-15.74 µmoL Trolox/100g d.m. after hot air drying (Table 2). A quadratic model ($p<0.05$; R^2: 0.812) was also determined for the variation of antioxidant

activity of white button mushroom dried with hot-air. Linear and quadratic effects of slice thickness on antioxidant activity of dried white button mushroom were found significant ($p<0.05$). Slice thickness had a negative impact on antioxidant activity. A decrease in the antioxidant activity was observed as the slice thickness was increased. Antioxidant activity decreased to 11.76 µmol Trolox/100g d.m. from 15.74 µmol Trolox/100g d.m. when the slice thickness increased to 6 mm from 2 mm at 50 ºC (Figure 2). Our findings are consistent with the other studies which asparagus (Nindo *et al*., 2003) and sour cherry (Wojdylo *et al*., 2014) dried at hot-air. The decrease in antioxidant activity of hot-air dried mushroom in this study can be attributed to the decrease in total phenolics in hot-air dried samples. Moreover, thicker samples exposure to temperatures longer than the other samples during drying. So, bioactive compounds degrade more in thicker samples. A better-quality product with higher scavenging activity was obtained in banana slices with lesser thickness values are also reported by Khawas *et al*. 2016.

In this study, the effects of temperature and the interaction between drying temperature and slice thickness on antioxidant activity were found insignificant ($p>0.05$).

Effect of microwave drying conditions

The antioxidant activity of mushroom dried at microwave ranged from 4.96 µmol Trolox/100g d.m. to 13.61 µmol Trolox/100g d.m. (Table 2). The variation in antioxidant activity of white button mushroom dried with microwave oven was best explained by the quadratic model ($p<0.05$, R^2: 0.815). Quadratic effects of slice thickness and microwave power were determined significant ($p<0.05$). Microwave power had also a significant linear effect on antioxidant activity of dried white button mushroom ($p<0.01$). An increase in the antioxidant activity was observed as the microwave power increased. As can be observed in Figure 2, rising microwave power from 90 W to 600 W promoted antioxidant activity significantly ($p<0.01$). Antioxidant activity increased to 12.75 µmol Trolox/100g d.m. from 9.2 µmol Trolox/100g d.m. when the microwave power increased to 600 W from 90 W at 2 mm (Figure 2). This increment could be explained that the deterioration of plant tissue increased with a rise in the power of microwave, causing more phenolic compounds to be released. So antioxidant activity increases (Inchuen *et al*., 2010). Similar results observed compared to the other studies related with dried asparagus (Nindo *et al*., 2003), strawberry (Wojdylo *et al*., 2009), Thai red curry powder (Inchuen *et al*., 2010) and sour cherry (Wojdylo *et al*., 2014). Those studies reported that antioxidant activity increased in proportional to the microwave power.

The results showed slice thickness had significant adverse effect on antioxidant activity ($p<0.05$). Antioxidant activity exhibited a negative relationship with slice thickness. As slice thickness declined, higher antioxidant activity was observed compared to thicker samples, which can be caused by much longer drying time needed. The antioxidant activity of white button mushroom with 4 mm thickness dried at 600 W was 10.71 µmol Trolox/100g d.m., while this value was raised to 12.75 µmol Trolox/100g d.m. in dried mushroom with 2 mm thickness at the same microwave power.

3.3. Phenolic Compounds

Effect of hot-air drying conditions

Pyrogallol (324.1 mg/kg d.m.), (+)-catechin (3.23 mg/kg d.m.), t-cinnamic acid (9.53 mg/kg d.m.), caffeic acid (3.39 mg/kg d.m.) and p-coumaric acid (0.69 mg/kg d.m.) were determined in fresh white button mushroom and pyrogallol was found to be the most abundant compound. When fresh sample was subjected to hot-air drying, increases in pyrogallol, (+)-catechin and p-coumaric acid were observed and the contents of these phenolics were obtained in the ranges of 333-963 mg/kg d.m., 46.3-88.1 mg/kg d.m. and 2.1-4.5 mg/kg d.m., respectively. On the other hand, caffeic acid declined in the range of 0.33-0.85 mg/kg d.m. Increases in t-cinnamic acid were only observed in samples dried at 60 °C, while at the other

drying temperatures t-cinnamic acid tended to be decrease. Representative HPLC chromatograms (280 and 320 nm) of a sample (4 mm slice thickness) dried at 50 ºC is depicted in Figure 3.

The quadratic model was found to obey for the variation of pyrogallol ($p<0.01$; R^2: 0.903), (+)-catechin ($p<0.05$; R^2: 0.831), t-cinnamic acid ($p<0.05$; R^2: 0.829), caffeic acid ($p<0.05$; R^2: 0.786) and p-coumaric acid ($p<0.05$; R^2: 0.801) contents of white button mushroom dried with hot-air. Pyrogallol was significantly affected by the linear term of temperature and slice thickness (Table 3). A significant increase was observed for the pyrogallol content of dried white button mushroom with the increase of drying temperature and slice thickness ($p<0.01$). Increase in hot-air temperature from 50° to 70 °C at a fixed thickness value (2 mm) enhanced the pyrogallol content about 2-fold (Table 2). The results showed t-cinnamic acid was the only phenolic acid which significantly affected by the quadratic term of temperature ($p<0.01$). It was observed t-cinnamic acid increased with the temperature up to 60 ºC. No significant linear effects of drying temperature and slice thickness on t-cinnamic acid and p-coumaric acid contents were observed ($p>0.05$) (Table 3). However, the linear effect of the interaction between drying temperature and slice thickness on p-coumaric acid was found significant ($p<0.05$). A decrease in the p-coumaric acid content was observed as the drying temperature and slice thickness were increased. The (+)-catechin content was found to be decreased with increasing temperature ($p<0.05$). The effect of cabinet drying at 50, 60, 70 and 80 °C on the content of phenolics especially catechins in apple pomace have been recently reported by Heras-Ramírez *et al*. (2012) and they found that the losses associated with catechin can be attributed to the epimerization reactions at higher drying temperatures. The results also revealed that slice thickness had adverse effect on caffeic acid content ($p<0.05$).

Similar results observed compared to the other studies in dried apricot (Igual *et al*. 2012), sage plants (Sellami *et al*., 2013) and sour cherry (Wojdylo *et al*., 2014). Wojdylo *et al*. 2014, also found a decrease in some phenolics ((-)-epicatechin, p-coumaric acid, chlorogenic acid etc.) and an increase in (+)-catechin with increasing temperature during convective drying. Valadez-Carmona *et al*. 2017 also reported that drying process affect the release of phenolic compounds from fruits and vegetables positively, negatively and neutral because of microstructural changes.

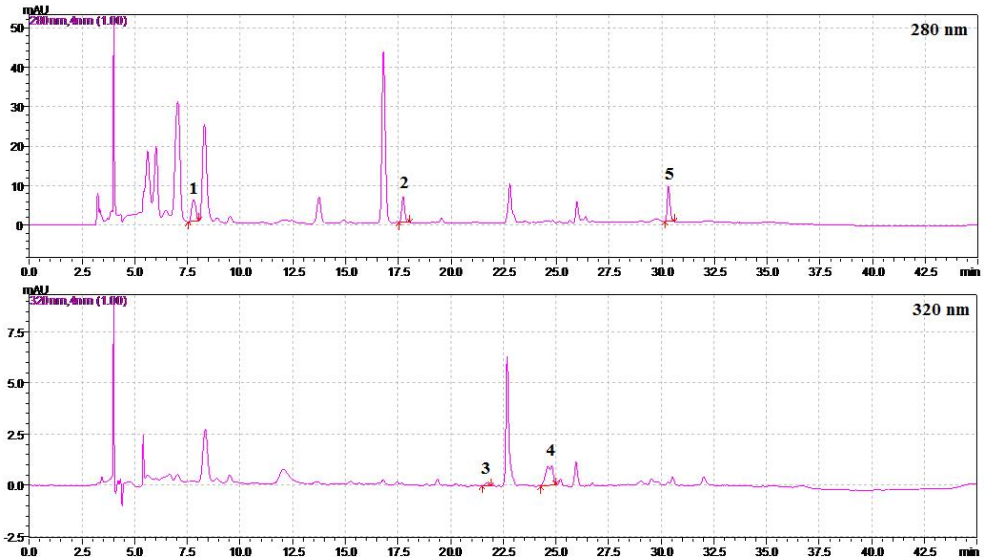

Figure 3. The HPLC chromatograms of phenolic compounds in white button mushrooms dried at 50 ºC, 4 mm with hot-air drier. Identified compounds 1, Pyrogallol; 2, (+)-catechin; 3, caffeic acid; 4, *p*-coumaric acid and 5, *t*-cinnamic acid

Effect of microwave drying conditions

Pyrogallol (324.1 mg/kg d.m.), (+)-catechin (3.23 mg/kg d.m.), t-cinnamic acid (9.53 mg/kg d.m.), caffeic acid (3.39 mg/kg d.m.) and p-coumaric acid (0.69 mg/kg d.m.) contents of fresh white button mushroom were determined in the ranges of 268-1359 mg/kg d.m., 19.3-39.2 mg/kg d.m., 0.29-10.74 mg/kg d.m., 0.09-0.24 mg/kg d.m. and 0.51-2.02 mg/kg d.m., respectively, when microwave drying was applied. It is relevant that (+)-catechin content enhanced about 10-13 fold; whereas caffeic acid declined 14-35 fold. This result revealed that microwave affected the phenolics differently.

Response surface methodology was also performed for exhibiting the effects of microwave power and slice thickness on phenolic compounds of white button mushroom dried with microwave oven. Data fitted to a quadratic model for the variation of pyrogallol ($p<0.01$; R^2: 0.978), (+)-catechin ($p<0.05$; R^2: 0.873), t-cinnamic acid ($p<0.01$; R^2: 0.958), caffeic acid ($p<0.05$; R^2: 0.852) and p-coumaric acid ($p<0.05$; R^2: 0.868) (Table 3).

The linear effects of microwave power and slice thickness were found significant for (+)-catechin and caffeic acid ($p<0.05$). The (+)-catechin and caffeic acid were found to be decreased with increasing slice thickness. An adverse significant effect of microwave power on (+)-catechin, leading the (+)-catechin decreased with the increase of microwave power except for the sample with the slice thickness of 2 mm. On the other hand, it was determined that increase in the caffeic acid was observed as the microwave power enhanced. The linear and quadratic effects of microwave power were determined significant upon a decrease in pyrogallol and t-cinnamic acid ($p<0.01$). The linear effect of slice thickness ($p<0.01$) and quadratic effect of microwave power ($p<0.05$) were observed significant for p-coumaric acid. The p-coumaric acid decreased as the slice thickness increased. The combined effect of microwave power and slice thickness significantly reduced pyrogallol and t-cinnamic acid contents of dried white button mushrooms ($p<0.05$).

Similar results observed compared to the other studies in dried strawberry (Wojdylo *et al*., 2009), sage plants (Sellami *et al*., 2013) and sour cherry (Wojdylo *et al*., 2014). The study by Wojdylo *et al*. (2009) indicated that variation in the phenolic compounds of Kent and Elsanta strawberry cultivars during microwave drying attributed to the higher microwave power. This research indicated that p-coumaroyl, ellagic acid, quercetin and (+)-catechin contents of Kent cultivar increased with increasing microwave power. Ellagic acid and (+)-catechin contents of Elsanta cultivar also increased by increasing microwave power. On the other hand, it was reported to be a decrease in p-coumaroyl as the microwave power increased.

4. Conclusions

By using quadratic models fitted in this study, total phenolics content, antioxidant activity and phenolic compounds of white button mushroom dried with hot-air drier and microwave oven can be predicted. The optimum conditions for maximum total phenolics content and antioxidant activity in drying of white button mushrooms in hot-air drier and microwave oven corresponded to temperature of 50 ºC at 2 mm and microwave power of 600 Watt at 2 mm, respectively. In this study, microwave drying is suggested as suitable method than hot-air drying because of least reducing the content of bioactive compounds and antioxidant activity and reducing drying time.

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ISOLATION, IDENTIFICATION AND COMPARISON OF SOME PROPERTIES OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* STRAINS FROM TRADITIONAL BULGARIAN AND ITALIAN YOGURTS

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ABSTRACT

An important step in the development of successful technological schemes for production of yogurt and other functional foods is the selection of appropriate *Lactobacillus strains* with useful properties that are resistant to antibiotics and bacteriocins. In the present study eleven *Lactobacillus* strains were isolated from fourteen homemade Bulgarian and Italian yogurts. The isolates were identified as *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) by 16S rDNA sequence analysis. The results from comparative 16S rRNA gene sequence-based phylogenetic analysis revealed 92-99% pairwise similarity of the isolates to the reference *L. bulgaricus* strains. The antimicrobial activity, antibiotic susceptibility and nisin resistance of the isolated *L. bulgaricus* strains were examined. Bulgarian *L. bulgaricus* strains 3-BG, 5-BG and 8-BG were characterized by highest antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Listeria ivanovii* ATCC 19119, *Listeria innocua* ATCC 33090, *Enterococcus faecalis* ATCC 19433 and *Enterococcus faecuim* ATCC 19434. *L. bulgaricus* 8-BG was active also against the Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 9027, *Proteus vulgaris* ATCC 6380, *Salmonella enteritidis* ATCC 13076, *Salmonella abony* NTCC 6017 and *Escherichia coli* ATCC 25922. In contrast, Italian *L. bulgaricus* strains demonstrated low antimicrobial activity. Bulgarian *L. bulgaricus* strains showed moderate sensitivity or resistance to most of the antibiotics used in the screening, while Italian *L. bulgaricus* strains were sensitive. Bulgarian *L. bulgaricus* strains 1-BG and 6-BG were resistant to 10 and 13 of a total of 24 antibiotics tested, respectively. Nisin resistance test showed that 10 of a total of 11 *L. bulgaricus* strains were highly sensitive to nisin (MIC values varying from 0.078 mg/mL to 0.156 mg/mL), except of Italian strain *L. bulgaricus* 6-IT which was resistant to nisin.

1. Introduction

Lactobacilli are lactic acid bacteria (LAB) that have been successfully used for many centuries in fermentation processes in the production of various foods. The life of modern people and associated "diseases of the 21-st century" have focused the scientific and commercial interests on the use of lactobacilli in a number of functional foods and probiotic products that have a positive impact on intestinal microflora, thus exert beneficial effect on the physiological status. Nowadays, they are widely accepted as useful component of the food chain, with a substantial role in

maintaining a healthy balance and preventing diseases in humans and animals.

Lactobacillus delbrueckii subsp. *bulgaricus* (*Lactobacillus bulgaricus*), isolated and described for a first time by the Bulgarian physician Dr. Stamen Grigoroff (1905), is the most economically important representative of the heterogeneous group of LAB with global application in the manufacture of fermented and functional foods, in particular yogurt. One of the most popular varieties of yogurt is the traditional Bulgarian yogurt, which has been consumed for millennia as a traditional food on the Balkans, known also as sour milk or "kiselo mlyako" due to its specific taste. Yogurt is recognized as a nutritious, natural and safe component of the healthy diet, which has beneficial effects on the gastrointestinal microflora and underlies the concept of probiotic foods. The benefits for human health from the consumption of yogurt are well known, and in recent years numerous studies have proven its therapeutic effects on various disorders (Adolfsson et al., 2004; McKinley, 2005; Fisberg and Machado, 2015).

The selection of appropriate *Lactobacillus* strains with useful properties is of paramount importance in the development of successful technological schemes in the dairy industry. *Lactobacillus* strains used as starter cultures or in composition of probiotic foods, should be carefully selected not only for their contribution to the organoleptic properties of the product (aroma, taste and texture), but also for their biological activities. In addition to the generally accepted requirement to be "generally recognized as safe" (GRAS), the probiotic strains must meet some other basic criteria related to their survival *in vivo* and probiotic role, such as resistance to the conditions in the gastrointestinal tract (Chen et al., 2017), adhesion to the intestinal epithelial cells, antimicrobial activity (Silva et al., 2020), and resistance to antibiotics (Havenaar et al., 1992; Amara et al., 2019).

The use of *L. bulgaricus* strains and their metabolites as biopreservatives in order to improve the shelf-life of various foods requires selection of strains that possess high antimicrobial activity (Mohammed et al., 2013). Another desired feature in the selection of *L. bulgaricus* strains is their resistance to antibiotics. The susceptibility of *L. bulgaricus* to different antibiotics used in the clinical practice for treatment of intestinal and other infections is considered undesirable, and in these cases antibiotic therapy reduces the effectiveness of probiotics and functional foods. On the other hand, the natural resistance of some *L. bulgaricus* strains may also have a negative side, as they can serve as sources of genes responsible for antibiotic resistance to be genetically transferred to the pathogenic bacteria, thus to threat human and animal health. Therefore, before including the lactobacilli strains in the composition of starter cultures or probiotic products, it is necessary to check whether these strains do not have transferable genes for antibiotic resistance (Danielsen and Wind, 2003; Karapetkov et al., 2011).

The application of some LAB bacteriocins (nisin) as biopreservatives in fermented products to prolong the shelf-life and control the pathogenic and spoilage microorganisms may have a negative impact on the strains in the starter culture and lactic acid fermentation. In this regard, the selection of *L. bulgaricus* strains resistant or weakly sensitive to nisin is also important for the technological process in the manufacture of quality fermented products.

Therefore, the aim of the present study was to isolate and identify *L. bulgaricus* strains from traditional Bulgarian and Italian yogurts, and to evaluate and compare some important properties of the strains such as antimicrobial activity, nisin resistance and antibiotic susceptibility.

2. Materials and methods

2.1. Materials

2.1.1. Yogurts

Fourteen samples of homemade yogurts (eight Bulgarian and six Italian) were used in the study.

2.1.2. Test microorganisms

Six Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Listeria innocua* ATCC 33090, *Listeria ivanovii* ATCC 19119, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecuim* ATCC 19434) and six Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Proteus vulgaris* ATCC 6380, *Salmonella enteritidis* ATCC 13076, *Salmonella abony* NTCC 6017, *Escherichia coli* ATCC 25922, *Klebsiella* sp. – clinical isolate) from the collection of the Department of Microbiology, University of Food Technologies, Plovdiv, Bulgaria were used in the antimicrobial screening.

2.1.3. Nisin

Nisin produced by *Lactococcus lactis* subsp. *lactis* - 2.5% (Sigma-Aldrich, USA), containing 1000000 IU active substance/g was used.

2.1.4. Culture media

Milk. Milk was prepared by the following prescription: 80 g of skim milk was dissolved in 1 L of deionized water, and then autoclaved at 112 °C for 45 min.

LAPTg10 broth. LAPTg10 broth was prepared by the manufacturer's (Laboratorios Conda S.A., Spain) prescription: 45 g of LAPTg10-solid substance mixture (containing 15 g peptone, 10 g yeast extract, 10 g tryptone and 10 g glucose) was dissolved in 1 L of deionized water. pH was adjusted to 6.6-6.8, and then 1mL Tween 80 (Sigma-Aldrich) was added. The medium was autoclaved at 121 °C for 20 min.

LAPTg10 agar. This medium was prepared by the following prescription: 45 g of LAPTg10-solid substance mixture (Laboratorios Conda S.A.) was dissolved in 1 L of deionized water. The final pH was adjusted to 6.6-6.8, and then 1mL Tween 80 (Sigma-Aldrich) and 15 g of agar (Sigma-Aldrich) were added. The medium was autoclaved at 121 °C for 20 min.

Luria-Bertani agar medium supplemented with glucose (LBG agar). LBG agar was prepared by the manufacturer's (Laboratorios Conda S.A.) prescription: 50 g of LBG-solid substance mixture (containing 10 g tryptone, 5 g yeast extract, 10 g NaCl, 10 g glucose and 15 g agar) was dissolved in 1 L of deionized water. The final pH was adjusted to 7.5, and then medium was autoclaved at 121 °C for 20 min.

de Man, Rogosa and Sharpe (MRS) agar. This medium was prepared by the manufacturer's (Merck, Germany) prescription: 55.2 g of MRS broth (containing 10 g peptone, 5 g yeast extract, 10 g beef extract, 20 g glucose, 2 g potassium phosphate, 5 g sodium acetate, 0.2 g magnesium sulphate, 0.05 g manganese sulfate, 1 g Tween 80 and 2 g ammonium citrate) was dissolved in 1 L of deionized water. The final pH was adjusted to 6.4, and then 15 g of agar (Sigma-Aldrich) was added. The medium was autoclaved at 121 °C for 15 min.

Modified de Man, Rogosa and Sharpe (mMRS) agar. The modified MRS agar medium was prepared by the following prescription: 55.2 g of MRS broth (Merck) and 0.05 g L-cysteine (Merck) were dissolved in 1 L of deionized water. The final pH was adjusted to 6.4, and then 15 g of agar (Sigma-Aldrich) was added. The medium was autoclaved at 121 °C for 15 min.

Modified skim milk (MSM) agar. This medium was prepared by the manufacturer's (Himedia®, India) prescription: 24.5 g of solid substance mixture (containing 5 g tryptone, 2.5 g yeast extract, 1 g glucose monohydrate, 1 g skim milk powder and 15 g agar) was dissolved in 1 L of deionized water. The final pH was adjusted to 7.0, and the medium was autoclaved at 121 °C for 15 min.

2.2. Methods

2.2.1. Isolation and cultivation of the strains

Samples from yogurts were first propagated in milk (1 mL sample + 9 mL milk) and incubated at 42 °C for 3-4 h (until coagulation). Then samples were streaked on LAPTg10 agar medium, and the Petri plates (d=90 mm; Gosselin™, France) were incubated at 37 °C for 48 h. Single colonies were cut and

transferred into 2 mL of LAPTg10 broth, stirred by vortex V-1 plus (Biosan, Latvia) for 5-10 s, and cultured at 37 °C for 48 h. Next, 1 mL of the biomass was transferred into 5 mL of LAPTg10 broth and incubated under identical conditions, then stored at 4 °C for further analyses. The cellular morphology of the isolated strains was determined by microscopic observation of colored smears. The colony morphology of the isolates was described by microscopic observation of single colonies grown on LAPTg10 agar, MRS agar, mMRS agar and MSM agar media.

2.2.2. Isolation of total DNA

The isolation of DNA was performed by the method of Delley et al. (1990).

2.2.3. 16S rDNA amplification

16S rDNA of the isolates was amplified using the universal primers 27F (5'AGAG TTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') according to the method of Lane (1991). The amplification program included: denaturation – 95 °C for 3 min; 40 cycles – 93 °C for 30 s, 55 °C for 60 s, 72 °C for 2 min; final elongation – 72 °C for 5 min.

2.2.4. Purification of the product of the PCR-reaction from TAE agarose gel

The purification of 16S rDNA was conducted using a DNA-purification kit in a Microspin^{TM} column following the standard protocol (Denkova et al., 2012).

2.2.5. Sequencing of the 16S rRNA gene

The partial sequencing of the 16S rRNA gene with two universal primers (27F and 1492R) was implemented according to the method of Sanger et al. (1977) at "Macrogen Europe Laboratory", The Netherlands.

The entire sequence of the 16S rRNA gene was obtained using the CLC Sequence Viewer software, and the resulting whole sequence was compared with the on-line database sequences via the BLASTn algorithm. Thus, the studied strains were identified to the species level with the corresponding confidence level.

2.2.6. Antimicrobial activity

The antimicrobial activity of *L. bulgaricus* strains was determined by the agar well diffusion method. The test bacteria were cultivated on LBG agar medium at 37 °C for 24 h. To prepare bacterial inocula, a small amount of biomass of each test microorganism was suspended in 5 mL of sterile 0.5% NaCl, and then vortexed (V-1 plus, Biosan) for 5-10 s. The number of viable cells in each bacterial inoculum was determined using the counting chamber Thoma (Poly-Optik GmbH, Germany), and the final concentration was adjusted to 1.0×10^8 cfu/mL. Next, bacterial suspensions were inoculated in preliminarily melted and tempered at 45–48 °C LAPTg10 agar media. The inoculated media were transferred in quantity of 18 mL in sterile Petri plates (Gosselin™) and allowed to harden. Then six wells (d=6 mm) per plate were cut.

L. bulgaricus strains (cultured in LAPTg10 broth at 37 °C for 48 h) were pippeted into the agar wells in quantity of 60 μL. As a control, the antibiotic Ampicillin (10 mg/mL) was used. The Petri plates (Gosselin™) were incubated at identical conditions. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zones around the wells at the 24-th and 48-th h after incubation. The results were interpreted as follows: high antimicrobial activity - inhibition zones of 18 mm or more; moderate antimicrobial activity - inhibition zones from 12 to 18 mm; low antimicrobial activity - inhibition zones under 12 mm (Tumbarski et al., 2018a).

2.2.7. Nisin resistance test and minimal inhibitory concentration

Nisin resistance and minimal inhibitory concentration (MIC) of nisin were determined by the dilution method. Two-fold serial dilutions of nisin in sterile distilled water, ranging from 10 mg/mL to 0.0049 mg/mL were prepared. 60 μL of dilution was pipetted in wells cut in LAPTg10 agar media inoculated with each *L. bulgaricus* strain. The Petri plates (Gosselin™) were incubated at 37 °C for 48 h. The MIC values were determined as the lowest concentration of nisin inhibiting completely the growth of each *L. bulgaricus* strain around the agar well (Tumbarski et al., 2017).

2.2.8. Antibiotic susceptibility test

Antibiotic susceptibility test was performed by *in vitro* disc diffusion method of Bauer et al. (1966) with impregnated paper discs of 24 antibiotics (Bul Bio - NCIPD Ltd., Bulgaria). The strain suspensions (0.1 mL) were spread plated on LAPTg10 agar medium, and then four discs of different antibiotics per Petri plate (Gosselin™) were put on the surface of the agar medium. The plates were incubated at 37 °C for 48 h. Zones of inhibition were measured and recorded at 24-th and 48-th h of incubation. Strains with no inhibition zones were considered resistant; with inhibition zones from 7 to 16 mm – intermediate sensitive; with zones >16 mm – sensitive.

2.2.9. Statistical analysis

Data from triplicate experiments for the antimicrobial activity were processed with MS Office Excel 2010 software, using statistical functions to determine the standard deviation (±SD), and maximum estimation error at significance level ά <0.05.

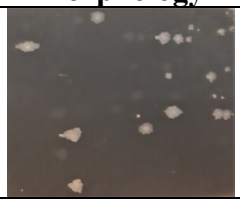
3.Results and discussions

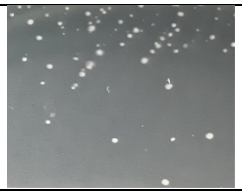
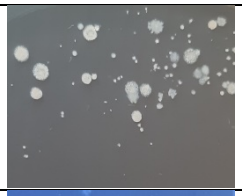
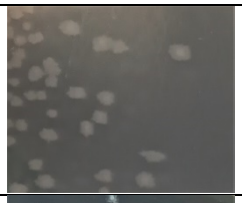
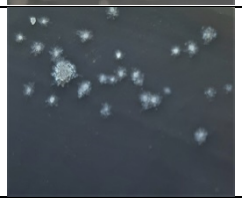
3.1. Strain isolation, cellular and colony morphology

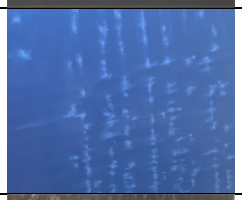
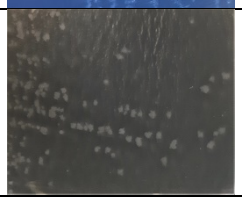
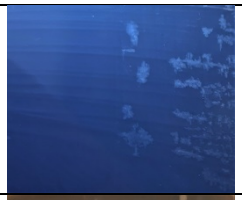
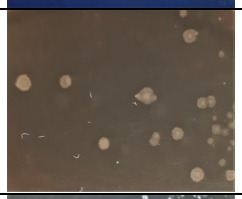
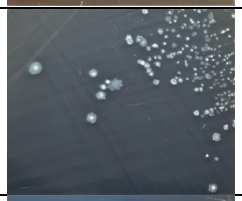
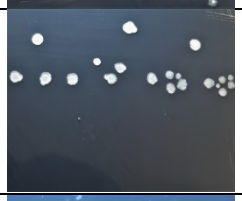
In the present study, six *L. bulgaricus* strains were isolated from eight traditional Bulgarian yogurts (1-BG, 3-BG, 5-BG, 6-BG, 7-BG and 8-BG) and five *L. bulgaricus* strains were isolated from six Italian yogurts (1-IT, 3-IT, 4-IT, 5-IT and 6-IT).

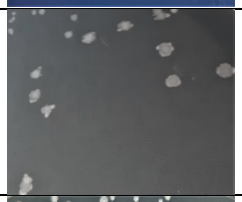
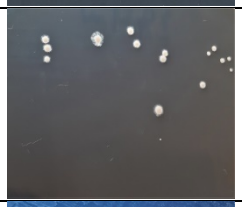
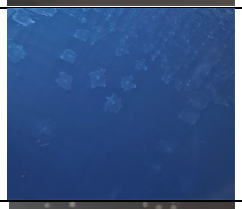
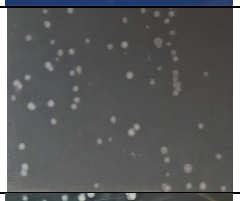
The microscopic observation of colored smears showed that all isolated strains possessed the typical cellular morphology for *L. bulgaricus* – Gram-positive, rod shaped with rounded ends (0.5–0.8 μm × 2–9 μm) or filamentous (0.5–0.8 μm × 20–25 μm) arranged singly, in short or longer chains (figures not provided), which depends on the age of the culture and the composition of the growth medium (Teixeira, 2014). The colony morphology of the isolates was determined by microscopic observation of single colonies. To study the influence of the medium composition on *L. bulgaricus* colony characteristics, the strains were cultured on different agar media used for isolation, enumeration and differentiation of lactobacilli from milk, yogurt, cheese, and other fermented milk products - LAPTg10, MRS, mMRS and MSM. The colonies of all isolated strains exhibited the typical characteristics for *L. bulgaricus colonies* – 1-6 mm in diameter, whitish, round or irregular shaped, flat profile, with or without dot-like center, serrated edges, rough surface, and soft texture, similar to those observed by Tabasco et al. (2007), Nwamaioha and Ibrahim (2018), and Oyeniran et al. (2020). In the present study, *L. bulgaricus* strains were affected by the type and composition of the agar media, and formed colonies with different morphology parameters (mainly size and shape), or did not grow on all agar media. Good growth of all strains was observed only on LAPTg10 agar medium (Table 1).

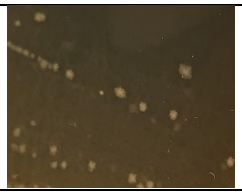
Table 1. Colony morphology of *L. bulgaricus* strains on different agar media for lactobacilli

Strain	Culture medium	Colony morphology	Colony description
1-BG	LAPTg10 agar		1-2 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture
	MRS agar		No growth
	mMRS agar		No growth
	MSM agar		No growth

3-BG	LAPTg10 agar		1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture
	MRS agar		No growth
	mMRS agar		1-3 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture
	MSM agar		1-3 mm in diameter, whitish, flat, irregular shaped, serrated edges, rough surface, soft texture
5-BG	LAPTg10 agar		1-4 mm in diameter, whitish, flat, irregular shaped, serrated edges, rough surface, soft texture
	MRS agar		No growth
	mMRS agar		2-3 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture
	MSM agar		No growth
6-BG	LAPTg10 agar		1-6 mm in diameter, whitish, flat, irregular shaped, serrated edges, rough surface, soft texture
	MRS agar		No growth
	mMRS agar		No growth
	MSM agar		No growth
7-BG	LAPTg10 agar		2-6 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture
	MRS agar		1-3 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture

	mMRS agar		1-5 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture
	MSM agar		1-2 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture
8-BG	LAPTg10 agar		1-2 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture
	MRS agar		No growth
	mMRS agar		No growth
	MSM agar		1-5 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture
1-IT	LAPTg10 agar		1-4 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture
	MRS agar		1-3 mm in diameter, whitish, flat, round or irregular shaped with dot-like center, serrated edges, rough surface, soft texture
	mMRS agar		1-4 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture
	MSM agar		1-2 mm in diameter, whitish, flat, round shaped with dot-like center, serrated edges, rough surface, soft texture
3-IT	LAPTg10 agar		1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture

	MRS agar		No growth
	mMRS agar		No growth
	MSM agar		1-4 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture
4-IT	LAPTg10 agar		1-6 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture
	MRS agar		1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture
	mMRS agar		1-4 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture
	MSM agar		2-6 mm in diameter, whitish, flat, round or irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture
5-IT	LAPTg10 agar		1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture
	MRS agar		1-2 mm in diameter, whitish, flat, round shaped with dot-like center, serrated edges, rough surface, soft texture
	mMRS agar		1-3 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture
	MSM agar		1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture

6-IT	LAPTg10 agar		1-4 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture
	MRS agar		No growth
	mMRS agar		No growth
	MSM agar		1-4 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture

3.2. Strain identification

Besides the standard methods used for characterization, the species and strain identification require application of some rapid and reliable molecular genetic techniques such as polymerase chain reaction (PCR), amplified ribosomal DNA restriction analysis (ARDRA) and nucleotide sequencing.

The results obtained from conventional PCR amplification of 16S rDNA, followed by nucleotide sequencing and comparative 16S rRNA gene sequence-based phylogenetic analysis revealed that all of the studied isolates belonged to the bacterial species *Lactobacillus delbrueckii* subsp. *bulgaricus*. The isolates exhibited 92% (strain 5-BG), 97% (strain 6-BG) and 99% (strains 1-BG, 3-BG, 7-BG, 8-BG, 1-IT, 3-IT, 4-IT, 5-IT and 6-IT) pairwise similarity of the sequence of 16S rDNA to the partial sequence of 16S rDNA of the relevant reference *L. bulgaricus* strains (Table 2).

Molecular genetic methods have been successfully applied in a number of previous studies related to the identification, genotyping and grouping of *Lactobacillus* strains isolated from different dairy and non-dairy fermented products (Andrighetto et al., 1998; Markiewicz et al., 2010; Denkova et al., 2012; Yu et al., 2015).

Table 2. Pairwise similarity of the sequences of 16S rDNA of the isolated *L. bulgaricus* strains and the partial sequences of 16S rDNA of the reference *L. bulgaricus* strains

Isolate	Reference strain	Identity/similarity	Gaps	Strand
1-BG	*L. delbrueckii* subsp. *bulgaricus* IMAU40160	1398/1407 (99%)	3/1407 (0%)	Plus/Plus
3-BG	*L. delbrueckii* subsp. *bulgaricus* 3641	1435/1445 (99%)	4/1445 (0%)	Plus/Plus
5-BG	*L.delbrueckii* subsp. *bulgaricus* A320	967/1053 (92%)	15/1053 (1%)	Plus/Minus
6-BG	*L. delbrueckii* subsp. *bulgaricus* 4640	707/731 (97%)	8/731 (1%)	Plus/Plus
7-BG	*L. delbrueckii* subsp. *bulgaricus* LJJ	1424/1434 (99%)	2/1434 (0%)	Plus/Minus
8-BG	*L. delbrueckii* subsp. *bulgaricus* 4133	1423/1431 (99%)	1/1431 (0%)	Plus/Plus

1-IT	*L. delbrueckii* subsp. *bulgaricus* 4119	1441/1449 (99%)	4/1449 (0%)	Plus/Plus
3-IT	*L. delbrueckii* subsp. *bulgaricus* 3789	1436/1454 (99%)	8/1454 (0%)	Plus/Plus
4-IT	*L. delbrueckii* subsp. *bulgaricus* KLDS1.1011	1410/1427 (99%)	6/1427 (0%)	Plus/Minus
5-IT	*L. delbrueckii* subsp. *bulgaricus* 3913	1447/1458 (99%)	8/1458 (0%)	Plus/Plus
6-IT	*L. delbrueckii* subsp. *bulgaricus* 4017	1419/1420 (99%)	0/1420 (0%)	Plus/Plus

3.3. Antimicrobial activity

Antimicrobial activity is an important criterion for selection of starter and probiotic cultures as natural antagonists of food spoilage and pathogenic microorganisms (Georgieva et al., 2015).

The results demonstrated that Bulgarian *L. bulgaricus* strains were characterized by higher antimicrobial activity compared to the Italian strains, expressed mainly against Gram-positive bacteria (Table 3). *L. bulgaricus* strains 3-BG, 5-BG and 8-BG exhibited the highest antimicrobial activity inhibiting the growth of *S. aureus* ATCC 25923, *L. monocytogenes* NBIMCC 8632, *L. ivanovii* ATCC 19119, *L. innocua* ATCC 33090, *E. faecalis* ATCC 19433 and *E. faecuim* ATCC 19434. *L. bulgaricus* strains 3-BG and 8-BG were active also against Gram-negative *P. aeruginosa* ATCC 9027, *P. vulgaris* ATCC 6380 (only 8-BG), *S. enteritidis* ATCC 13076, *S. abony* NTCC 6017 and *E. coli* ATCC 25922. Among Italian strains, *L. bulgaricus* 4-IT showed highest antimicrobial activity against all Gram-positive bacteria used in the study, and Gram-negative *S. enteritidis* ATCC 13076 and *S. abony* NTCC 6017. The rest of the *L. bulgaricus* strains (1-BG, 6-BG, 7-BG, 1-IT, 3-IT, 5-IT and 6-IT) demonstrated weak antimicrobial effect. None of *L. bulgaricus* strains inhibited the test microorganism *Klebsiella* sp.

In recent years, several studies have revealed the technological properties and probiotic characteristics of *L. bulgaricus* strains such as antimicrobial activity, nisin resistance, and antibiotic susceptibility. Akpinar et al. (2011) ascertained that 17 of

25 *L. bulgaricus* strains isolated from homemade yogurts possessed antimicrobial activity against *Klebsiella pneumoniae*, 16 of 25 were active against *Bacillus cereus* and *Pseudomonas fluorescens*, 18 of 25 were active against *L. monocytogenes*, 6 of 25 were active against *S. aureus*, 3 of 25 were active against *Bacillus coagulans*, and all of them inhibited the growth of *E. coli*.

In contrast, Erdogrul and Erbilir (2006) reported weak antimicrobial activity (inhibition zones <12 mm) of *L. bulgaricus* strain isolated from the probiotic dairy product against *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 18833, *Salmonella typhimurium* ATCC 13311 and *Enterobacter cloacae* ATCC 13047. The strain was inactive against *Bacillus subtilis*. Georgieva et al. (2015) investigated antimicrobial activity of five *L. bulgaricus* strains isolated from homemade yogurts, and found that all isolates had significant inhibitory effect on all tested bacteria - *S. aureus* NBIMCC 3703, *B. cereus* NBIMCC 1085, and *E. coli* NBIMCC 3702.

Table 3. Antimicrobial activity of the isolated *L. bulgaricus* strains

Test microorganism	*L. bulgaricus strains*, inhibition zones (mm)											Control *
	1-BG	3-BG	5-BG	6-BG	7-BG	8-BG	1-IT	3-IT	4-IT	5-IT	6-IT	
Gram (+) bacteria												
***S. aureus* ATCC 25923**	8±0.15**	13±0.21	12±0.23	8±0.17	-	11±0.26	8±0.11	10±0.23	9±0.12	8±0.11	8±0.17	40±0.29
***L. monocytogenes* NBIMCC 8632**	-	12±0.23	12±0.25	8±0.18	8±0.12	12±0.29	-	8±0.15	8±0.18	-	-	38±0.27
***L. ivanovii* ATCC 19119**	8±0.11	10±0.2	12±0.29	-	10±0.23	12±0.24	10±0.26	10±0.29	10±0.24	8±0.16	10±0.2	40±0.24
***L. innocua* ATCC 33090**	-	11±0.24	12±0.2	8±0.16	11±0.28	13±0.2	8±0.17	10±0.21	10±0.28	8±0.13	10±0.23	40±0.22
***E. faecalis* ATCC 19433**	-	10±0.22	10±0.19	10±0.27	9±0.19	12±0.22	8±0.13	9±0.14	10±0.22	8±0.18	8±0.15	37±0.2
***E. faecuim* ATCC 19434**	8±0.14	9±0.16	8±0.18	8±0.14	9±0.2	10±0.21	-	-	8±0.16	-	-	30±0.21
Gram (-) bacteria												
***P. aeruginosa* ATCC 9027**	8±0.12	8±0.17	-	-	-	8±0.18	-	-	-	-	-	13±0.19
***P. vulgaris* ATCC 6380**	-	-	-	-	-	11±0.25	-	-	-	-	-	25±0.2
***S. enteritidis* ATCC 13076**	-	9±0.14	8±0.13	-	-	10±0.22	8±0.16	-	8±0.19	8±0.12	-	32±0.25
***S. abony* NTCC 6017**	-	11±0.25	8±0.11	8±0.15	-	10±0.28	-	-	8±0.14	-	-	29±0.28
***E. coli* ATCC 25922**	-	8±0.19	-	-	-	8±0.15	-	-	-	-	-	30±0.23
***Klebsiella* sp. (clinical isolate)**	-	-	-	-	-	-	-	-	-	-	-	-

*Ampicillin (10 mg/ml); **standard deviation (±SD); d_{well}=6mm

3.4. Nisin resistance and minimal inhibitory concentration (MIC)

In our study, nisin resistance test showed that 10 of a total of 11 *L. bulgaricus* strains were highly sensitive to the action of nisin (MIC values varying from 0.078 mg/ml for *L. bulgaricus* 3-BG, 6-BG, 1-IT, 3-IT, and 4-IT to 0.156 mg/ml for *L. bulgaricus* 1-BG, 5-BG, 7-BG, 8-BG, and 5-IT) with the exception of Italian strain *L. bulgaricus* 6-IT which was resistant to nisin (Table 4).

Table 4. Nisin resistance of the isolated *L. bulgaricus* strains and minimal inhibitory concentration (MIC)

Nisin	***L. bulgaricus* strains**										
	1-BG	**3-BG**	**5-BG**	**6-BG**	**7-BG**	**8-BG**	**1-IT**	**3-IT**	**4-IT**	**5-IT**	**6-IT**
MIC, mg/ml	0.156	0.078	0.156	0.078	0.156	0.156	0.078	0.078	0.078	0.156	Resistant

Lactobacilli, in particular *L. bulgaricus* strains are essential for food fermentation processes and food preservation, whether they are part of the natural microflora of the products or added as starter cultures.

One of the advanced approaches in food biopreservation is the application of bacteriocins – peptides with antimicrobial activity produced by some LAB and members of genus *Bacillus*. Nisin (E-234) is a bacteriocin synthesized by *Lactococcus lactis* subsp. *lactis*, which is officially approved as a "Generally Recognized as Safe" (GRAS), and widely used as a food additive and biopreservative in the food industry. Nisin is known to possesses strong inhibitory activity against Gram-positive bacteria, including LAB (Tumbarski et al., 2018b). However, the application of bacteriocins can disrupt the fermentation by inhibiting the LAB. In this regard, the selection of *L. bulgaricus* strains resistant to nisin is of great importance for the technological process in the production of stable and high-quality fermented products.

Inhibitory effect of nisin and sensitivity of *L. bulgaricus* strains have been demonstrated in some previous studies. Durlu-Özkaya et al. (2007) examined the exopolysaccharide (EPS) production of 20 *L. bulgaricus* isolates from homemade yogurt and raw milk, and the correlation between EPS production and sensitivity of the strains to bacteriophages and nisin. The authors found that *L. bulgaricus* strains with high EPS-producing capacity were resistant to phages and nisin, and concluded that they are perspective as starter cultures in commercial yogurt production. On the other hand, the antimicrobial activity of nisin against pathogenic microorganisms and LAB depends on the dosage and storage conditions. Benkerroum et al. (2002) stated that in yogurt with addition of nisin at a dose of 10 RU/mL, no *L. monocytogenes* survived at 24-th hour of storage at refrigeration conditions (7 °C). However, the pathogen survived 13 days of storage at the same temperature in the controls (without addition of nisin). The authors concluded that nisin inhibited the yogurt fermentation at concentration higher than 50 RU/mL, and recommended its application in lower doses in order to control the growth of *L. monocytogenes*, and to prevent the excessive acidification normally observed in the end of the storage life of yogurt.

3.5. Antibiotic susceptibility

In addition to antimicrobial activity and nisin resistance, the antibiotic resistance is another substantial criterion for selection of appropriate functional strains. The results showed that Bulgarian *L. bulgaricus* strains were moderately sensitive (inhibition zones 7-16 mm) or resistant to most of the 24 antibiotics used in the screening (Table 5). The most resistant among the Bulgarian strains was *L. bulgaricus* 6-BG, which was insensitive to 13 of 24 antibiotics tested (bacitracin, penicillin, oxacillin, amoxicillin, tetracycline, doxycycline, gentamicin, amikacin, rifampin, clarithromycin, chloramphenicol, ciprofloxacin and nalidixic acid), followed by *L. bulgaricus* 1-BG, which was resistant to 10 antibiotics (penicillin, ampicillin, amoxicillin, tetracycline, gentamicin, amikacin, clarithromycin, chloramphenicol, novobiocin and norfloxacin). *L. bulgaricus* 3-BG was resistant to penicillin, ampicillin, tetracycline, lincomycin, novobiocin, ciprofloxacin, nalidixic acid and sulfamethoxazole/trimethoprim. *L. bulgaricus* 5-BG was resistant to bacitracin, penicillin, amoxicillin, vancomycin, tetracycline, gentamicin, rifampin and nalidixic acid. *L. bulgaricus* 8-BG exhibited resistance to piperacillin, vancomycin, cefuroxime, doxycycline, rifampin, erythromycin, clarithromycin and sulfamethoxazole/ trimethoprim, while *L. bulgaricus* 7-BG was resistant to 5 antibiotics – rifampin, lincomycin, tobramycin, novobiocin and norfloxacin. In contrast, Italian *L. bulgaricus* strains were sensitive to almost all of total of 24 antibiotics tested. *L. bulgaricus* 5-IT was resistant to ampicillin, levofloxacin and sulfamethoxazole/trimethoprim; *L. bulgaricus* 1-IT was resistant to levofloxacin and sulfamethoxazole/trimethoprim; *L. bulgaricus* 3-IT and *L. bulgaricus* 4-IT – to sulfamethoxazole/ trimethoprim; *L. bulgaricus* 6-IT – to levofloxacin.

The antibiotic susceptibility of *L. bulgaricus* strains varies widely. Georgieva et al. (2015) examined antibiotic susceptibility of five *L. bulgaricus* isolates to nine antibiotics, and found that all tested strains were susceptible toward ampicillin, gentamicin, erythromycin and tetracycline. The results reported by Erdogrul and Erbilir (2006) demonstrated that *L. bulgaricus* strain isolated from probiotic product was moderately sensitive to ampicillin, vancomycin, oxacillin, cephalothin, and cefodizime, but highly sensitive to tobramycin. Kyriacou et al. (2008) investigated the antibiotic resistance of 91 *L. bulgaricus* strains isolated from different commercial Greek yogurts, and found that 97.8% from them were resistant to ciprofloxacin, 65.9% resistant to kanamycin, 62.6% resistant to amikacin, 1.1% resistant to vancomycin, and 1.1% resistant to bacitracin.

Table 5. Antibiotic susceptibility of the isolated *L. bulgaricus* strains

Antibiotic			***L. bulgaricus* strains**										
	µg/disc	**MA*****	**1-BG**	**3-BG**	**5-BG**	**6-BG**	**7-BG**	**8-BG**	**1-IT**	**3-IT**	**4-IT**	**5-IT**	**6-IT**
Bacitracin	0.07*	Inhibitors of the cell wall synthesis	S	S	R	R	S	S	S	S	S	S	S
Piperacillin	100		SR	S	S	S	S	R	S	S	S	S	S
Penicillin	10*		R	R	R	R	SR	S	S	S	S	S	S
Ampicillin	10		R	R	SR	S	SR	SR	SR	S	SR	R	SR
Oxacillin	1		S	SR	S	R	S	SR	S	S	S	SR	S
Amoxicillin	25		R	S	R	R	SR	S	S	S	S	S	S
Vancomycin	30		S	S	R	S	S	R	S	S	S	S	S
Cefuroxime	30		S	S	S	S	SR	R	SR	SR	S	S	S
Tetracycline	30	Inhibitors of the protein synthesis	R	R	R	R	S	S	S	S	S	S	S
Doxycycline	30		S	SR	S	R	S	R	S	S	S	S	S
Gentamicin	10		R	SR	R	R	S	S	S	S	S	S	S
Amikacin	30		R	S	S	R	S	S	S	S	SR	S	S
Rifampin	5		SR	SR	R	R	R	R	S	S	S	S	S
Lincomycin	15		S	R	SR	S	R	S	SR	S	S	S	SR
Tobramycin	10		SR	S	S	S	R	SR	SR	S	SR	S	SR
Erythromycin	15		S	SR	SR	S	SR	R	S	S	S	S	S
Clarithromycin	15		R	S	SR	R	SR	R	S	S	S	S	S
Chloramphenicol	30		R	S	SR	R	S	S	S	S	S	S	S
Novobiocin	5	Inhibitors of the DNA synthesis or cell division	R	R	SR	SR	R	S	S	S	S	S	S
Ciprofloxacin	5		S	R	SR	R	SR	S	SR	SR	S	S	SR
Norfloxacin	10		R	SR	SR	S	R	SR	SR	SR	S	S	SR
Levofloxacin	5		SR	SR	SR	S	SR	SR	R	S	S	R	R
Nalidixic acid	30		S	R	R	R	SR	SR	SR	SR	SR	SR	SR
Sulfamethoxazole/ Trimethoprim**	23.75/ 1.25		SR	R	S	S	SR	R	R	R	R	R	SR

*E/disc; ** - inhibits also the protein synthesis; ***MA – mechanism of action; R – resistant; SR – intermediate (zone 7-16 mm); S – sensitive (zone >16 mm); d_{disc}=6mm

4. Conclusions

In the present study, 11 *L. bulgaricus* strains from traditional Bulgarian and Italian homemade yogurts were isolated and identified, and their antimicrobial activity, nisin resistance and antibiotic susceptibility were determined and compared. The antimicrobial screening demonstrated that Bulgarian *L. bulgaricus* strains were characterized by higher antimicrobial activity compared with the Italian strains, which was most pronounced in *L. bulgaricus* 3-BG, 5-BG and 8-BG. The results from nisin resistance test showed that only one of the isolates was resistant to nisin (*L. bulgaricus* 6-IT). The antibiotic susceptibility test revealed that Bulgarian *L. bulgaricus* isolates were resistant or moderately sensitive to most of the antibiotics used, in comparison with the Italian strains, which were sensitive. The obtained results are important from a practical point of view, in the selection of suitable *L. bulgaricus* strains for use as starter cultures, probiotics, or in order to improve the food quality and safety. The future perspectives of the present research will be directed to further investigation of other probiotic and technological properties of the newly isolated *L. bulgaricus* strains, and development of technological schemes for their successful application in the composition of probiotic products.

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RELATIONSHIP OF LECTIN PROTEINS AND HEALTH: STUDIES ON PRODUCT DEVELOPMENT WITHOUT LECTIN

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ABSTRACT

Foods that contain lectin protein prevent the body from reaching the required nutritional content, thus not completely benefit, when consumed. In the literature, it is reported that people with lectin sensitivity could have health problems. Among those are insulin resistance, obesity, leptin sensitivity, gastrointestinal system disorders due to various toxic effects. Germination, soaking, fermentation and cooking processes are important processes in reducing the amount of lectin in foods. In some studies, it has been reported that lectin-free diet shows improvement in autoimmune diseases. While there are many studies in the literature aimed at decreasing the lectin level, research on developing the lectin-free product is ongoing. The aim of our study was to develop alternative food and snack formulas free of lectin protein. Therefore, traditional cookies, pretzels and cakes were used as the basis as the commonly consumed snacks. In the formulas, instead of white flour, Almond and Coconut flour without lectin protein was used. The differences in perceptions between consuming lectin and non-lectin products were evaluated according to descriptive statistics methods. In conclusion, a significant difference was found between perception of the lectin-free snacks and the lectin-containing snacks. When examined according to gender with regards to the taste and interest; while women were more interested in gluten-free and lectin-free products than men, $p < 0.05$), there was no difference in flavor scores ($p > 0.05$).

1.Introduction

Apart from pathological factors such as, bacteria, viruses, fungi and parasites; food poisoning is a metabolic reaction that can also be caused by different structures contained in food products (Peumans and Van Damme, 1995). These different active structures in the natural structure of plants, while providing the necessary opportunities for their survival, sometimes can cause serious toxic effects when consumed (Tamimi et al., 2008). The main function of lectins in plants is to protect the seeds of the plant from harmful effects and ensure the survival of its species. They function as powerful insecticides and microorganism killers. (Barooah, et al., 2017) One of the most important factors that contributes to health hazard is considered anti-nutrients when a food products is not properly prepared. Anti-nutrients are structures that prevent the absorption of consumed food by the body. Due to the nature of these substances, the digestive system is unable to absorb the food received and thus cannot benefit from the food as intended. The most common known anti-nutrients are; phytate, saponin, tanin and lectin (Adebanke et al.,2015; Petroski and Minich, 2020; Popova and Mihaylova, 2019; Oke et al., 2020; Vasconcelos

and Oliveira, 2004). Lectins are substances in protein or glycoprotein structure that can bind specifically to glycosides in the cell membrane and organelles (Akande et al., 2010; Hamid et al., 2013). The reactions of these proteins when bound to carbohydrates in the cell membranes are thought to play a role in gaining excessive weight and developing certain diseases (Day et al., 2002; Freed, 1999; Sharon and Lis, 1997; Werz and Seeberger, 2005;). Many lectin proteins exhibit inflammation-inducing immunotoxic, neurotoxic and cytotoxic effects. Some lectin proteins hemagglutinate, hence increase blood viscosity, affect gene expression, and disrupt endocrine function (Zwolak, 2014). Because these effects of lectins cannot be felt during nutrition, they can remain hidden, but later revealed during absorption in intestinal cells when reaction occurs with antibodies. It is reported that health-related threats to lectins could arise unless most of the plant-based food is carefully consumed (Mishra, et al., 2019). Reducing the amount of lectin in food products should minimize the damages of lectin to the body. Methods used to reduce the amount of lectin are; germination, soaking, fermentation and cooking thoroughly (Ibrahim et al., 2002). High-pressure cooking of the food provides the decomposition of lectins. Food cooking or applications as such before cooking are proved effective in their digestibility. The volume of water used in the cooking process, the properties of the food to be cooked, the temperature used in cooking and the cooking methods are the factors contribute to the nutritional content (Larrosa et al., 2015; Yu-Wei and Wei-Hua, 2013;). According to Van der Poel, (1990), the starch digestibility is attributed to the swelling and tearing of starch granules, the separation of various bean components during cooking and the inactivation of α-amylase inhibitors. The inhibitors in the structure of heat sensitive proteins can inactivate anti nutritional agents such as lectins and various volatile compounds during the process of cooking. It is known that trypsin inhibitors contained in pods, chickpeas and lentils, which are kept in 121˚C degree hot water for 30 minutes, are completely suppressed. A significant decrease was observed in proteolytic enzyme inhibitory activities of beans prepared by the fast cooking method. In this context, the aim of our study is to develop non-lectin food products and compare them with similar food products consumed in daily life.

2. Materials and methods

The ingredients used in this study are; almond flour which was milled in Datça region Village Products Company and Coconut Flour, which was obtained from a chain grocery. End products are classified as follows:

- Product A, cookies produced with lectin-free and gluten-free almond flour with tahini and walnut,
- Product B, cookies produced with lectin-containing white flour with tahini and walnut,
- Product C, cheese-herbal sesame bagels produced with lectin-free and gluten-free almond flour,
- Product D, cheese herbal sesame bagels produced with lectin-containing white flour,
- Product E, cake produced with lectin-free and gluten-free almond and coconut flour,
- Product F, cake produced with lectin-containing white flour.

The study was conducted with a total of 30 healthy volunteers (15 females and 15 males) aged between 35-45 years old and selected randomly using the free panellist method (Kilcast, 2010). In the experiment, 30 panellists were informed about panel questions first. In this study, the volunteers have been served with both packaged, lectin-rich products and similar lectin-free products obtained from the market. Experiments were performed using a completely randomized design. Data were subjected to one-way analysis of variance (ANOVA). Mean separations were performed using Tukey's least significant difference (LSD) procedure in Matlab 7.12.0 (R2011a) software (MathWorks Inc., 130 Natick, MA, USA). Differences at $p < 0.05$ were considered significant (Tarlak et al., 2020).

Humidity content, dry matter percentage, carbohydrate percentage, protein percentage, ash percentage, dietary fibre percentage, energy value, fat percentage, and gluten content were

also analysed using proper standard methods and products were compared.

3. Results and discussions

Table 1 and Table 2 show the participants' evaluation of the products A and B, C and D, E and F in terms of differences, and the results they found to be different in terms of flavour. While 50% (15 people) stated that there was a taste difference between A and B cookie products, the remaining 50% (15 people) stated that there was no difference. In addition, 80% of 15 people (12 people) stated that the A product was richer in flavour. 20% (3 people) think that B is more delicious in terms of flavour.

While 63.3% (19 people) stated that there was a taste difference between C and D products, 36.7% (11 people) stated that there was no difference between the two products. In addition, while 63.2% (12 people) stated that C product was more delicious than D product, 36.8% (7 people) stated that they had the opposite opinion and said that the white flour cheese-herb sesame product was more delicious.

90% (27 people) stated that there was a difference in flavour between E product and F products, while 10% (3 people) stated that there was no difference between the two products. 74.1% (20 people) of the 27 people who stated that there was a difference between the two products stated that the E product was richer in flavour. 25.9% (7 people) think that F is more delicious in terms of flavour.

People seem to think that lectin-free and gluten-free products are more delicious in flavour. Individuals participating in the study think that the shape of the products produced with lectin-free and gluten-free ingredients is smooth and round, the shell is smooth and crisp, the volume is light and proportionate, the texture is elastic and light, the pores are smooth and thin-walled and the product colour is in accordance with the ingredient material used. Mert et al. (2015) has investigated the attributes of different flour varieties on gluten-free wafer leaf quality. In his study where wafer layers' texture profiles were assessed with regard to rigidness, only rice corn-based samples and corn wheat products have more rigidity compared to others. Romero, 2017, in their research on the viscoelastic properties of the gluten-free pasta, it was concluded that dough mixture with the addition of casein has been lighter than the egg white color and the redness-yellowness values have decreased. Furthermore, when XG (Xanthan Gum) and KMS (carboxymethyl cellulose) were used as gum, they reached a structure closer to the desired level when making pasta. Turkut et al. (2015), in his research on dough structure and product properties in sour yeast and gluten-free bread production, concluded that added quinoa flour increased viscosity, elastic modulus (G') and viscous modulus values. The addition of kinoa and buckwheat increased the protein value of the breads, decreased the brightness value of the bread and crust, and increased the redness value ($p < 0.05$). Yildiz and Dogan (2014), in their study of the effects of different formulation, baking and storage times on gluten-free cake quality, found out that cakes produced by partial baking method had higher inner property scores, whereas outer property scores, specific volumes, hardness and chewability values were lower. It was concluded that there was no statistical difference between fresh cakes prepared for each cake and full or partially cooked cakes, considering all of the cake characteristics. Martinescu et al. (2020) have concluded that gluten-free flour and almond flour cookies are more favourable than other cookies with respect to their appearance, flavour, texture and smell, in their study based on the averages. The purchase intention also confirms this appreciation. As a result of cookies made with gluten-free flour and almond flour ($n = 47$), it is observed that cookies made with gluten-free flour and almond flour are more preferred in terms of purchase intention.

Tests were carried out in the accredited food analysis laboratory to evaluate the products' nutritional properties with different contents that we designed and produced. The product results in terms of humidity, dry matter percentage, carbohydrate percentage, protein percentage, ash percentage, dietary fibre percentage, energy value, fat percentage, and gluten content are shown in Table 3.

There was significant difference ($p < 0.05$) between A, C and E products which are lectin and gluten free and B, D and F commercial products which are including lectin and gluten in terms of carbohydrate percentage, protein percentage and dietary fibre percentage. A, C and E products have higher protein percentage and dietary fibre percentage and lower carbohydrate percentage than B, D and F. Especially dramatic increasing in dietary fibre percentage is led to considered healthy food. Additionally, lower carbohydrate percentage and higher protein contents obtained for A, C and E products increase nutrient property.

Table 1. Participants' perceptions when compared; A and B, C and D, E and F products in terms of differences and taste evaluation.

		YES	NO	Which is more delicious?							
		# of Subjects	Percentage	# of Subjects	Percentage	Product	# of Subjects	Percentage	Product	# of Subjects	Percentage
1	**Is there a difference between A and B?**	15	50%	15	50%	A	12	80%	B	3	20%
2	**Is there a difference between C and D?**	19	63,3%	11	36,7%	C	12	63,2%	D	7	36,8%
3	**Is there a difference between E and F?**	27	90%	3	10%	E	20	74,1%	F	7	25,9%

Table 2. Total Quality and Sub-parameter Score Averages of Participants who have tried A, C and E Products.

	Shape	Shell	Volume	Texture	Pores	Color	Taste	Total Average
A Product	8.67	8.70	8.73	8.13	8.43	8.37	36.77	87.80
C Product	8.10	8.00	8.27	8.20	8.30	8.77	35.63	85.27
E Product	8.83	8.77	8.83	8.90	9.07	8.87	36.97	90.23

Table 3. Nutritional content analysis result of the product.

Analyses	Products						Method
	A product	B product	C product	D product	E product	F product	
Moisture (%)	35.6	47	17.65	49.1	9.89	44.5	AOAC 925.10 (2005)
Dry Matter (%)	64.4	53	82.32	50.9	90.11	61.5	AOAC 925.10 (2005)
Carbohydrate (%)	8.78	28.24	0.58	29.10	31.28	60.9	FAO (2003)
Protein (%)	10.35	5.15	24,14	4.54	18.56	7.9	NMKL 6 (2003)
Ash (%)	4.09	0.96	24.14	0.85	2.38	5.4	NMKL 173 (2005)
Dietary Fibre (%)	11.55	1.35	6.69	0.84	11.10	5.2	NMKL 129 (2003)
Energy (kcal/100g) – (kj/100g)	366 - 1530	292 - 1220	404 - 1692	276 - 1155	462 - 1913	323 - 1352	FAO (2003)
Fat Content (%)	29.63	17.3	30.02	15.57	26.79	32.4	NMKL 160 (1998)
Gluten Control	Not detected	detected	Not detected	detected	Not detected	detected	ISO 21415–1 (2006)

4. Conclusions

In our study; it was concluded that women had more interest in gluten-free and lectin-free products than men. When evaluated according to the gender as perception of taste and interest; while women were more interested in gluten-free and lectin-free products than men ($p < 0.05$), there was no difference in flavour scores ($p > 0.05$). There was no gender-related differentiation between almond and coconut flour lectin-free and gluten-free cake and white flour cake. It was concluded that the changes in taste and nutritional quality of the products have a significant effect ($p < 0.05$) on panellists choosing. Our intention is to contribute to the literature with our study, due to the limited number of studies in this specific area. It is highly recommended to conduct more scientific studies within this context and to increase the efforts to develop alternative products.

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HEAT STABILITY OF ANTIBIOTICS RESIDUES IN MILK

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ABSTRACT

The main aim of study was to assess heat stability of antibiotics residues in the milk. Milk samples were divided into five equal parts and encoded with A, B, C, D and E. Samples encoded A were spiked with penicillin G, B with oxytetracyline, C with gentamycin and D with sulfonamide each of at 500, 750 and 1000 ppm concentration. Samples encoded E were kept as control and no antibiotic was spiked. All the spiked and control samples were sub-divided into four parts. Spiked and control samples under part one, two and three were observed for the effect of heat treatments (60 °C for 15 sec, 65 °C for 30 min and 110 °C for 10 min) on the stability of antibiotics, while samples under part four were kept as non-heated for comparison purpose. Results showed mean recovery of sulfonamide (81.73 ± 1.44) significantly higher than penicillin G (80.63 ± 0.92%), gentamycin (76.32 ± 1.29) and oxytetracycline (73.94 ± 1.56%) from spiked milk samples (65 °C) compared of non-heated antibiotics spiked milk samples. The mean recovery for sulfonamide residues (64.48 ± 1.41%) was remarkably ($P < 0.05$) higher compared to residues of penicillin G (62.93 ± 1.24%), oxytetracycline (58.90 ± 1.13%) and gentamycin (40.00 ± 1.26%) from the heated (110 °C) antibiotics spiked milk compared to non-heated spiked milk samples with similar drugs (13.80 ± 1.28, 26.27 ± 0.79, 18.43 ± 1.04, 15.60 ± 1.39 mm). On the basis of findings of this study it could be concluded that oxytetracyline, gentamycin and penicillin G residues in milk are significantly ($P < 0.05$) reduced with the pasteurization (at 65 °C) and sterilization (at 110°C). Further, sulfonamide is more thermo stable and oxytetracycline is the less at 110 °C. The prevention of antibiotics residues in milk and heat applications have strong correlation with each other.

1.Introduction

Milk is a very nutritious food with higher level of carbohydrates, protein, fats, vitamins and minerals. It can be associated with health risks to consumers due to presence of zoonotic pathogens and antimicrobial drug residues. Consumers are much conscious about their safe food supply, which should be free from contamination, herbicides, pesticides, drugs and antibiotics, though otherwise may create severe health hazards, allergic reactions, carcinogenicity and bacterial resistance (Abbas *et al*. 2013). The misuse of antibiotics in animals lead to the development of resistant bacterial strains, and their transmission to human beings result significantly reduced

efficacy of antibiotics. The antibiotics residues in milk are of great concern for dairy farmers, milk processors, consumers and regulatory agencies. The inactivation of these antibiotics residues is utmost important due to possible development of antibiotics resistance in humans (Berends *et al*. 2001).

Antibiotics are commonly used in domestic animals for the prevention and treatment of diseases. They are also used as growth promoters for enhancing the production of food producing animals. Excessive or improper use of antibiotics is of great concern due to appearance of residues in the milk (Shetandi and Sternesjo 2004). Antimicrobial residues in milk may pose potential health threats to the consumers in form of antibiotics resistance and allergies. They also influence the dairy industry by impairing the bacteriological processes used for manufacturing of dairy products (Conesa *et al*. 2008). The presence of antimicrobial substances in milk even in low concentrations is one of the main concerns of milk industry, as it poses risk of toxicity to public health, and can seriously influence the technological properties of milk and dairy products. For example, in the production of fermented milk products such compounds inhibit the growth of starter cultures (Jones 2008). Concentration of 1 ppb delays the starter activity and decreases the acid and flavors the production during yoghurt or butter making, and also causes improper ripening of cheese. In past studies, heat treatment has been applied to reduce the level of antibiotics residues in milk (Khopaibool 2015). However, other study showed that antibiotics residues may not be totally destroyed under normal cooking procedures (Ghidini *et al*. 2002). The control of residues of veterinary drugs in food producing animals and animal products has been a cornerstone of the present agricultural and food policies for providing assurance to consumers about the safety and wholesomeness of their food (Moats 2007). In order to be safeguard human health, many countries have set up maximum residue limits (MRLs) of some antibiotics in milk. Milk samples exceeding prescribed MRL for penicillin G 4 μg/l, sulfonamide MRL 100 μg/l and oxytetracyline 100 μg/l, must be excluded from human consumption (Brady 2016). Failure to adhere these recommended periods has been reported to be the primary cause of violate levels of veterinary drugs in food (KuKanich *et al*. 2005).

Present study was therefore planned in order to observe the extent of antibiotics residues in the market milk and assess influence of various heat treatments on stability of antibiotics residues in milk.

2.Materials and methods

The present study was conducted during the year 2018, whereby antibiotics free milk samples were collected from the local markets of Sindh province and brought to the laboratory of department of Animal Products Technology for analysis purpose.

2.1.Preparation of milk samples

Milk samples were divided into five equal parts and encoded as A, B, C, D and E. Samples encoded A were spiked with penicillin G (Amino-Vet, manufac. ICI, Pakistan), B with oxytetracyline (Oxytetracycline, manufac. ICI, Pakistan), C with gentamycin (Refobacin, manufac. ICI, Pakistan), D with sulfonamide (Trisolizin, manufac. ICI, Pakistan) each of at concentration of 500, 750 and 1000 ppm (AOAC 2000)[11], however samples encoded E were kept as control and no antibiotics were spiked. All the spiked and control samples were again sub-divided into four parts. Spiked and control samples under part one, two and three were observed for the effect of heat treatment (thermization; 60 °C for 15 seconds, pasteurization; 65 °C for 30 min and sterilization; 110 °C for 10 min) on the stability of antibiotic residues, while under part four samples were kept as non-heated for comparison purpose. Turbidity test was used in order estimate microbial growth.

2.2.Preparation of media and isolation of *Bacillus subtilis*

Nutrient agar was used against *Bacillus subtilis* [(Ehrenberg) Cohn, ATCC® 6051™]. Petri dishes containing nutrient agar were inoculated with test *Bacillus subtilis*. Milk samples were streaked on nutrient agar and petri dishes were incubated at 35°C under aerobic condition for 24 hours. Isolated colonies of *Bacillus subtilis* were picked-up and kept on clean glass slides. Gram staining was used for morphological characteristics of the isolates. The plates with purified colonies were stored in cryotube for further use. Moreover, purified colonies were picked from nutrient agar and inoculated in nutrient broth (HiMedia M002-100G Nutrient Broth, USA). Broth was incubated (36 ± 1 °C) for 24 hours. Before screening of test samples, this medium (0.1 ml) was mixed with soft agar (3 ml) and spread over nutrient agar (DeJonghe *et al*. 2010).

2.3.Screening of milk samples for the presence of antibiotics residues

A total of 10 samples were prepared and processed for assessing the effect of different heat treatments on stability of penicillin G, oxytetracyline, gentamycin and sulfonamide residues in milk. The blank disc of filter papers (Whatman 1, 12 mm, CAT No. 1002-125, Bharat Instruments & Chemicals Company, India) were completely dipped into each antibiotics spiked and control milk samples using forceps, and placed on the surface of agar medium containing the sensitive test organism (*Bacillus subtilis*). The plates were incubated (36 ± 1 °C) for 24 hours. The positive results (the presence of antibiotic residues) were manifested by formation of transparent zones around the disc and compared with control group. The zone size around each positive sample was measured with help of Vernier caliper. Since no any zone was appeared in control samples (no addition of antibiotics), the data on the zone size of heated and non-heated spiked milk samples were gathered for comparison purpose.

2.4.Statistical analysis

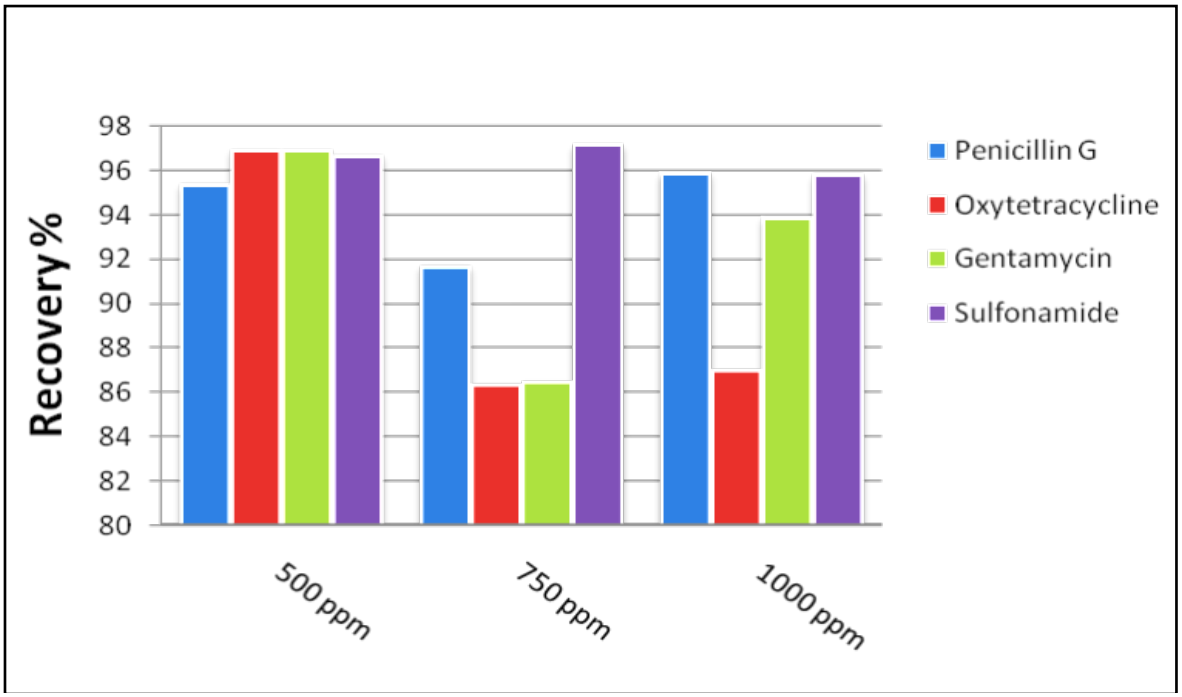
A computerized statistical package i.e. Student Edition of Statistix (SXW), Version 8.1 (Copyright 2005, Analytical Software, USA) was applied to assess the data. Statistical procedure of completely randomized analysis of variance (ANOVA) under linear models was used to observe the significant variations among the variables, and in case of the significant differences found among the means, the least significant difference (LSD) test was applied (Gomez and Gomez 1984). Difference was considered significant at ($P < 0.05$).

3.Results

In present study, the stability of antibiotics residues in milk on different heat treatments was assessed. The results obtained are given in the subsequent sections.

3.1.Penicillin G

It was observed that the zones size of penicillin G spiked (500 - 1000 ppm) milk samples were significantly ($P < 0.05$) reduced when heated at 60, 65 and 110 °C compared to non-heated spiked (penicillin G) milk samples. The average recovery percentages of penicillin G spiked milk samples with concentration of 500, 750 and 1000 ppm heated at 60 °C were 95.36, 91.67 and 95.86% (reduction level) compared to non-heated (23.60, 26.40 and 28.80 mm) spiked milk samples with similar concentrations (Figure 1).



LSD (0.05) = 3.28, S.E = 1.61 (750 ppm)
LSD (0.05) = 3.53, S.E = 1.74 (1000 ppm)
Data are the average of ten samples and each in duplicate

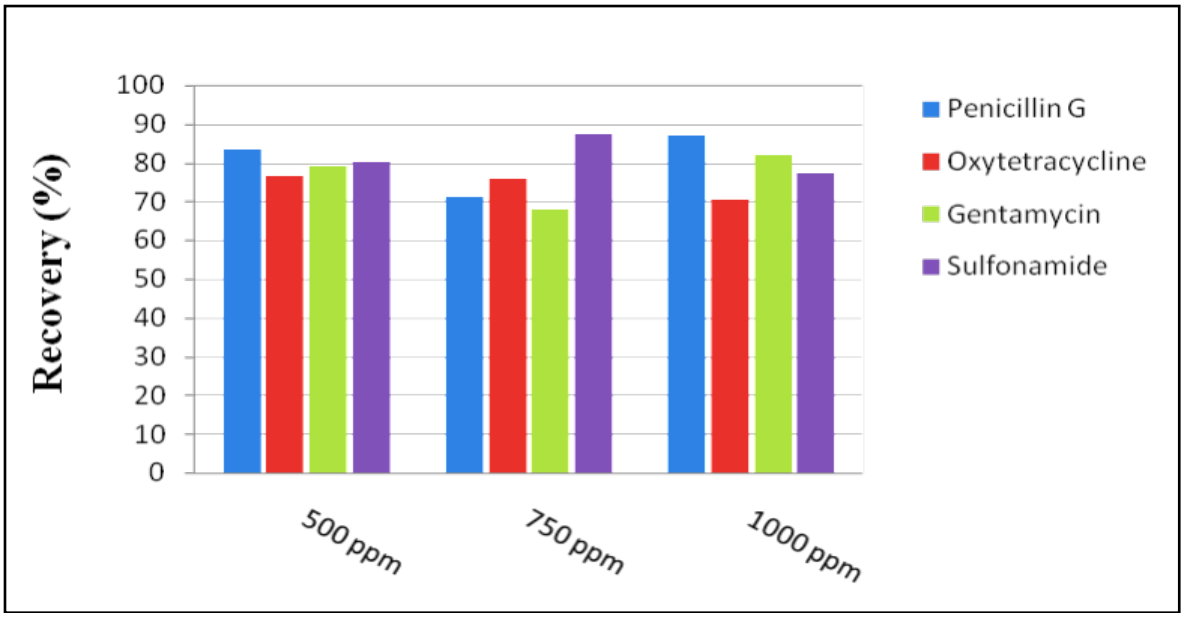
Figure 1. Recovery (%) of antibiotics (penicillin G, oxytetracyline, gentamycin and sulfonamide) residues from spiked milk (500, 750, 1000 ppm) heated at 60 °C for few seconds.

The recovery percentages in zones size of penicillin G spiked (500, 750 and 1000 ppm) milk samples heated at 65 °C were found 83.53, 71.22 and 87.16%, respectively with overall reduction of 16.47, 28.78 and 12.84%, respectively as shown in Table 1. However, at 110 °C recovery percentages in zone size were recorded 73.31, 64.06 and 51.41%, respectively (Figure 2) with average reduction of 26.69, 35.94 and 48.59% (Table 1). Mean reduction percentage in zone size of penicillin G spiked (500 - 1000 ppm) samples was highest (37.07%) at 110 °C followed by at 65 °C (19.36%) and at 60 °C (5.70%) compared to zone size of non-heated penicillin G spiked milk samples (500 - 1000 ppm) as shown in Table 2.

3.2.Oxytetracycline

In the present study, average zones size of oxytetracycline spiked milk samples (500, 750 and 1000 ppm) were detected as 12.60, 16.00 and 18.20 mm, respectively. The zones size significantly ($P < 0.05$) reduced in oxytetracycline spiked milk samples heated at 60, 65 and 110 °C. The average recovery levels in oxytetracycline spiked milk (with concentration of 500, 750 and 1000 ppm) were 96.91, 86.31 and 86.96% with average reduction level of 3.09, 13.69 and 13.04%, respectively at 60 °C (Figure 1 and Table 1). Antibiotics residues in oxyteracycline spiked milk heated at 65 °C were observed as 76.44, 75.84 and 70.36% with reduction in zones size of 23.56, 24.16 and 29.64%, respectively (Figure 2). However, at 110 °C residues reduced from 58.86, 60.68 and 57.77% to 41.14, 39.32 and 42.23%, respectively compared to non-heated oxytetracycline spiked milk samples (12.60, 16.00 and 18.20 mm) with similar concentration level (Figure 3 and Table 1). The average zone size of oxytetracycline spiked (500 - 1000 ppm) milk samples heated at 110 °C was reduced up to 41.10%. Sample heated at 65 °C and 60 °C showed reduction in zones size up to 26.06 and

10.18%, respectively compared to average zone size of non-heated oxytetracycline spiked milk samples with similar concentration (15.60 mm) as tabulated in Table 2. Present results suggested that heating of milk up to 110 °C does not completely eliminate oxytetracycline residues level.

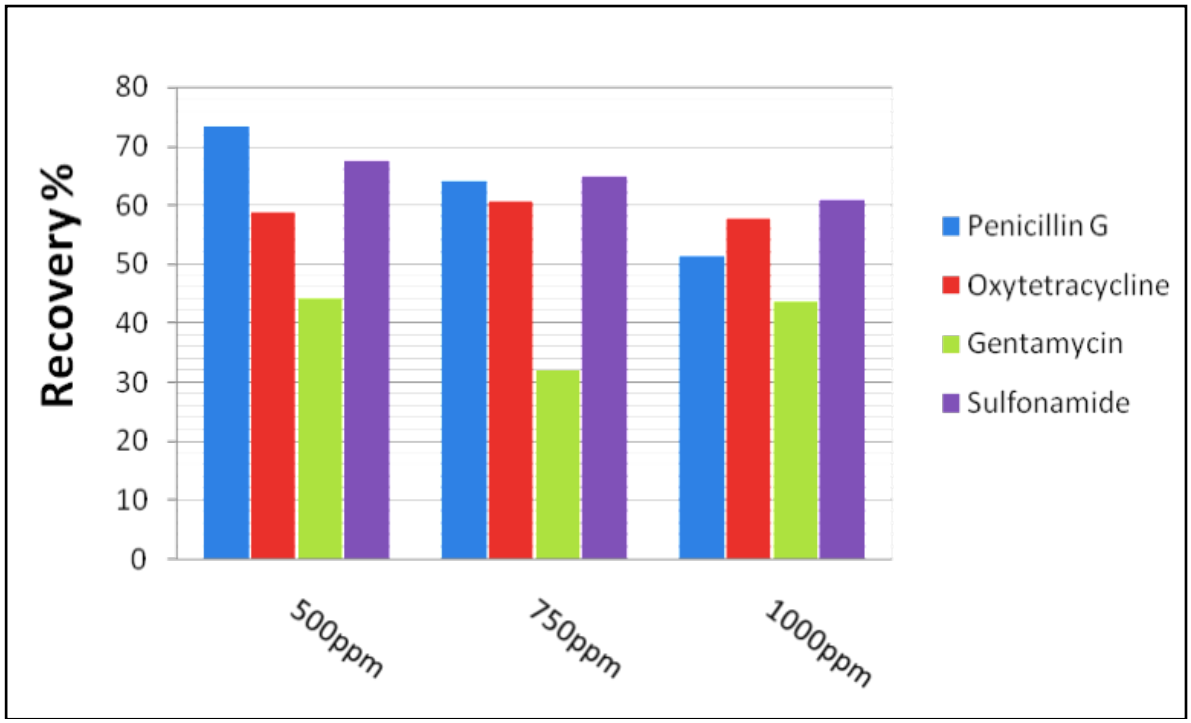


LSD (0.05) = 4.91, S.E = 2.42 (500 ppm)
LSD (0.05) = 3.72, S.E = 1.83 (750 ppm)
LSD (0.05) = 2.92, S.E = 1.44 (1000 ppm)
Data are the average of ten samples and each in duplicate

Figure 2. Recovery (%) of antibiotics (penicillin G, oxytetracyline, gentamycin and sulfonamide) residues from spiked milk (500, 750, 1000 ppm) heated at 65 °C for 30 min.

3.3.Gentamycin

Average zones size of non-heated gentamycin spiked milk samples (500, 750 and 1000 ppm) in the present study appeared as 15.80, 18.40 and 21.10 mm, respectively (Table 1). The zones size were remarkably ($P < 0.05$) reduced in gentamycin spiked milk samples heated at 60, 65 and 110 °C. The average recovery percentages of antibiotics residues from gentamycin spiked milk (500, 750 and 1000 ppm) at 60 °C appeared 96.87, 86.43 and 93.87% with decreased zone size of 3.13, 13.57 and 6.13% (Figure 1). The recovery percentages of residues from gentamycin spiked milk samples heated at 65 °C revealed as 79.20, 67.98 and 82.07% with zone size reduction 20.80, 32.02 and 17.93% (Figure 2 and Table 1). Whereas spiked milk heated at 110 °C (Figure 3) revealed recovery of antibiotics residues 44.33, 32.08 and 43.61% with zones size reduction up to 55.77, 67.92 and 56.39%, respectively compared to non-heated gentamycin spiked milk samples (500, 750 and 1000 ppm). The average reduction in zones size of gentamycin spiked milk samples (500 - 1000 ppm) heated at 110 °C was highest (60.03%) followed by heated at 65 °C (23.58%) and at 60 °C (7.61%) as shown in the Table 1.



LSD (0.05) = 3.96, S.E = 1.95 (500 ppm)
LSD (0.05) = 4.17, S.E = 2.05 (750 ppm)
LSD (0.05) = 3.01, S.E = 1.48 (1000 ppm)
Data are the average of ten samples and each in duplicate.

Figure 3. Recovery (%) of antibiotics (penicillin G, oxytetracyline, gentamycin and sulfonamide) residues from spiked milk (500, 750, 1000 ppm) heated at 110 °C for 10 min.

3.4.Sulfonamide

Sulfonamide spiked milk (500, 750 and 1000 ppm) heated at 60 °C appeared as 96.66, 97.14 and 95.77% (decreased to 3.34, 2.86 and 3.33%) of total non-heat zone size respectively (11.60, 13.40 and 16.40 mm) (Figure 1 and Table). The recovery residues percentages were 80.34, 87.47 and 77.42% and that reduced to 19.66, 12.53 and 22.58% when heated at 65 °C. At 110 °C observed residues recovery percentage 67.44, 65.00 and 61.00% reduced to 32.56, 35.00 and 39.00%, respectively (Figure 2 and Figure 3). The mean reduction level in zones size of sulfonamide spiked milk (500 - 1000 ppm) samples was higher at 110 °C (32.52%) and lower at 60 °C (3.48%) with intermediate level of 18.25% at 65 °C compared to mean zones size of non-heated sulfonamide spiked milk samples (13.80 mm) as summarized in Table 2. Findings further indicate that thermization process (60 °C for 15 seconds) has no significant ($P > 0.05$) effect on the reduction of residues in sulfonamide contaminated milk. While sterilization process (110 °C for 10 min) reduces up to 50% level of residues in sulfonamide spiked milk. Severe heating is required for complete reduction of sulfonamide residues from milk.

Table 1. Effect of various heat treatments on antibiotics (penicillin G, oxytetracycline, gentamycin and sulfonamide) spiked milk samples (500, 750 and 1000 ppm)

Concentration	Zone size of penicillin G (mm) 500 ppm				Zone size oxytetracycline (mm) 500 ppm			
	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)
Maximum	24.00	23.00	20.00	18.00	14.00	13.00	11.00	8.00
Minimum	23.00	22.00	19.00	16.00	12.00	11.00	9.00	7.00
Mean	23.600	22.500	19.700	17.300	12.60	12.200	9.600	7.400
SE±	0.163	0.163	0.152	0.213	0.221	0.2000	0.221	0.163
Concentration	**Zone size of penicillin G (mm) 750 ppm**				**Zone size oxytetracycline (mm) 750 ppm**			
	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)
Maximum	27.00	25.00	20.00	19.00	17.00	15.00	13.00	10.00
Minimum	26.00	23.00	18.00	15.00	15.00	13.00	11.00	9.00
Mean	26.400	24.200	18.800	16.900	16.00	13.800	12.00	9.700
SE±	0.163	0.249	0.249	0.504	0.210	0.2000	0.210	0.152
Concentration	**Zone size of penicillin G (mm) 1000 ppm**				**Zone size oxytetracycline (mm) 1000 ppm**			
	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)
Maximum	29.00	28.00	26.00	16.00	19.00	17.00	14.00	10.00
Minimum	28.00	27.00	24.00	14.00	17.00	15.00	12.00	11.00
Mean	28.800	27.600	25.100	14.800	18.200	15.800	12.800	10.400
SE±	0.133	0.163	0.233	0.200	0.2000	0.2000	0.2000	0.163
Concentration	**Zone size of gentamycin (mm) 500 ppm**				**Zone size sulfonamide (mm) 500 ppm**			
	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)
Maximum	16.00	16.00	13.00	8.00	13.00	12.00	10.00	8.00
Minimum	15.00	15.00	12.00	6.00	11.00	10.00	9.00	7.00
Mean	15.800	15.300	12.500	7.00	11.600	11.300	9.300	7.800
SE±	0.133	0.152	0.166	0.210	0.221	0.213	0.152	0.133
Concentration	**Zone size of gentamycin (mm) 750 ppm**				**Zone size sulfonamide (mm) 750 ppm**			
	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)
Maximum	19.00	17.00	13.00	7.00	13.00	12.00	10.00	8.00
Minimum	18.00	15.00	12.00	5.00	11.00	10.00	9.00	7.00
Mean	18.400	15.900	12.500	5.900	11.600	11.300	9.300	7.800
SE±	0.163	0.179	0.166	0.233	0.221	0.213	0.152	0.133
Concentration	**Zone size of gentamycin (mm) 1000 ppm**				**Zone size sulfonamide (mm) 1000 ppm**			
	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)	Non heated	Heated (60 °C)	Heated (65 °C)	Heated (110 °C)
Maximum	22.00	21.00	18.00	10.00	17.00	17.00	14.00	11.00
Minimum	20.00	19.00	17.00	8.00	16.00	15.00	12.00	9.00
Mean	21.100	19.800	17.300	9.200	16.400	15.800	12.700	10.00
SE±	0.179	0.249	0.152	0.249	0.163	0.200	0.213	0.210

Table 2. Effect of various heat treatments on antibiotics (Penicillin G, Oxytetracycline, Gentamycin and Sulfonamide) spiked milk samples (500 - 1000 ppm)

Heat treatment at 60 °C					
Antibiotics	No heat zone size (mm)	zone size (mm)	Recovery (%)	Reduction (%)	Standard error
Penicillin G	26.27	24.76	94.29	5.76	0.79
Oxytetracycline	15.60	13.93	90.0	10.18	1.39

			6		
Gentamycin	18.43	17.00	92.39	7.61	1.04
Sulfonamide	13.80	13.34	96.52	3.48	1.28
Heat treatment at 65 °C					
Antibiotics	No heat zone size (mm)	zone size (mm)	Recovery (%)	Reduction (%)	Standard error
Penicillin G	26.27	21.20	80.63	19.36	0.92
Oxytetracycline	15.60	11.47	73.94	26.06	1.55
Gentamycin	18.43	14.10	76.32	23.58	1.29
Sulfonamide	13.80	11.23	81.73	18.25	1.44
Heat treatment at 110 °C					
Antibiotics	No heat zone size (mm)	zone size (mm)	Recovery (%)	Reduction (%)	Standard error
Penicillin G	26.27	16.33	62.93	37.07	1.24
Oxytetracycline	15.60	9.17	58.90	41.10	1.13
Gentamycin	18.43	7.37	40.00	60.00	1.26
Sulfonamide	13.80	8.83	64.48	32.52	1.41

3.5.Discussions

Current study was carried out for observing the stability of antibiotics residues in the milk with respect to different heat treatments. It was observed that average recovery percentage of penicillin G spiked milk samples (500, 750 and 1000 ppm) heated at 60 °C was 95.36, 91.67 and 95.86% compared to non-heated spiked milk samples. Recovery percentages of zones size 65 °C were 83.53, 71.22 and 87.16%. However, at 110 °C zones size were recorded 73.31, 64.06 and 51.41%. Mean reduction percentage in zones size of penicillin G spiked (500 - 1000 ppm) samples was highest at 110 °C followed by at 65 °C and at 60 °C compared to non-heated spiked milk samples. Our results are not in consistent with the findings of (Loksuwan 2002) who reported non-significant ($P > 0.05$) reduction in penicillin G spiked milk (500 - 1000 ppm) heated at 65 °C for 30 min. The variation in the results may be due to change in the efficacy of antibiotics with respect to producing company as well experimental site. Change in the results may also because of heat treatment methods used during the research. Our results are supported by another research, where effects of heat treatments on stability of β-lactams in milk were studied. Their results indicated that in sterilization, the heat treatment of 120°C for 20 min leads to high degradation of β-lactam antibiotics including penicillin G. The degradation rates of penicillin remains between 47.6% and 84% (Roca *et al*. 2011). Results obtained by Zorraquino *et al*. (2008) are also supportive to present study. They indicated that pasteurization at 63 °C for 30 min shows low antimicrobial activity loss for penicillin (between 7% and 11%) compared to cephalosporins (between 6% and 18%). Our

results show significant relevance with (Konecˇny 2007), who observed low-heat inactivation percentage (10%) in penicillin G–fortified milk samples heated at 83 °C for 10 min. This percentage increased to 30 and 32% when milk was treated at 70 °C for 30 min and 100 °C for 30 min, respectively. Jacquet and Auxepaules (2012) carried out a low temperature–long-time pasteurization (63 °C for 30 min). They obtained low inactivation percentages for ampicillin (1.7%) and penicillin G (2.6%); values supportive to those that appear in this study. Tropilo (2015), on the other hand, obtained an inactivation percentage of 15.2% in aqueous penicillin solutions at pH 7.0 when heated at 80 °C for 15 min. However, when these solutions were heated to 121 °C for 15 min, the percentage increased greatly to 81.5%. If we compare them to what is obtained using milk samples, then this increase in the inactivation percentage could be attributed to a possible protective effect of the milk's fat content on the antimicrobial molecules. The work of Broggler *et al*. (2015) indicated that if a moderate dose of penicillin (50,000 units) is administered, a significant amount (0.14 units/ ml) is detectable in milk from the treated animal 72 hours later. Further, penicillin has been reported in one of the first reported surveys of antibiotics in market milk which was conducted in New York State in 1951 (Kosikowsky *et al*. 2013). A total of 1,794 samples of fresh, blended and pasteurized whole milk were obtained from dairy plants or route wagons in 36 counties. Six percent of the samples tested contained antibiotics and they were present in the range of 0.05 - 0.1 unit per ml of milk. Penicillin was found most frequently appeared antibiotic in the milk during the spring of the year. The first survey was made on both raw and pasteurized milk obtained from seven of the 16 Food and Drug Administration districts and showed that 3.2% of 94 samples contained penicillin and 1.07% contained bacitracin. These all reports strongly support to our results (Welch 2005; Welch *et al*. 2010).

In present study, the average recovery levels in oxytetracycline spiked milk (500, 750 and 1000 ppm) were observed 96.91, 86.31 and 86.96% with average reduction levels of 3.09, 13.69 and 13.04% at 60 °C. Antibiotics residues at 65 °C were observed 76.44, 75.84 and 70.36%, however at 110 °C residues reduced. The average zones size of oxytetracycline spiked (500 - 1000 ppm) milk samples heated at 110 °C showed reduction up to 41.10%, while at 65 °C and 60 °C reduction was up to 26.06 and 10.18%. Loksuwan (2002) studied the effects of low-temperature long-time (LTLT) pasteurization (63 °C/30 min) on oxytetracycline residues in raw milk. The oxytetracycline residues were in samples with concentration of 100 $\mu g \cdot l^{-1}$ inactivated to such an extent that they could not be detected. The starting oxytetracycline concentrations were found to be dropped by 86.7%. Hsieh *et al*. (2011) studied the effects of the heat treatments on tetracycline thermostability, using double-distilled water as a matrix. They used two different heating temperatures (100 °C, 121 °C) with the same time of exposure (15 min). Their findings showed that higher temperatures (121 °C/15 min) cause tetracycline degradation of up to 99%. At 100 °C the degradation was less extensive, amounting to as little as 54.4%. The results of this study clearly show that the degree of oxytetracycline degradation is temperature dependent and these same results have been observed in our study. In support our study, Hassani *et al*. (2008) set out to determine the thermostability of oxytetracycline in McIlvaine buffer with varying pH value (pH 7.0, 5.5 and 4.0) and ultra-high temperatures ranging from 110 to 140 °C. The results of the study showed that sterilization (118 °C per 30 min and 121 °C per 20 min) reduces the concentration of oxytetracycline to negligible amounts (less than 0.01%). The ultra-high temperature treatment on the other hand, reduces oxytetracycline concentrations by more than 40%. At 135 °C per 15 sec the ultra-high temperature treatment inactivate oxytetracycline residues by 44%. While the sterilization process degrades oxytetracycline in

milk by more than 98%. Few others reported that pasteurization of milk which contained antibiotics did not inactivate chlortetracycline, chloramphenicol, streptomycin and oxytetracycline (Overby 2015). These results are somewhat change from our study. Difference may be related to the variation in heat treatment methods. Shahani (2011) reported a reduction of 9.3, 14.2, 18.4, 22.5 and 25.4 percent in the potency of chlortetracycline in milk which were pasteurized at 143 °F for 10, 20, 30, 40 and 50 minutes, respectively. The loss in potency was 13.5, 20.1, 27.1, 32.5 and 38.2 percent when milk were pasteurized at 160 °F for the same time periods. These results strongly agree with our reports. More recently it has been found that pasteurization at 143 °F for 30 minutes result 30 percent loss of oxytetracycline activity when 0.84 to 1.0 ug of the antibiotics is present per ml of milk. An increase in pasteurization temperature to 160 °F results in a 4 < percent loss in activity after 30 minutes of heating. Oxytetracycline in milk is completely inactivated by heating to 160 °F for 190 minutes, 175 °F for 92 minutes and 185 °F for 60 minutes (Shahani 2009). These results strongly support to our findings regarding oxytetracycline.

Further, the average recovery percentages of antibiotics residues from gentamycin spiked milk (500, 750 and 1000 ppm) at 60 °C appeared 96.87, 86.43 and 93.87% with decrease zones size of 3.13, 13.57 and 6.13%. The recovery percentages of residues at 65 °C were 79.20, 67.98 and 82.07%, whereas, at 110 °C were 44.33, 32.08 and 43.61%. The average reduction in zones size of gentamycin spiked milk samples (500 - 1000 ppm) heated at 110 °C was highest followed by heated at 65 °C and 60 °C. Our findings are in consistent with the study of (Omar and Eltinay 2008). They reported same reduction pattern of gentamycin as investigated in current research. Few other authors reported that heat treatment at 60 °C for 30 min does not inactivate the residues of gentamycin, while classic sterilization (120 °C for 20 min) shows high heat inactivation (> 95%) for the residues of gentamycin.

Sulfonamide spiked milk (500, 750 and 1000 ppm) heated at 60 °C appeared as 96.66, 97.14 and 95.77%. The recovery residues percentages were 80.34, 87.47 and 77.42% at 65 °C, while 67.44, 65.00 and 61.00% at 110 °C. Present findings indicated that thermization process has no significant effect on the reduction of residues in sulfonamide contaminated milk. While sterilization process (110 °C for 10 min) reduces up to 50% level of residues in sulfonamide spiked milk. Our results are supported by (Yassin *et al*. 2015) who stated that N^4 aromatic group of sulfonamide reacts with reducing sugar to form a sugar sulfonamide complex which is more stable to heating at 100 °C. Results are also supported by (Malik 2014), who reported that heat treatment up to 100 °C causes lower reduction of sulfonamide residues. It may be due to binding of sulfamethazine to protein and reducing sugars.

4.Conclusions

On the basis of present findings it could be concluded that thermization (60 °C) poorly reduces antibiotics residues in milk. However penicillin G, oxytetracyline and gentamycin residues in milk are considerably reduced with the pasteurization (65 °C) and sterilization (110 °C). Thermostability of sulfonamide residues remains higher compared to penicillin G, oxytetracyline and gentamycin residues. It was further concluded that complete elimination of antibiotics residues from milk by thermization, pasteurization and sterilization is not possible, however residues levels and their efficacy can be reduced. It is suggested to boil the milk at higher temperature in order to reduce the concentration as well as efficacy of antibiotics residues in milk, though otherwise may develop antimicrobial resistance in the consumers.

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CHITOSAN/CUMIN (*CUMINUM CYMINUM* L.) ESSENTIAL OIL EDIBLE BIODEGRADABLE COATING: ITS EFFECT ON MICROBIAL, PHYSICAL AND SENSORY PROPERTIES OF CHICKEN MEAT DURING REFERIGERATION

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ABSTRACT

Chicken meat is a popular food around the world due to its high nutrient content, low fat content and relatively low cost. Perishable and enrich chicken meat caused it sensitive spoilage and fat oxidation, so reduce the shelf-life of the product. The aim of this study was to investigate the effect of chitosan (Ch) and cumin essential oil (CEO) on the quality and shelf life chicken meat. Ch-CEO coatings were prepared in three treats covered chitosan, cumin essential oil / chitosan and essential oil of cumin 0.2, 0.4 and 0.6%. The microbial tests (Total count, *Enterobacteriaceae*, *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli)*, mold and yeast), the chemical tests (pH, Total volatile nitrogen (TVN), Thiobarbituric acid (TBA), Peroxide value (PV) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and sensory properties were assessed in 2, 5, 9 days. There was a significant difference in microbial load between control and treated samples with Ch-CEO (0.6%). The most antioxidant activity, TBA and PV have been shown to be CEO (0.6%). In all of concentration of CEO, pH and TVN decreased. Sensory properties in treating samples with Ch-CEO were acceptable in the second day, but in final storage period showed significant differences with the control sample. The results show that due to the antioxidant activity of CEO and the high antimicrobial activity of chitosan coating and the synergistic effect of both of them improved of sensory properties and increase shelf life chicken meat at the refrigerator temperature.

1. Introduction

Increasing the shelf life of food products, especially perishable products such as meat and dairy products have an important role in maintaining the quality and safety of food products. For this reason, researchers are looking for new methods and packaging owing to use of chemical preservative reduce the product quality and safety (Azlin-Hasim *et al*., 2018; Lomate *et al.*, 2018). Knowing that synthetic packaging materials derived from petroleum products are widely used in the food packaging due to their lower price, comfortable, extensive availability and desirable characteristics such as, brightness, plasticity and transparency, but they are not suitable in many

aspects and even dangerous. Nevertheless, the main concerns usage of these materials for food packaging includes the environmental pollution, non-degradability and environmental incompatibility, the migration of compounds from packaging to product, which can be endanger for safety of products and consumer health (Tharanathan, 2003). Therefore, finding materials and methods on new packaging, has attracted greatly for researchers. So, edible, biodegradable and friendly environmentally films for coatings are a new attitude (Alizadeh-Sani *et al*., 2020; Bagheri, *et al*., 2019). Edible coatings and films have been proposed as an appropriate packaging due to cheapness, biodegradability, environmental compatibility, nutritional value, renewable potential (Alizadeh-Sani *et al*., 2018; Noshirvani *et al*., 2018). Also, films and edible coatings are suitable carriers for additives and antimicrobial compounds, enzymes, preservatives, etc (Azizi-lalabadi *et al*., 2020; Salari *et al*., 2018; Sani *et al*., 2017). As well as biodegradable coatings and films, mainly made of natural compounds, such as proteins, lipids and polysaccharides alone or in combination with other compounds. Therefore, to use of films and coatings composite will promote achieving coating features beside that the keeping properties of the maintained products (Azlin-Hasim *et al*., 2018).

Chicken as a perishable product, is used throughout the world because of its reasonable cost and its high nutritional values (Chouliara *et al*., 2007). Fresh chicken is mainly stored at a refrigerated temperature and is freshly consumed (2-5˚C). While, microbial decay or oxidative rancidity are the main reasons the spoilage of these products. It will be worthy to improve new processing and packaging solutions to prolong shelf life of the poultry products (Babuskin *et al*., 2014). Hence, it is recommended to apply natural food preservatives such as essential oils, chitosan, nisin, etc. to be assisted in keeping poultry from spoilage and pathogenic microorganisms, because of these compounds have low processing side effects on the products (Petrou *et al*., 2012). So, it is recommended that use the polysaccharides, such as Chitosan (Ch), is considered as an excellent biopolymer, for biodegradable and edible films and coatings composite due to its non-toxic, biodegradable, biocompatible, antimicrobial properties and commonly regarded as a safe food additive (Xu *et al*., 2005). Chitosan is a cationic polysaccharide consisting of (1, 4)-linked-2-amino-deoxy-b-D-glucan, and is the deacetylated form of chitin (Petrou *et al*., 2012; Siripatrawan *et al*., 2012; Yuan *et al*., 2016). Chitosan is recognized as Generally Recognized as Safe (GRAS) by FDA and possess good antimicrobial properties against wide range of microorganisms *(Rhim et al., 2006; Yuan, et al.*, 2016). Also, it has antioxidant activity that prevents of lipid oxidation and acting as a secondary natural antioxidant for product keeping (Yuan *et al*., 2016). Chitosan films are suitable system to be used as active compounds carriers (Rhim *et al*., 2006; Yuan *et al*., 2016). Many studies reported the benefits of Ch have being applied either individually or in combination with other compounds such as essential oils in food systems. Giatrakou *et al*. (2010) extended cooked chicken shelf-life by using of Ch and thyme oil (Giatrakou *et al*., 2010). Also, Vasilatos *et al*. (2013) demonstrated the effects of Ch or rosemary oil, singly or combined, to prolong the shelf-life of turkey meat (Vasilatos *et al*., 2013); while Petrou *et al*. (2012) studied Ch dipping or oregano oil, individually or combined, on modified atmosphere packaged chicken breast meat (Petrou *et al.*, 2012).

With this attitude, EO can be considered as a good additive for production of combined chitosan films. EOs, as a natural additive, have antibacterial, antioxidant, antiviral and antifungal activities (Kedia *et al.*, 2014; Petrou *et al*., 2012; Sani *et al*., 2017). The most important characteristic of an EO is bactericidal or bacteriostatic properties against a broad range of microorganisms and/ or preventing the oxidation process (Ribeiro-Santos *et al*., 2017). Cumin also, is an annual herb that belongs to the family Apiaceae. It used extensively and afterward black pepper, is known as the second

commonly used spice in the world (Kedia *et al*., 2014; Ruby *et al*., 2012). Cumin is native to Iran, Egypt, Turkistan and East Mediterranean, China, India, Morocco, South Russia, Japan, Indonesia, Algeria and Turkey (Ruby *et al*., 2012). CEO seed exhibits antibacterial, antioxidant properties (Jirovetz *et al*., 2005; Kedia *et al*., 2014).

Based on what was said, the application of Ch with CEO, has not been reported to date, in fresh chicken meat. Thus, the purpose of this study was to evaluate the effects of Ch and CEO, applied individually or simultaneous combination use of physicochemical, microbiological and sensory properties of chicken breast meat during refrigeration.

2. Materials and methods

2.1. Chicken meat

Fresh chicken breast fillet meat was purchased from a local poultry processing company. Samples were transferred to the laboratory using insulated polystyrene boxes on ice flasks and then were divided (ca.220 g or 16cm × 8cm for each sample). Chicken meat samples were kept at refrigerated temperature for other tests.

2.2. Preparation of chitosan coating solution

Low molecular weight chitosan powder (MW; 340) with moisture content less than 10% and a deacetylation degree of 75–85% (Manufacturer's data) obtained from crab shells was purchased from Sigma Aldrich company. Chitosan coating was prepared according to Vasilatos *et al*. (2013) method with some modifications (Vasilatos *et al*., 2013). Coating-forming solution of chitosan was prepared by dissolving 1.5 g chitosan powder in 100 mL of glacial acetic acid solution (1% v/v) (as plasticizer) and was stirred 8 h at room temperature (final chitosan concentration was 1.5% w/v) (Siripatrawan *et al*., 2012).

2.3. Preparation of Cumin essential oil

To prepare CEO, about 100 g of powdered cumin seed was placed in a blender containing 500 mL distilled water for 24 h and then was transferred to our hydro-distillation facility. The distillation was performed by Clevenger apparatus for 4 h. The obtained EO dehydrated and dried using anhydrous Na_2SO_4, and then stored in dark glass bottles at 4°C for later use (Oroojalian, Kasra-Kermanshahi, Azizi, & Bassami, 2010). Different concentrations of EO (0.2, 0.4 and 0.6% (v/w) were prepared by stock concentration. Tween 80 (0.1% w/v) was added to the solution as a surfactant to assist EO dissolution in coating forming solution (Peng *et al*., 2013). The solution stirred continually for 20 min at room temperature for better homogenization.

2.4. Preparation of samples

The chicken meat samples were coated with Ch and CEO solutions, singly or in combination. Samples of meat (ca.200 g) were immersed separately and were placed inside sterile packaging pouch, containing 100 mL of Ch solution (1.5% w/v) for 1.5 min. After immersing, the excess solution was drained off on a sterilized rack (incubator) under aseptic conditions. Then, samples packaged into a clean sterile pouch. CEO in various concentration (0.2, 0.4 and 0.6% w/v) was added into the chicken meat samples (0.25 mL of EO into 100 g of chicken meat) (Petrou *et al*., 2012; Vasilatos *et al*., 2013). Finally, the same above method was used for combine Ch and CEO for samples.

2.5. Packaging of samples

Chicken breast meat samples treated with coating solutions individually (~200 g) and were transferred aseptically into the low-density polyethylene pouches. Treatments included the following groups: Blank or control (in the absence of Ch or CEO) , Ch: (samples treated with Ch 1.5% w/v), CEO: (samples treated with cumin oil 0.2, 0.4 and 0.6%), Ch-CEO: (samples treated with combined Ch 1.5% and CEO 0.2, 0.4 and 0.6%). All specimens were stored at the refrigerator temperature during the test period (9 days).

2.6. Microbiological analysis

Chicken meat samples (25 g) were blended with 225 mL of sterile peptone water (0.1%) (Merck, Darmstadt, Germany) in a stomacher bag and homogenised for 3 min. The serial dilution method was applied for microbial test. For microbial analysis, 0.1 mL from serial dilutions of homogenized chicken meats were spread on the surface of agar plates. Total viable counts (TVCs) were determined in Plate Count agar medium (PCA, Merck, Darmstadt, Germany) by incubation for 48-72 h at 30˚C (Giatrakou *et al*., 2010). *Staphylococcus aureus* count was determined in Baird-Parker agar medium (BPA, Merck, Darmstadt, Germany) by incubation for 48 h at 37˚C. To count moulds and yeasts, duplicate 0.1 mL of suitable dilutions were pour–plated on Sabouraud Dextrose agar medium (Merck, Darmstadt, Germany) and incubated at 25˚C for 3-4 days (Siripatrawan *et al*., 2012). *Enterobacteriaceae* were determined by pour-overlay method using Violet Red Bile Glucose (VRBG) agar medium by incubation for 48 h at 37˚C (Merck, Darmstadt, Germany) (Petrou *et al*., 2012; Vasilatos *et al*., 2013).

2.7. Chemical and sensory characteristics

2.7.1. pH

The pH value determined by a pH meter (Kent EIL 7020). About 25 g of chicken meat sample was homogenised with 225 mL of distilled water and the homogenised samples were used for pH estimation (Petrou *et al*., 2012).

2.7.2. DPPH assay

DPPH test is the most commonly used method for measuring antioxidant capacity. 1 mL of the CEO in different concentration was added to 0.5 mL of a standard DPPH (Sigma-Aldrich) methanolic solution. The mixture was shaken and left standing in the dark at room temperature for 30 min. The absorbance of the resulting solution was then measured at 517 nm (Mahdizadeh *et al*., 2020; Rebey *et al*., 2012). The Butylated hydroxytoluene (BHT) (Merck, Darmstadt, Germany) was used as standard and control sample. The capacity scavenging DPPH radical calculated by the following equation:

DPPH scavenging effect (%) = $((A_0 - A_1) / A_0) * 100$

A_0: absorbance of the control

A_1: absorbance of the sample

2.7.3. Peroxide value

Five g of meat samples and 30 mL of acid acetic and chloroform (Sigma-Aldrich) (ratio 3:2) were added to 0.5 mL Potassium iodide (KI) (Merck, Darmstadt, Germany) and was left for 1 min. Titration was performed with sodium thiosulfate ($Na_2S_2O_3$) (0.1 N) until yellow color appeared, and 0.5 mL of starch solution was added to appear purple color. Peroxide value (PV) is characterized as milliequivalents (meq) peroxide oxygen per 1 kg of lipids (Karakaya *et al*., 2011). The peroxide value calculated by the following equation:

$$\mathrm{PV}\left(\frac{\mathrm{meq}}{\mathrm{kg}}\right) = \frac{(\mathrm{S}-\mathrm{B}) * \mathrm{N} * 1000}{\mathrm{W}} \qquad (1)$$

S: The volume of titrant ($Na_2S_2O_3$ standard solution) consumed by sample (mL)

B: The volume of titrant ($Na_2S_2O_3$ standard solution) consumed by control sample (mL)

N: Normality titrant ($Na_2S_2O_3$)

W: weight sample (fat extracted, g)

2.7.4. TBARS assay

The 2-thiobarbituric acid (TBA) assay commonly used to assess lipid oxidation and expressed as mg of malondialdehyde (MDA) per kg chicken meat samples (Xiong *et al*., 2015). Ten grams of the meat sample with 50 mL of distilled water were mixed in a 100 mL tall beaker, then were stirred by a glass bar for several seconds and left for approximately 30 min. The samples were homogenized at high speed as possible for 15 sec by mixer. Then added 20 mL of 20% TBA and was placed for 10 min in ambient condition. Samples filtered through a Toyo filter paper No.42 with suction, and added distilled water until the solution level equals 100 mL. Then, the absorbance of the obtained solution was measured at 532 nm by spectrophotometry (Ultrospec 2000, Scinteck, UK) (Alizadeh-Sani *et al*., 2020). TBA content was expressed as µg MDA per g chicken meat. The ability to lipid oxidation calculated by the following equation:

MDA (µg/g) = E532 * 12.9

2.7.5. Total volatile nitrogen

To determine total volatile nitrogen (TVN), the samples (10 g) were boiled for 25 min with Magnesium oxide (MgO) (2 g), the distilled water and Ammonia (NH_3) were taken up in 0.04 M boric acid (Merck, Darmstadt, Germany) which was back titrated with 0.1 M sulfuric acid (H_2SO_4) (Merck, Darmstadt, Germany) with methyl red as indicator (Alizadeh-Sani *et al*., 2020). The control sample was without chicken meat. TVN value calculated by the following equation:

$$\mathrm{TVN}\left(\frac{\mathrm{mg}}{100\mathrm{g}}\mathrm{sample}\right) = v\,(titrant) * 14 \qquad (2)$$

2.8. Sensory analysis

For the sensory evaluation of the samples, a semi-trained 7-person panel was used (laboratory – trained and postgraduate students). Panellists were asked to assess the odour, appearance, colour and overall acceptance of uncooked chicken meat samples during the storage period. Chicken meat samples were evaluated using a 0-9 ranging score from 9 (highest score) to 1 (lowest score) (Petrou *et al*., 2012).

2.9. Statistical analysis

The statistical analysis was performed using R software. Data were subjected to analysis of nonparametric Kruskal–Wallis one-way analysis of variance. P-values less than 0.05 were considered statistically significant. All tests of this study were performed in triplicate. Results are reported as mean values ± standard deviation (S.D)

3. Results and Discussion

Seven type samples of Ch and CEO (0.2, 0.4 and 0.6% (w/v)) alone or in combination (Ch-CEO at 0.2, 0.4 and 0.6% (w/v)) were prepared and microbial and physiochemical tests were examined.

3.1. Microbiological analysis

All of microbiological results were shown in Figures 1 to 5.

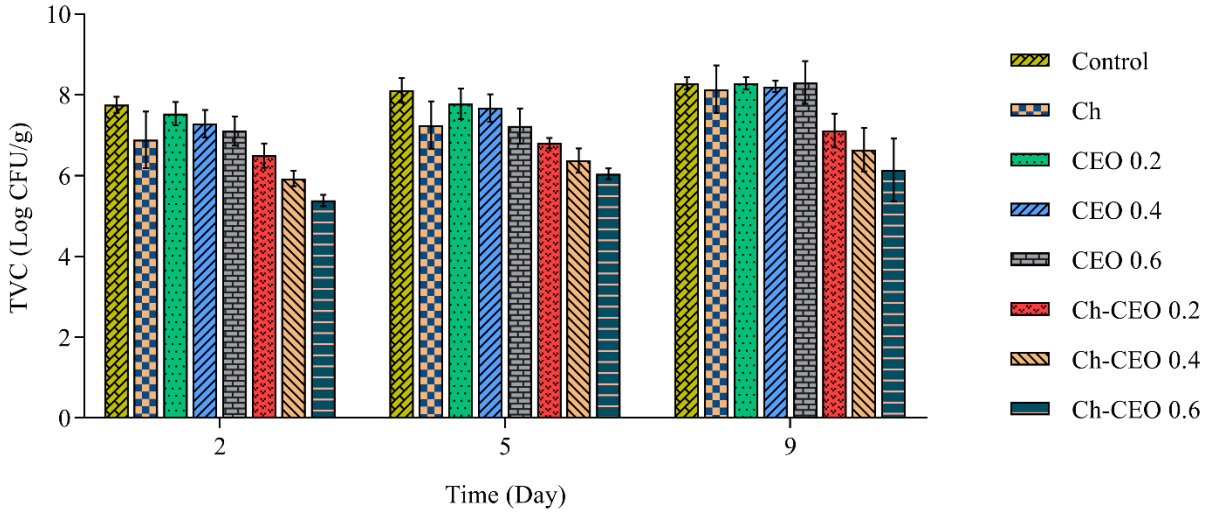

Figure 1. Effect of edible biodegradable coating containing Chitosan and CEO on the total viable counts (TVC) of chicken meat during refrigerated storage.

3.1.1. Total viable count

On the second day, the initial total count was assessed and result was shown 7.8 log cfu/g, increasing the final population during storage up (9 days) to reached ca. 8.3 log cfu/g (Chitosan samples) (Figure 1). These results indicated that

counts for treatments were about 0.5 – 1.3 log cfu/g lower than in the control samples. Data analysis was released a significant difference ($p < 0.05$) for TVC between control samples and wrapped Ch-CEO 0.2, Ch-CEO 0.4 and Ch-CEO 0.6%. TVC values of chicken meat samples exceed of 8.5 log cfu/g, which was considered as the upper acceptability limit for fresh meat on days 9th in control, Ch, CEO 0.2, 0.4 and 0.6% treatments. While samples treated with Ch-CEO 0.4 and Ch-CEO 0.6 never reached the limit value after 7days. Thus, in comparison with control samples, an increase in microbiological shelf life of 9th was achieved for Ch-CEO 0.4 and Ch-CEO 0.6 samples. This shelf life extension of these two groups could be due to the antimicrobial action CEO components (especially, cuminic alcohol) and of Ch, which increases antimicrobial activity (Allahghadri *et al*., 2010).

Recently, in a related study, a 9th microbiological shelf life increment was obtained for a fresh chicken breast meat treated with modified atmosphere (70/30 CO_2/N_2) and oregano oil (0.1%) (Chouliara *et al*., 2007). In other studies, Giatrakou *et al*. (2010) indicated that microbiological shelf life for a ready-to-eat chicken pepper kebab treated by either thyme oil (0.2% v/w) or chitosan (1.5% w/v) increased after 5 days (Giatrakou *et al*., 2010). Siripatrawan *et al*. (2012) reported a reduction of microbial counts by an average of 2.52 log cfu/g for pork sausages treated by chitosan incorporating green tea extract (20% w/v) on day 20th (Siripatrawan *et al*., 2012). Petrou *et al*. (2012) reported a shelf life extension of 5-6 days for a chicken breast meat treated with chitosan 1.5% (w/v) or oregano oil 0.25% (v/w) and modified atmosphere packaging than control samples (Petrou *et al*., 2012). Also, the combined use of chitosan and rosemary oil on the preservation of turkey meat led to a reduction of TVC by 1.0 log cfu/g, extending their shelf life at 2˚C (Vasilatos *et al*., 2013).

Similar to previous studies in present study, among all the treatments, Ch-CEO 0.6 and Ch-CEO 0.4 were the most effective on the growth inhibition of TVC (Figure 1a) in the storage period. Different antimicrobial effects were detected when using an edible coating based on chitosan combined with 0.2, 0.4 and 0.6% (v/w) of CEO against various microbial groups in fresh chicken meat stored at 4˚C. Ch prevents growth and spore germination bacteria due to the absorption of minerals and in particular calcium (Plascencia-Jatomea *et al*., 2003). The use of Ch has been proven to be a very effective way to control the microbial growth rate on chicken meat than the use of the direct addition of CEO.

3.1.2. Enterobacteriaceae count

In our study, *Enterobacteriaceae* (Coliforms), the most important part of the microbial flora of chicken meat with a psychotropic facultative anaerobic bacterial group and final counts reached to ca 8.4 log cfu/g on after 9 days (Figure 2). As previously noted, Ch-CEO 0.2, 0.4 and 0.6% treatments caused a significant reduction in coliform counts (approximately 1.5-2.2 log cycles) compared to control samples on day 9th ($p < 0.05$). Petrou *et al*. (2012) reported that a decrease of microbial counts by an average of 3-4 log cfu/g for chicken breast meat treated with chitosan/oregano oil and modified atmosphere than control samples on day 12th (Petrou *et al*., 2012). Giatrakou *et al*. (2010) showed that Enterobacteriaceae growth in ready to cook chicken product were inhibited by use of chitosan 1.5% w/v and thyme oil 0.2 w/v under aerobic packaging (Giatrakou *et al*., 2010). Chantarasataporn *et al*. (2014) showed that the total Enterobacteriaceae in minced pork control samples significant increase of 5 to 6 log cfu/g during storage while samples containing oligochitosan 0.2 and 0.4% reduced about 1 and 2 log cfu/g (Chantarasataporn *et al*., 2014).

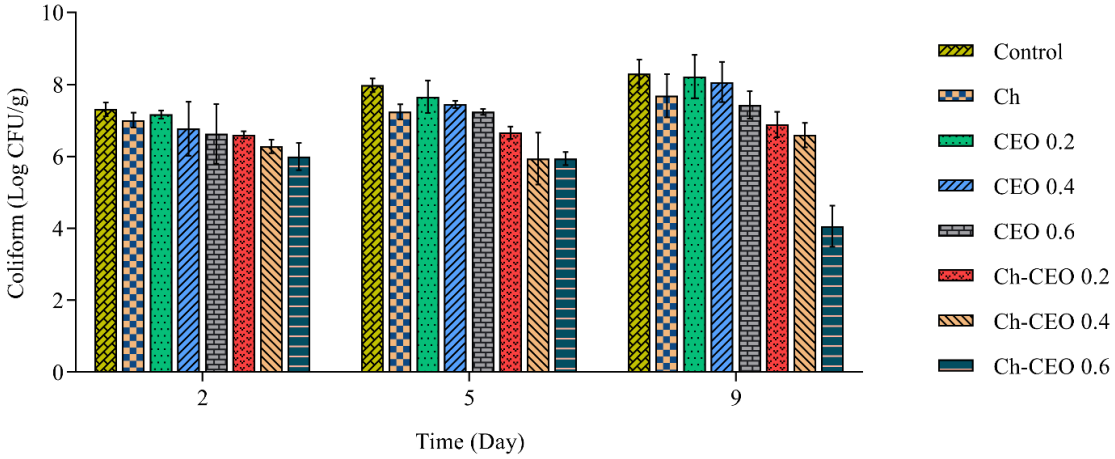

Figure 2. Effect of edible biodegradable coating containing Chitosan and CEO on the Coliforms of chicken meat during refrigerated storage.

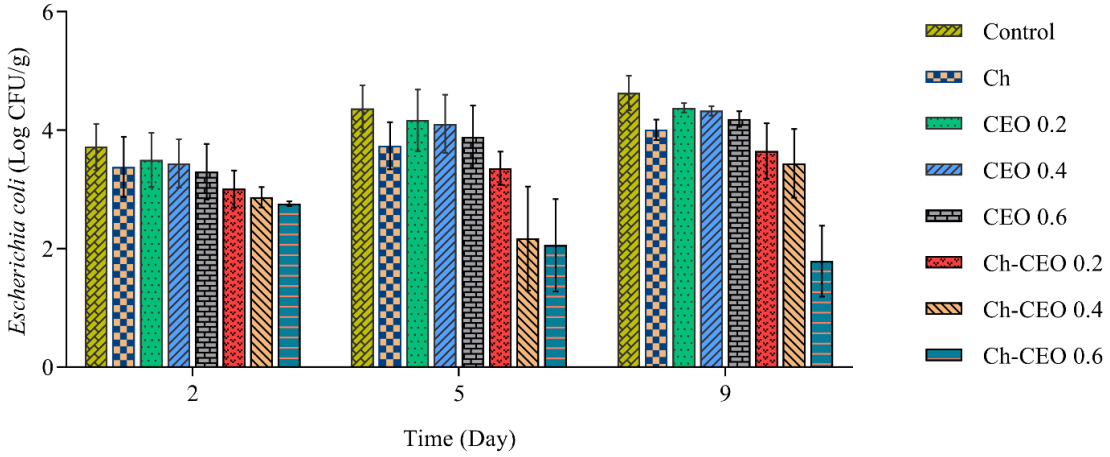

Figure 3. Effect of edible biodegradable coating containing Chitosan and CEO on the *E. coli* of chicken meat during refrigerated storage

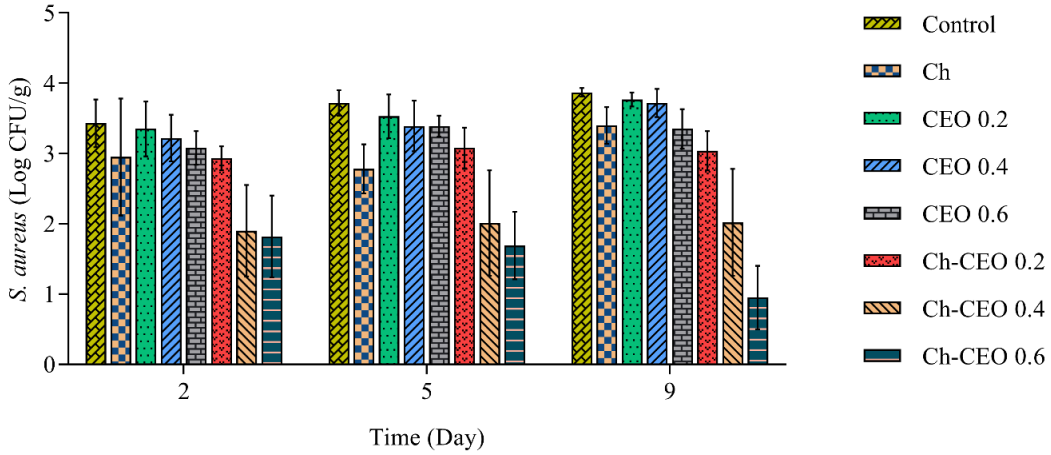

Figure 4. Effect of edible biodegradable coating containing Chitosan and CEO on the *S. aureus* of chicken meat during refrigerated storage.

3.1.3. Escherichia coli count

The initial population of *E.coli* was ca. 3.8 log cfu/g and increased to 4.7 log cfu/g at the end of the storage period (day 9th) (Figure 3).

Although, lower *E.coli* counts ($p < 0.05$) were recorded for Ch-CEO 0.6% samples stored at 4˚C. Of all the antimicrobial treatments in our study, Ch-CEO 0.6% and Ch-CEO 0.4% groups demonstrated to be the greatest effect inhibitory of growth of *E.coli* in samples, approximately resulting in a 2.1 log cycle decrease during the storage period.

These antimicrobial effects are usually attributed to the effective compounds in the CEO and the antimicrobial properties of Ch. Allahghadri *et al*. (2010) demonstrated that CEO dilutions had strong antimicrobial effects against the *E. coli* and *E. coli* was the most sensitive

3.1.4. Staphylococcus aureus count

S. aureus count in second day was 3.5 log cfu/g, increased during storage and reached final population ca. 3.9 log cfu/g for control sample (Figure 4). In contrast, counts related for Ch-CEO 0.4% and Ch-CEO 0.6% were about 0.7 – 1.2 log cfu/g lower than the control samples. *S.aureus* population was significantly ($p < 0.05$) lower in Ch-CEO 0.6% samples compared to all the other treatments. The direct addition of CEO without the use of a chitosan, in general, did not improve the microbial quality chicken meat samples. *S.aureus* count was not significantly difference ($p > 0.05$) for CEO 0.2%, CEO 0.4% and CEO 0.6% samples compared to the control samples. García-Díez *et al*. (2017) indicated that CEO inhibited the growth of *S. aureus* associated to dry-cured meat products (García-Díez *et al*., 2017). Also, Sadegi *et al*. (2012) showed that the use of cumin essential oil significantly inhibited the growth of *S. aureus* bacteria in Iranian white brined cheese (Sadeghi *et al*., 2013). In another study that investigated the effects of electro-spun chitosan-based nanofibers, it was shown that chitosan significantly inhibited the growth of *S. aureus* in meat samples (Arkoun *et al*., 2017).

bacteria to the CEO with the lowest MBC value (1 µL/mL) (Allahghadri *et al.*, 2010).

In another study, García-Díez *et al*. (2016) indicated that CEO inhibited the growth of *E. coli* related to dry-cured meat products (García-Díez *et al*., 2017). In addition, accordant with the results of this study, Shekarforoush *et al*. (2015) showed that using chitosan and oregano CEO in combination are more effective in reducing the number of spoilage and pathogenic bacteria such as *E. coli* O157:H7 in cured chicken meat (Shekarforoush *et al*., 2015).

Arkoun *et al*. (2017) also, proved that electro-spun chitosan-based nanofibers reduced the growth of spoilage and pathogenic bacteria, including *E. coli*, and resulted in an increase in the shelf life of the meat samples for one week (Arkoun *et al*., 2017)

3. 1. 5. Moulds and yeasts

Eventually, with regard to moulds and yeasts known species to be involved at the spoilage of poultry meat (Petrou *et al*., 2012). The antimicrobial treatments Ch-CEO 0.2%, Ch-CEO 0.4% and Ch-CEO 0.6% led to a significant reduction ($p < 0.05$) in yeasts and moulds count compared to the control group up to day 9th of storage (Figure 5). On the other hand, moulds and yeasts populations were significantly lower for Ch-CEO 0.6% samples compared to the wrapped chitosan samples during the storage ($p < 0.05$). Thus, while the Ch-CEO 0.6% showed effectiveness on the moulds and yeasts after 9 days, but the same result was shown in CEO 0.6% treatment without chitosan coating while did not show this parameter in microbial counts. In other studies, involving preservation of chicken breast meat treatments with chitosan or oregano oil (Petrou *et al*., 2012) led to a reduction two cycles in compared with control samples. Also, Giatrakou *et al*. (2010) reported the effects of chitosan and thymol oil beside aerobic packaging that led to count remained below 4.0 log cfu/g during the entire storage period than the control samples (Giatrakou *et al*., 2010). In another study, similarly, Siripatrawa *et al*. (2012) indicated that chitosan incorporation with green tea extract

caused shelf life extension of pork sausages 3 and 2 log cycles, respectively, compared to control samples and chitosan wrapped samples (Siripatrawan *et al*., 2012).

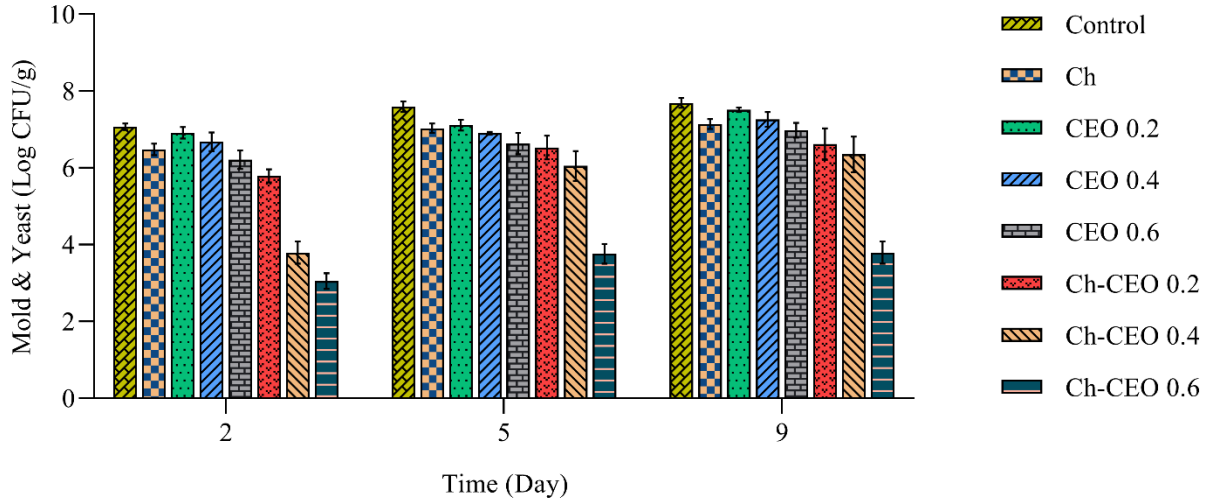

Figure 5. Effect of edible biodegradable coating containing Chitosan and CEO on the mold and yeast of chicken meat during refrigerated storage.

3.2. Physicochemical changes

3.2.1. pH value

The pH value of control and treated chicken meat samples during storage at 4˚C are shown in Table 1. The primary pH of the chicken meat samples was 6.1, whereas at the end of storage final pH was 7.5 at control samples. The pH value of treated chicken meat samples decreased during 9 days; although, between control and CEO groups, no significant difference was observed ($p > 0.05$) in this time. Treatments containing Ch-CEO 0.2, 0.4 and 0.6% resulted in lower pH value ($p < 0.05$) compared to the other groups.

Our results seem to be in agreement with those reported by Giatrakou *et al*. (2010) for reduced ready to cook chicken product containing chitosan (Giatrakou *et al*., 2010). Whereas, Petrou *et al*. (2012) reported no significant difference between chitosan, thymol oil and the combination of chitosan and thymol oil for chicken breast meat (Petrou *et al*., 2012). Also, Soultos *et al*. (2008) reported no significant difference in pH value between pork sausages samples treated with chitosan and nitrite, separately or in combination (Soultos *et al*., 2008).

Table 1. Effect of edible biodegradable coating containing Chitosan and EO on the chemical properties of chicken meat during refrigerated storage

Days	Groups	pH	PV (%)	TBARS (µg MDA/g)	TVN (mg/100g)
2	Control	6.1 ± 0.264^{a}	1.9 ± 0.115^{a}	0.006 ± 0.001^{a}	13 ± 8.08^{a}
	Ch	5.6 ± 0.251^{bcd}	1.5 ± 0.115^{b}	0.005 ± 0.0005^{a}	7.7 ± 3.89^{ab}
	CEO 0.2%	5.8 ± 0.152^{ac}	1.3 ± 0.230^{b}	0.003 ± 0.004^{ab}	9.8 ± 3.70^{ab}
	CEO 0.4%	5.7 ± 0.152^{bc}	0.9 ± 0.115^{ce}	0.002 ± 0.0006^{bc}	9.1 ± 1.85^{ab}
	CEO 0.6%	5.6 ± 0.152^{bcd}	0.4 ± 0.1^{d}	0.0004 ± 0.0004^{c}	6.3 ± 1.32^{b}
	Ch-CEO 0.2%	5.4 ± 0.057^{bde}	1.1 ± 0.1^{c}	0.004 ± 0.0005^{ad}	8.1 ± 2.65^{a}
	Ch-CEO 0.4%	5.3 ± 0.057^{de}	0.8 ± 0.05^{e}	0.004 ± 0.001^{ad}	6.9 ± 1.40^{a}
	Ch-CEO 0.6%	5.2 ± 0.057^{e}	0.4 ± 0.11^{d}	0.0026 ± 0.0005^{bcd}	5.6 ± 1.27^{b}
5	Control	6.7 ± 0.251^{a}	3.13 ± 0.230^{a}	0.010 ± 0.001^{a}	28 ± 0.00^{a}
	Ch	5.5 ± 0.076^{b}	1.8 ± 0.115^{b}	0.006 ± 0.004^{b}	6.5 ± 2.13^{bc}
	CEO 0.2%	6.6 ± 0.251^{a}	1.2 ± 0.2^{c}	0.001 ± 0.00^{cd}	14.6 ± 5.12^{d}

	CEO 0.4%	6.4 ± 0.251^{a}	1 ± 0.1^{c}	0.0007 ± 0.0001^{c}	15.8 ± 2.91^{d}
	CEO 0.6%	5.3 ± 0.115^{b}	0.6 ± 0.2^{d}	0.0004 ± 0.0001^{c}	10 ± 1.06^{b}
	Ch-CEO 0.2%	5.6 ± 0.180^{b}	1.2 ± 0.2^{c}	0.006 ± 0.001^{b}	8.1 ± 1.02^{bc}
	Ch-CEO 0.4%	5.4 ± 0.152^{b}	0.9 ± 0.05^{ce}	0.005 ± 0.001^{b}	7.3 ± 1.49^{bc}
	Ch-CEO 0.6%	5.3 ± 0.115^{b}	0.7 ± 0.1^{de}	0.004 ± 0.001^{bd}	5.4 ± 2.27^{c}
9	Control	7.5 ± 0.152^{a}	4.6 ± 0.577^{a}	0.02 ± 0.00^{a}	48.5 ± 0.80^{a}
	Ch	5.9 ± 0.229^{bd}	2.5 ± 0.503^{b}	0.01 ± 0.005^{b}	9.8 ± 1.4^{b}
	CEO 0.2%	7.2 ± 0.152^{ac}	1.5 ± 0.1^{c}	0.002 ± 0.001^{cd}	35.4 ± 14.02^{ac}
	CEO 0.4%	7.1 ± 0.115^{c}	1.2 ± 0.1^{cd}	0.001 ± 0.0008^{cd}	33.3 ± 14.09^{c}
	CEO 0.6%	7.0 ± 0.115^{c}	0.9 ± 0.057^{d}	0.0008 ± 0.001^{c}	26.6 ± 9.18^{c}
	Ch-CEO 0.2%	6.1 ± 0.346^{b}	1.6 ± 0.152^{c}	0.01 ± 0.00^{be}	12.8 ± 0.40^{b}
	Ch-CEO 0.4%	5.7 ± 0.115^{d}	1.3 ± 0.1^{cd}	0.007 ± 0.006^{de}	7 ± 1.4^{b}
	Ch-CEO 0.6%	5.6 ± 0.152^{d}	0.9 ± 0.152^{d}	0.006 ± 0.004^{ce}	4.8 ± 1.4^{b}

Any two means in the same column followed by the same letter are not significantly ($p > 0.05$) different from Duncan's multiple range tests. Ch: Chitosan, EO: Essential oil, CEO: Cumin essential oil, pH, peroxide value (PV), Malondialdehyde (MDA), Total volatile nitrogen (TVN), Thiobarbituric acid (TBARS).

3.2.2. DDPH assay

Antioxidant properties, especially radical scavenging activity, it's too important due to the deleterious role of free radicals in food and biological system (Wang *et al*., 2015). Figure 6 shows DPPH free radical scavenging activity of CEO with different concentrations compared to BHT (synthetic antioxidant). In the present study was observed statistically significant difference ($p < 0.05$) between BHT and CEO 0.2, 0.4 and 0.6%. The variety EO showed high antioxidant activities. Solvent nature had significant effect on DPPH scavenging activity of CEO. Rebey *et al*. (2012) reported highest antioxidant activities water extract cumin (Rebey *et al*., 2012). Also, Martins *et al*. (2012) stated chitosan film in combination α-tocopherol with concentration 0.1 and 0.2% the highest DPPH scavenging activity, whereas no significant difference between two α-tocopherol concentration (Martins *et al*., 2012).

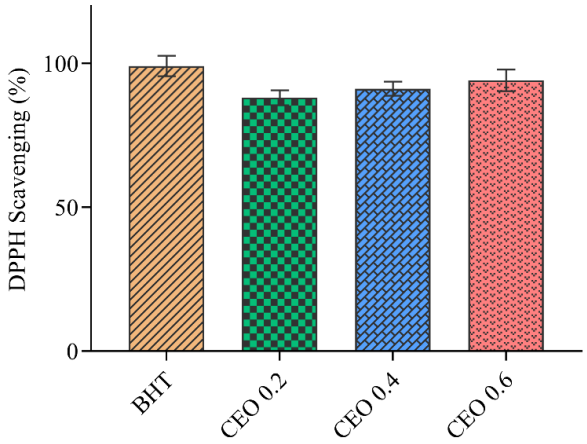

Figure 6. Antioxidant activities of Chitosan and CEO. BHT was used as standard samples. Each point represents the mean ± SD.

3.2.3. Thiobarbituric acid value

The lipid oxidation changes (TBA value) in control and treatment chicken meat samples are shown in Table1. At during storage, results were released significantly higher ($p < 0.05$) TBA in control samples than those wrapped with EO 0.2, 0.4 and 0.6%, respectively. Therefore, combination of CEO and chitosan coating increased the antioxidant properties of the coating. The TBA values of Ch-CEO 0.6% wrapped samples were lower than those wrapped with chitosan coating, whereas not showing statistically difference significantly ($p > 0.05$).

Similarly, Siripatrawan *et al*. (2012) showed that the antioxidant activities of Ch film with incorporation green tea extract increased in pork sausages (Siripatrawa *et al*., 2012). Furthermore, Ch with oregano oil and modified atmosphere packaging was shown to the lowest TBA values in 12 days of storage in chicken breast meat (Petrou *et al*., 2012). Soultos *et al*. (2008) shows low levels of lipid oxidation in sausage samples treated with chitosan concentration 0.5 and 1% in combination nitrite 150 ppm (Soultos *et al*., 2008).

3.2.4. Peroxide value

Concentration of elementary oxidation products in the lipid breakdown of the chicken meat sample measured as PV after 9 days storage at 4 ˚C are presented in Table 1. Samples containing chitosan and combination (CEO 0.2, 0.4 and 0.6%) together with those containing only CEO exhibited the lowest ($p < 0.05$) values for PV compared to the control samples. The best anti-oxidative effect ($p < 0.05$) was obtained by the combination of Ch and CEO 0.6% for the PV values that had lower at the end of storage period.

In this study, was not seen statistically difference significantly between control samples with combination of Ch and CEO 0.2 or samples containing only CEO 0.2% ($p > 0.05$).

Georgantelis *et al*. (2007) observed a decrease in the peroxide value of pork sausage samples containing Ch with rosemary EO, α- tocopherol and samples containing only Ch compared α-tocopherol and control samples (Georgantelis *et al*., 2007).

3.2.5. Total volatile nitrogen value

Changes in total volatile nitrogen value (TVN) content during the storage time are shown in Table 1. The primary TVN value was about 13 mg/100g in second day, and then it increased with time of refrigerator storage for the control samples on day 9^{th}. The control samples had the highest TVN values, while the treatment sample Ch-CEO 0.4 and 0.6% had lowered values ($p < 0.05$). In the current study was not observed statistically difference significantly between control samples with combination of Ch and CEO 0.4, 0.6% and samples containing only CEO 0.2, 0.4% with combination Ch and CEO 0.6 ($p \geq 0.05$).

Fan *et al*. (2009) reported that TVN contents increased from an initial value to 18.8 mg/100g in fish samples were given a dip treatment in 2% chitosan solution than to 30.2 mg/100g in control samples (Fan *et al*., 2009). The study conducted by Chantarasataporn *et al*. (2014) showed the amount of biogenic amines in minced pork samples of treated oligo-chitosan in the first day was about 50 mg/kg by increasing the concentration of oligo-chitosan to 0.2 and 0.4 the amount of biogenic amines reached the acceptable level on the second day (under 50 mg/kg) (Chantarasataporn *et al*., 2014).

3.2.6. Sensory analysis

Means of sensory analysis scores including odour, colour and overall acceptance of control chicken meat sample and those wrapped with Ch coating, treated (CEO 0.2, 0.4 and 0.6%) and treated (Ch-CEO 0.2%, Ch-CEO 0.4%, Ch-CEO 0.6%) during storage at 4˚C are shown in Table 2. The sensory analysis results showed significant differences ($p < 0.05$) for odour between control samples with treated samples (Ch-CEO 0.2, 0.4, and 0.6%), and colour was shown between control samples with treated samples (Ch-CEO 0.4 and 0.6%) and overall acceptance. Results showed significant differences ($p < 0.05$) for overall acceptance between control samples with treated samples that were Ch-CEO 0.4 and 0.6%.

In this study the existence of chitosan (1.5% w/v) in Ch-CEO 0.2, 0.4 and 0.6% treated samples a very desirable odour and appearance color in the chicken meat, increasing the natural freshness of the chicken meat, while addition of cumin oil (0.2, 0.4 and 0.6%) in chicken meat samples caused to off odour and slime. According to Giatrakou *et al*. (2010) study, the addition of chitosan with thyme oil to cook chicken product gave a more acceptable taste and odour as compared to the untreated samples (Giatrakou *et al*., 2010).

Table 2. Effect of edible biodegradable coating containing Chitosan and CEO on the sensory analysis of chicken meat during refrigerated storage

Sensorial indexes				
Days	**Groups**	**Colour**	**Odour**	**Acceptability**
2	Control	7.8^{a}	7.2^{b}	8.0^{a}
	Ch	7.8^{a}	8.0^{b}	8.2^{a}
	CEO 0.2%	7.8^{a}	7.8^{b}	7.8^{a}
	CEO 0.4%	7.1^{a}	7.1^{b}	7.5^{a}
	CEO 0.6%	7.0^{a}	6.8^{c}	6.5^{b}
	Ch-CEO 0.2%	8.2^{a}	8.1^{b}	8.4^{a}
	Ch-CEO 0.4%	8.2^{a}	8.2^{b}	8.5^{a}
	Ch-CEO 0.6%	8.2^{a}	8.5^{b}	9.0^{a}
5	Control	3.5^{ab}	1.4^{e}	4.1^{a}
	Ch	7.5^{d}	8.1^{f}	7.4^{b}
	CEO 0.2%	4.5^{ab}	2.4^{e}	2.4^{a}
	CEO 0.4%	3.5^{ab}	2.0^{e}	1.8^{a}
	CEO 0.6%	2.0^{ab}	1.5^{e}	1.7^{a}
	Ch-CEO 0.2%	7.8^{d}	7.7^{f}	7.5^{b}
	Ch-CEO 0.4%	8.4^{d}	8.0^{f}	8.0^{b}
	Ch-CEO 0.6%	8.4^{d}	8.2^{f}	8.5^{b}
9	Control	1.4^{g}	1.1^{c}	1.0^{ab}
	Ch	7.8^{h}	8.1^{d}	7.0^{g}
	CEO 0.2%	2.2^{g}	2.1^{c}	2.0^{ab}
	CEO 0.4%	1.8^{g}	1.0^{c}	1.5^{ab}
	CEO 0.6%	1.2^{g}	1.0^{c}	1.0^{ab}
	Ch-CEO 0.2%	8.0^{h}	8.5^{d}	8.0^{g}
	Ch-CEO 0.4%	8.4^{h}	8.4^{d}	8.2^{g}
	Ch-CEO 0.6%	8.7^{h}	8.4^{d}	8.2^{g}

Any two means in the same column followed by the same letter are not significantly ($p > 0.05$) different from Duncan's multiple range tests. Ch: Chitosan, CEO: Cumin essential oil.

4. Conclusions

The results of this study showed that the combined use of chitosan and CEO prevented the growth of spoilage and foodborne pathogenic microbial, delayed lipid oxidation and increased shelf life of chicken meat at 4˚C. The samples wrapped with Ch and CEO 0.4 and 0.6% was the most effective of all, inhibiting the growth of the microbial spoilage, decreasing lipid oxidation of 9 days. In conclusion, the use of antimicrobial coatings has been indicated to be an effective method to preserve microbial and sensory quality of meat. On the other hand, using various preservative factors in small amounts is a preferred approach, because it has physicochemical characteristics, sensory properties and economic advantages.

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HOVENIA DULCIS - DEVELOPMENT AND EVALUATION OF JELLY AND DEHYDRATED FRUIT

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ABSTRACT

The aim of this study was to develop and evaluate *Hovenia dulcis* as jelly and dehydrated. The jellies were made in four formulations, following the steps of reception, selection and classification, washing and sanitizing, freezing, thawing, pre-tests, formulation, concentration, filling\sealing of the packaging, cooling, labeling and storage. The dehydrated pseudofruit was prepared with four treatments according to, the following steps: defrosting, pre-treatments, dehydration (70 ºC) from 90 to 120 min., conditioning, packaging, labeling, storage. The products were characterized according to: color (L*, a* and b*), texture, pH, soluble solids (SS), titratable acidity, moisture, ash, phenolic compounds, vitamin C, and carotenoids. The prepared jellies showed good color and texture, high content of SS, total phenolics and vitamin C. Formulations F1 and F3 are more suitable, as they have more bioactive compounds. Dehydrated *Hovenia dulcis* showed high SS, ash, carotenoids, phenolic compounds and vitamin C, with treatments 3 and 4 being recommended.

1.Introduction

Hovenia dulcis Thunb, is a deciduous tree originating in East Asia and distributed mainly in China and Korea (Hyun et al., 2010). Its pseudofruits are globose, approximately 7 mm wide, with three seeds. When ripe the fruit is brown in color, the ripening phase occurring from late summer to autumn. When the contorted "fruit" stems are fully ripe, they are fleshy and sweet due to their high sucrose content (De Biaggi et al., 2019).

Studies have reported that *Hovenia dulcis* pseudofruits are a good alternative source to improve the nutritional quality of modern diets as they are a source of fiber, Cu, Ca and Mn (Maieves et al., 2015). They also have health properties, among which the following stand out: antioxidant effect (considerable content of phenolic compounds, acids and tocopherols), anti-tumor, anti-diabetic and hepatoprotective. The fruit is also efficient at disinfection after alcohol intoxication, besides helping to prevent alcoholic steatosis and inflammation. It also has excellent colloidal properties with the potential to serve as a thickening, gelling and stabilizing agent, attributes that increase its potential for applicability in the food, pharmacological and cosmetic industries (Yang et al., 2020; Yang et al., 2019; Choi et al., 2017; Morales et al., 2017; Hyun et al., 2010).

Although the benefits of consuming *Hovenia dulcis* pseudofruit (popularly known as Japanese grape) are proven, there are few studies reporting its application as a raw material in the production of food products. Some studies indicate its use as a raw material in the preparation of alcoholic beverages (Pinto et al., 2017); cookies made from their fermented biomass (Cunha, Reineri & Loss, 2015); dehydrated fruit (Maieves et al., 2015), justifying the performance of studies that expand its possibilities of processed

consumption. Among these, jelly and dehydration can be highlighted.

Jelly is defined as a product obtained by cooking whole or in pieces, pulp or fruit juice, with sugar and water and concentrated to a gelling consistency. It must not be excessively sweet, sticky or viscous, and must retain the flavor and aroma of the original fruit, in addition to the consistency. When extracted from its container, the product must able to remain in the semi-solid state, without dripping ans be, soft when cutting, however firm (Brasil, 2005).

Jelly is consumed by a good part of the population because it presents a sweet and pleasant taste on the palate, which makes it widely accepted by consumers of different age groups, being part of important daily meals (Scolforo & Silva, 2013).

Dried or dehydrated fruit, on the other hand, is defined as the product originating from the partial loss of water from the ripe fruit, whole or in pieces, by appropriate technological processes and with a maximum moisture of 25% (Brasil, 1978). In addition to increasing the product shelf life, dehydration provides a characteristic and refined flavor to vegetables, as with dried tomatoes, which have a high market price.

In this context, this work aimed to develop and evaluate jelly and pseudofruits from *Hovenia dulcis*.

2. Materials and methods

The work was carried out at the Federal Institute of Southeast Minas Gerais, *campus* Barbacena, industrialization and processing of vegetables, and a physical-chemical analysis laboratory.

2.1. Materials

2.1.1. Obtaining and preparing the raw material

The ripe pseudofruits collected from the Barbacena *campus* of IF *Southeast* MG, were selected and classified according to the stage of maturation (reddish brown color and slightly soft texture) and imperfections. After removing the seeds from the ends of the pseudofruits; washing was carried out under running water, sanitized in sodium hypochlorite solution (NaCIO) 100 mg/L for 10 minutes, packed in low density polyethylene (LDPE) plastic packaging, identification and freezer freezing (-18ºC). Freezing was necessary, since the pseudofruits are seasonal and obtained by extractivism, so it was possible to store a sufficient quantity for the pre-tests and development of the jelly and dehydrated fruit. Defrosting was performed in refrigeration and followed by pre-tests to define the best treatments.

2.1.2. Manufacture of jellies

After performing the pre-tests, the proportions of the ingredients were defined considering the legislation on ingredients and additives for the manufacture of fruit jellies. The jelly formulations are shown in table 1.

Table 1. Formulations of *Hovenia dulcis* jellies

Formulations	Pulp (%)	Sugar (%)	Citric acid (%)	Water (%)	Orange juice (%)	Screening
F1	79.7	20	0.3	-	-	-
F2	60	20	0.3	19.7	-	Yes
F3	60	20	0.3	19.7	-	-
F4	34.85	20	0.3	10	34.85	Yes

The thawed pseudofruits were chopped and crushed to obtain the pulp. Then, the weighing and mixing process of the ingredients was started according to each formulation, cooking and concentration until the final point was obtained, observing the consistency that enables the formation of the gel. The jellies were packed in identified polyethylene containers and analyzed for color and texture on the same day of manufacture. The other samples, equally identified, were stored refrigerated until the other analyzes were carried out at a, maximum of 10 days.

2.2. Manufacture of dehydrates

The dehydrated products were prepared following the treatments shown in table 2.

Table 2. Treatments applied to the pseudofruit of *Hovenia dulcis* for dehydration

	Treatments
1	Fresh fruit
2	Water Vapor bleaching
3	Water Vapor bleaching + 3 % citric acid
4	Water Vapor bleaching + 3 % ascorbic acid

After defrosting the pseudofruits, dehydration was carried out using a temperature of 70ºC for a time ranging from 90 to 120 min. When the pseudofruits reached room temperature, they were packed in high density polyethylene packages and closed for 48 hours for moisture standardization, and the batches were identified with the corresponding and dated treatments, then they were stored refrigerated until the time of analysis by maximum 10 days.

2.3. Analyzes performed on jellies and dehydrated fruit

2.3.1. Coloration

Determined using a konica Minolta CR400 colorimeter, using the L*, a* and b* color scale system (CIELAB), previously calibrated.

2.3.2. Texture

Test conditions: a) jellies: cylindrical aluminum probe sensor with a diameter of one inch, test speed 2.0 mm/s, distance 10.0 mm and force 0.10 N. For compression analysis, 5 g of jelly were used for each measurement. The parameters of firmness, cohesiveness, elasticity, adhesiveness, guminess and chewability were evaluated (Pons & Fiszman, 1996). b) dehydrated: Probe TA39 (cylindrical), pre-test, test speed, post-test of 5.0 mm/s, penetration distance of 5.0 mm, time of 5.00 s and contact force of 1 g.

Only the firmness parameter, expressed in Newton, was considered.

2.3.3. pH

TEKNA T-1000 pH-meter was used according to the methodology proposed by Instituto Adolfo Lutz (IAL, 2008). The pH meter was previously calibrated using buffer solutions (pH 4.0 and 7.0).

2.3.4. Titratable acidity

Determined by titration with standardized 0.1 N sodium hydroxide solution, using phenolphthalein as an indicator. According to the methodology proposed by the Analytical Standards of (IAL, 2008).

2.3.4. Soluble solids (% SS)

Determined by refractometry using an IONLAB benchtop refractometer (evem).

2.3.5. Moisture

Made by gravimetry in a drying oven and sterilization Deleo A4SE sterilization at 105 ° C until constant dry mass, according to the Instituto Adolfo Lutz (IAL, 2008).

2.3.6. Ash

Made after incineration in a 550ºC muffle, by gravimetry according to the Adolfo Lutz Institute (IAL, 2008).

2.3.7. Total phenolic compounds

The extracts were obtained as described by Brand Williams; Cuvelier and Berset (1995) and adapted by Rufino et al. (2007) and determined as described by Waterhouse (2002). Gallic acid was used as a reference standard and the results were expressed in milligrams of gallic acid equivalents (mg GAE 100 g-1 fresh matter).

2.3.8. Vitamin C

Determined by the Balentine method, which is based on the oxidation of ascorbic acid by potassium iodate (Tavares, 1999).

2.3.9. Total carotenoids

Carotenoid extraction and determination were performed as described by Rodriguez-Amaya (1999).

Experimental design and statistical analysis: Dehydrated pseudofruits and jellies were prepared with 4 treatments, with 3 repetitions and the results were submitted to analysis of variance. When there was a significant difference ($p < 0.05$), the averages were compared using the Tukey test at 5% significance using the Sisvar 5.3 program (Ferreira, 2010).

3. Results and discussions

The results of the physical analysis of the *Hovenia dulcis* pseudofruit jellies are shown in Table 3.

Table 3. Physical characterization of *Hovenia dulcis* jellies

Parameters	F1	F 2	F 3	F 4
Color				
L*	34.33 a	36.61 a	34.57 a	33.94 a
a*	8.57 a	8.71 a	8.73 a	9.68 a
b*	13.95 a	19.79 a	16 a	15.40 a
Firmness (N)	5.65 a	5.33 a	5.35 a	0.92 b
Adhesiveness (N)	-10.25 a	-9.80 a	-7.84 a	-2.28 b
Elasticity (N)	0.0095 a	0.0091 a	0.0094 a	0.0092 a
Chewability (N)	5.57 a	4.29 a	4.12 a	0.61 b
Gumness (N)	5.74 a	4.03 a	5.61 a	0.65 b
Cohesiveness (N)	0.0083 a	0.0081 a	0.0076 a	0.0070 a

Means followed by the same letters on the line are statistically equal by the Tukey test at 5% significance.

The parameter L* that represents (luminosity or clarity), varying from white (L* = 100) to black (L* = 0) did not differ statistically between the formulations analyzed (average of 34.86) indicating that the jellies were dark, which was expected considering the characteristic brown color of the fruit, associated with cooking heat that favors oxidation reactions of ascorbic acid and Caramelization, resulting in browning (Fellows, 2006). Curi et al. (2017) when analyzing jam from different loquat cultivars, reported L* values ranging from 52.47 - 76.03.

Positive values of a* indicate reddish color, and when negative indicate green color. The prepared jellies showed no statistical difference regarding this parameter (mean of 8.92) indicating that they have a reddish color. Curi et al. (2017) when analyzing jam from different loquat cultivars, report values varying between 1.23-8.35 for the same parameter, thus *Hovenia dulcis* jelly has a color similar to that of loquat jelly.

The value of b*, when, positive indicates yellow color and when negative, blue. There was no difference between the jellies analyzed for this variable (average of 16.28). Curi et al. (2017) when analyzing jam from different loquat cultivars, report values ranging from 6.27 - 33.53 for the same parameter. These results

showed that the jelly staining parameters are specific to each species and influenced by the heat treatments applied.

Instrumental firmness is defined as the force necessary to obtain a certain deformation, however in sensory analysis it is described as being the force necessary to compress a substance between the teeth (molars, when it comes to solids) or between the tongue and the palate (when semi-solid) (Szczesniak, 2002). The jam from treatment F4 (with the addition of orange juice) differed from the others with a firmness of 0.92 N. Lemos et al. (2019), when analyzing the mixed prebiotic jabuticaba and acerola jelly, obtained values ranging from 0.96 to 4.60 N. Already Vieira et al. (2017), reports higher values ranging from 10.11-13.69 N, for the same parameter when evaluating mixed jabuticaba and pitanga jam.

Adhesiveness is related to the force expended to remove material adhered to the mouth during chewing (Szczesniak, 2002). Only the F4 treatment jelly differed from the others in terms of adhesion.

Elasticity, the ability of a deformed material to return to its initial state after removing the deformation force (Szczesniak, 2002), did not show any difference between the formulations elaborated. Higher values for the same parameter are reported by Lemos et al. (2019), when analyzing the mixed prebiotic jabuticaba and acerola jelly, who describes values ranging from 0.98 to 1N for this parameter.

The energy required to chew a solid food to a state capable of swallowing, defines chewability (Szczesniak, 2002). Only the F4 treatment differed from the other formulations analyzed regarding this parameter. The values for this parameter, with the exception of formulation 4, are higher than those reported by Lemos et al. (2019), when analyzing the mixed prebiotic jabuticaba and acerola jelly, which obtained values ranging from 0.33 to 2.04 N.

Gumminess can be understood as the energy necessary to disintegrate a semi-solid food at a stage where it is able to swallow (being a product with a low degree of hardness and a high degree of cohesion (Szczesniak, 2002). The F4 treatment jelly differed from the other formulations. The results of the present study are superior to those described by Lemos et al. (2019), when analyzing the mixed prebiotic jabuticaba and acerola jelly, which reports values ranging from 0.33 to 1.97N.

Table 4. Physico-chemical characterization and bioactive compounds of *Hovenia dulcis* jellies

Parameters	F1	F2	F3	F4
pH	4.97 a	4.78 a	5.03 a	4.72 a
Titratable acidityl – AT (g/100 g)	0.29 a	0.36 a	0.24 a	0.29 a
Soluble solids - SS (g/100 g)	76.4 a	73.73 ab	69 a	71.20 ab
Humidity (g/100 g)	56.38 a	52.94 ab	50.90b	53.22 ab
Ashes (g/100 g)	1.47 ab	1.56 ab	1.73 a	1.21 b
Phenolics (mg GAE 100 g)	1960.59 ab	1489.27 c	2328.03 a	1767.26 bc
Vitamin C (mg/100 g)	21.51 a	25.43 a	17.60 a	19.55 a
Carotenoids (mg/100 g)	121.52 a	47.16 c	74.02 a	15.56 c

Means followed by the same letters, are statistically equal by Tukey's test at 5% significance. Formulation 1 (jelly without added water), Formulation 2 (jelly with added water and sieving), Formulation 3 (jelly with added water and no sieving), Formulation 4 (jelly with added water, sieving and added juice) orange)

Cohesiveness is defined as the level of deformation of the sample between the teeth until its rupture, or the maximum level of deformation supported by the food until its rupture (Szczesniak, 2002). There was no significant difference between the samples under study. The values obtained in this study are lower than those reported by Vieira et al. (2017), who obtained values ranging from 0.44-0.69 N, for the same parameter when evaluating mixed jabuticaba and pitanga jelly, and by Lemos et al. (2019), when analyzing the mixed

prebiotic jabuticaba and acerola jelly, who report values ranging from 0.34 to 0.52 N.

The texture of the jellies was similar, as were most parameters, with only formulation 4 highlighted, the only one with added with orange juice, which possibly was a determinant of the differences observed.

The formulations studied did not differ from each other, regarding the pH parameter, obtaining an average of 4.87; a lower value is reported by Ribas, Buratto and Pereira (2017) when analyzing Thompson Seedless grape jelly, who report a value of 3.53 for the same parameter.

There was no difference between the jellies studied in terms of titratable acidity, obtaining an average of 0.29 g/100 g of citric acid. Higher values for the same parameter are reported by Ribas, Buratto and Pereira (2017), when analyzing Thompson Seedless grape jelly (0.75 g/100 g), Vieira et al. (2017), in mixed pineapple peel and peach pulp jelly (0.40 g/100 g). The higher acidity in jellies can be influenced, among other factors, by the original acidity of the raw material, which in this case favored the lower acidity of the jellies. This characteristic can be altered by new studies to improve the formulations of pseudofruit jellies, using percentages greater than 0.3% of citric acid.

The levels of soluble solids did not differ between the formulations under study, obtaining an average of 72.58 g/100 g, so all formulations are in accordance with that established by the Brazilian Standard of Identity and Quality of jams and fruits (Brasil, 1978), which stipulates a minimum total solids content of 62-65 g/100 g. A little lower value for the same parameter is reported by Ribas, Buratto and Pereira (2017), when analyzing Thompson Seedless grape jelly which obtained a value of 69 g/100 g for SS. Considering that *Hovenia dulcis* has high sugar content, this characteristic certainly favored the high content of soluble solids in jellies. However, further studies to improve the formulation can be done, because even the proportion of the lowest sugar content used in the formulations (20%), previously defined in pre-tests, can be reduced, complying with Brazilian legislation and additionally obtaining final product with less caloric value, characteristic of healthiness currently sought by consumers.

As for moisture, all formulations had a higher content than that defined by the Brazilian Standard of Identity and Quality of fruit jellies regarding this parameter (Brasil, 1978), which defines a maximum moisture content of 38 g/100 g. Lower values are reported by Ribas, Buratto and Pereira (2017), when analyzing Thompson Seedless grape jelly which obtained a value of 30.49 g/100 g for moisture. Higher moisture levels are related to greater ease of alteration of the final product, since it allows greater water activity, compromising the product' durability.

Regarding the ash content, formulations F3 and F4 were statistically different, which can be justified by the addition of orange juice in formulation F4, which diluted the sample and favored lower ash content. A similar value for the same parameter is reported by Vieira et al. (2017) in mixed pineapple peel and peach pulp jelly (1.44 g/100 g) and a lower value is described by Ribas, Buratto and Pereira (2017), when analyzing Thompson Seedless grape jelly which obtained 0,49 g/100 g of ash. This demonstrates that jams made with *Hovenia dulcis* can be significant sources of minerals.

The jams of the *Hovenia dulcis* pseudofruit showed high levels of phenolic compounds. Formulations F1 and F3 are statistically equal and have the highest levels of these compounds. When comparing its values with other studies, even the jelly with the lowest phenolic content, formulation F2 presented a content (1489.27 mg/100 g) higher than that of other jellies, such as araçá red (117.59 mg/100 g) (Reissig et al., 2016); palm jelly (*Butia odorata* Barb. Rodr.) in Which values ranging from 280.5 to 398.50 mg/100 g were found (Beskow et al., 2015).

The vitamin C content did not vary statistically between the jelly formulations. The results of this study showed that pseudofruit jams have higher levels of vitamin C than mixed umbu and mangaba jams (1.7 to 1.9 mg/100 g); passion fruit jelly with flaxseed (average of 3.61

mg/100 g) (Souza et al., 2018; Moura et al., 2019) and that bocaiuva jelly with passion fruit (1.70 to 3.61 mg/100 g) (Souza et al., 2019). In this sense, it can be said that the jam of the pseudofruit of *Hovenia dulcis* is an important source of vitamin C.

Formulations F1 and F3 are statistically equal and have the highest levels of carotenoids (average of 97.77 mg/100 g) in the jellies. The carotenoid values found in the present study are higher than those of passion fruit jelly with flaxseed seed (0.02 mg/100g), bocaiuva jam with passion fruit (11.44 mg/100g), and palm jelly (*Butia odorata* Barb Rodr.) (2.8 to 4.08 mg/100g) (Moura et al., 2019; Souza et al., 2019; Beskow et al., 2015).

In general, formulations without sieving (F1 and F3) had a higher content of phenolic and carotenoid compounds when compared to those that underwent such process (F2 and F4). This is possibly because most of the simple phenolics, which represent a large part of the plant phenolics, have relatively low molecular weight and variable solubility depending on the polarity and chemical structure. Some phenolic compounds, however, can be attached to the cell wall. Depending on the nature of the ester bonds, such compounds can be solubilized in alkaline conditions or can be retained in the fiber matrix (Bravo, 1998). Either way, in both cases, the sieving can induce the elimination of bioactive compounds adhered to the fibers by different types of chemical bonds and forces of attraction, consequently decreasing these compounds in sieved foods in general.

The dehydratyed *Hovenia dulcis* analyzes are shown in Table 5.

Table 5. Analysis of dehydratyed *Hovenia dulcis* according to the treatments applied

Parameters	**T 1**	**T 2**	**T 3**	**T 4**
Color				
L*	33.66 a	31.48 b	31.78 b	31.29 b
a*	3.32 a	2.39 a	2.91 a	2.04 a
b*	4.93 a	3.27 b	4.06 a b	3.22 b
Firmness (N)	1.67 a	1.49 a	1.36 a	1.52 a
pH	5.66 a	5.61 a	5.51 a	5.57 a
Titratable acidity (g/100 g)	0.10 a	0.10 a	0.14 a	0.10 a
Soluble solids (g/100 g)	60 a	60 a	60 a	60 a
Humidity (g/100 g)	44.55 a	44.01 a	38.03 a	39.51 a
Ashes (g/100 g)	3.34 a	3.44 a	3.88 a	3.56 a
Phenolics (mg GAE 100 g)	17.21 b	9.24 b	72.77 a	27.21 b
Vitamin C (mg 100 g)	5.87 a	5.86 a	5.86 a	5.86 a
Carotenoids (mg /100 g)	10.12 a	5.41 b	6.32 ab	7.09 ab

Averages followed by the same letters on the line are statistically equal by the Tukey test at 5% significance. T1-Treatment 1 (*in natura*), T2-Treatment 2 (Water vapor), T3-Treatment 3 (Water vapor + citric acid), T4-Treatment 4 (water vapor + ascorbic acid).

The results of the L* value that expresses the luminosity or clarity of the sample indicated that only T1 differed statistically from the other treatments, presenting a higher L* value, indicating that dehydrated *in natura*, that is, without any pre-treatment, presented lighter color compared to other treatments.

Considering the scarcity of works in the literature evaluating the dehydrated Japanese grape, this work will be compared with the traditional raisin, as this is the fruit that most resembles the dried *Hovenia dulcis*.

Melo et al. (2018) when studying the grape raiseing *BRS Victory*, submitted to drying methods for obtaining raisins, reported L* value of 25.26, indicating that the dehydrated grape have a darker color compared to the dehydrated pseudofruits from *Hovenia dulcis*.

As for the results of the value of a*, the treatments are statistically equal, indicating that dehydrated *Hovenia dulcis* passes has a reddish color regardless of the treatment applied. Melo et al. (2018) report a value of 5.67 for the same parameter when evaluating raisins.

The b* value of dehydrated pseudofruits showed that T1 was statistically equal to T3, differing from the other treatments (T2 and T4). The results for this parameter are similar to those of Melo et al. (2018) for raisins, who reported a b* value of 3.59, showing the similarity in color of *Hovenia dulcis* raisin with raisin.

Firmness was not influenced by the treatments applied, with an average of 1.51 N. Melo et al. (2018) when evaluating raisins (*BRS Victory*) reports lower average firmness than this study (0.23 N). Considering that texture is an important attribute of influence in the acceptance of processed fruits and vegetables, it would be interesting to evaluate the sensorial acceptance of dehydrated *Hovenia dulcis* as a way to evaluate its possible introduction in the consumer market of dehydrated fruits.

Regarding pH, there was no difference between the treatments under study, obtaining an average of 5.58, and it can be inferred that both the use of citric acid or ascorbic acid in the steam-forming water did not influence this parameter. This pH is higher than that reported by Martineli et al. (2018) when analyzing three cultivars processed raisins produced in the semiarid region of Brazil (4.51-5.56), Machado, Souza and Novaes (2015), when analyzing Isabel pass (4.13), and Melo et al. (2018) when analyzing BRS *Victory* raisins (4.03), this may be due to the low natural acidity of the fresh pseudofruit.

The titratable acidity of dehydrated *Hovenia dulcis* did not differ statistically between treatments, obtaining an average of 0.14 g/100 g, correlating with the pH value found. Higher value for the same parameter is reported by Melo et al. (2018), when analyzing *BRS Victory* raisins (2.10 g/100 g), Machado, Souza and Novaes (2015), when analyzing *Isabel* grape raisin (1.31 g/100 g); and Slva et al (2015), when analyzing the *Crimson grape* raisin (2 g/100g). This variation can be justified by intrinsic differences specific to each species of fruit evaluated.

The soluble solids content did not vary ($p < 0.05$) between the treatments under study (average of 60 g/100 g). The higher SS content in dehydrated foods is due to the concentration of sugars and organic acids due to the loss of water that occurs during dehydration. The result of this research is much higher than that reported by Silva et al. (2015), (17.3g/100 g of SS) when analyzing the dehydrated *Crimson* grape, and by Machado, Souza and Novaes (2015), when analyzing the *Isabel* grape raisin (46.38 g/100 g of SS), and lower than that reported by Melo et al. (2018), when analyzing *BRS Victory* grape raisins (66.3 g/100 g), this difference can be justified by the differences in the species evaluated, in the cultivars, soil type, climate and, cultural treatments, among others.

Regarding moisture, there was no statistical difference between the treatments under study (average 32.53 g/100 g), however it was higher than that indicated in the Brazilian legislation (Brasil, 2005), which stipulates a maximum of 25 g/100 g for dried or dehydrated fruits. However, considering the higher firmness identified in the dehydrated pseudofruit, when compared to previously presented studies on dehydrates, lower moisture content could negatively influence the sensory acceptance of this product. On the other hand, lower moisture values are reported for the Isabel raisin (19.87 g/100g) (Machado, Souza & Novaes, 2015).

The studied samples were statistically equal in relation parameter Ashes (average 3.55 g/ 100g of ash).

The levels of phenolic compounds in treatment T3 (water vapor + citric acid) were statistically higher than other treatments. A higher value for the same parameter is reported by Melo et al. (2018), when analyzing *BRS Victory* raisins (254.55 mg/100g). The author states that the increase in temperature and the discontinuity of the tissue, promoted by heating, increase the bioavailability of compounds with antioxidant activity. Interestingly, when comparing the phenolic content of the

treatments applied in the pseudofruit jelly formulations (Table 2), we observe much higher values than for the pseudofruit in the dehydrated form. Tjis makes, it possible to infer that the combination of ingredients, association with sugar, crushing or shorter heating time (average of 30 min for jellies) favored greater extraction or retention of these compounds.

Regarding vitamin C, there was no significant difference between the treatments under study (average of 5.86 mg/100g). The low content of vitamin C in dehydrated pseudofruits may be due to the high instability of this compound under heating processes such as that used by a cabin dehydrator with an air system heated to 70 ºC used in the elaboration of the studied treatments. When comparing with the results found for jellies (Table 2), there are also higher levels of vitamin C in jellies (average 21.02 mg/100 g) than in dehydrated pseudofruits.

The carotenoid content of the dehydrated pseudofruit varied between the treatments applied, with the T2 treatment being statistically lower (5.41 mg/100 g than the others (average of 7.84 mg/100 g). Possibly the bleaching process (T2, T3 and T4) may have influenced the color and the phenolic compounds content of the dehydrateds, and treatment 3 retained a higher content of phenolic compounds.

In general, when comparing the content of bioactive compounds studied, it can be indicated that the pseudofruit in the form of jelly favors a higher content of these compounds than when in the dehydrated form.

4. Conclusions

The jellies made with the pseudofruit of *Hovenia dulcis* showed good color, texture, high content of soluble solids, ash and bioactive compounds, phenolic compounds, vitamin C and carotenoids, with emphasis on formulations F1 and F3 because they have a higher content of bioactive compounds.

The elaborated dehydrated *Hovenia dulcis* had a high content of SS and ash and a good source of carotenoids, phenolic compounds and vitamin C. Treatments 3 and 4 obtained a higher content of bioactive compounds, these treatments being the most indicated. Aiming at a higher content of bioactive compounds, the processing of the pseudofruit in the form of jelly should be preferred.

Further studies are recommended for the development and improvement of jam and dehydrated *Hovenia dulcis* and also the sensory analysis of the products to adjust the formulations in order to meet consumer expectations.

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CARAWAY, CHINESE CHIVES AND CASSIA AS FUNCTIONAL FOODS WITH CONSIDERING NUTRIENTS AND HEALTH BENEFITS

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ABSTRACT

Since ancient times, the medicinal properties of plant material improve the quality of life. Medicinal plants and foods may provide phytotherapy a new dimension and enable their application to treat and prevention of diseases with the advantage of reducing chemical drugs. Historically, caraway, Chinese chives and cassia have enjoyed a rich tradition of use for flavouring, and medicinal purposes, because of wide range of secondary metabolites with potent antibacterial, antioxidant, antimicrobial, anti-inflammatory, anticancer and other tremendous benefits. In many countries, medicinal plants are widely used as functional foods and daily supplements with the aim of promoting public health and both preventing and curing diseases. The main characteristics, components, active substance and important pharmacological and health benefits of caraway, Chinese chives and cassia was reviewed.

1. Introduction

Medicinal and aromatic plants are the prospective source of bio-molecules in curative drug formulations (Shahrajabian et al., 2020a,b). Medicinal plants application dates back to the origin of human civilization (Sun et al., 2019a,b; Shahrajabian et al., 2019a,b), which works to prevent and treatment of diseases by boosting immune system (Shahrajabian et al., 2020c). Caraway (*Carum carvii* L.) of the *Apiaceae* family, appears to have its origin in Asia minor. It is mainly cultivated in the Netherlands, Finland, Hungary, Morocco, Iran, India and Russia (Laribi et al., 2011). Caraway is basically a biennial but usually treated as an annual from crop production techniques. Chinese chives (*Allium tuberosum*) are an herb-like perennial which is indigenous to Eurasia and North America. Chinese chives are low in calories but high in beneficial nutrients such as vitamins, minerals and antioxidants. Cassia (*Cinnamomum cassia*) is a tree, native to Southern China, Laso, Vietnam and Sumatra. *Cinnamomum cassia* from Lauraceae family is considered as one of the 50 fundamental herbs in traditional Chinese medicine. It is used in food products, such as liqueurs, flavourings and perfumes and regularly used in therapeutic aromatherapy products. The goal of this manuscript is review of important pharmacological benefits of caraway, Chinese chives and cassia.

2. Caraway (*Carum carvi*)

2.1. Introduction and chemical constituents of caraway

Caraway oil is more effective when topically applied than when supplemented in the diet (Shwaireb, 1993). The dried fruit contains 2-8% essential oil with carvone and limonene the principal components (Bailer et al., 2001; Lopez et al., 2008). Morphological characteristics of the caraway flowers influenced by temperatures and photoperiod (Nemeth et al., 1998). Other important oil

components of caraway are β-myrcene, *trans*-dihydrocarvone, *trans*-carveole, α-pinene, sabinene, n-octanal, *trans*-β-ocimene, γ-terpinene, linalool, *cis*- and *trans*-limonene oxide, *cis*-dihydrocarvone, *cis*-carveol, perillaldehyde, *trans*-anethole, and *trans-β*-caryo-phyllene (Raal et al., 2012). *Carum carvi* (caraway) seed have shown that they contain large numbers of terpenes in different concentrations which vary greatly with geographical origin, climate, and harvest time (Grevsen et al., 2009; Laribi et al., 2012). The major constituents of caraway essential oil isolated by hydrodistillation (HD) are limonene (43.5%), carvone (32.6%), and apiole (15.1%); and by microwave-assisted hydrodistillation (MHD) are apiole (12.3%), carvone (31.1%), and limonene (48.4%), consisting of 99.6% of total essential oil, respectively (Jiang et al., 2011). Putievsky et al. (1994) reported that two main compounds in the various caraway oils were limonene (34%-50%), and carvone (47%-62%). Galambosi and Peura (1996) reported that the carvone/limonene ration of the wild populations from the northern parts of Finland was higher than that from the Southern parts of the country. Abdalaziz et al. (2017) reported that from the eight identified constituents, representing 100% of the oil, the most abundant compounds detected were L-Fenchone (55.01%), *p*-Methoxy benzaldehyde (19.15%), and *p*-Methoxy allyl benzene (9.46%), and *Carum carvi* L. seeds are rich sources of oils containing diverse group of phytochemicals.

2.2. Pharmaceutical benefits in traditional and modern pharmaceutical sciences of caraway

Carvone as the main constituent of caraway essential oil is used as a natural inhibitor of sprouting mainly in stored potatoes and onions (Bouwmeester et al., 1998; Laribi et al., 2009), which has been shown to be biologically active, inhibiting germination of seeds, and promoting the growth of certain fungi and microbes (Toxopeus and Bouwmeester, 1992; Iacobellis et al., 2005; Khan and Sastry, 2009). The monoterpenoid limonene has antibacterial, anticancer, antispasmodic, expectorant, fungistatic and other properties (Duke et al., 2003). The traditional use of caraway as a hypoglycemic agent is proved and its extract shows a dose-dependent hypoglycemic activity (Eidi et al., 2010).

In traditional Persian medicine, Ibn Sina traditionally used caraway for weight loss, stomach ache, burping, flatulence and intestinal spasms (Mahboubi, 2019). Carvone has various applications, such as fragrance, and flavor, potato sprouting inhibitor, antimicrobial agent and also in the medical field (Baysal and Starmans, 1999; Laribi et al., 2013). Carvone (*p*-mentha-6,8-dien-2-on, $C_{10}H_{14}O$), molecular weight 150,21, density 0.965, boiling point 230-231^{o}C) is a colorless or yellow oil, insoluble in water and miscible with ethanol (Bailer et al., 2001). Wichtl (1994) reported that caraway promotes gastric secretion, stimulates appetite, and is used as a remedy for colic, loss of appetite and digestive disorders. Caraway oil inhibited the motor activities of SMC of the gallbladder, stomach, trachea and ileum (Boskabady et al., 2003; Micklefield et al., 2003). It is beneficial effect in relieving gastrointestinal symptoms associated with dyspepsia was reported (Al-Essa et al., 2010). Caraway extract has positive role in the management of obesity (Kazemipoor et al., 2013).

Colon premalignant lesions induced by 1,2-dimethylhydrazine (DMH) is mediated by interference of caraway oil components in the activities of the main hepatic xenobiotic metabolizing enzymes (Dadkhah et al., 2011). The aqueous extract of *Carum carvi* may exhibit a potent lowering activity in both normal and severe hyperglycemic rats after repeated oral administration of *Carum carvi* aqueous extract (Lemhadri et al., 2006).

The chronic intake of caraway essential oil influences the pharmacokinetic properties of both orally and intraperitoneally applied paracetamol (Samojlik et al., 2012). Treatment with the black caraway seed essential oil sub-acute toxicity study attenuated histopathological changes in lung, liver, kidney, testes and spleen tissues, but its essential oil can not affect the

immune and blood system (Tabarraei et al., 2019). Pharmaceutical benefits of caraway are shown in Table 1.

Table 1. Pharmaceutical benefits of caraway.

Pharmaceutical benefits	Mechanism and impacts	References
Anti-oxidant activity	a. Black caraway seed oil may be used as a natural antioxidative food additive for improving food quality and stability. b. Caraway seeds and by-product flour can improve the antioxidant potential and overall quality of protein bread.	Yu et al., 2005 Polovka and Suhaj, 2010 Kozlowska et al., 2016 Ahmad et al., 2018
Anti-microbial activity	a. Caraway essential oil has effective antimicrobial agents against *Escherichia coli* and *Staphylococcus aureus*.	Simic et al., 2008 Dimic et al., 2009 Seidler-Lozykowska et al., 2013 Hormis et al., 2015 Khalil et al., 2018
Anti-bacterial activity	a. Caraway essential oil may have the inhibiting effect to reduce the number of *Staphylococcus aureus* and *Escherichia coli*.	Singh et al., 2002 Kwiatkowski et al., 2015
Anti-ulcerogenic activity		Khayyal et al., 2001
Anti-proliferative activity		Nakano et al., 1998
Anti-mutagenic activity		Akram et al., 2020
Anti-cancer activities	a. Caraway may inhibit tumorigenesis though the effect of the intermediary dose of 60 mg/kg body weight was clear. b. Thymoquinone (TQ) from black caraway seeds has several anticancer activities. c. TQ may enhance cisplatin- and docetaxel-induced cytotoxicity.	Naderi-Kalali et al., 2005 Kamaleeswari et al., 2006 Sutton et al., 2014
Anti-hyperglycaemic		Eddouks et al., 2004 Ene et al., 2007 Tahraoui et al., 2007
Anti-diabetes	a. Caraway maybe useful in the control of postprandial rise of blood glucose particularly in diabetic condition. b. Caraway may exhibit blood glucose and lipid lowering activities in diabetes without any effect on C-reactive protein level.	Li et al., 2004 Sushruta et al., 2006 Sadjadi et al., 2014
Anti-inflammatory	a. Combined treatment with peppermint and caraway oil modulates post-inflammatory visceral hyperalgesia synergistically.	Adam et al., 2006 Lacatusu et al., 2017
Improve sleep quality	a. Consuming caraway with aerobic exercise has positive effects on the level of C-reactive protein and sleep quality.	Mohammadkhani et al., 2019
Bio-herbicide	a. Oil-in-water emulsion containing 2.5% of caraway essential oil can be considered as a foliar applied botanical herbicide against barnyardgrass in maize cultivation.	Synowiec et al., 2017 Synowiec et al., 2019

3. Chinese chives (*Allium tuberosum*)

3.1. Chemical components and pharmacological benefits

Chinese chives (*Allium tuberosum*) is a perennial plant cultivated in different parts of Asia, with smelly odor which had been caused by the sulfur-containing compounds (Block, 2013). Its aerial parts of Chinese chive are one of the daily edible green vegetable for Chinese cuisine which is widely cultivated and used for both food and medicine (Mnayer et al., 2014). Chinese chives can have different nutritional and functional components depending on their harvest time (Kim et al., 2018). It is a long-day plant, similar to leek (*A. ampeloprasum* L.) or rakkyo (*A. chinense* G. Don), which are both in the same genus, *Allium* (Kamenetsky and Rabinowitch, 2002). The leaves and seeds of this plant are often used in traditional folk medicines for the treatment of impotence and nocturnal emission in China (Hu et al., 2009). Polysaccharide which is an important type of natural biopolymers has posses various nutritional value and health functions (Zhang et al., 2016). The green leaves are smooth, slim, linear, flat, have a distinctive mild garlic flavor, and are rich in vitamins, fiber, mineral compounds and sulfur compounds that have antibiotic properties (Imahori et al., 2004), but they are highly perishable and quickly lose freshness after harvest (Jia et al., 2017). The major pest of Chinese chives in china is the oligophagous insect *Bradysia odoriphaga* Yang & Zhang (Diptera: Sciaridae) (Feng and Zheng, 1987; Chen et al., 2019), which is distributed in the soil layer at 0-5 cm (Shi et al., 2016), and the rhizomes particularly damaged by Chinese chive (Zhang et al., 2015).

Analysis of the amino acid content of Chinese chive seed revealed that it is a good source of the essential amino acids, isoleucine, tryptophan and lysine with high levels of nutritionally important components, such as oil, minerals and essential amino acids (Hu et al., 2006). Its seeds contain amounts of steroidal saponins (Sang et al., 2001; Zou et al., 2001). The most important constituents isolated from various parts of *Allium tuberosum* are thymidine, adenosine, daucosterol, dimethyl disulfide, allyl methyl disulfide, dimethyl trisulfide, allyl methyl trisulfide, diallyl disulfide, tuberosine A, and tuberoside A. *A. tubersoum* L. contains thiosulfinates which are unstable intermediates in the enzymatically initiated degradation of *S*-alk(en)yl-$_{L}$-cysteine sulfoxide (Ashe and Berry, 2003). Chinese chive contains high concentrations of organic sulfur compounds, which confer characteristics flavors (Randle and Lancaster, 2002), and human health benefits (Griffiths et al., 2002). Chinese chive is used for kidney protection in traditional Chinese medicine (Guohua et al., 2009; Pandey et al., 2014). Chinese chive polysaccharides (CCP) could improve the kidney functions of adenine-induced chronic renal failure (CRF) mice and the renoprotective effect might be associated with its antioxidant, anti-inflammatory and anti-fibrosis activities (Li et al., 2018). Combined extracts from *Lepidium meyenii* (maca) root and *Allium tuberosum* Rottl. (Chinese chive) seed produce better synergistic effects on male sexual function than maca extract or Chinese chive extract alone, and the positive effects may involve the up-regulation of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) concentrations in penis (Zhang et al., 2019). Results suggest that *n*-BuOH extract preparation of *Allium tuberosum* seeds possesses aphrodisiac property (Guohua et al., 2009). *Allium tuberosum* has a significant hepatoprotective and antioxidant activity which may be useful for adjuvant chemotherapy doxorubicin (Sutejo and Efendi, 2017). Ferulic acid from *A. tuberoum* Rottl is the strong choline acetytrasferase (ChAT) activator (Kim et al., 2007). It is also possess sexual enhancing properties (Tang et al., 2017). It is also reported that its extract possess strong hair growth promoting potential which controls the expression of insulin like growth factor-1 (IGF-1) (Park et al., 2015). Pharmaceutical benefits of Chinese chives are presented in Table 2.

Table 2. Pharmaceutical benefits of Chinese chives.

Pharmaceutical benefits	Mechanism and impacts	References
Anti-cancer activity	a. Thiosulfinates from A. tuberosum L. inhibit cell proliferation and induce apoptosis in PC-3 cells, which may be mediated via both caspase-dependent and –independent pathways. b. Thiosulfinates from *A. tubersoum* L. inhibited cell proliferation and activated both the caspase-dependent and caspase-independent apoptotic pathways in HT-29 cells.	Kim et al., 2008 Lee et al., 2009
Anti-diabetic activity	a. The anti-diabetic and hepatoprotective effect of *Allium tuberosum* maybe associated with its antioxidant and its ability to inhibit the pro-inflammatory mediators.	Tang et al., 2017
Antifungal activity	a. It exhibited antifungal activity against *Rhizoctonia solani*, *Fusarium oxysporum*, *Coprinus comatus*, *Mycosphaerella arachidicola*, and *Botrytis cinerea*.	Lam et al., 2000 Benkeblia and Virginia, 2007 Rattanachaikunsopon and Phumkhachorn, 2009 Kocevski et al., 2013
Anti-bacterial activity	a. *S*-Methyl methanethiosufinate and *S*-methyl 2-propene-1-thiosulfinate separated from Chinese chive showed significant antibacterial activities against *E. coli* O-157:H7 including spoilage microorganism in food.	Seo et al., 2001 Nauman et al., 2014 Sharifi-rad et al., 2016
Anti-oxidant activity	a. It can be concluded that ethanolic extract of *Allium tuberosum* has a significant hepatoprotective and antioxidant activity.	Sutejo and Efendi, 2017
Antiparasitic activity	a. It has nematicidal property against root-know nematodes particularly against *Meloidogyne incognita* J2 which attacks root.	Yong-hong et al., 2016
Aphrodisiac	a. It has traditional usage for its aphrodisiac property.	Baljinder et al., 2010 Cinara et al., 2012 Ramandeep et al., 2012
Hypolipidemic	a. It may process sulfur ameliorated liver dysfunction and reduce serum LDL level and intra-abdominal fat.	Eun-Jeong et al., 2012
Anti-inflammation activity	a. Polysaccharides from *Allium* plants have been shown to be effective in resisting oxidation and alleviating inflammation. b. Caraway extract has preventive and anti-inflammatory impact against vascular disease and has potential for supporting prevention against the early process of atherosclerosis.	Lee et al., 2009 Mladenovic et al., 2011 Nikolova et al., 2013 Hur and Lee, 2017

4. Cassia (*Cinnamomum cassia*)

4.1. Introduction and chemical constituents

The genus *Cinnamomum* comprises of several hundreds species which are distributed in all over the world. Cassia, which is the bark of the evergreen tree, is a similar spice to cinnamon but of an inferior quality, which is native to Myanmar, China, Indonesia, and central America. Cassia bark is coarser and less fragrant than cinnamon and is sometimes used as a substitute. *Cinnamomum cassia* from Lauraceae family is considered as one of the 50 fundamental herbs in traditional Chinese medicine. Cinnamon is a spice obtained from the inner bark of several tree species from the genus *Cinnamomum*. Cinnamon has been reported to have significant benefits for human health, especially as an anti-inflammatory, antitumor, anticancer, anti-diabetic and anti-hypertriglyceridemia agent (Muhammad and Dewettinck, 2017; Han and Parker, 2017; Kaur et al., 2019). Other important derivatives are cinnamaldehyde, cinnamic acid and cinnamate (Rao and Gan, 2014). Liu et al. (2018) reported that 39 isolated compound of *C. cassia*, including 12 flavonoid glycosides, three cinnamic acid amides, 12 lignans, five sesquiterpenoids, three cinnamaldehyde derivatives, two phenols, and two indole derivatives. The dominant constituents found in the essential oils of *C. cassia* were aromatic compounds (> 90%) including (E)-cinnamaldehyde (CAL) and (*E*)-cinnamyl acetate (CAC) as the major components (Dong et al., 2013; Jeyaratnam et al., 2016; Le et al., 2020). He et al. (2016) reported three new compounds, including two new diterpenoids, named epianhydrocinnzeylanol, cinnacasiol H., and one hydroxylasiodiplodin, (3R,4S,6R)-4,6-dihydroxy-de-*O*-methyllasiodiplodin, together with five known diterpenoids, and two know phenolic glycosides isolated from the barks of *Cinnamomum cassia*. Zeng et al. (2017) isolated two new glycosides, cinnacassides F, and G from the barks of *Cinnamomum cassia*.

4.2. Pharmaceutical benefits of *Cinnamomum cassia*

Some *Cinnamomum* species such as *C. cassia* is consumed as a spice in cooking to add flavor (Mendis Abeysekera et al., 2019) or some other are cultivated as landscape plants and sidewalk trees (Wang et al., 2020). In traditional medicine in countries like China, India, Indonesia and Vietnam, cassia leaves are used to treat headache, chills, abdominal pain, dysentery, vomiting, cold stomachache, chest tightness, diarrhea, frostbite and cough, and twigs are used to treat blood circulation disturbances, diabetes, dyspepsia and gastritis (Ngoc et al., 2009), the stem barks are used to treat tussis, gastrointestinal neurosis, diarrhea, amenorrhea, dysmenorrheal, impotency, arthralgia, edema, and cardiac palpitation (Liao et al., 2009), and the buds are used to treat cardiothoracic pains, cold pain in the stomach and abdomen nausea, vomiting, belch, hiccup, cough and dyspnea (Zhou et al., 2019). Prasanth et al. (2020) showed that the nine phytochemicals of *Cinnamon* are very likely against the main protease enzyme of COVID-19. Promising antimutagenic and antimicrobial properties were revealed by the cinnamon bark ethanolic extract and cinnamaldehyde, respectively (Vijayan and Mazumder, 2018). Both in vivo and in vitro findings suggest that the EOCC possesses significant spasmolytic effect on uterine contraction which can be a good candidate for the prevention and treatment of primary dysmenorrheal (Sun et al., 2017). *C. cassia* inhibits fibrogensis, followed by HSC-T6 cell activation and may increase restoration of liver function, ultimately resulting in acute liver injury (Lim et al., 2010). Kim et al. (2015) found that the water extract of *Cinnamomum cassia* (CCWE) was a potent inhibitor of angiogenesis. Combined treatment with cinnamaldehyde and β-TCP promoted bone formation and angiogenesis in osteoporotic bone defects, which provides a promising new strategy for repairing bone defects in osteoporotic conditions (Weng et al., 2019).

Table 3. Pharmaceutical benefits of cassia.

Pharmaceutical benefits	Mechanism and impacts	References
Anti-microbial activity	a. *C. cassia* oil is a promising natural antimicrobial for food industry.	Cava et al., 2007 Andrade et al., 2014 Sheng and Zhu, 2014
Anti-fungal activity	a. Trans-Cinnamaldehyde showed the highest antifungal activity, also thymol and carvacrol had additive effect with trans-cinnamaldehyde in preventing the mycelia growth of *Sclerotinia sclerotiorum*. b. *C. cassia* extracts showed notable antifungal potential towards *Penicillium italicum* and *Penicillium digitatum*. c. The antifungal effects of acetone extracts of *C. cassia* against five kinds of plant pathogenic germ including *Alternaria solani*, *Alternaria alternate*, *Fusarium decemcellulare*, *Botrytis cinerea* and *Colletotrichum glycines* were reported.	Jiang et al., 2013 Liu et al., 2015 Pekmezovic et al., 2015 Lu et al., 2019
Anti-bacterial activity	a. Total polyphenols in the non-volatile parts of *C. cassia* branches and leaves showed antibacterial activity in vitro against Gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*), and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria. Its hexane extract of bark may inhibit MRSA, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Acinetobacter baumannii*.	Hossan et al., 2018 Li et al., 2018 Vaillancourt et al., 2018 El Atki et al., 2019 Song et al., 2019
Anti-oxidant activity	a. *Cinnamomum* leaf can be used potentially as a readily accessible source of natural antioxidants. Its ethanol extract has significant anti-oxidant properties.	Hwa et al., 2012 Yang et al., 2012
Anti-inflammation activity	a. The essential oil (EO) from the twigs of C. cassia demonstrates the antinociceptive and anti-inflammatory properties. b. It can significantly attenuated danger signals-induced inflammatory responses via regulation of inflammasome activation. c. Ethyl acetate extracts of cinnamon could alleviate the lung injury of endotoxin-poisoned mice by antagonizing the activation of the NLRP3 inflammasome.	Xu et al., 2019
Anti-viral activity	a. *C. cassia* prevents airway epithelia from human respiratory syncytial virus (HRSV) infection through	Yeh et al., 2013

	inhibiting viral attachment, internalization and syncytium formation.	
Anti-diabetes activity	a. Sesquiterpenoids may be the active compounds in its bark on diabetic nephropathy. b. The cinnamon is effective in controlling blood sugar of people with type 2 diabetes. c. Its extracts may enhance blood sugar lowering effect as compared with standard extracts, and better effects on lipid profile, liver enzymes and other biochemical parameters.	Soni and Bhatnagar, 2009 Gruenwalk et al., 2010 Yan et al., 2015 Kaur et al., 2018
Anti-cancer activity	a. *C. cassia* essential oil and its main component cinnamaldehyde had anti-oral cancer properties, and they could significantly reduce the viability of human oral squamous cell carcinoma HSC-3 cells, and induce DNA damage as well as G2/M cell cycle arrest and apoptosis. b. The cinnamon extract possess cytotoxicity at very low concentrations, and cinnamon extract has the potential use as a part of the food regime in patients suffering from gastric and colon cancer. c. Water-extracted branch of *C. cassia* (WBCC) and cinnamic acid can be potential candidates for developing novel anti-cancer drugs through glycolysis metabolism. d. Its extract may exhibit cytotoxic activity against HepG2.	Park et al., 2002 Wang et al., 2016 Chang et al., 2017 Anju et al., 2018 Lee et al., 2018
Anti-tyrosinase activity	a. It has inhibitory effects against tyrosinase	Chang et al., 2013 Chou et al., 2013
Anti-allergy activity	a. Inhibitory effects of cinnamaldehyde on phospholipase C (PLC) signaling pathway in human embryonic kidney cells have been shown. b. Inhibition of mucosal mast cell activation via suppression of PLCγl signaling pathway.	Kim et al., 2008
Anti-depressant activity	a. It might be an effective anxiolytic agent by regulating the serotonergic and GABAergic system. b. The standardized methanolic extract of *C. cassia* demonstrated antidepressant activity that can be attributed to rise in serotonin levels.	Yu et al., 2007 Jung et al., 2012 Zada et al., 2016
Lactation	a. Supplementing lactating goats rations with garlic, cinnamon or ginger oils has positive effect on milk yield, milk composition and milk fatty acids profile.	Kholif et al., 2012

Cardiovascular protective activity	a. Cinnamic acid, eugenol and cinnamyl alcohol are identified as the active components of cardiovascular protective. b. The water extract of *C. cassia* may have preventive and protective effects on diabetic cardiomyopathy through significantly increasing the content of PCR, ATP and ADP in myocardial tissue as well as improving cardiac energy metabolism to a certain extent.	Kim et al., 2015 Kwon et al., 2015 Wei et al., 2018
Anti-arthritic activity	a. *C. cassia* bark hydroalcoholic extract significantly reduce MDA levels and may lead to decrease in TNF-α receptor expression.	Sharma et al., 2018
Cytoprotective activity	a. The aqueous extract of *C. cassia* showed the *in vitro* cytotoxic effects of *cis*-diammine dichloroplatinum (CDDP), which was achieved by suppressing the increased expression of CDDP-induced mitochondrial Bax protein, releasing mitochondrial cytochrome *c*, activating caspase-3, making DNA fragmentation and generating ROS and up-regulating expression of cytoprotective gene (heme oxygenase (HO)-1).	El Kady and Ramadan, 2016
Anti-nematodes activity	a. Cinnamaldehyde was found to be the most potent chemical derived from Cinnamon.	Kong et al., 2007

5. Conclusions

Medicinal plants have been shown to have tremendous health benefits, such as digestive stimulant action, anti-inflammatory, antioxidant activity, antimicrobial, antimutagenic, hypolipidemic activities, anticarcinogenic potential and etc. Nutraceutical substances obtained from medicinal plants have demonstrated physiological benefits or are capable of providing some sort of protection against diseases. The most important pharmaceutical and health benefits of caraway are anti-oxidant, anti-microbial, anti-bacterial, anti-ulcerogenic, anti-proliferative, anti-mutagenic, anti-cancer, anti-hyperglycaemic, anti-diabetes, anti-inflammatory activities, improve sleep quality and bio-herbicide. The most important pharmacological properties of Chinese chives are anti-bacterial, anti-fungal, anti-parasitic, aphrodisiac, anti-cancer, hypolipidemic, pesticidal, renoprotective, promotion of hair growth, anti-coagulant, regulate hormonal balance, and mediate sensory perception. The most important health benefits of cassia are anti-microbial, anti-fungal, anti-bacterial, anti-oxidant, anti-inflammation, anti-viral, anti-diabetes, anti-cancer, anti-tyrosinase, anti-allergy, anti-depressant, cardiovascular protective activity, anti-arthritic, cytoprotective, anti nematodes activity and improve lactation. More researches are needed to find the potential challenges and benefits of incorporating these medicinal plants in the diet which may offer prospective opportunities for future drug development.

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EFFECT OF MICROENCAPSULATION AND COATING ON THE SURVIVABILITY OF LACTOBACILLI PROBIOTICS IN YOGURT AND GASTROINTESTINAL CONDITIONS

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ABSTRACT

Microencapsulation of probiotics is an efficient way that can improve the viability rate of them in dairy products like yogurt as well as in lumen tract conditions. The viability of free and microencapsulated forms of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* were evaluated in yogurt and under simulated gastrointestinal conditions. Microencapsulation and double coating process carried out by alginate-chitosan and Eudragit S100 nanoparticles and by the extrusion method. Bacterial count (cfu g-1) of *L. acidophilus* reduced from 7.0×10^8 to 4.2×10^6 in day 0 and in day 42 in yogurt containing free bacteria, while the bacterial count of microencapsulated bacterium showed a reduction from 3.3×10^7 to 2.5×10^7. Microencapsulation of *L. rhamnosus* could also increase the viability of this bacterium; 3.2×10^9 to 5.8×10^6 bacterial count by reduction of free-form storage, and 7.6×10^9 to 3.4×10^8 bacterial count by reduction of microencapsulated form in 42 days. On day 14 (first day of bacterial count in gastrointestinal condition) *L. acidophilus* count was 1.3×10^3 and 5.0×10^7 which reached 2.0×10^0 and 2.8×10^4 on day 42 in free and microencapsulated forms respectively. The bacterial count of *L. rhamnosus* decreased from 1.2×10^3 to 5.0×10^0 in free form, and from 2.5×10^7 to 2.8×10^4 in microencapsulated one. The results of this study suggest that this method of microencapsulation can improve the viability of *L. rhamnosus* and *L. acidophilus* in yogurt and in the simulated human gastrointestinal tract.

1. Introduction

Probiotics are identified as live microbial foodstuff supplements which benefit the host via improving its intestinal microbial equilibrium. These microorganisms can be formulated in several special kinds of manufactured goods including foodstuffs, medicines, and nutritional complements (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). Identified healthiness advantages of probiotic strains comprise suppressing the development of unwanted microbes in the small intestine and

colon, immunomodulating the immune system, reducing serum cholesterol heights, improving lactose consumption, and so on (Huang, Shen, Liang, & Jan, 2016; Rijkers et al., 2010; Tabrizi et al., 2019; Ansari, Pourjafar, Tabrizi, & Homayouni, 2020).

Foodstuffs including at least 107 cfu g-1 probiotic microorganisms at the time of consumption are called "probiotic food products". Dairy products like yogurt, buttermilk, cheese, and ice-cream are common probiotic foods. Fermented dairy products with some specific properties like proper taste, aroma, and oral sense are appealing to all age groups, therefore they can be considered as appropriate carriers for probiotic microorganisms (Anal & Singh, 2007; Granato, Branco, Cruz, Faria, & Shah, 2010).

Lactic acid bacteria, especially Lactobacilli are the main probiotic microorganisms of the human gastrointestinal (GI) lumen. The proper adhesion of Lactobacilli to the enterocytes and their advanced health effects has led this genus of probiotic bacteria to be the most illustrated and applicable among other probiotic genera (Bernet, Brassart, Neeser, & Servin, 1994; Kandasamy et al., 2016).

Generally, for applying every probiotic microorganism in dairy products some features need to be addressed, for instance, the viability of the microbes in the dairies, the chemical, physical and organoleptic characteristics of the final product, and the probiotic's healthiness and outcomes. The viability of these microbes during the processing and storage time has a significant role in the induction of their asserted healthiness effects. Exposure to acid and bile, oxidative stress, osmotic pressure, and cold stress may possibly diminish the number of probiotic bacteria under the effective threshold (Frederico et al., 2016; Ranadheera, Evans, Adams, & Baines, 2012).

Microencapsulation of probiotics is an efficient method that can improve the survival rate of these microorganisms in dairy products like yogurt as well as in GI tract conditions. As a matter of fact, microencapsulation permits the probiotics to be separated from their surroundings via a protective covering (Rocha, 2016). Some investigations have reported the method of the microencapsulation by using calcium alginate and coating it with chitosan, which can provide protection for probiotic microorganisms. These materials have also been used widely for immobilization of probiotic microorganisms due to the reason of ease of use, its non-toxic characteristic, and its low cost (Ansari, Pourjafar, Jodat, Sahebi, & Ataei, 2017; Chávarri et al., 2010; Crcarevska, Dodov, & Goracinova, 2008; Kanmani et al., 2011).

Chitosan is a linear polysaccharide with a positive charge that is structured through deacetylation of chitin. Chitosan is water soluble in lower than pH 6 and makes coagulation via ionotropic gelation. This polysaccharide is able to cross-link with anions or polyanions, such as Eudragit substance (Abouhussein, El-bary, Shalaby, & El Nabarawi, 2016; Ahmed & Aljaeid, 2016).

Eudragit (Eu) is a trade name for Rohm GmbH & Co. KG. Darmstadt in Germany, originally marketed during the 1950s. This product is prepared by the polymerization of acrylic and methacrylic acids or their esters, such as dimethylamino ethyl ester or butyl ester. Eu powder products are unique polymers with different grades of solubility. Eu polymers are non-toxic and food-grade polymers. Alternatives to Eu polymer are employed to coat solid medicines which used orally, for instance, granules, pills or capsules. Eu S100 is an anionic copolymer (one kind of the Eu polymers) derived from methacrylic acid and methyl methacrylate (1:2 proportion). This material is insoluble in water and acids, but soluble in aqueous solution at pH 7 or higher. Therefore, this pH-sensitive polymer doesn't release its contents in the stomach (pH 1.5-2), but in the distal small intestine and in the colon (pH 7) as an aimed organ, and it seemed that this polymer can carry probiotic bacteria similar to it carrying solid medicines to colon in a safe way (Badhana, Garud, & Garud, 2013; Hu, Liu, Chen, Li, & Zhao, 2012; Thakral, Thakral, & Majumdar, 2013).

Double coating of calcium alginate beads by chitosan (first coat) and Eu S100 nanoparticles (second coat) that contains probiotic bacteria, is one of the newest kind of microencapsulation methods that we applied for achieving suitable strength in the bead's wall with a smooth surface (smother surface more strength in bead's wall). Nanoparticles in preference to Eu powder is founding of a thin nanosize layer in the coating of the beads. This particularly thin layer is potentially able to enhance the strength of beads with no increase in the size of them. Smaller beads perhaps may reduce the oral sense of beads in a food carrier as well as diminishing use of Eu powder (Younis, Shaheen, & Abdallah, 2016).

Yogurt is one of the high-consumption product and favorite dairy can be used as a probiotic carrier. Several factors possibly will have an effect on the continued existence of probiotic microorganisms in yogurt. Final pH at the end of yogurt fermentation shows to be the main significant factor influencing the growth and survival of probiotic microorganisms (Akın, Akın, & Kırmacı, 2007; Hekmat & Reid, 2006; Mortazavian et al., 2007).

The goal of this study is to evaluate the effects of calcium alginate-chitosan and Eu S100 nanoparticles microencapsulation on the viability of probiotic bacteria (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) under simulated GI conditions and during storage in yogurt. (Body text TNR 12, normal, indent first line 0.66 cm, line spacing Single)

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 *et al.*, 1979; Caglar *et al.*, 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, 2010; Pochart and Desjeux, 1988).

2. Materials and methods

2.1. Preparation of probiotic bacteria

Probiotic cultures of L. rhamnosus (PTCC 1469) and L. acidophilus (PTCC 4356) were achieved as of Iranian Research Organization for Science and Technology (IROST) and inoculated into MRS-broth (de Man-Rogosa-Sharpe) and incubated at 37±2 °C for 24 h in aerobic conditions. The probiotic growth in late-log phase was collected by means of centrifugation (Centrion Centrifuge, Model 2010, West Sussex, BNI8OHY, UK) at 5,000 rpm for 10 min, and afterward it was washed two times in sterilized distilled water before employing in the microencapsulation procedure (Mirzaei, Pourjafar, & Rad, 2011).

2.2. Preparation of chitosan solution

For the preparation of chitosan solution, 0.4 g low-molecular-weight chitosan (Sigma, USA) blended with 90 mL distilled water and acidified using 0.4 mL of glacial acetic acid (Merk, Darmstadt, Germany). Then, the pH was regulated in 5.6–5.8 using adjoining 1 mol L-1 NaOH, and the solution was filtered through Whatman #4 paper filter and the extent was adjusted to 100 mL before sterilizing into the autoclave (121 °C, 15 min). Finally, the chitosan solution was held at 5 °C overnight (Crcarevska et al., 2008; Kanmani et al., 2011; Lee, Cha, & Park, 2004).

2.3. Preparation of Eudragit S100 nanoparticles

For preparing the Eu S100 nanoparticles from, Eu S100 copolymer powder (EvonikPharma Polymers, Darmstadt, Germany), we used Supercritical Antisolvent Technique (SAS), this technique was employed and option of acetone was applied as a solvent for Eu powder (as a modified SAS process; we utilized homogenization power as a replacement for using high pressure). In this method, 4 mg mL-1 of Eu solution was applied in distilled water slowly as a supercritical fluid that had been held below homogenization pressure (Wisetise, DAIHAN Scientific Co., Ltd, Korea) at 26,000 rpm at 35 °C for 10 min. Also, distilled

water as a surfactant included 15 mg L-1 Tween 80 (Merk, Hohenbrunn, Germany). Lastly, the acetone solvent was evaporated. The particle size of the Eu and PDI (polydispersibility/polydispersivity index) were assessed using Laser Particle Size Analyzer device (Brookhaven Instruments Corporation, USA) (Hari, Lu, Narayanan, Wang, & Zheng, 2016; Hu et al., 2012; Pourjafar, Noori, Gandomi, Basti, & Ansari, 2018; Yoo, Giri, & Lee, 2011).

2.4. Microencapsulation process

2.4.1. Primary microencapsulation process

In this process, 4 g 100 mL-1 sodium alginate (Sigma, USA) was blended with distilled water and then sterilized and kept in 5 °C overnight. Following day, 10 mL of probiotic suspension (2×1010 cfu mL-1) was added to the sodium alginate liquid. Subsequently, the mixture of the bacterial suspension and sodium alginate was injected into sterile 0.1 mol L-1 CaCl2 (Merk, Darmstadt, Germany) fluid by means of sterile insulin syringes (0.2 mm) (extrusion technique was used for encapsulation process). After applying the drops into CaCl2 solution, the drops immediately turned into clot balls (the space between the CaCl2 solution and syringe needle was roughly 20 cm, and we applied as much pressure as possible to the syringe to force the solution out extremely fast), and in 60 minutes, the entire beads were gathered and washed with distilled water (Abdolhosseinzadeh, Dehnad, Pourjafar, Homayouni, & Ansari, 2018; Ghasemnezhad, Razavilar, Pourjafar, Khosravi-Darani, & Ala, 2017). (See Fig. 1)

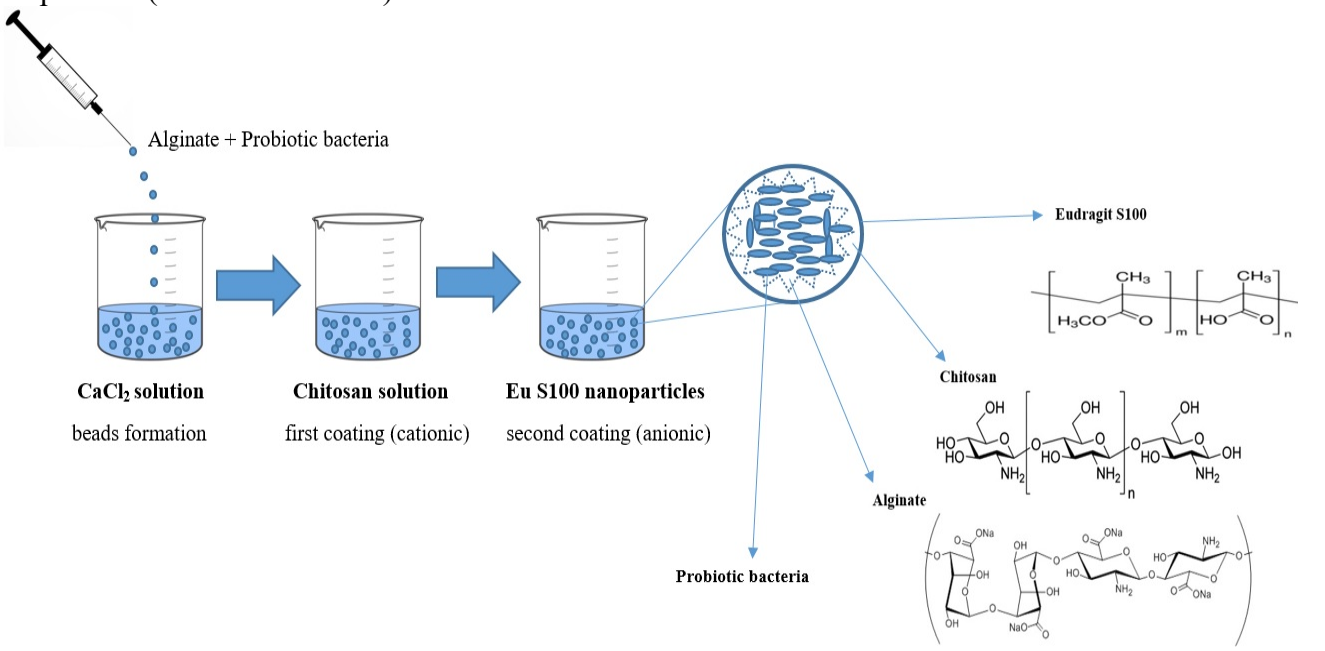

Figure 1. Microencapsulation process: Primary microencapsulation, the first coating of beads with chitosan solution, and the second coating of beads by Eudragit S100 nanoparticles. The final structure of formed beads has been illustrated.

2.4.2. The first coating of beads with chitosan solution

For the primary coating of the beads, they were submerged in 100 mL of chitosan solution lightly shaken at 100 rpm for 40 min on a magnetic stirrer (IKA Labortechnik, Model 79219 staufen, KG, Germany). Then, the chitosan coated beads (single coated) were collected and rinsed with distilled water (Chávarri et al., 2010; Kanmani et al., 2011; Mirzaei et al., 2011). (See Fig. 1)

2.4.3. The second coating of beads by Eudragit S100 nanoparticles

For second coating of beads previously coated by chitosan (single coated beads), the

beads were immersed in 100 mL Eu S100 nanoparticles solution (4 mg 100 mL-1) and held for 4 h on the shaker (100 rpm) (Badhana et al., 2013; Hu et al., 2012; Yoo et al., 2011). Finally, the double-coated beads were washed thoroughly with distilled water and applied on the same day. (See Fig. 1)

2.5. Probiotic yogurt preparation

Yogurt was manufactured by heating reconstituted skimmed milk (13% w/v) at 90 °C for 20 min and after cooling to 45 °C, the milk was inoculated (1 unit 10 L-1) of each of *Lactobacillus delbrueckii* subsp. bulgaricus and *Streptococcus thermophilus* until the pH reached 4.5. The inoculated milk was separated into equivalent portions; one part was added free probiotic cells (approximately 1010 cfu g-1), whereas the other part was added beads (1 g beads per 10 g yogurt, 1 g beads containing ~1010 cfu g-1). Finally, all yogurt portions were stored at 4 °C for 42 days.

2.6. Survey of the viability of free and microencapsulated probiotic bacteria into yogurt

Analysis of bacterial enumeration was concluded through plate count on MRS-Glucose-vancomycin-agar (MRS agar; QUELAB, Canada, Glucose; Merk, Germany and Vancomycin; Sigma, USA) for L. rhamnosus and MRS-Salicin-agar (MRS agar; QUELAB, Canada and Salicin; Sigma, USA) for L. acidophilus straight following the production of probiotic yogurt at time at 0 and during the 42 days period with one week interval time (the storage temperature was 5 °C) (Ansari & Pourjafar, 2019b; Homayouni et al., 2018; H Pourjafar, Mirzaei, Ghasemnezhad, & Homayouni rad, 2012; H Pourjafar, Noori, Gandomi, & Akhondzadeh Basti, 2016; Shah, 2000).

Samples of two type yogurts (10 g of yogurt contains free cells and 10 g of yogurt contains beads) were diluted into 90 mL peptone water (0.1 g 100 mL-1) and 1 mL aliquot dilutions were introduced to all plates of the MRS-Glucose-vancomycin-agar and MRS-Salicin-agar. For a production of the MRS-Glucose-vancomycin-agar, Glucose (10 mL solution at 10% w/v) and vancomycin (50 µg mL-1) were added to 90 mL of pure MRS agar. For a production of the MRS-Salicin-agar, Salicin (10 mL solution at 10% w/v) was added to 90 mL of pure MRS agar. Finally, each medium was sterilized at 121.1 °C for 15 min. The entire plates of L. rhamnosus and *L. acidophilus* were incubated at 37±2 °C for 48 h in the aerobic situation. The standards were expressed as colony-forming units per gram of sample (cfu g-1) (Pourjafar et al., 2016; Saxelin et al., 2010; Shah, 2000).

To enumerate the microencapsulated probiotic bacteria within yogurt, the arrested probiotics were released from the beads. Ten grams of yogurt were blended with 90 mL of phosphate buffer (0.1 mol L-1, pH 7.0) followed by 60 min shaking in a bag blender (netech-laboratory, Bag Tech®). The yogurt sample counting free probiotic bacteria were treated in a similar fashion so to remain the same analogous action order. (See Fig. 2)

2.7. Survey of the viability of free and microencapsulated probiotic bacteria under simulated gastrointestinal circumstances

The survival rate of probiotic bacteria in simulated GI fluid was studied in 14, 28 and 42 days following inoculation of bacteria (in two types; free and encapsulated with double coating) in yogurt. In each study period (14, 28, and 42) the samples (10 g of yogurt contains free cells and 10 g of yogurt contains beads) were placed separately in a tube counted by 100 mL of sterilized simulated gastric juice (0.08 mol L-1 HCl, including 2 g L-1 NaCl, with 3 g L-1 pepsin, pH 1.5) and incubated for 30, 60, 90, and 120 min at 37±2 °C. Following the incubation, aliquots of 10 g of beads or 10 mL of free cell suspensions from the previous stage were transferred to 100 mL of sterilized simulated intestinal liquid (0.05 mol L-1 KH2PO4, with 10 g L-1 bile salt, pH 7.5). Subsequently, these tubes were incubated for 150 min at 37±2 °C. Then samples were diluted with sterilized peptone water and 1 mL aliquot dilutions were

dispensed in every plate of the MRS-Salicin-agar and MRS-Glucose-vancomycin-agar. All counting plates of *L. rhamnosus* and *L. acidophilus* were incubated at 37±2 °C for 48 h in aerobic condition. To enumerate the microencapsulated bacteria, the arrested cells were released from the beads. The beads re-suspended in 90 mL of phosphate buffer (0.1 mol L-1, pH 7.0) followed by 60 min shaking in a bag blender (netech-laboratory, Bag Tech®) (Ansari & Pourjafar, 2019a; Mirzaei et al., 2011; H Pourjafar et al., 2012; H Pourjafar et al., 2016; Shima, Morita, Yamashita, & Adachi, 2006; Sultana et al., 2000). (See Fig. 2)

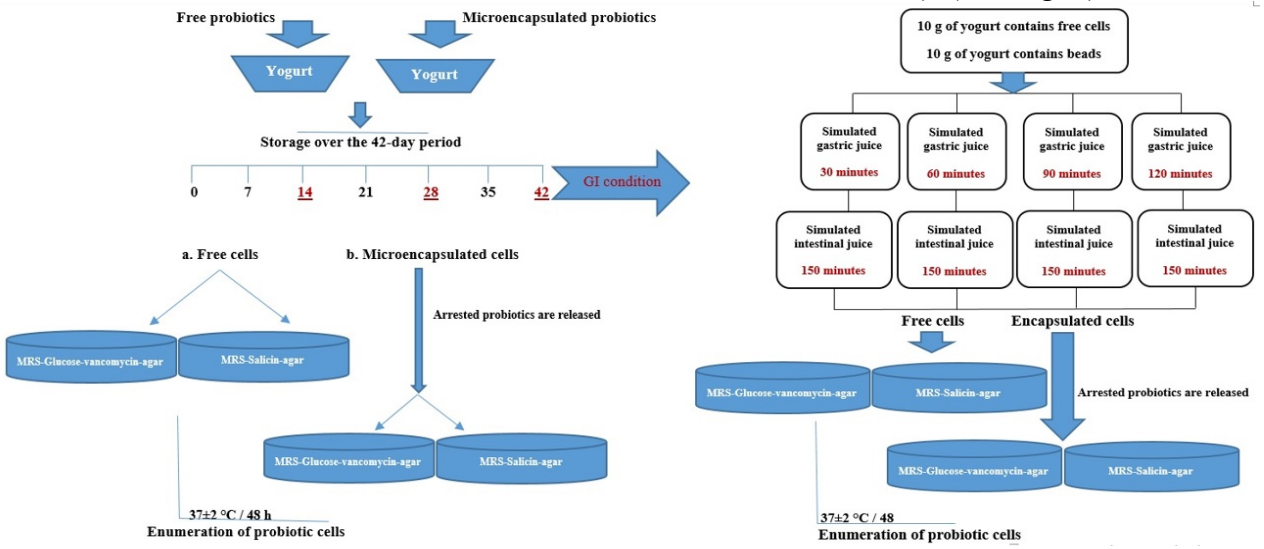

Figure 2. Survey of the viability of free and microencapsulated probiotic bacteria into yogurt following the production of probiotic yogurt at time at 0 and during the 42 days period with one week interval time (the storage temperature was 5 °C) **(Left)**, and survey of the viability of free and microencapsulated probiotic bacteria under simulated gastrointestinal circumstances in 14, 28 and 42 days following inoculation of bacteria (in two types; free and encapsulated with double coating) in yogurt **(Right)**

2.8. Assessment of acidity, pH, and organoleptic characteristics

Acidity and pH of each product were determined in periods of 0, 7, 21 and 42 days (at the same time of examination the survival rate of free and encapsulated bacteria). For acidity and pH measurement, Dornic method and pH meter (AZ-8601, Taiwan) were employed respectively. The assessments of the organoleptic characteristics of each product were done via 32 experts (taste panel) in the same condition as locality, lightness, and containers in periods of 7, 21 and 42 days.

2.9. Statistical analyses

The viability of bacteria in samples of yogurt was assessed in 42 days storage period using Repeated Measures ANOVA test. The viability of bacteria in the GI simulation environment evaluated in periods of 14, 28 and 42 days after inoculation of bacteria by Repeated Measures ANOVA test. Friedman none-parametric test carried out for comparison of the mean of acidity, pH, and organoleptic scores in different days and the mean values of yogurt containing free or coated probiotics and control group on each day were compared using Kruskal-Wallis test. The total assessments were obtained in triplicate.

3.Results and discussions

3.1. Manufacture of Eudragit S100 nanoparticles and characteristics of beads

In this study, 100-150 nm sized encapsulated particles were prepared through the homogenization of Eu S100 powder (26000 rpm, 10 min). Hu et al. (Hu et al., 2012) also used Eu S100 powder and acetone solvent through the SAS technique to create nanoparticles of Eu S100. They attained regular

and uniform nanoparticles with satisfactory size (147 nm). This study was performed at 35 °C and at 15 MPa pressure. In our investigation, we utilized the homogenization process to break particles instead of rising environmental pressure. In this way, the size of the obtained nanoparticles by our method was comparable to Hu et al. (Hu et al., 2012) examination. After preparation of the Eu S100 using SAS technique, the particle size and PDI of Eu S100 particles were 100 nm and 0.410 respectively. The ending diameter of the double coated beads was at about 80–200 μm.

3.2. The viability of free and microencapsulated probiotics in yogurt during storage time

Bacterial counts in yogurt containing free and microencapsulated probiotic bacteria are displayed in table 1. The bacterial count was taken twice for each sample and the mean of these repetitions is shown. The viability of bacteria decreased significantly during the study ($P=0.027$), and there were not any significant differences between microencapsulated and free-form bacteria in this case ($P=0.360$). Also, there was not any significant difference between the viability of two species of bacteria ($P=0.408$). In a similar study, Krasaekoopt et al. (2003, 2004) (Krasaekoopt, Bhandari, & Deeth, 2003, 2004) assessed the viability of *L. acidophilus* 574, *L. casei* 01 and *B. bifidum* 1994 microencapsulated in the only chitosan-coated alginate beads in yogurt product during storage time. The survival rate of the microencapsulated mentioned probiotics was higher than that of the free bacteria just about 1 log. The count of Lactobacilli was maintained higher than the 107 cfu g-1 (suggested therapeutic minimum) during storage, but not for the Bifidobacteria.

Calcium alginate makes a tender membrane between probiotics and harsh environmental circumstance; therefore we employed chitosan as an external layer to improve the strength of beads. Chitosan itself is vulnerable to deterioration via acids in low pH situations; therefore we coated a second layer of anionic Eu around cationic chitosan layer. This second layer is thin and improves the resistance of coated beads in the acidic state without major alteration in size of beads (Badhana et al., 2013; Chávarri et al., 2010; Kanmani et al., 2011; Liserre, Re, & Franco, 2007).

3.3. The viability of free and microencapsulated probiotics under simulated gastrointestinal conditions

In this study, to determine the effect of the acidic juice of the stomach and the bile of the intestine on the viability of microencapsulated probiotic bacteria, an in vitro method was employed. Bacterial count in simulated GI conditions is displayed in table 2. The viability of bacteria decreased significantly during the study period ($P<0.01$) and during the measurement time ($P<0.01$). *Lactobacillus acidophilus* was more stable in comparison with *Lactobacillus rhamnosus* ($P<0.01$) and mirocoated bacteria were more stable than free bacteria ($P<0.01$).

There are several studies on the viability of the free and encapsulated of probiotic microorganisms under the simulated GI conditions (Chávarri et al., 2010; Hansen, Allan-Wojtas, Jin, & Paulson, 2002; Kanmani et al., 2011; Krasaekoopt & Watcharapoka, 2014; Mirzaei et al., 2011). In our investigation, we had an efficient new and modified approach to producing beads. We employed a second layer of nanoparticle Eu S100 which could enhance probiotic bacteria (*L. acidophilus* and *L. rhamnosus*) survival rate through storage time as well as simulated GI situation. We assessed the survival rate of mentioned probiotics under simulated GI circumstance in elected days throughout the storage time which has not been considered in previous studies. In these studies, beads were moved to simulate GI situation accurately the following production. Nevertheless, in our investigation, we primarily inserted beads into the yogurt and monitored the survival rate of probiotic microorganisms into the product itself. We also gathered beads from the yogurt in elected days and studied the viability of probiotics in simulated GI situations at the equivalent time.

Table 1. Bacterial viability (Mean ± SD) comparison of yogurt in lab environment

Experimental Group	Bacteria	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Free	*Lactobacillus acidophilus*	$7.0\times10^{8}\pm9.9\times10^{7}$	$2.3\times10^{8}\pm2.1\times10^{6}$	$1.9\times10^{8}\pm1.8\times10^{7}$	$4.2\times10^{7}\pm9.9\times10^{6}$	$1.8\times10^{7}\pm1.1\times10^{7}$	$6.9\times10^{6}\pm3.4\times10^{6}$	$4.2\times10^{6}\pm2.2\times10^{6}$
	Lactobacillus rhamnosus	$3.2\times10^{9}\pm2.8\times10^{8}$	$2.7\times10^{8}\pm1.0\times10^{8}$	$1.9\times10^{8}\pm5.5\times10^{7}$	$5.1\times10^{7}\pm5.1\times10^{7}$	$2.7\times10^{7}\pm1.1\times10^{6}$	$1.3\times10^{6}\pm1.6\times10^{6}$	$5.8\times10^{6}\pm6.3\times10^{6}$
Encapsulated	*Lactobacillus acidophilus*	$3.3\times10^{7}\pm1.1\times10^{7}$	$5.2\times10^{7}\pm1.6\times10^{7}$	$4.1\times10^{7}\pm2.0\times10^{7}$	$1.9\times10^{7}\pm1.3\times10^{7}$	$1.5\times10^{7}\pm8.4\times10^{5}$	$2.7\times10^{7}\pm1.3\times10^{6}$	$2.5\times10^{7}\pm1.1\times10^{6}$
	Lactobacillus rhamnosus	$7.6\times10^{9}\pm4.2\times10^{9}$	$2.0\times10^{9}\pm2.5\times10^{8}$	$2.2\times10^{9}\pm4.9\times10^{7}$	$2.4\times10^{9}\pm4.7\times10^{8}$	$1.5\times10^{9}\pm3.7\times10^{8}$	$3.2\times10^{8}\pm9.9\times10^{7}$	$3.4\times10^{8}\pm9.9\times10^{7}$

Table 2. Bacterial viability (Mean ± SD) comparison of yogurt in the simulated gastrointestinal conditions

Day	Experimental Group	Bacteria	0 min	30 min	60 min	90 min	120 min
14	**Free**	*Lactobacillus acidophilus*	$2.0\times10^{8}\pm0.0\times10^{1}$	$1.0\times10^{7}\pm7.1\times10^{5}$	$7.4\times10^{6}\pm1.4\times10^{5}$	$3.1\times10^{4}\pm7.1\times10^{2}$	$1.3\times10^{3}\pm1.4\times10^{2}$
		Lactobacillus rhamnosus	$1.3\times10^{8}\pm2.1\times10^{7}$	$8.8\times10^{6}\pm4.2\times10^{5}$	$3.2\times10^{5}\pm0.0\times10^{1}$	$2.8\times10^{4}\pm7.1\times10^{2}$	$1.2\times10^{3}\pm1.4\times10^{2}$
	Microencapsulated	*Lactobacillus acidophilus*	$5.0\times10^{7}\pm7.8\times10^{6}$	$4.3\times10^{7}\pm2.8\times10^{6}$	$7.6\times10^{6}\pm7.1\times10^{4}$	$6.8\times10^{6}\pm4.2\times10^{5}$	$5.6\times10^{5}\pm3.5\times10^{4}$
		Lactobacillus rhamnosus	$2.6\times10^{9}\pm7.8\times10^{8}$	$1.0\times10^{9}\pm0.0\times10^{1}$	$7.3\times10^{8}\pm2.4\times10^{8}$	$1.7\times10^{8}\pm1.4\times10^{7}$	$2.5\times10^{7}\pm1.5\times10^{7}$
28	**Free**	*Lactobacillus acidophilus*	$1.5\times10^{7}\pm0.0\times10^{1}$	$2.4\times10^{5}\pm7.1\times10^{3}$	$8.6\times10^{4}\pm1.4\times10^{3}$	$3.3\times10^{3}\pm2.8\times10^{2}$	$2.9\times10^{2}\pm7.0\times10^{1}$
		Lactobacillus rhamnosus	$2.5\times10^{7}\pm0.0\times10^{1}$	$9.3\times10^{5}\pm2.8\times10^{4}$	$3.5\times10^{4}\pm2.8\times10^{3}$	$2.7\times10^{3}\pm2.8\times10^{2}$	$1.3\times10^{2}\pm2.8\times10^{1}$
	Microencapsulated	*Lactobacillus acidophilus*	$1.6\times10^{7}\pm1.4\times10^{6}$	$1.3\times10^{7}\pm2.8\times10^{6}$	$8.2\times10^{6}\pm2.1\times10^{5}$	$2.6\times10^{6}\pm8.5\times10^{5}$	$6.8\times10^{5}\pm4.9\times10^{4}$
		Lactobacillus rhamnosus	$1.5\times10^{9}\pm7.1\times10^{7}$	$1.0\times10^{9}\pm7.1\times10^{7}$	$7.3\times10^{8}\pm2.1\times10^{7}$	$9.5\times10^{6}\pm7.1\times10^{5}$	$1.2\times10^{5}\pm7.1\times10^{3}$
42	**Free**	*Lactobacillus acidophilus*	$2.7\times10^{6}\pm1.4\times10^{5}$	$2.7\times10^{4}\pm6.4\times10^{3}$	$3.9\times10^{2}\pm1.2\times10^{2}$	$1.9\times10^{1}\pm1.4\times10^{0}$	$2.0\times10^{0}\pm1.4\times10^{0}$
		Lactobacillus rhamnosus	$1.1\times10^{6}\pm7.1\times10^{4}$	$6.0\times10^{3}\pm6.4\times10^{2}$	$2.1\times10^{2}\pm4.9\times10^{1}$	$4.0\times10^{1}\pm2.1\times10^{1}$	$5.0\times10^{0}\pm4.2\times10^{0}$
	Microencapsulated	*Lactobacillus acidophilus*	$1.5\times10^{7}\pm4.9\times10^{6}$	$1.6\times10^{6}\pm1.4\times10^{5}$	$5.1\times10^{6}\pm5.8\times10^{6}$	$4.3\times10^{5}\pm1.2\times10^{5}$	$2.8\times10^{4}\pm1.8\times10^{4}$
		Lactobacillus rhamnosus	$3.1\times10^{8}\pm1.4\times10^{7}$	$6.0\times10^{7}\pm4.4\times10^{7}$	$4.5\times10^{6}\pm1.6\times10^{6}$	$3.4\times10^{5}\pm1.3\times10^{5}$	$2.8\times10^{4}\pm4.9\times10^{3}$

3.4. Acidity and pH of yogurt samples during 42 days storage

Acidity and pH of yogurt samples were evaluated on days 0, 7, 21 and 42 following incubation and results are shown in Table 3. Throughout the storage period the acidity and pH were diminished and increased respectively in all samples. In yogurt samples containing the free-form of bacteria acidity and pH, alterations were more dramatic than those of encapsulated and control groups. After the day 21, the differences between pH and acidity of yogurt containing the free and microencapsulated form of probiotics were considerable and the latter remained its pH and acidity at a value close to the control group.

3.5. Organoleptic assessments

Organoleptic scores of yogurt containing the free form of bacteria were the best on the first day but reduced substantially during 42 days of storage. Yogurt containing microencapsulated probiotics, however, maintained its acceptability during the experiment. The flavor of this group was significantly better than yogurt containing the free form of bacteria at day 42, so the maintenance of the flavor was the main organoleptic characteristic improved by microencapsulation (See Table 4).

Table 3. Acidity and pH of 0, 7, 21 and 42 days old yogurt (Mean ± SD) in different experimental groups

Time of measuring (day)	Group	pH	Acidity (°D)
0	Free Bacteria	4.49 ± 0.10^{ABDa}	90.75 ± 1.06^{ABa}
	Microencapsulated bacteria	4.49 ± 0.00^{Aa}	90.50 ± 0.70^{Aa}
	Control	4.50 ± 0.00^{Aa}	89.95 ± 1.34^{Aa}
7	Free Bacteria	4.41 ± 0.00^{BCa}	95.50 ± 0.42^{Ba}
	Microencapsulated bacteria	4.44 ± 0.05^{Aa}	94.25 ± 0.35^{Aa}
	Control	4.45 ± 0.00^{Aa}	93.60 ± 0.56^{Aa}
21	Free Bacteria	3.82 ± 0.00^{Cb}	116.90 ± 0.14^{Ab}
	Microencapsulated bacteria	4.14 ± 0.00^{Aa}	99.00 ± 0.00^{Aa}
	Control	4.16 ± 0.02^{Aa}	98.50 ± 0.00^{Ac}
42	Free Bacteria	3.66 ± 0.01^{DCa}	152.00 ± 0.00^{Db}
	Microencapsulated bacteria	4.07 ± 0.02^{Aa}	103.75 ± 0.35^{Aa}
	Control	4.05 ± 0.56^{Aa}	101.40 ± 0.56^{Ac}

Different lowercase letters indicate significant differences between groups in each day, and different uppercases indicate significant differences in each group between days.

Table 4. Organoleptic scores of 7, 21 and 42 day old yogurt (Mean ± SD) in different experimental groups

Time of measuring (day)	Group	Color (from 5)	Texture (from 5)	Flavor (from 10)	Total (from 20)
7	Free Bacteria	4.75 ± 0.44^{Aa}	4.63 ± 0.49^{Aa}	9.22 ± 1.2^{Aa}	18.59 ± 1.68^{Aa}
	Microencapsulated bacteria	4.53 ± 0.67^{Aa}	3.13 ± 0.98^{Ab}	8.84 ± 1.35^{Aa}	16.47 ± 2.30^{Ab}

	Control	4.59 ± 0.50^{Aa}	4.50 ± 0.57^{Aa}	8.97 ± 1.23^{Aa}	18.06 ± 1.64^{Aa}
21	Free Bacteria	4.81 ± 0.40^{Aa}	4.69 ± 0.47^{Aa}	9.28 ± 0.96^{Aa}	18.75 ± 1.59^{Aa}
	Microencapsulated bacteria	4.66 ± 0.54^{Aa}	3.06 ± 1.01^{Ab}	8.69 ± 1.31^{Aa}	16.44 ± 2.09^{Ab}
	Control	4.72 ± 0.46^{Aa}	4.47 ± 0.62^{Aa}	8.88 ± 1.18^{Aa}	18.06 ± 1.72^{Aa}
42	Free Bacteria	4.34 ± 0.54^{Ba}	4.00 ± 0.76^{Ba}	7.88 ± 1.13^{Ba}	16.19 ± 1.71^{Ba}
	Microencapsulated bacteria	4.47 ± 0.51^{Aa}	3.03 ± 1.06^{Ab}	8.67 ± 1.00^{Ab}	16.13 ± 1.91^{Aa}
	Control	4.38 ± 0.71^{Aa}	4.31 ± 0.69^{Aa}	8.09 ± 1.61^{Aab}	16.78 ± 2.21^{Aa}

Different lower-case letters indicate significant differences between groups in each day and capital case letters indicate significant differences between days of measurement for each item in each group.

4. Conclusions

In this study, we presented a method of microencapsulation to produce an efficient probiotic yogurt. *L. rhamnosus* and *L. acidophilus* were microencapsulated with calcium alginate, and then double layer coating of these beads with chitosan and Eu S100 nanoparticles carried out. The results of this study suggest that this technique of microencapsulation can improve the viability of *L. rhamnosus* and *L. acidophilus* in yogurt and in the simulated human GI tract. This method can also reduce the metabolic activity of the contained bacteria; consequently, pH and acidity of the final product stayed at an acceptable level during storage time. The consistency of pH and acidity of the product had considerable effects on maintaining taste and flavor during this period, and may also improve the viability of bacteria by decreasing bacterial cell damages. The second coating layer (Eu S100nanoparticles) adds resistance to the beads and can help them to reach their target functional place (Colon). The final product had appropriate acceptability; however, its texture was not competitive with other experimental groups, so there is a need to do more researches to improve the acceptability of final products. It is also necessary to investigate the application of this method of microencapsulation in other dairy products such as cheese, ice cream, fruit yogurt, and kefir as well as under in vivo GI conditions.

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EFFECTS OF PUMPKIN (*CUCURBITA MOSCHAT*) / SOYBEAN (*GLYCINE MAX*)FLOUR BLENDS ON FUNCTIONAL, PHYSIC-CHEMICAL PROPERTIES AND SENSORY ATTRIBUTES OF BREADS PRODUCED FROM WHOLE WHEAT (*TRITICUM AESTIVUM L*)

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ABSTRACT

Bread is an important staple food in many countries. Bread making is currently limited to wheat and a few other commonly used cereal seeds in many countries. This study was initiated with the objective of determining the effect of pumpkin/soybean flour blends on functional, physicochemical properties and sensory attributes of composite wheat, pumpkin and soy bread. The result indicated that the proximate compositions of breads produced with the different mix ratios of wheat, pumpkin and soybean flours were ranged from 11.07 to 13.19% for moisture, 1.15 to 2.15% for ash, 1.18 to 1.54% for fiber, 16.31 to 18.74% for fat, 14.22 to 17.33% for protein, 60.24 to 66.78% for carbohydrates and 470.79 to 478.95 kcal/100g for energy. The result showed that as the supplementation level of pumpkin and soybean flour increases, the ash, fiber, fat and protein of the composite bread produced were increased. However, the carbohydrate contents of the produced bread were decreased. The sensory evaluation of bread produced were in acceptable range even though, there were decreasing as the supplementation level of the pumpkin and soybean increased. These finding produced bread of acceptable nutritional, functional and sensory qualities from 3.5 to 10.5% of pumpkin and 1.5 to 4.5% of soybean flour with wheat, therefore, these research findings have shown new windows for further utilization of pumpkin and represents one way of cutting down on the large amounts of wheat importation for bread making and other wheat flour based products.

1.Introduction

Bread is among the most common foods prepared through fermentation and is a major food for mankind; thus bread making is one of the oldest processes known (Plessas *et al*., 2005). The word bread is used to describe the whole range of different bread varieties which may vary in weight, shape, crust hardness, crumb cell structure, softness, colour and eating quality (Collins, 1995).

In Ethiopia, bread is an important staple food. However, bread making is currently limited to wheat and a few other commonly cereal seeds in any countries. Anjum *et al* (2005) reported that wheat is the main ingredients in bread preparation being used as a dietary staple, averaging that two-third of total consumption in the world.

Although, no other crop can achieve the absolute baking properties of wheat, composite flours have become the subject of numerous studies, for the developing countries. The use of composite flours have several advantages: saving of hard currency, promotion of high-yielding native plant species, a better supply of protein for human nutrition and a simple

production technology (Bugusu *et al*., 2001). Also, the formulation of composite flour with local staple crop results in value-added product. Thus, there is a need for studies on proper utilization of composite flours, including pumpkins and soybeans. This became a necessity of over reliance on imported wheat (Akpapunam and Darbe, 1999). Ethiopia, moreover, grows staple crops other than wheat such as cassava, sweet potato, potato, pumpkins and cereals that can be used for bakery foods. It would therefore be economically advantageous if imported wheat could be reduced and the demand for baked foods such as bread could be met by the use of domestically grown products other than wheat.

Pumpkin is from genus *Cucurbita* of the family Cucurbitaceae. It includessquash and cucumbers which are grown throughout the tropical and sub tropicalcountries. There are three common types of pumpkin world-wide, namely *Curcurbitapepo*, *Curcurbita maxima* and *C. moschata* (Lee *et al.,* 2003).The yellow-orange characteristic colour of pumpkin is due to the presence ofcarotenoid. Carotenoids are natural pigments responsible for the yellow, orange andred colour of many foods.

According to Lee *et al.* (2002) stated that by adding certain amount of pumpkin powder the Beta-carotene content in noodles was clearly enhanced. Pumpkin flour is a good source of food containing high and healthy amount of dietary fiber (Kulaitiene *et al*, 2014). Moreover, pumpkin contains high content of Beta-carotene, pectin, some vitamin, material salts. Pumpkin also included the various source of carotenoids and ascorbic acids (Caili, 2006) which have important roles in nutrition as provitamin A (antioxidant).

According to research, they claim that foods include high level of Beta-carotene that may reduce the risk of cancer, protect heart, delay aging and body generation (Krinsky and Johnson, 2005). Beta-carotene may help lower the risk of metabolic syndrome (Sugiura *et al*. (2015), may protect the skin from the sun's damaging UV rays (Stahl and Sies, 2012). Vitamin A is known as a strong natural antioxidant and it is necessary for preserve the integrity of skin and mucosa and also an essential vitamin for good eyesight. Besides pumpkins also provide lots of riboflavin, potassium, copper, manganese and smaller significant amount of niacin, folate, phosphorus and iron.

Although there is surplus production of pumpkins in Ethiopia, consumption of pumpkin based fermented foods like bread is not widely practiced like that of wheat in rural and urban areas. Thus, implementation of composite flour technology is thought to be an appropriate intervention to improve the physicochemical composition of bread, there by showing an alternative way of utilizing pumpkins by humans which is limited only for stew preparation. The aim of the research is, therefore, to study the effects of progressive supplementation of pumpkin/soybean flour blends on physicochemical properties and sensory attributes of breads produced from wheat composite flours. The outcomes of the study is used to generate baseline information for subsequent studies that focus on pumpkin-based value added products improvement and related programmes in the country.

2.Materials and methods

2.1.Experimental materials

The experiment was conducted at Wollega University Shambu Campus in Food science and Nutrition laboratory and Jimma University College of Agriculture and Veterinary Medicine Department of Food Science and Postharvest Technology laboratory.The raw materials used for bread production pumpkin (*Cucurbita moschat*) was obtained at Bako market and soybean (*Glycine max*) (Didessa) variety was obtained from Bako agricultural research center. Whole wheat flour, salt, sugar and oil were purchased from Jimma city.

2.2.Production of pumpkin flour

Fresh pumpkin were washed, trimmed and peeled to make them free from soil, rotting or insect damage. The pumpkin pieces were then cut into slices. The slices of pumpkins were washed, spread evenly on different trays and then dried in an oven at 55^0C for 24 hours. The

dry pumpkins were milled into flour using laboratory grinder and passed through 250 µm sieve and obtain uniform sized flours. The flour was then packed in sealed plastic bag and stored at ambient temperature till further used.

2.3.Production of soybean flour

The soybean were thoroughly sorted and cleaned to remove foreign materials from the lot. Then ,the soybeans were cleaned by clean water and soaked in boiled water at 100^0C for 30 minutes to facilitate the removing of coat and separated the beans from the hull in cold water. Then, the soybeans were dried in an oven at 100^0C for 6 hours. The dried soybeans were grinded by laboratory grinder into flours. Then, the flours were sieved through 250 µm and obtained uniform sized flours. Finally, the flours were packed in plastic large and stored at ambient temperature till further used (Edema *et al*., 2005).

2.4.Experimental design

The experiment were carried out by completely randomized design with a single factor of four blending ratio with three replications were used. The blending ratio with one control (100% wheat flours) and the other is pumpkin, soybean and wheat flours with different supplementation level. The proportion wheat: pumpkin: soybean flour, 100:0:0; 95: 3.5: 1.5; 90: 7: 3; and 85: 10.5: 4.5, respectively.

Table 1. Experimental Formulation

Blending ratio	Pumpkin flour (%)	Soybean flour	Wheat flour (%)
B0	00	00	100
B1	3.5	1.5	95
B2	7.0	3.0	90
B3	10.5	4.5	85

Where; B0= 100% wheat flour, B1= 3.5% pumpkin flour +1.5% soybean flour +95% wheat flours, B2= 7% pumpkin flour +3% soybean flour +90% wheat flours, B3= 10.5% pumpkin flour +4.5% soybean flour +85% wheat flours

2.5.Bread preparation

Bread was prepared by straight dough methodof AACC optimized straight dough bread making method 10-10B(AACC, 10th edition, 2000**).**Bread production process (mixing and kneading,bulk fermentation, molding, rounding, intermediate proofing, molding, final proofing, baking,cooling and packaging). The control flour (100% wheat flour (B0)), blended at ratios of 3.5% Pumpkin flour: 1.5%soy flour: 95% wheat flour;7% pumpkin flour: 3% soy flour: 90% wheat flour and 10.5% pumpkin flour:4.5% soy flour: 85% wheat flour were employed for the production of bread. The baking formula were 100g of flour, wheat flour or the blend, 2g yeast, 1g bread improver, 2g salt and 180ml water. All ingredients were mixed in a bowl manually for 15 minutes. All ingredients were placed in plastic bowls and mixed for exactly 10 to 15 minutes until non-stickyhomogeneous dough were formed. The dough was fermented in a bowl covered with polyethylene plastic in fermentation cabinet which was maintained at 27^0C and 75% relative humidity for 60minutes. After 1hour of fermentation the dough were taken out of the fermentation cabinet and remixed until its size was reduced almost to its original size. The dough was then kept back in the fermentation cabinet for second fermentation for 20minutes. After 20minutes the dough was taken out of the fermentation cabinet, sheeted and molded. After sheeting and molding process were completed, the dough was placed on pan which was already greased by oil and baked in oven maintained at 200^0C for 15minutes. Finally the bread were removed and cooled for 1hour and packed in polyethylene bag and stored in a cool and dry area.

2.6.Chemical composition

The chemical composition of the compositebread produced and the flour of wheat, pumpkin and soybean, including moisture, crude fat, crude protein, crude fiber and total ash were determined using AOAC official methods of 925.09, 4.5.01, 979.09 and 923.03, respectively (AOAC, 2000). Total carbohydrate was determined by difference. Results were expressed as g/100 g of dry matter. Energy value was calculated using Atwater's conversion factors, where carbohydrates and proteins give 4kcal/g while lipids give 9 kcal /g (Osborne and Voogt, 1978).

2.7.Total carotenoid contents

Total carotenoids content of pumpkins flour and bread samples were determined according Bandyopadhyay *et al.* (2008). Pumpkin flour and composite bread sample (5g) were mixed with 37.5 ml methanol and 12.5 ml of 50% Potassium hydroxide solution in a flask for saponification. Then unsaponifiable materials were extracted twice with Diethyl ether (20 ml each time) and the ether extract was washed twice with distilled water (40 ml each time).Next, the extract was dried over anhydrous sodium sulfate. The diethyl ether was evaporated on steam bath and the dried residue was then re-dissolved in petroleum ether (20 ml). The yellow to orange color of the petroleum ether was measured at wavelength of 450 nm with a Spectrophotometer. The Total Carotenoids content of samples was computed using the formula shown below and the result was reported in mg equivalent of β- carotene per kg of sample.

2.8.Functional properties of flours

2.8.1.Bulk density

Bulk density was determined following the method described by Eleazu and Ironua (2013) and Onabanjo and Dickson (2014). A 10ml graduated cylinder, previously tarred, was gently filled with 5g of sample. The bottom of the cylinder was gently tapped on a laboratory bench several times until there was to a constant. The bulk density of the sample (g/ml) was calculated as weight of the sample per unit volume of sample.

2.8.2 Water absorption capacity

Water absorption capacity was determined according to the method used byAbdlwahab *et al*. (2009).10% suspension of the sample was mixed with glass rod in centrifuge tube for 2 min at room temperature. After 20 min of shaking the suspension was centrifuged for 30 min at 4000 rpm at room temperature. The free water was decanted into a 10 ml graduated cylinder and the volume was recorded. Water absorption capacity (WAC) was estimated as the amount of water retained by 100 grams materials.

2.8.3.Oil absorption capacity

The oil absorption capacity was estimated by the method used by Abdlwahab *et al*. (2009) and wasexpressed as the amount of oil bound by 100 grams dry matter. 10% suspensions of the sample were mixed with glass rod in centrifuge tube for 2 min at room temperature. After 20 min of shaking the suspension was centrifuged for 30 min at 4000 rpm at room temperature. The freed water was decanted into a 10 ml graduated cylinder and the volume was recorded.

2.9.Sensory evaluation of bread

Sensory evaluation of bread samples were carried out according to the method described by Barnes *et al*. (1991) by 45 untrained panelists at Jimma University College of Agriculture and Veterinary Medicine in the department of Food Science and Postharvest Technology laboratory. The samples were served in random order, identified by three digits codes.Panelists were advised to avoid strong odorous materials, such as soaps, lotions and perfumes prior to participating on panels and to avoid eating, drinking or smoking at least 30 minutes prior to a sensory test. Consumers were asked to fill questionnaire prepared for the evaluated sensory attributes of the bread samples, i.e.,color, appearance, body and texture, flavor and overall acceptability using a 9- point hedonic scale (1 dislike extremely, 5 = neither like nor dislike, 9 = like extremely). Packaged drinking water was provided for rinsing their mouth between samples.

2.10.Statistical analysis

All the data obtained were subjected to analysis of variance (ANOVA). The mean ± standard error of mean were determined for all the data and were separated by least significance difference (LSD) at P≤0.05, using SAS computer software.

3. Results and discussions

The results of functional properties of wheat, pumpkin and soybean flour are presented in the (Table 2). The functional properties determine the application and use of food material for various products. For instance properties are very important for the appropriateness of the diet, particularly for growing children (Omueti *et al*., 2009). The respective results showed significant (*P<0.05*) differences between wheat, pumpkin and soybean flour.

The bulk density value of wheat flour was 0.61 g/ml whereas those of pumpkin and soybean flours amounted to 0.51 and 0.55 g/ml respectively. There were significant (*P<0.05*) difference among all the flours. This finding was in agreement with work of Van Toan and Thuy (2018) on their work of production of high quality flour and made biscuits from pumpkin. Bulk density is affected by the particle size and density of the flour and it is very important in determining the packaging requirement, material handling and application in wet processing food industry (Karuna *et al*., 1996). The lower the bulk desnity, the higher the amount of flour particles that can bind together leading to higher energy values (Onimawo and Egbekum, 1998).

The water absorption capacity and oil absorption capacity of the wheat flour were 2.01 and 1.39 g/ml respectively. Oil absorption capacity (OAC) of flour is important as it improves the mouth feel and retains the flavor. Flours of pumpkin and soybean had 0.51 and 0.55 g/ml water absorption capacity and 0.81 and 2.62 ml/g of oil absorption capacity. There were also significant differences (*P<0.05*) between the flours in their water absorption and oil absorption capacity.The highest and lowest swelling index were recorded in wheat flour and soybean flour with values of (3.70 and 2.79) respectively. There were significant (*P<0.05*) different among all the flours.

The highestvalue oftotal carrotenoid content was recorded in pumpkins flour with value of 5.66 mg/kg and the lowest has recorded in wheat flour with value of 0.07 mg/kg. The wheat, pumpkin and soybean flour showed significance (*P<0.05*) difference. This an indication that pumpkin flour contains higher total carrotenoids as compared to wheat and soybean flours used for this study. According to many scholars, they claim that foods that have high level of beta-carotene reduce the risk of cancer, protect heart and delay aging and body generation (Krinsky and Johnson, 2005).

Table 2. Functional properties and Total carotenoids of wheat, pumpkin and soybean flours

Sample	Bulk Density (g/ ml)	Water Absorption Capacity (ml/g)	Oil Absorption Capacity (ml/g)	Swelling Index (%)	Total Carotenoids (mg/kg)
wheat flour	0.61 ± 0.01^{a}	2.01 ± 0.02^{b}	1.39 ± 0.02^{c}	3.70 ± 0.01^{a}	0.07 ± 0.01^{b}
pumpkin flour	0.51 ± 0.01^{c}	0.81 ± 0.01^{c}	1.63 ± 0.01^{a}	3.61 ± 0.01^{b}	5.66 ± 0.02^{a}
soybean flour	0.55 ± 0.01^{b}	2.62 ± 0.02^{a}	1.51 ± 0.02^{b}	2.79 ± 0.01^{c}	0.11 ± 0.01^{c}
LSD	0.03	0.06	0.05	0.04	0.04
CV	2.47	1.62	1.71	0.65	1.06

Where, LSD = least significance differences, CV = Coefficient of variation. Values are means ± standard error of three replicates and means with the same letter in the column are not significantly different at 5% level of significance.

Table 3. Proximate composition of wheat, pumpkin and soybean flour

Sample	Moisture (%)	Ash (%)	Fiber (%)	Fat (%)	Protein (%)	Carbohydrate (%)	Energy (kcal/100g)
wheat flour	10.37 ± 0.19^{b}	1.92 ± 0.02^{c}	0.60 ± 0.01^{c}	2.46 ± 0.01^{b}	10.46 ± 0.04^{b}	84.56 ± 0.02^{b}	402.25 ± 0.12^{b}
pumpkin flour	11.52 ± 0.04^{a}	2.39 ± 0.01^{b}	1.77 ± 0.02^{b}	0.56 ± 0.01^{c}	2.31 ± 0.02^{c}	92.96 ± 0.03^{a}	386.13 ± 0.05^{c}
soybean flour	9.61 ± 0.02^{c}	4.08 ± 0.05^{a}	5.35 ± 0.02^{a}	16.47 ± 0.06^{a}	34.98 ± 0.05^{a}	39.12 ± 0.07^{c}	444.65 ± 0.56^{a}
LSD	0.40	0.11	0.05	0.13	0.14	0.16	1.15
CV	1.92	1.93	1.03	1.02	0.44	0.11	0.14

Where, LSD = Least significance difference, CV = Coefficient of variation. Values are means ± standard error of three replicates and means with the same letter in the column are not significantly different at 5% level of significance.

3.1.Proximate composition of wheat, pumpkin and soybean flour

The proximate composition of the wheat, pumpkin and soybean flour were analyzed for moisture content, ash, protein, fat, fiber, carbohydrates and energy (Table 3). The moisture contents of wheat, pumpkin and soybean flour showed significance ($P<0.05$) differences and the highest value was recorded in pumpkin with value of 11.52% and the lowest was recorded in soybean flour with value of 9.61%. The variation of moisture contents may be attributed to the genetic composition and also agro cultural practices (Ingabire and Hild, 2011).

The ash contents of wheat, pumpkin and soybean flour were showed significance ($P<0.05$) differences from each other having the highest value in soybean flour 4.08% and the lowest value in wheat flour with the value of 1.92%. Similar works have been reported by Gerhard and Saeleaw (2010) who worked on composition, physicochemical and morphological characterization of pumpkin flour.

The fiber contents of wheat, pumpkin and soybean flour were found significantly ($P<0.05$) different from each other. Soybean had greater crude fiber contents (5.35%) than wheat (0.60%) and pumpkin (1.77%) flour. People who consume generous amounts of dietary fiber have health protective effect in comparison with those who have minimal fiber intake. Dietary fiber has recently gained much importance as it is said to reduce colon cancer, diabetes, heart diseases and the level of low density lipoprotein cholesterol in blood (Kulaitiene, 2014).

The crude fat contents were showed significance ($P<0.05$) differences among each other and the highest value were found in soybean flour with value of 16.47% and the lowest was found in pumpkin flour with the value of 0.56%. Similar findings was reported by Mesfin and Shimelis (2013) on their work of bread produced for soybean and quality maize flour.

The protein contents of wheat, pumpkin and soybean flour showed significant ($P<0.05$) different among each other. The highest protein content was found in soybean flour (34.98%) and the lowest were recorded in pumpkin with value of 2.31%. Pumpkin contains the lowest protein content as compared to wheat and soybean flours. Thus, pumpkin flours are recommended for those people of gluten intolerance.

The carbohydrate content of wheat flour, pumpkin and soybean flours were 84.56, 92.96 and 39.12% respectively. The result of wheat, pumpkin and soybean flour exhibited significant ($P<0.05$) differences between them. Pumpkin flour contains higher carbohydrate contents than wheat and soybean flour.On the other hand, the energy contents of wheat, pumpkin and soybean flours were 402.25, 386.13 and 444.65 kcal/100g, respectively and exhibited significant ($P<0.05$) differences among each other. The energy content differences could be due to

variation in their protein, fat and carbohydrate contents (Giami *et al*., 2000).

3.2.Proximate composition of breads from blends of wheat, pumpkin and soybean flour.

The proximate compositions of breads produced with the different mix ratios of wheat, pumpkin and soybean flours are presented in (Table 4). The highest (13.19%) and lowest (11.07%) moisture contents of composite bread produced from wheat, pumpkin and soybean flour were observed from samples with (10.5% pumpkin, 4.5% soybean and 85% wheat) and 100% wheat breads. There was significance (*P<0.05*) difference in moisture content of whole wheat bread as compared to the all treatments. However, there was no significance (*P<0.05*) differences between 3.5% and 7% pumpkin flour supplementations. Similar works were reported by Adriana and Simona 2014 on their works of physicochemical and sensory evaluations of wheat bread with pumpkin.

The highest ash contents were recorded in 10.5% pumpkin and 4.5% soybean flour supplementation and the lowest were recorded in 100% wheat flour breads with value of 2.15% and 1.51% respectively. The results of ash contents showed significance (*P<0.05*) differences among the treatments and the ash contents of whole wheat bread were significantly (*P<0.05*) different from those of pumpkin and soybean treatments. As the supplementation of pumpkin and soybean flour increases the ash contents were increased which is an indication of increment in mineral contents. This finding was in agreement with the work of Aniedu and Agugo, (2010) who reported that the ash content increased with progressive increase in supplementation of fruit flour in wheat for bread production.

The crude fiber result obtained in 10.5% pumpkin and 4.5% soybean flour were higher than those of pure wheat bread samples, implying that addition of pumpkin and soybean flour increases crude fiber. The highest values were recorded in 10.5% pumpkin and 4.5% soybean flour supplementation with value of 1.54%. The crude fiber contents of the composite breads made by adding flours of both pumpkin and soybean exhibited significant (*P<0.05*) differences from that of whole wheat breads. The crude fiber contents increased progressively with increased proportion of pumpkin and soybean flour. This increase in crude fiber content of the breads of the composite flours is the effect of the relatively higher percentage (1.77 and 5.35%) of crude fiber present in pumpkin and soybean flours (Table 3).

The highest and lowest fat contents were recorded in 10.5% pumpkin flour, 4.5% soybean flour and 100% whole wheat flours with value of 18.74% and 16.31% respectively. The result showed significance (*P<0.05*) differences between the whole wheat bread and among all the treatments. The results revealed that fat content of the breads of composite flours increased as the proportion of pumpkin and soybean flour addition increased. This due to high level of fat in soybean flour (Table 3).

The lowest protein content was found in whole wheat bread with value of 14.22% and highest were recorded in composite breads having 10.5% pumpkin and 4.5% soybean flour with value of 17.33%. There were significant (*P<0.05*) different among the composite breads produced and with whole wheat breads. The protein content increased as percentage of pumpkin and soybean flours increased. This may have been due to the high protein content of soybean flours. This is similar to the works of Olaoye *et al*., 2006 on their work of quality characteristics of bread produced from composite flours of wheat, plantain and soybeans.

Regarding carbohydrate contents of the breads made from the composite flours of wheat, pumpkin and soybean flours, significantly higher values were recorded in whole wheat breads as compared to the other treatments. As the supplementation level of pumpkin and soybean flour increases, the carbohydrate contents was decreased. Similar findings were reported by Olaoye *et al*., 2006.

The energy contents of breads made from composite flours of wheat, pumpkin and soybean ranged from 470.79 to 478.95 kcal/100g, with significant (*P<0.05*) differences

among them. The energy content increased with increase in proportion of pumpkin and soybean flour. The lowest energy content was recorded for whole wheat breads and the highest was recorded in 10.5% pumpkin and 4.5% soybean supplementation respectively.

Table 4. Proximate composition of breads produced from blends of wheat, pumpkin and soybean flour

Sample	Moisture (%)	Ash (%)	Fiber (%)	Fat (%)	Protein (%)	Carbohydrate (%)	Energy (kcal/100g)
B0	11.07 ± 0.11^{c}	1.51 ± 0.02^{d}	1.18 ± 0.01^{c}	16.31 ± 0.01^{d}	14.22 ± 0.02^{d}	66.78 ± 0.06^{a}	470.79 ± 0.10^{d}
B1	12.54 ± 0.08^{b}	1.71 ± 0.02^{c}	1.22 ± 0.02^{c}	17.11 ± 0.01^{c}	15.14 ± 0.03^{c}	64.81 ± 0.06^{b}	473.83 ± 0.12^{c}
B2	12.76 ± 0.05^{b}	1.80 ± 0.01^{b}	1.35 ± 0.01^{b}	17.65 ± 0.02^{b}	16.22 ± 0.02^{b}	62.98 ± 0.04^{c}	475.65 ± 0.07^{b}
B3	13.19 ± 0.08^{a}	2.15 ± 0.03^{a}	1.54 ± 0.01^{a}	18.74 ± 0.04^{a}	17.33 ± 0.03^{a}	60.24 ± 0.08^{d}	478.95 ± 0.20^{a}
LSD	0.28	0.07	0.04	0.08	0.08	0.20	0.42
CV	1.20	1.99	1.88	0.24	0.28	0.17	0.05

Where, LSD; Least significance difference, CV= Coefficient of variation. Values are means ± standard error of three replicates and means with the same letter in the column are not significantly different at 5% level of significance. B0 = 100% wheat Flour; B1 = 3.5% pumpkin flour + 1.5% soybean flour + 95% wheat flour; B2 = 7% pumpkin flour + 3% soybean flour + 90% wheat flour; B3 = 10.5% pumpkin flour + 4.5% soybean flour + 85% wheat flour.

Table 5. Sensory evaluation of breads made of wheat, pumpkin and soybean flour

Sample	Taste	Aroma	Texture	Crust color	Crump color	Overall Acceptability
B0	7.09 ± 0.02^{a}	6.78 ± 0.02^{a}	6.42 ± 0.02^{a}	6.58 ± 0.02^{a}	6.80 ± 0.01^{a}	7.09 ± 0.03^{a}
B1	6.48 ± 0.01^{b}	6.38 ± 0.01b^{a}	6.19 ± 0.02^{b}	6.36 ± 0.03^{b}	6.18 ± 0.01^{b}	6.61 ± 0.01^{b}
B2	5.77 ± 0.02^{c}	5.97 ± 0.33^{b}	5.70 ± 0.01^{c}	6.48 ± 0.01^{c}	6.41 ± 0.01^{c}	6.42 ± 0.01^{c}
B3	5.36 ± 0.03^{d}	5.18 ± 0.03^{c}	5.40 ± 0.01^{d}	5.58 ± 0.01^{d}	5.76 ± 0.01^{d}	5.49 ± 0.02^{d}
LSD	0.08	0.55	0.05	0.06	0.04	0.06
CV	0.68	4.78	0.46	0.54	0.34	0.51

Where, LSD; Least Significance Difference, CV= Coefficient of Variation, B0 = 100% wheat Flour; B1 = 3.5% pumpkin flour + 1.5% soybean flour + 95% wheat flour; B2 = 7% pumpkin flour + 3% soybean flour + 90% wheat flour; B3 = 10.5% pumpkin flour + 4.5% soybean flour + 85% wheat flour. Values are means ± standard error of three replicates and means with the same letter in the column are not significantly different at 5% level of significance.

3.3.Sensory evaluation of breads made of wheat, pumpkin and soybean flour

Sensory acceptability data of breads produced by blending wheat, pumpkin and soybean flours are presented in (Table 5). The taste acceptability score ranges from 5.36 to 7.09. There were significant ($P<0.05$) differences among the treatments and also with whole wheat breads. The aroma acceptability score ranges from 5.18 to 6.78 in 10.5% pumpkin and 4.5% soybean supplementation and 100% wheat breads produced. Statistically aroma didn't showed significance differences between the whole wheat breads and 3.5%

pumpkin and 1.5% soybean composite breads. However, there was significance (*P<0.05*) differences between the whole wheat breads and composite breads supplements with 7%, 10.5% pumpkin and 3% and 4.5% soybean flour breads.

The texture acceptability scores ranged from 5.40 to 6.42 and exhibited significance (*P<0.05*) differences between the whole wheat breads and among all the treatments in the composite breads produced. The highest texture score was recorded in 100% wheat bread and the lowest was recorded in 10.5% pumpkin and 4.5% supplementation of soybean flour.The crust and crumb color of composite bread produced by supplementing pumpkin and soybean decreased when the addition of pumpkin and soybean flour increased. As the supplementation level of pumpkin and soybean flour increased, the brightness of the crust and crumb color of composite were recorded in the whole wheat breads and the lowest was recorded in 10.5% pumpkin and 4.5% soybean flour.

The overall acceptability evaluation of the breads which are presented in the same table showed significant (*P<0.05*) difference between breads whole wheat breads and composite breads produced from addition of pumpkin and soybean at different level. The highest overall acceptability of breads was recorded in whole wheat breads and the lowest were recorded in 10.5% pumpkin and 4.5% soybean supplemented. In general, the overall acceptability of breads decreased, as the supplementation level of pumpkin and soybean flour increased.

4. Conclusions

The use of pumpkin and soybean in supplementing wheat flour in bread production has significant benefits in countries like Ethiopia where inadequate wheat is grown. Therefore, incorporation of pumpkin and soybean flour in bread production will reduce the quantity of wheat that will be imported, thereby reducing the cost of production of breads. These finding produced bread of acceptable nutritional, functional and sensory qualities from 3.5 to10.5% of pumpkin and 1.5 to 4.5% of soybean flour with wheat, therefore, these research findings have shown new windows for further utilization of pumpkin and represents one way of cutting down on the large amounts of wheat importation for bread making and other wheat flour based products.It is recommended that up to 10.5% pumpkin flour and 4.5 % soybean flour substitution could be adopted in bread making processes, without affecting the quality adversely.

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THE EFFECT OF SUBSTITUTING COCOA AND CONVENTIONAL SUGAR WITH CAROB POWDER AND HONEY RESPECTIVELY ON THE PHYSICOCHEMICAL, RHEOLOGICAL AND SENSORY PROPERTIES OF DARK CHOCOLATE

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ABSTRACT

The effect of replacing cocoa and conventional sugar with carob powder and honey respectively on the physico-chemical, rheological, and sensory properties of dark chocolate was evaluated. The ash content and crude fiber content of the dark chocolate increased significantly ($p \leq 0.05$) whereas, total sugar content decreased with the increasing carob but decreasing honey concentration in the formulation. The total phenolic content (TPC) increased nearly four-fold and caffeine content reduced to trace amount (0.03 mg /g) with 100% replacement of cocoa with carob. Chocolate melt exhibited a non-Newtonian flow behavior with Casson viscosity ranging from 1.61 to 7.51 Pa.s. The increase in carob content enhanced the storage modulus (G′) and loss (G″) modulus with a dominance of elastic nature. Dark chocolate with good acceptable sensory scores, good dark color appearance and with trace amounts of caffeine and high fiber content can be prepared using carob powder and honey as substituent ingredients. The carob, which is relatively underutilized for food applications, although in recent past its application profile in food applications has increased, can be better exploited by the food industry for the development of novel food products like chocolate. The high nutrient profile of carob and its better antioxidant potential in comparison to cocoa can make it a better healthy food ingredient in various types of processed food products.

1. Introduction

Cocoa is the main ingredient in chocolate manufacture and its value and quality are related to complex flavors and to its distinct sensory properties. Dark chocolate has a more intense cocoa flavour than milk chocolate and this stronger sensory signal may lead to a stronger sensory satiety response. Dark chocolate can have 70-85% cocoa solids and therefore, flavanol content of dark chocolate is five times more than that of milk chocolate (Katz *et al.,* 2011). The polyphenolic profile of cocoa mainly comprises of catechins (37%), anthocyanins (3%) and proanthocyanidins (58%) (Lamuela-Raventós *et al*., 2005). However, chocolate is a common culprit in heart burn as it contains concentrations of theobromine, an alkaloid that is closely related to the caffeine, which relaxes the esophageal sphincter muscle, letting stomach acid squirt up into the esophagus (Murphy *et al.,* 1988). Cocoa also contains tyramine that may trigger migraine headache. Apart from this, high sugar in chocolate may also has various health implications such as blood pressure, heart diseases, and tooth decay, obesity and increased blood glucose and insulin levels. Thus, increasing consumer's demand for the

caffeine free chocolate with low glycemic index coupled with overwhelming demand of cocoa powder for chocolate manufacturing has increased the quest for suitable substitutes for cocoa and sugar in dark chocolate.

Carob is the fruit of an evergreen tree (*Ceratonia siliqua* L.) cultivated in the Mediterranean area. The pulp of the carob pod is mainly used for animal feed, but recently its utilization has been reported as a valuable food ingredient (Nasar-Abbas *et al*., 2016; Goulas *et al*., 2016). In contrast to cocoa, carob contains relatively very high sugar and fiber content but negligible amounts of theobromine and caffeine. Unlike to that of cocoa polyphenols, the carob polyphenolic compounds are abundant in phenolic acids mainly gallic acid and flavonoids and tannins (Goulas *et al*., 2016; Papagiannopoulos *et al*., 2004) contributing to its good antioxidant activities (Nasar-Abbas *et al*., 2016), anticancer and antiproliferation effects (Saura-Calixto *et al*., 2010), antidiabetic effect (Ba˜nuls *et al*., 2016) and cholesterol lowering effect (Ruiz-Roso *et al*., 2010). Carob pulp is gaining popularity for its organoleptic properties, aroma, color, and taste, and for its dietary quality as roasting of carob generates cocoa like aroma, enhancing its suitability as cocoa substitute (Ayaz *et al*., 2009). Therefore, the current trend is to utilize roasted carob powder as a substitue for cocoa powder in different food formulations (Pawłowska *et al*., 2018; Moreira *et al*., 2017; Srour *et al*., 2016; Salem and Fahad, 2012). The implication of using carob in chocolate resides in its caffeine and theobromine free nature (Bengoechea *et al*., 2008).

The high sugar content of chocolate has adverse health effects particularly for diabetic people and therefore, studies have been focused on substitution of sucrose with other sweetening agents such as tagatose and inulin (Shourideh *et al.* (2010b), sucralose and maltodextrin (Farzanmehr and Abbasi, 2009), jaggery (Chand *et al*., 2011), stevia and agave nectar (Vahedi and Mousazadeh, 2016) in chocolate in order to reduce the calorie content or glycemic index of the chocolate. Another, sugar alternate can be honey, which is having low glycemic index and greater health and nutritional properties in comparison to sucrose. Since, rheological properties of chocolate are affected by both formulation and processing conditions (Schantz and Rohm, 2005), it is imperative to study the rheological properties in light of the process design and final product quality. Therefore, keeping in view of the above facts, the present investigation was undertaken to explore the suitability of carob powder and honey as replacement for cocoa and sucrose respectively for dark chocolate making and its quality characterization.

2. Materials and methods

Carob powder was purchased from the Urban Platter Food Company, Mumbai (India), Cocoa butter was purchased from Cosmix stores, Telangana (India) and soya lecithin (emulsifier) was purchased from Baskerville India Pvt. Ltd, Madhya Pradesh. Carob powder contained 0.0g fat, 4.0g protein and 7.0g sugar per 15.0g as per the specification given. Honey and other ingredients such as cocoa powder and vanilla were purchased from the local market in Rohtak. All the other chemicals and regents used in this study were of analytical grade.

2.1. Formulation and preparation of dark chocolate

Dark chocolates were prepared with 30, 50, 70 and 100% replacement level of cocoa powder with carob powder in the formulation along with varying concentration of honey. The formulations used for the development of final products are as given in the Table (1). The process flow chart for the preparation of dark chocolate is as given in the Fig.1. The developed chocolate samples were stored under refrigeration temperature for 28 days to assess the stability of chocolates with reference to the rancidity and sensory quality attributes.

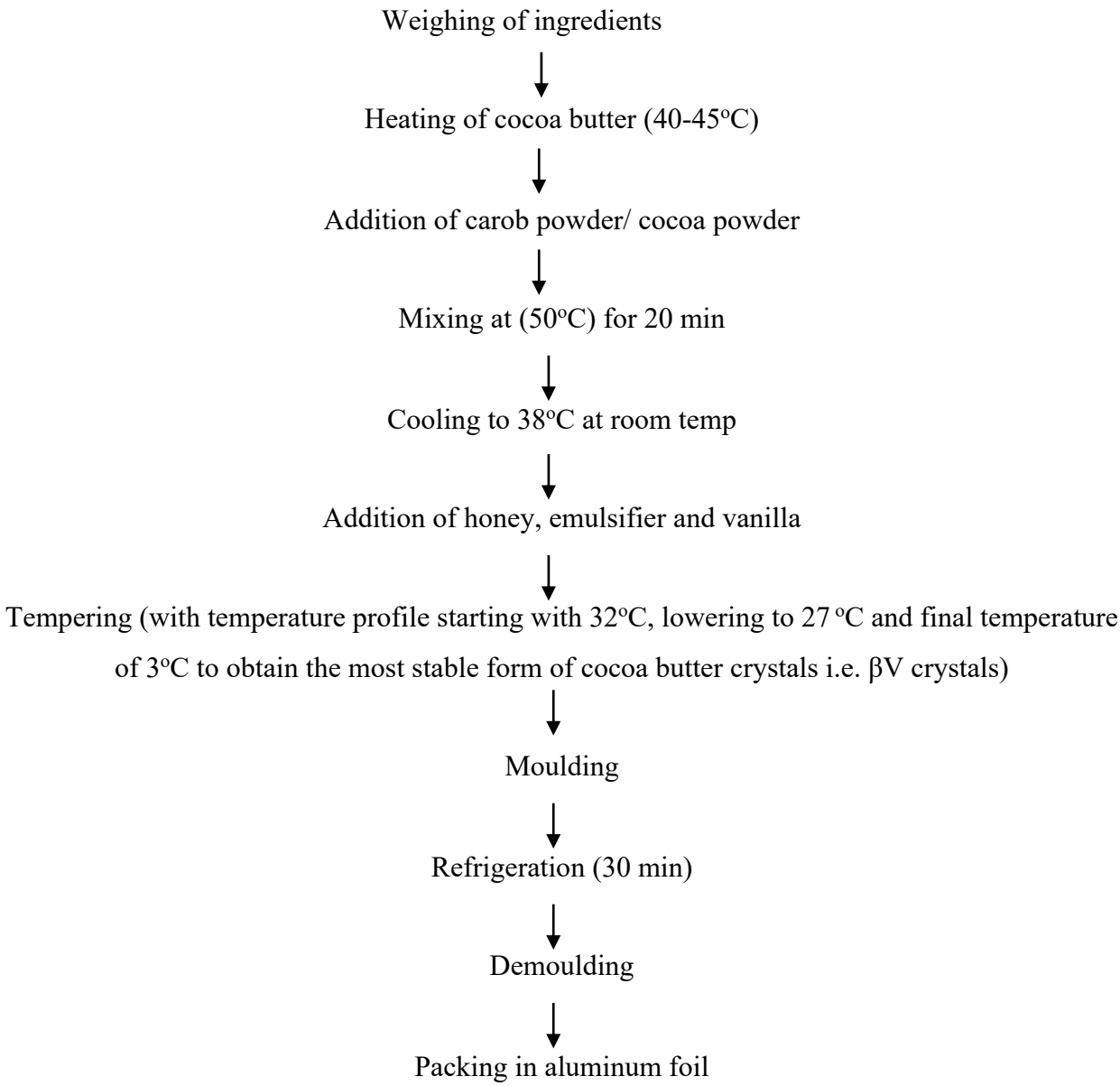

Figure 1. The process flow diagram for the preparation of dark chocolate

Table 1. Formulations for the development of dark chocolates

Sample	Cocoa powder (g)	Carob powder (g)	Cocoa butter (g)	Honey (g)	Vanilla (g)	Emulsifier (g)
A	20.0	-	20.0	13.0	0.5	1.0
B	14.0	6.0	20.0	11.5	0.5	1.0
C	10.0	10.0	20.0	9.5	0.5	1.0
D	6.0	14.0	20.0	7.0	0.5	1.0
E	-	20.0	20.0	5.0	0.5	1.0

A=100% Cocoa; **B**= 70% Cocoa and 30% carob; **C**= 50% Cocoa and 50% carob; **D**=30% Cocoa and 70% Carob; **E**= 100% Carob.

2.2. Chemical composition of dark chocolate

The moisture, fat, ash, crude fiber and protein and sugar content of the developed chocolate samples were determined using standard AOAC (2000) methods.

2.2.1. Total phenolic content (TPC)

TPC was determined by Folin-Ciocalteu assay using gallic acid as standard phenolic acid and the results were calculated as gallic acid equivalent (mg GAE/g) as suggested by the regression equation ($y = 0.0039x + 0.0406$; $R^2 = 0.998$) derived for the standard curve of gallic acid, where 'x' is the concentration and 'y' is the absorbance.

2.2.2. Caffeine content

Caffeine content of chocolate was determined by UV-VIS spectrometric method (Dobrinas *et al.,* 2013). For the preparation of standard curve, working standard solutions (3, 6, 9, 12, 15 and 18 $mg \cdot L^{-1}$) were prepared using the standard stock solution (0.0100 g of caffeine /100 ml of double distilled water). The caffeine content of the various samples of dark chocolate was measured using the obtained regression equation ($y = 0.0222x - 0.0122$; $R^2 = 0.9994$), where 'x' is the concentration and 'y' is the absorbance.

2.3. Rancidity

Rancidity of the chocolate samples was determined by IS: 7679 – 1978 method. 10.0 g of chocolate sample was taken and 10 ml of 0.1% phloroglucin dihydrate solution was added to it. The appearance of pink colour was indicative of rancidity.

2.4. Color analysis of dark chocolate

The colour measurement of chocolate samples was carried out using a Hunter Lab Colorimeter (Colour flex EZ). The measurements were made with a D65 illuminant, 25.4 mm diameter of the measurement hole. The 'L*' scale showed light Vs dark, where a low number (0-50) indicated dark and a high number (51-100) indicated light. The 'a*' scale showed red vs green, where a positive number indicated red and a negative number indicated green. The 'b*' scale showed yellow vs blue, where a positive number indicated yellow and a negative number indicated blue. The chroma and hue values were determined using the mean of the measured values by different formulas mentioned below

Chroma (C) = $(a^{*2} + b^{*2})$ 0.5 (Giese, 2000)

Brown index (BI) = (100-L*) (Jimoh *et al*., 2009)

2.5. Rheological properties of chocolate melt

The chocolates were placed in container and melted in a hot water bath at 70°C. Rheological properties of the samples were measured using a dynamic rheometer (MCR 102, Anton Paar GmbH, Germany) with cone and plate geometry (1° cone angle, 40mm diameter and 0.08 mm gap). For dynamic viscoelastic determinations two different experiments were performed at 37°C. (1) Deformation sweep at a constant frequency (10 rad/S) to determine linear viscoelastic range and (2) Frequency sweep at a constant deformation at (0.01% stain) over a frequency range 0.1-100 rad/s. The storage modulus (G′), loss modulus (G″) and loss factor (tan δ= G″/ G′) as tangent a function of frequency (ω) were obtained.

Steady flow tests were also performed at 25°C to obtain shear rate versus shear stress curves. The cone was programmed to increase the shear rate from $0\text{-}300^{s-1}$ using the equipment software. The experimented data were fitted to the Casson equation:

$$\tau^{1/2} = \tau_o{}^{1/2} + \eta_a \varepsilon^{1/2} \quad (1)$$

Where,

τ_o = Casson yield stress (Pa)

η_a = Viscosity (Pa.s)

ε = Shear rate (s^{-1}).

2.6. Sensory evaluation

The sensory analysis was performed on a 9 point hedonic scale by a panel of 15 semi-trained members (8 males and 7 females), with an age group between 21 and 25 years (mean

age: 23 years). Before performing the sensory evaluation, the chocolate samples stored under refrigeration were kept at room temperature for 30 min. The chocolate samples were evaluated for their appearance, taste, color, odour and after taste. The sensory evaluation was done at an interval of 7 days during the storage period of 28 days.

2.7. Statistical analysis

The data was analyzed using 'SPSS' statistical software. ANOVA was applied in a completely randomized model. The values were represented as mean ±S.D. of four independent determinations. The mean was compared at 5% level of significance.

3. Results and discussion

3.1. Physico-chemical analysis of dark chocolate

The results of physico-chemical analysis of different samples of dark chocolate are given in Table (2). The moisture content of various dark chocolate samples varied from 5.3-10.0 % and it was significantly higher than those reported in the literature, which is normally around 1 percent (Tran *et al*., 2016). This higher moisture content could be due to the use of honey in the formulation and the chocolate samples containing higher concentration of honey had higher moisture content. The fat content ranging from 45.2 to 51.5% as observed in the present study for different chocolate samples was reasonably in agreement with the reported values of 36.5 to 53.26% by Gao *et al.* (2015) for various dark chocolates. Expectedly, it decreased in the order of A > B > C > D > E with the decreasing amount of cocoa. A similar trend of decreasing fat content with the increasing carob concentration in milk chocolates has also been reported by Salem and Fahad (2012). The ash content and crude fiber content of the dark chocolate increased in a highly significant manner ($p \leq 0.05$) as with the increasing carob concentration in the chocolate formulation and it increased to a maximum of 5.4 % and 9.2% respectively when compared to respective values of 1.4% and 1.9% in the control sample. The total sugar content of different chocolate samples varied from 13.46 to 24.48 %, and it decreased as the concentration of carob powder was increased and honey decreased. Although carob powder is known to contain relatively very high amount of sugars than cocoa powder (Loullis and Pinakoulaki, 2017) but it seems that in the current study, the sugar content was much affected with the addition of honey. However, Salem and Fahad (2012) reported higher values of total sugar content in milk chocolates containing carob. The pH of different dark chocolate samples ranged from 4.96 to 6.97. Highest pH was found for sample A while lowest for sample E. The pH decreased as the carob concentration was increased. Vahedi and Mousazadeh (2016) reported the pH for different dark chocolates in the range of 6.94 to 7.0. This decrease in pH might be due to higher tannin content in carob powder

Table 2. Physico-chemical properties of dark chocolates

Sample	Moisture (%)	Fat (%)	Ash (%)	Crude Fiber (%)	Protein (%)	Sugar Content (%)	pH
A	10.0±0.18 ^{d}	51.5±0.3 ^{c}	1.41±0.1 ^{a}	1.93±0.1^{a}	4.66±0.18^{a}	24.8±0.2^{d}	6.97±0.05^{c}
B	8.01±0.44^{c}	50.4±0.2 ^{c}	2.32±0.1^{b}	3.70±0.1^{b}	4.74±0.11^{a}	20.5±0.2^{c}	6.86±0.02^{c}
C	7.66±0.25 ^{c}	48.3±0.2^{b}	3.01±0.2^{c}	5.74±0.1^{c}	4.79±0.20^{a}	18.8±0.2^{c}	6.52±0.11^{bc}
D	6.66±0.29^{b}	47.8±0.2 ^{b}	4.10±0.1^{d}	7.71±0.2^{d}	4.83±0.22^{a}	16.3±0.1^{b}	6.13±0.21^{b}
E	5.30±0.10^{a}	45.3±0.2 ^{a}	5.43±0.1^{e}	9.20±0.2^{e}	4.86±0.25^{a}	13.5±0.3^{a}	4.96±0.07^{a}

Values are mean ± SD of four independent determinations; Values with the same superscripts in a particular column do not differ significantly ($p<0.05$) **A**=100% Cocoa; **B**= 70% Cocoa and 30% carob; **C**= 50% Cocoa and 50% carob; **D**=30% Cocoa and 70% Carob; **E**= 100% Carob.

3.2. TPC and caffeine content of dark chocolate

The TPC for different dark chocolate samples is given in Table (3). The results showed that the sample containing higher amount of carob powder had higher TPC and it varied from 5.33 to 23.32 mg GAE/g representing nearly four-fold variation. The carob powder in comparison to cocoa is considered as richer in phenolic acids mainly gallic acid as main polyphenolic compounds. The TPC of carob extracts from various varieties/cultivars grown in different geographic regions have been found to contain polyphenolic compounds ranging from 11.6 to 41.3 mg GAE/g (Loullis and Pinakoulaki, 2017). Further, the processing operations such as fermentation of cocoa beans and alkalinization of cocoa powders may also greatly affect the polyphenol content in chocolates (Wollgast and Anklam, 2000). The most abundant polyphenols in carob pods are phenolic acids (gallic acid), flavonoids particularly flavonols and isoflavones and tannins (Stavrou *et al*., 2018). Roasting of carob pulp may also degrade certain phenolic compounds and some polyphenolic compounds may also be released from polymeric compounds enhancing their available and total phenolic content as well as antioxidant activity of roasted carob (Vitali Čepo *et al*., 2014; Srour etal., 2016). Markis and Kefalas (2004) reported carob powder as a more potential source of antioxidants in comparison to red wines. Thus, it can be suggested that the carob chocolate might be a potential nutraceutical resource considering its high phenolic content. The antioxidant potential of carob flour could also be important in delaying the lipid peroxidation, thus enhancing the product shelf life.

The cocoa free chocolate revealed only trace amount of caffeine (0.03 mg /g) and it varied up to 3.62 mg/g in various dark chocolate samples. The results clearly indicated that with the replacement of cocoa with carob powder, the caffeine content of the dark chocolate can be reduced to very less amount and therefore, such type of chocolate can be a boon for the persons for whom very less or caffeine free products have been recommended. Caffeine comes under Methylxanthines, and generally, theobromine is a caffeine metabolite (Stavric, 1988), which is considered as an alkaloid compound that acts as a stimulant. The content of polyphenols and methylxanthines may vary and it depends upon the source of cocoa variety, its cultivation conditions, process parameters during fermentation and drying, and the chocolate making process (Meng *et al*., 2009).

Table 3. Total phenolic content and caffeine content of dark chocolates

Sample	TPC (mg GAE/g)	Caffeine (mg / g)
A	5.33 ± 0.08^{a}	3.62 ± 0.13^{d}
B	7.30 ± 0.37^{b}	2.57 ± 0.07^{c}
C	15.66 ± 1.00^{c}	2.01 ± 0.08^{b}
D	19.77 ± 0.94^{d}	0.14 ± 0.04^{a}
E	23.32 ± 2.65^{e}	0.03 ± 0.015^{a}

Values are mean ± SD of four independent determinations

Values with the same superscripts in a particular column do not differ significantly ($p<0.05$)

A=100% Cocoa; **B**= 70% Cocoa and 30% carob; **C**= 50% Cocoa and 50% carob; **D**=30% Cocoa and 70% Carob; **E**= 100% Carob.

3.3. Rheological properties of dark chocolate

3.3.1. Steady shear stress rheological properties

The rheological properties play an essential role not only in determining the efficiency of the process such as mixing and flowing of materials but also application of chocolate melt for coating and molding. The steady flow properties of molten dark chocolate samples were determined using Casson model. The values for the Casson parameters for the chocolate samples are given in Table (4) and the steady flow curves of five different molten chocolates are shown in Fig. 2. Casson yield stress is the force required to initiate the flow of molten chocolate. It represents the low shear-rate properties of chocolate and is affected by particle–particle interaction (Servais *et al.,* 2004; Afoakwa *et al.,* 2007; Aidoo *et al.,* 2013). The steady shear results showed that yield stress ranged from 10.39 to 13.99 (Pa) and it was found that there was an increase in the Casson yield stress with the increasing carob concentration. Carob powder contains a sufficient amount of fibers (Bengoechaea *et al.,* 2008), which would have contributed to the increased viscosity and increased yield stress of the carob containing chocolates. The addition of carob powder is one of the means of increasing solid particles, which being in contact with cocoa butter dominantly interacted with each other yielding to great collisions, friction and resistance, which are required to be broken down to make the chocolate begin to flow (Servais *et al.,* 2004).

The viscosity of molten chocolate decreased as the shear rate was increased (Fig. 1b). The molten chocolate displayed a shear-thinning non-Newtonian behavior as its viscosity decreased with the increasing shear rate. This can be explained by the fact that with the increasing shear rate, the disruption of entanglements dominated over the formation of new ones and the particles aligned in the direction of flow leading to a reduction in the viscosity. The Casson viscosity of chocolate melt increased exponentially ranging from 1.61 to 7.51(Pa.s). The lowest viscosity was found for sample A while highest for sample C. Servais *et al*. (2002) suggested that chocolate viscosity should be attributed to several factors such as fat content, shape of particles and particle size distribution. The increase in viscosity could be due to the addition of carob powder in the chocolate. The water capturing ability of carob assisted in the increased viscosity of the chocolates. Syafiq *et al.* (2015) reported an increase in yield stress and viscosity with the addition of xanthan gum and glycerin in chocolates. The authors hypothesized that with water holding ability, the trapped moisture (bound water) acted as sticking agent and agglomerated sugar particles to form gritty clods and the moisture attached on sugar particles directly increased the friction and apparent viscosity (Afoakwa *et al.*, 2007). The flow curves of carob containing chocolates were not linear which could be due the presence of carob particles which might have increased sample roughness and its attrition to the spindle surface.

Table 4. Casson model parameters for dark chocolates

Sample	Casson yield stress (Pa)	Casson viscosity (Pa.s)
A	10.39 ± 0.18^{a}	1.61 ± 0.23^{a}
B	10.87 ± 0.26^{ab}	6.06 ± 0.17^{b}
C	11.27 ± 0.11^{b}	7.51 ± 0.28^{c}
D	11.39 ± 0.06^{b}	6.17 ± 0.14^{b}
E	13.99 ± 0.15^{c}	6.97 ± 0.21^{c}

Values are mean ± SD of four independent determinations

Values with the same superscripts in a particular column do not differ significantly ($p<0.05$)

A=100% Cocoa; **B**= 70% Cocoa and 30% carob; **C**= 50% Cocoa and 50% carob; **D**=30% Cocoa and 70% Carob; **E**= 100% Carob.

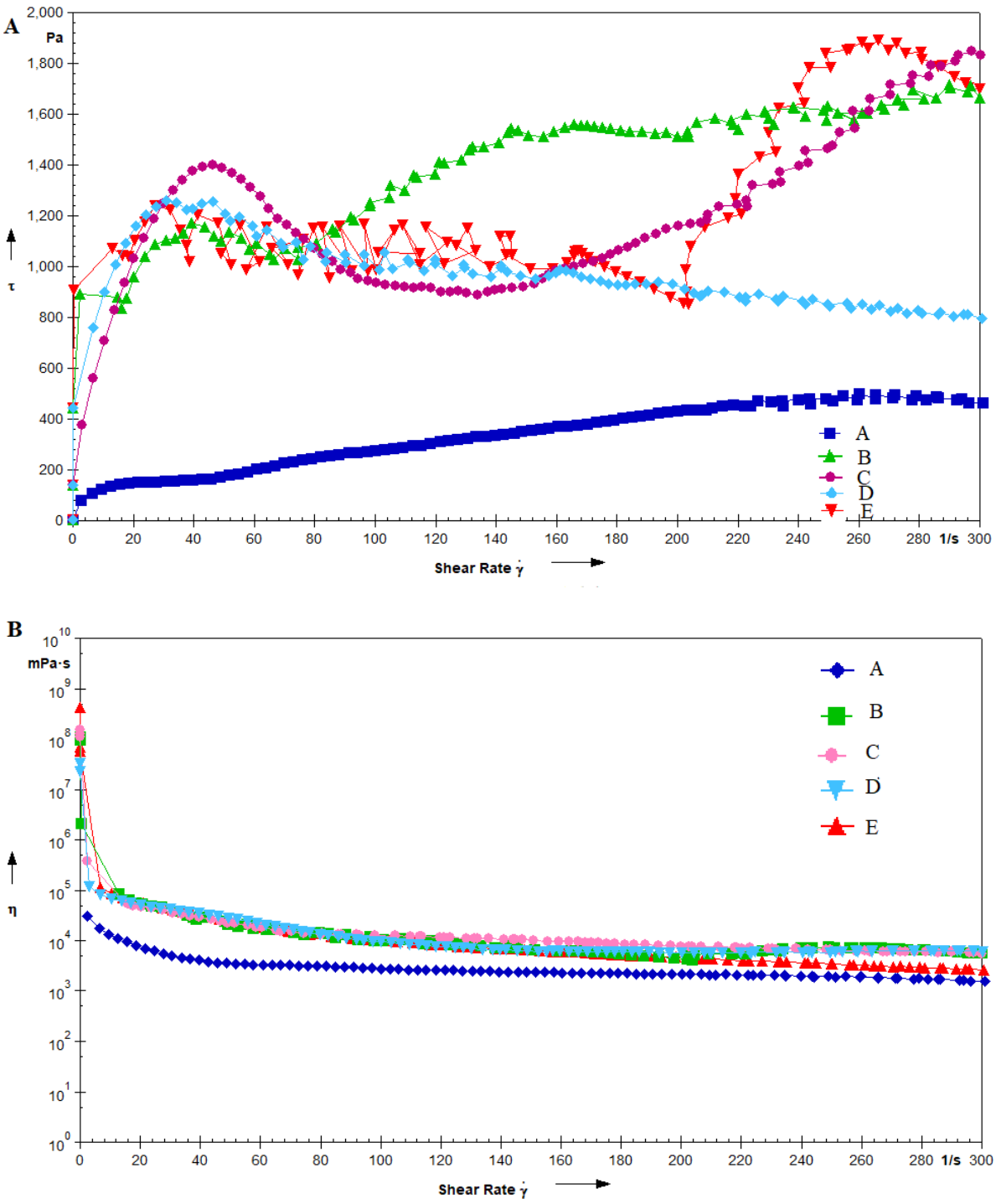

Figure 2. Steady shear rheological properties, (a) flow curve and (b) viscosity curve of dark chocolates.

3.3.2. Dynamic visco-elastic properties

The typical curves of dynamic oscillation test data concerning G′, G″ and tan δ are presented in Fig. 3. G′ is the storage modulus expressing the elastic nature and G″ is the loss modulus, expressing the viscous nature of the chocolate. All chocolate formulations showed an elastic modulus G′, greater than viscous

modulus G″, throughout the measured frequency. These results showed that the chocolate samples had more elastic nature than viscous suggesting that under non-destructive conditions the elasticity had a predominant effect on viscosity (Peressini *et al.,* 2006). The increase in carob content caused a shift in G′ and G″ towards higher values. Both moduli increased with frequency, thus exhibiting a solid like behavior. The increase in G′ and G″ values in carob chocolates could be due to the addition of carob powder in the chocolates. Carob powder contained fiber rich constituents that might act as strengthening and elastifying agent for the structure which in terms of rheology is closer to a solid material (Spend *et al.,* 2009). Lower values of G′ and G″ for the sample A might be because of the weakly structured system due to the lubricating and emulsifier effect of fat and lecithin. These results are in agreement with the studies of Johansson and Bergensthål (1992). Similar increase in the G′ and G″ values with carob flour addition has also been reported for gluten free breads (Tsatsaragkou and Krokida, 2014).

The loss factor, tan δ = G″/ G′ is a dimension less value that compares the amount of energy lost during a test cycle to the amount of energy stored during the same time. The dynamic mechanical loss tangent (tan δ) is shown in Fig. 3 for all the samples. For all samples, tan δ values were less than 1 indicating a predominant elastic nature. The values for tan δ decreased with a decreasing frequency up to 1 rad/s after which values fluctuates gently which may be due to the increased relaxation times. The increase in carob content caused a shift in the tan δ values towards lower side suggesting that carob addition caused an increase in elasticity of the chocolate. The elastic nature of chocolates is determined by the extent of particle interactions during the recovery of deformed structure and it could be suggested that the presence of fibers in carob powder might have led to increased inter-particle associations in chocolates.

3.4. Color characteristics of dark chocolates

Composition and processing parameters are the key factors affecting the color characteristics of chocolate. In the present study, while increasing the level of carob powder, level of honey as a sweetener was decreased. The results obtained from the instrumental color analysis of the chocolate samples are given in Table (5). L^* value of approximately 28.54 is considered to be acceptable for dark chocolate as reported by Aidoo *et al.* (2014). The lower value of L* indicates a darker appearance. The L* value ranged from 21.22 to 18.12, which indicated a good dark color appearance of the chocolate. The highest L* value was found for the control sample followed by the sample E and C. Sample B and D were comparatively darker in color. Normally, it has been suggested that the chocolate sample containing higher concentration of sugar substitutes represented a darker color (Shah *et al*., 2010; Aidoo *et al*., 2014). However, in the present study a consistent trend in the change of color parameters with the changing levels of honey was not obtained. This could be ascribed to the reason that as the level of honey was decreased in the formulation, simultaneously the level of carob powder was increased. The results suggested that there was a possible interaction effect between the sugar rich carob powder and honey during caramelization and maillard reactions to impart the characteristic dark appearance to the chocolate sample, which is evident from the fact that the sample B and D containing reasonably higher concentration of either of the component (carob powder or honey) and lower concentration of other were observed to be darker in color. Positive values of a* and b* indicated the slightly reddish brown color of chocolates. The samples showing lower L* values also showed higher a* and b* values, which indicated their increased dark brown color. The values of chroma which describes the vividness or dullness of a color, ranged from 2.61 to 5.74 with sample B and D having lower values of L showing the higher values of chroma i.e. these chocolates exhibited more brown color with

higher shine. Similarly, the browning index, which closely associated with the degree of browning on account of maillard browning or caramelization of sugars in chocolate processing, was also higher for the samples B and D.

Table 5. Color characteristics of the freshly prepared dark chocolates

Sample	L*	a*	b*	Chroma	Brown index
A	21.2 ± 0.2^{b}	2.97 ± 0.12^{c}	1.92 ± 0.20^{b}	3.53 ± 0.11^{c}	78.8 ± 0.1^{a}
B	18.1 ± 0.4^{a}	3.04 ± 0.19^{c}	2.55 ± 0.26^{c}	3.96 ± 0.07^{c}	81.9 ± 0.3^{b}
C	20.5 ± 0.2^{b}	2.18 ± 0.24^{b}	1.45 ± 0.13^{a}	2.61 ± 0.15^{b}	79.5 ± 0.1^{a}
D	18.7 ± 0.3^{a}	4.17 ± 0.14^{d}	3.95 ± 0.34^{d}	5.74 ± 0.22^{d}	81.3 ± 0.2^{b}
E	20.6 ± 0.1^{b}	1.74 ± 0.21^{a}	1.12 ± 0.19^{a}	2.07 ± 0.19^{a}	79.4 ± 0.2^{a}

Values are mean ± SD of four independent determinations

Values with the same superscripts in a particular column do not differ significantly ($p<0.05$)

A=100% Cocoa; **B**= 70% Cocoa and 30% carob; **C**= 50% Cocoa and 50% carob; **D**=30% Cocoa and 70% Carob; **E**= 100% Carob.

3.5. Sensory quality characteristics of dark chocolates

The dark chocolates prepared with substitution of cocoa powder with carob powder are as depicted in Fig 5. The sensory attributes of the chocolate are the most important quality attributes as the consumer enjoys chocolate as a delicacy item for sensory appeal and satisfaction and not as an usual food stuff. The sensory scores for various parameters of the tested dark chocolate samples are depicted in Fig. 4. As indicated, although the sensory scores for most of the parameters generally decreased with the increase in replacement level but still there was no significant difference in most of the sensory parameters of the various freshly prepared cocoa substituted chocolate samples, particularly up to a substitution level of 70 % to that of the control sample. However, in case of complete substitution of cocoa powder with carob powder, the sensory scores for all the parameters except appearance reduced significantly but still in the acceptable range. It was found that the sensory scores for the taste and after taste of the sample E containing 100% carob powder was slightly bitter in comparison to control sample containing 100% cocoa powder. This could be ascribed to the presence of higher tannin content in carob powder. Appearance, which is related to the smoothness and brilliance of the surface, is the key sensory attribute of the dark chocolate and this attribute remained most consistent in various samples. Many visual attributes such as shape, gloss, surface smoothness or roughness, haze, translucency and color can contribute to the overall appearance of chocolate (Briones *et al*., 2006). The grayish appearance of the dark chocolate may be caused by fat bloom problem, which is normally observed on account of either the transformation of cocoa butter crystals or the mixing of other fats with cocoa butter while processing the chocolate. Regarding the overall acceptability, the sample C obtained highest score while sample E obtained the lowest score. Interestingly, during storage period of 28 days, there was no significant reduction in the sensory scores for various parameters for different chocolate samples was observed when measured at an interval of 7 days. Only, a slight decrease in sensory attributes of different chocolate samples was observed after the storage of 28 days and sample C containing 50% carob powder and 50% cocoa powder was found to be most acceptable chocolate among all the samples.

Rancid chocolate in its strictest sense i.e. the oxidation of the fats in the cocoa butter, will taste somewhat sharply cheesy and soapy. Results showed that there was no rancidity development during the 28 days of storage period and hence, no off flavors got developed. The higher total phenolic content of the dark chocolate could have been influential in

delaying the lipid peroxidation (Buzzini *et al.,* 2008).

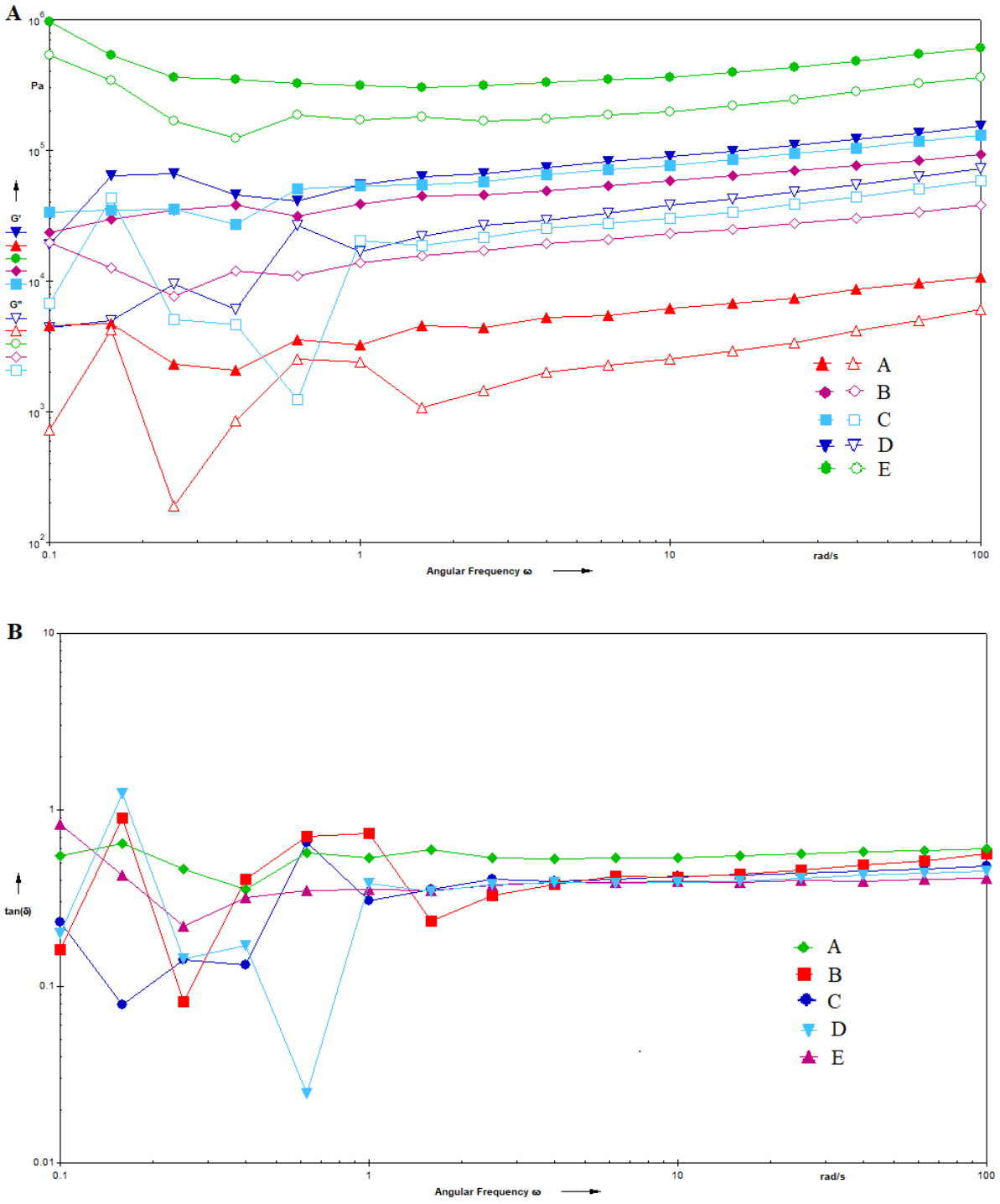

Figure 3. Dynamic rheological properties, (a) frequency sweep spectra and (b) tan δ of different dark chocolate samples measured at 37°C.

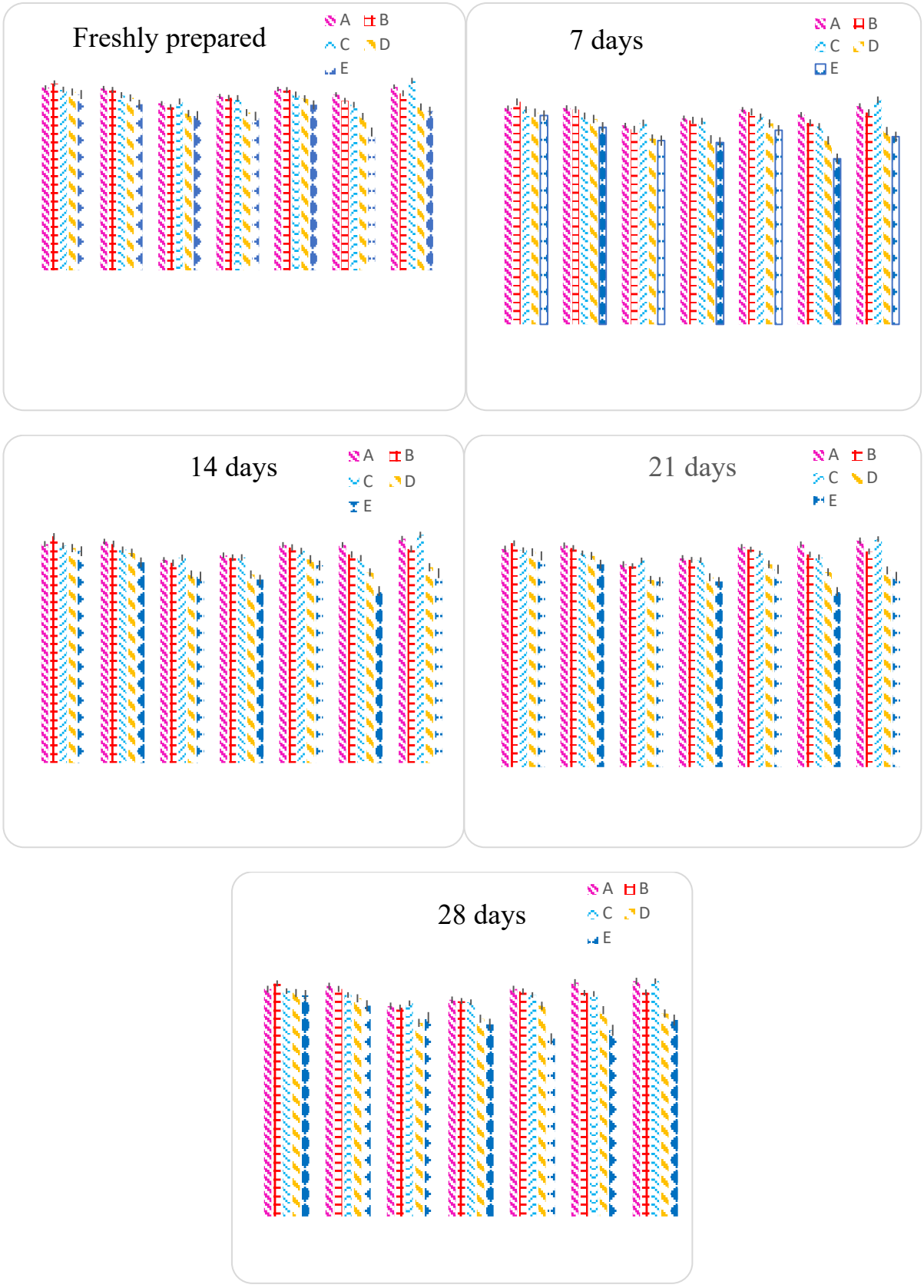

Figure 4. Sensory attributes of different chocolate samples

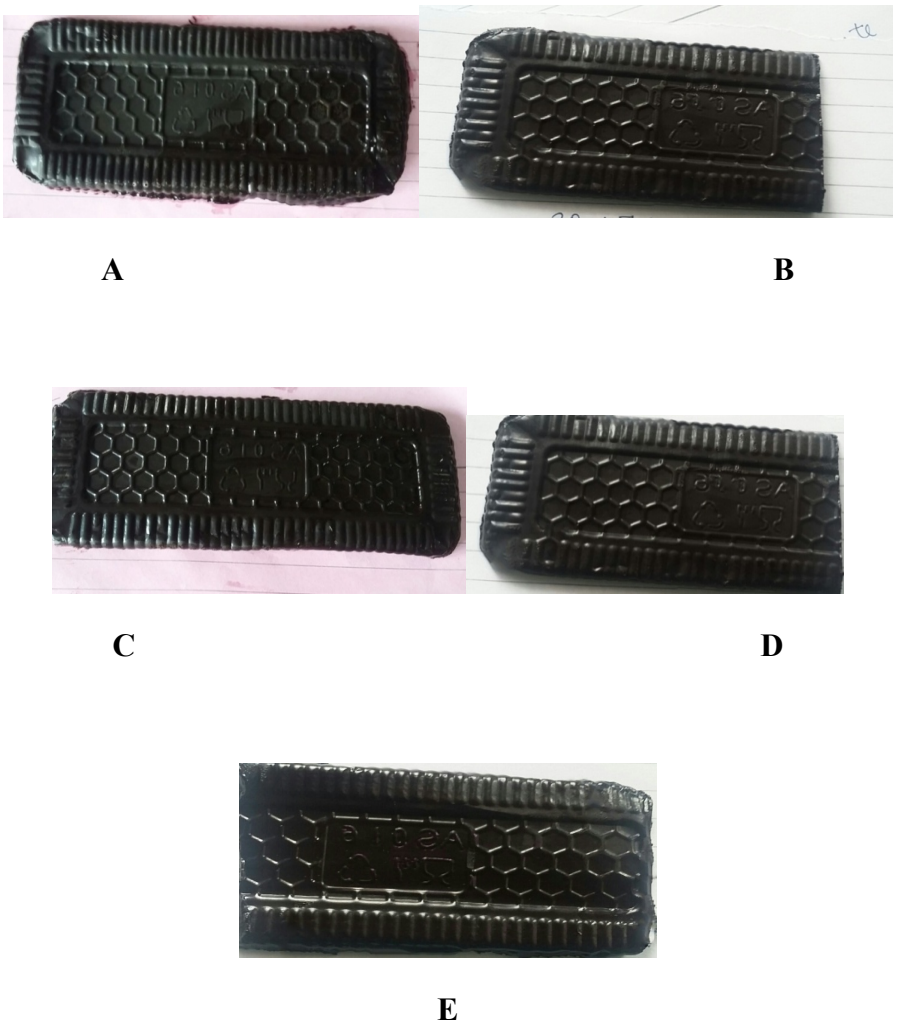

Figure 5. Different types of dark chocolates prepared with varying substitution level of cocoa powder with carob powder

4. Conclusions

Dark chocolates were prepared with 30, 50, 70 and 100% replacement level of cocoa powder with carob powder along with varying concentration of honey. The dark chocolate prepared with 100% replacement of cocoa with carob powder exhibited only trace amounts of caffeine and about four –fold increase in TPC and significantly higher fiber content, which suggested that dark chocolate with added functionality can be produced with carob powder. The addition of carob powder to the formulation increased the Casson yield stress of the dark chocolate melt. The increase in G′ and G″ was observed with the addition of carob powder, which indicated that carob powder acted as elastifying agent attributed to its high fiber content. Good color darkness was attributed to the addition of honey as well as high content of free sugars in carob powder. Sensory attributes like colour, appearance, taste, texture, flavour and overall acceptability scored well and were in acceptable range even after a storage period of 28 days. It can be concluded from the results of the study that good quality dark chocolate with acceptable sensory attributes coupled with added functional attributes like higher amount of TPC and fiber content and negligible amount of caffeine can be produced with partial or complete replacement of the cocoa with carob powder and conventional sugar with honey.

This type of product can be very beneficial for the persons who prefer caffeine free products.

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OPTIMIZATION OF SPRAY-DRYING PARAMETERS FOR 'BINTANGOR' ORANGE (*CITRUS RETICULATA* BLANCO X *CITRUS AURANTIUM* L.) JUICE

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ABSTRACT

'Bintangor' orange is a type of mandarin-orange hybrid, that has orange-colored pulp, but green-colored peel. The objective of this study was to determine the effects of maltodextrin concentrations (5% w/w to 25% w/w) and inlet temperatures (140°C to 180°C) on the properties of spray-dried 'Bintangor' orange powder. Color, moisture content, hygroscopicity, bulk density and wettability of powder were analyzed. Also, 'Bintangor' orange juice and the reconstituted powder were compared in color, viscosity, total soluble solids (TSS), and pH value. When 'Bintangor' orange juice was spray-dried with different maltodextrin concentrations, moisture content, color, hygroscopicity, bulk density and wettability increases as the maltodextrin concentration increase. On the other hand, there was no significant difference ($p>0.05$) in the color, moisture content, and hygroscopicity of spray-dried 'Bintangor' orange powder produced at different inlet temperatures, except bulk density. Optimum inlet temperature (170°C) and optimum maltodextrin concentration (20% w/w) produced powder with 0.11±0.00 water activity and 20.02±1.00 g/ 100 g hygroscopicity. When comparing reconstituted optimized powder with 'Bintangor' orange juice, the color, viscosity, TSS, and pH value of 'Bintangor' orange juice were higher than the optimized reconstituted powder. 'Bintangor' orange powder produced have 2.21±1.91% fat, and 2.44±0.00% protein.

1.Introduction

Mandarin is one type of commercial citrus fruit that belongs to the separate species, hybrids that are available worldwide (Laszlo, 2008). It is well known for Mandarins are well known for their attractive appearance, pleasant taste, and convenience due to easy peeling characteristics (Rizza et al., 2002). Besides, vitamin C and flavonoids in the citrus juice are providing good health effects to our body, such as anti-allergic and anticarcinogenic (Ladanyia and Ladaniya, 2010). Mandarin is a seasonal fruit that is not available throughout the year, like oranges (Jackson et al., 2011). 'Bintangor' orange is believed to be one of the hybrids between common mandarin and bitter orange, which is a hybrid between *Citrus reticulata* Blanco with *Citrus aurantium* L. (Urgi, 2014). The flesh of the 'Bintangor' orange is more sour and bitter taste than the other mandarin species. It is more prone to damage as they have thinner and looser skins. In addition, with shorter shelf life, there is a need to convert the fruit into more stable products such as fruit powder, which has a lower moisture content (Phisut, 2012).

In addition to food preservation, drying can help to minimize the packaging requirement, reduce the shipping costs, and makes the food supply more economical (Hui et al., 2010). Among different drying methods, spray-drying has been employed in the drying of liquid suspensions such as dairy products, coffee, eggs, protein, and fruit juice (Karel and Lund, 2003). Spray-drying is the technique which is usually used to produce the fruit juice powder (Chew et al., 2019), this includes watermelon, acai, pineapple, orange, blackberry, pomegranate, mango, and jackfruit (Quek et al., 2007; Angel et al., 2009; Tonon et al., 2010; Jittanit et al., 2010; Goula and Adamopoulos, 2010; Ferrari et al., 2012; Chng et al., 2020; Pui et al., 2020a).

However, the physicochemical properties of the final product obtained will depend on the parameter of spray-drying such as types and concentration of carrier agent, inlet temperature, airflow rate, feed flow rate, and atomizer speed (Phisut, 2012). Fruit powder, when spray-dried without a carrier, has a low glass transition temperature. Besides that, the product will also have an unfavorable characteristic such as high hygroscopicity, low melting point, and high-water solubility, which in turn results in a highly sticky product (Angel et al., 2009; Jittanit et al., 2010). Hence, carrier agents such as maltodextrin, waxy starch, liquid glucose and microcrystalline cellulose and Arabic gum were added to facilitate the drying process, reducing the stickiness and hygroscopicity of the powder (Phoungchandang and Sertwasana, 2010; Phisut, 2012). Among these carrier agents, maltodextrin is the most common carrier agent had used for spray-drying as it is considered cheaper and has high water solubility (Tuyen et al., 2010; Jittanit et al., 2010). Spray-drying with maltodextrin as a carrier has been used to convert juice from tropical fruit such as 'Terung Asam', papaya, pineapple, 'Cempedak' and 'Kuini' (Chang et al., 2020a; Chang et al., 2020b; Wong et al. 2015, Gopinathan et al., 2020; Gan et al., 2021).

Hence, this research aims to work on the spray-drying of mandarin juice in producing mandarin powder. This research will work on the effect of different concentrations of maltodextrin as carrier agents, and the effect of different inlet temperatures on the production of mandarin powder. Besides that, the physicochemical properties and also the proximate compositions of the spray-dried mandarin powder and reconstituted powder will be investigated.

2. Materials and methods

2.1. Materials

2.1.1. Fruit samples

The fruit that is used in this study was 'Bintangor' orange. Figure 1 shows the fresh 'Bintangor' orange and the peeled 'Bintangor' orange. These oranges were bought from the local market in Sibu, Sarawak. The selected oranges were free from external defects and were uniform in size (6.0 cm to 6.5 cm in diameter). The 'Bintangor' orange juice was extracted, and physicochemical properties (color, viscosity, total soluble solids (TSS), and pH) determined. ***2.2.1 Color***

The color of the 'Bintangor' orange juice was analyzed, according to Chang et al. (2020a), by using a colorimeter (ColorFlex Ez, Hunter Associates Laboratory Inc., USA), equipped with EasyMatch QC-ER software. Standardization was conducted using black tile first and followed by white tile. The juice was poured into the Quartz sample cup and placed on top of the sample port, covered, and measurements were taken. The values were expressed in terms of L*, a*, and b*.

2.2.2. Viscosity

The viscosity of the samples was analyzed according to Grabowski et al. (2006) with a viscometer (DV-II+ Pro, Brookfield, USA) coupled with Rheocale software program and Ultra Lower Adapter (ULA) spindle. Juice (15 mL) was poured into the sample tube, and measurements were taken at a rotational speed of 100 rpm.

Figure 1. Fresh 'Bintangor' orange (left) and peeled 'Bintangor' orange (right).

2.2. *Analysis of 'Bintangor' orange juice*

2.2.3. Total soluble solid (TSS) and pH value

Total soluble solids (TSS) of the samples were measured with a digital refractometer (MA 871, Milwaukee Instrument, USA), (0- 85 °Brix range) (Pui et al., 2018). On the other hand, the pH values of the samples were measured with a digital pH meter (Jenway, UK) (Pui et al., 2020b).

2.3. *Spray-drying of 'Bintangor' orange juice*

The spray-drying process was performed with mini spray-dryer (B-290, Büchi, Switzerland) (Loo and Pui, 2020). The setting was fixed at aspirator rate 100%, nozzle speed 5, and air compressor 40 nm. Maltodextrin was added into juice at different concentrations of 5, 10, 15, 20, and 25% (w/w), respectively, with a spray-drying temperature fixed at 180°C.

The 'Bintangor' orange powders were recovered in the vessel, which is the collecting area of the spray-dryer. It was collected, weighed, and vacuum packed immediately. The weight of the spray-dried powders, total time taken, and also the condition of powders was recorded for spray-drying of each inlet temperature. Physicochemical analyses and the reconstitution properties of spray-dried powders were performed after all the spray-dried powders with different inlet temperatures were collected.

The spray-drying process was repeated to determine the effect of inlet temperature (140, 150, 160, 170, and 180°C) used in the production of 'Bintangor' orange powder, with maltodextrin concentration fixed at 20 % (w/w).

2.4. *Physicochemical analyses of spray-dried 'Bintangor' orange powder*

2.4.1. Color

The color determination of the 'Bintangor' orange powders was conducted, according to Chang et al. (2020a).

2.4.2. Moisture content

The moisture content of the 'Bintangor' orange powders was determined, according to AOAC (2000). Approximately 2 grams of the powders were weighed into the pre-dried aluminum plates before drying at 105°C for 24 hours (Memmert, Germany). The readings of powders were collected after a constant weight was obtained.

2.4.3. Hygroscopicity

'Bintangor' orange powders (2 g) was placed in a desiccator containing saturated ammonium sulfate at room temperature for 1 week. The hygroscopicity of the powders was calculated by using the following equation (Cai and Corke, 2000):

Equation 1

Hygroscopicity (g/100 g) = (Weight of sample after a week (g)- Initial Weight of sample (g))/(Initial weight of sample (g)) x 100. (1)

2.4.4. Bulk density

'Bintangor' orange powder (5 g) was transferred into a 20 mL measuring cylinder and tapped by hand for 5 times. The volume occupied that was recorded, and bulk density was calculated according to equation 2 (Tonon et al., 2011). Bulk density was expressed in grams per milliliter (g/mL).

Equation 2

Bulk density (BD) = (Weight of powder (g))/(Volume of powder (mL)). (2)

2.4.5. Wettability

The wettability of the 'Bintangor' orange powder was determined according to Chauhan and Patil (2013). 'Bintangor' orange powder (1 g) was spread about 6 cm (in diameter) on a cloth, and the time for the powder to be completely wet as recorded.

2.5. *Physicochemical analyses of reconstituted 'bintangor' orange powder*

The spray-dried 'Bintangor' orange powder was added with water, to a similar total soluble solid (TSS) content as the 'Bintangor' orange fruit juice (Pui et al., 2021). 'Bintangor' orange powder (5 g) was added with warm water (45 g), stirred until all powder is dissolved. This is followed by the gradual addition of powder until TSS is reached. Analysis such as color, pH, and viscosity, were carried out on reconstituted powders.

2.6. *Proximate analysis of spray-dried 'Bintangor' orange powder*

The proximate analyses for ash, fat and protein of spray-dried 'Bintangor' orange powder were determined according to the Association of Official Analytical Chemists (AOAC) methods (2000) with some modifications.

2.7. *Statistical Analysis*

All the experiments were carried out in triplicate (n=3). The data collected was expressed in terms of mean ± standard deviations. All the data in the analysis of powder were analyzed by One-Way ANOVA (IBM SPSS software 22). The significant differences at $p \leq 0.05$ were determined by Tukey's test (Chng et al., 2020). On the other hand, paired-samples T-test was used to compare the physicochemical analyses between 'Bintangor' orange juice and optimized reconstituted 'Bintangor' orange powder.

3.Results and discussions

3.1.Physicochemical properties of 'bintangor' orange juice

Table 1 showed the physicochemical properties of the 'Bintangor' orange juice used for the spray-drying process, including color measurement, pH values total soluble solid (TSS), and viscosity. The lightness (L*), redness (a*), and yellowness (b*) of the 'Bintangor' orange juice were 47.92±1.46, 10.44±0.33 and 38.53±0.88, respectively. When compared to the fresh orange juice in Cortés et al. (2008), the lightness and yellowness of the fresh orange juice were slightly higher than the 'Bintangor' orange juice which was 51.36 and 50.73, respectively, while the redness of the orange juice was slightly lower than the 'Bintangor' orange juice which was 4.56. This can be possibly explained as the growing environment and the ripeness of the mandarin tree (Ladanyia and Ladaniya, 2010). Besides that, the viscosity of 'Bintangor' orange juice recorded in Table 1 was 4.87±0.60 centiPoise (cP). This viscosity is low enough to prevent clogging in the atomizer during the spray-drying process (Chegini and Ghobadian, 2007; Phisut, 2012).

From Table 1, the total soluble solids (TSS) of the 'Bintangor' orange juice was 12.00±0.35 °Brix, while the TSS for the other types of mandarin was around 12.5-14.5 °Brix (Roussos et al., 2011). This TSS was slightly lower than the other types of mandarins. This difference may due to different species of mandarins have different types of breeding goals. Hence, there will be some differences in the quality of different species of mandarin (Jenks and Bebeli, 2011).

Table 1. Physicochemical properties of 'Bintangor' orange juice (spray-dryer feeds)

Properties	Values
L* value	47.92±1.46
a* value	10.44±0.33
b* value	38.53±0.88
Viscosity (cP)	4.87±0.60
Total soluble solids, TSS (°Brix)	12.00±0.35
pH	4.29±0.03

Data on TSS, pH, viscosity, and color are means ± standard deviations of triplicate determination. Abbreviations: TSS = total soluble solid, °Brix = degree Brix, pH = potential of hydrogen, cP = centipoise, L* = degree of lightness and darkness, a* = degree of redness or greenness and b* = degree of yellowness or blueness.

The pH value was less susceptible to microbial growth as the value lower than 4.5 is considered as a low acid fruit (Chukwuka et al., 2010). However, the pH value of 'Bintangor' orange juice was higher than the other species of mandarins like Clementine, Encore, and Kara, which are ranged from 3.71 to 3.86 (Roussos et al., 2011). This may be attributed to the different environment for mandarin growing (Ladanyia and Ladaniya, 2010). Besides the environment, the storage temperature and time also can influence the sensory quality of mandarins such as TSS, acidity, and aroma volatile composition (Sinha et al., 2012).

3.2. Effect of maltodextrin concentration on physicochemical analyses of spray-dried 'Bintangor' orange powder

The physicochemical analyses of spray-dried 'Bintangor' orange powder at different maltodextrin concentrations were exhibited in Table 2.

L* value of spray-dried powder was ranged from 87.45±1.02 to 92.71±0.45, and a* value was ranged from 2.75±0.21 to 6.18±0.56. Besides that, the b* value of the spray-dried 'Bintangor' orange powder was ranged from 14.28±0.67 to 27.63±0.48. All L* values in the table were close to 100. Hence, it can be said that the spray-dried 'Bintangor' orange powders appeared to be lighter or brighter in color. The L* value increased from the lowest value at 5% (w/w) concentration, which was 87.45±1.02 to the highest value at 25% (w/w) maltodextrin concentration, which was 92.71±0.45. The possible explanation for the increase of value is due to the addition of maltodextrin, which is whitish, which affects the lightness of the spray-dried powder (Tuyen et al., 2010).

Table 2. Physicochemical analyses of spray-dried 'Bintangor' orange powder under different maltodextrin concentrations of maltodextrin

Maltodextrin concentration (w/w)	Color (L*)	Color (a*)	Color (b*)	Moisture content (%)	Hygroscopicity (g/100 g)	Bulk density (g/mL)	Wettability (secs)
5	87.45±1.02^{a}	6.18±0.56^{a}	27.63±0.48^{a}	7.39±1.03^{a}	29.09±0.79^{a}	0.37±0.01^{a}	112.56±6.50^{a}
10	90.63±0.66^{b}	3.88±0.40^{b}	19.92±0.88^{b}	6.03±0.35^{ab}	25.02±0.06^{b}	0.45±0.03^{b}	125.22±4.02^{a}
15	91.54±0.32^{c}	3.40±0.21^{bc}	17.33±0.64^{c}	3.81±0.99^{bc}	22.99±0.37^{c}	0.49±0.03^{b}	149.22±5.67^{b}
20	92.22±0.29^{bc}	2.99±0.09^{bc}	15.54±0.67^{cd}	2.43±0.78^{c}	21.64±0.43^{d}	0.48±0.03^{b}	166.78±5.19^{c}
25	92.71±0.45^{c}	2.75±0.21^{c}	14.28±0.67^{d}	2.38±0.85^{c}	20.79±0.49^{d}	0.48±0.03^{b}	177.22±3.35^{c}

Data on all analyses are means ± standard deviations of triplicate determination. Within the same column, different superscripts are significantly different at $p \leq 0.05$, as measured by Tukey's test. Abbreviations: % = percent, L* = degree of lightness, a* = degree of redness, b* = degree of yellowness, g/mL = gram per milliliter, g/100 g = gram per 100 g, and secs = seconds.

In Table 2, there was a significant effect ($p \leq 0.05$) of maltodextrin concentration on the value of a* and b* of the spray-dried 'Bintangor' orange powder. All a* value obtained were in positive. The a* value, indicating the 'Bintangor' orange powder's redness, decreased from maltodextrin concentration of 5% (w/w) to 25% (w/w). The positive a* value indicates the powders are more in red color and less in green color. The b* values in the table were also in positive value, where it is increased from 5% (w/w) concentration of maltodextrin to 25% (w/w) concentration of maltodextrin. All powders obtained are yellowish, as all the b* values are in positive value. As mentioned by Tuyen et al. (2010), the color intensity of the spray-dried powder was lost due to increase concentration maltodextrin.

The moisture content of spray-dried powder at 5% (w/w) maltodextrin was 7.39±1.03%, decreased to 6.03±035% and 3.81±0.99%, respectively with the addition of maltodextrin at 10% (w/w) and 15% (w/w). It was further decreased to 2.43±0.78% and 2.38±0.85% for spray-dried powder with 20% (w/w) and 25% (w/w) concentration of maltodextrin, respectively. The moisture content was in agreement with Jittanit et al. (2010) and Tuyen et al. (2010), who reported the range of 4-4.8% and 4.06-4.87 pineapple and gac fruit. Besides that, Quek et al. (2007) also reported that powders with lower moisture content could be obtained by increasing the concentration of maltodextrin added.

When the maltodextrin concentration increases, maltodextrin addition to the feed will increase the total solid content, leading to a reduction in the amount of water for evaporation. This, in turn, will lead to a decrease in moisture content (Quek et al., 2007). In addition, maltodextrin covers the sugars content, which is highly hygroscopic in the powder, thus reducing the ability to absorb the humidity in the surrounding air (Phoungchandang and Sertwasana, 2010; Phisut, 2012). Hence, decreasing moisture content in the powder cause the powder less likely to get sticky as the maltodextrin concentration increases.

According to Table 2, it was found that there was a reduction of hygroscopicity of spray-dried powder with 5% (w/w) to 25% (w/w) maltodextrin concentration, which was reduced from 0.79 g/100 g to 29.09±0.79 g/100 g. Maltodextrin is a material with low hygroscopicity (Tuyen et al., 2010). When it acts as the coating agent, it could reduce the hygroscopicity of the spray-dried powder (Cai and Corke, 2000).

The bulk density of the spray-dried 'Bintangor' orange powder obtained with different concentrations of maltodextrin can be observed in Table 2. The bulk density of spray-dried powder varied from 0.37±0.01 g/mL to 0.49±0.03 g/mL, which are achieved by addition of 5% (w/w) concentration and 15% (w/w) concentration of maltodextrin added, respectively. However, bulk density of spray-dried powder with 20% (w/w) and 25% (w/w) maltodextrin added decreased to 0.48±0.03 g/mL. This is because maltodextrin added can minimize the thermoplastic particles from sticking (Goula and Adamopoulos, 2010).

From Table 2, it can be observed that the wettability of the spray-dried powder was decreased from 112.56±6.50 seconds to 177.22±3.35 seconds. 'Bintangor' orange powder with 5% (w/w) maltodextrin have the highest wettability value (112.56±6.50 seconds), while with 25% (w/w) maltodextrin, it has the lowest value (177.22±3.35 seconds). Wettability can be defined as the ability of a powder to be penetrated by a liquid. The wettability value is higher than those of blackberry powder (82.20±12.30 seconds to 134.20±12.52 seconds). With higher wettability value, a longer time is needed to wet the powder. This may be due to smaller particles are less porous, making it difficult for liquid penetration to happen. It will result in poor reconstitution properties (Ferrari et al., 2012).

The spray-dried powder with 20% (w/w) maltodextrin concentration achieved the lowest bulk density value and the highest L* value, which were 0.48±0.03 g/mL and 92.22±0.29

g/mL, respectively. For the moisture content, the spray-dried powder with 25% (w/w) maltodextrin achieved the lowest value. However, there was no significant difference ($p>0.05$) between the moisture content of 20% (w/w) and 25% (w/w) concentration. The value of the moisture content for 20% (w/w) maltodextrin concentration was 2.43±0.78%. Apart from that, the water activity of the spray-dried powder with 20% (w/w) maltodextrin concentration achieved the lowest value, which is 0.08±0.01.

3.3. Effect of inlet temperature on Physicochemical analyses of spray-dried 'Bintangor' orange powder

Table 3 describes the physicochemical analyses of spray-dried 'Bintangor' orange powder at different inlet temperatures ranged from 140°C to 180°C under 20% (w/w) maltodextrin concentration. From Table 3, there was no significant difference ($p>0.05$) observed for all the physicochemical analyses except bulk density. In Table 3, it can be observed L* values, a* and b* value of 'Bintangor' orange powder were in the range of 92.23-92.81, 1.76-1.98, and 13.99-15.05, respectively. The powders have low moisture contents (2.82-3.66%), the hygroscopicity of 20.02-20.85 g/100g and wettability of 198 to 220 sec.

Table 3. Physicochemical analyses of spray-dried 'Bintangor' orange powder with optimum maltodextrin concentration at different inlet temperatures

Inlet temperature (°C)	Color (L*)	Color (a*)	Color (b*)	Moisture content (%)	Hygroscopicity (g/100 g)	Bulk density (g/mL)	Wettability (secs)
140	92.27±0.27^{a}	1.92±0.29^{a}	13.99±0.44^{a}	3.66±0.61^{a}	20.77±1.53^{a}	0.48±0.01^{b}	198.33±0.58^{a}
150	92.49±0.13^{a}	1.88±0.14^{a}	14.51±0.37^{a}	3.27±0.42^{a}	20.85±0.64^{a}	0.49±0.00^{bc}	198.33±0.58^{a}
160	92.81±0.17^{a}	1.76±0.23^{a}	14.43±0.85^{a}	3.00±0.50^{a}	20.71±0.75^{a}	0.46±0.00^{a}	205.89±0.19^{a}
170	92.44±0.10^{a}	1.98±0.26^{a}	15.48±0.90^{a}	2.88±0.50^{a}	20.02±1.00^{a}	0.50±0.01^{c}	211.11±1.50^{a}
180	92.23±0.52^{a}	1.69±0.29^{a}	15.05±0.66^{a}	2.82±1.04^{a}	20.15±1.42^{a}	0.50±0.00^{c}	220.00±0.67^{a}

Data on all analyses are means ± standard deviations of triplicate determination. Within the same column, different superscripts are significantly different at $p\leq0.05$, as measured by Tukey's test. Abbreviations: % = percent, L* = degree of lightness, a* = degree of redness, b* = degree of yellowness, g/mL = gram per milliliter, g/100 g = gram per 100 g, °C = degree Celcius and secs = seconds.

The bulk density of the spray-dried 'Bintangor' orange powder ranged from 0.46±0.00 g/mL to 0.50±0.01 g/mL. The lowest bulk density was achieved by the spray-dried powder at inlet temperature 160°C, while the highest bulk density was achieved by the powder that spray-dried at 170°C. The bulk density of the spray-dried powder at inlet temperature 140°C and 150°C increased from 0.48±0.01 g/mL to 0.49±0.00 g/mL. While the bulk density of the powder spray-dried at 180°C was 0.50±0.00 g/mL, which is less than the bulk density of powder that is spray-dried at inlet temperature 170°C.

From the finding of other studies, it was found that increasing inlet temperature causes the bulk density of the spray-dried powder to decrease (Cai and Corke, 2000; Chegini and Ghobadian, 2007; Tuyen et al., 2010). This reduction was due to evaporation rates are faster and products dry to a more porous or fragmented structure when the inlet temperature increases (Goula and Adamopoulos, 2005). Hence, it can be said the optimum inlet temperature with optimum maltodextrin concentration for the spray-drying of 'Bintangor' orange powder is 170°C with 20% maltodextrin concentration.

3.4. Reconstitution of optimized 'Bintangor' orange powder

The optimized reconstituted powder was compared with the 'Bintangor' orange juice in Table 4. The reconstituted powder was darker (with a reduction in L* value). In addition, the yellow and red values were lesser as compared to the fresh 'Bintangor' orange juice. This may be due to browning reactions occurring during spray-drying and maltodextrin addition (Jittanit et al., 2010). The viscosity of the optimized reconstituted powder was 2.92±0.22 cP, while the viscosity of the 'Bintangor' orange juice was 4.87±0.60 cP.

The difference between the TSS of the optimized reconstituted powder and 'Bintangor' orange juice might due to the optimized powder produced at high temperature was increased in particle size. The particles produced were uneven in size. Powder with larger size will sink while smaller size particles are dusty and resulted in uneven wetting and reconstitution (Fazaeli et al., 2012). The pH of the optimized reconstituted powder was 4.20±0.02, which was slightly lower than the pH of the 'Bintangor' orange juice with pH 4.29±0.03. This difference may be the result of concentration accompanied by the release of sugars and acids from maltodextrin during drying (Patil et al., 2014).

Table 4. Physicochemical analyses of 'Bintangor' orange juice and optimized reconstituted powder

Analyses	'Bintangor' orange juice	Optimized reconstituted powder
Color (L*)	47.92±1.46[a]	37.16±0.26[b]
Color (a*)	10.44±0.33[a]	-3.28±0.37[b]
Color (b*)	38.53±0.88[a]	15.03±1.85[b]
Viscosity (cP)	4.87±0.60[a]	2.92±0.22[b]
TSS (°Brix)	12.00±0.35[a]	11.67±0.25[b]
pH	4.29±0.03[a]	4.20±0.02[b]

Data on TSS, pH, viscosity, and color are means ± standard deviations of triplicate determination. Abbreviations: TSS = total soluble solid, °Brix = degree Brix, pH = potential of hydrogen, cP = centipoise, L* = degree of lightness and darkness, a* = degree of redness or greenness and b* = degree of yellowness or blueness.

3.5.Proximate analyses spray-dried 'Bintangor' orange powder

The proximate analyses of spray-dried 'Bintangor' orange powder are shown in Table 5. There was no ash content had been observed. Besides that, the fat content and the protein content of the 'Bintangor' orange powder were 2.21±1.91% and 2.44±0.00%, respectively. The fat and protein content of the 'Bintangor' orange powder was very low. As compared to the spray-dried sweet potato powder, the ash content of the spray-dried 'Bintangor' orange powder was much lower than the ash content of spray-dried sweet potato powder, which is 2.4 g/100 g (Grabowski et al., 2008). While for the protein and fat content, the fat and protein content of the 'Bintangor' orange powder is higher than the berry powders (strawberries, blueberries, raspberries, and blackberries). The fat content of berries fruit powder was ranged from 0.30% to 1.00% while the protein content for berries fruit powder was ranged from 0.60% to 1.30% (Khalloufi et al., 2000).

Table 5. Proximate analyses of spray-dried 'Bintangor' orange powder

Properties	Values (%)
Ash	0.00±0.00
Fat	2.21±1.91
Protein	2.44±0.00

Data on ash, fat, and protein are means ± standard deviations of triplicate determination. Abbreviations: g/100 g = gram per 100 g and % = percent.

4. Conclusions

'Bintangor' orange is believed to be the hybrid between a common mandarin and bitter orange. This research study was conducted to produce 'Bintangor' orange powder through the spray-drying process, with different maltodextrin concentration and different inlet temperatures. The water activity of spray-dried powder was decreased with the addition of maltodextrin (5 to 15% w/w), with no further decrease from 15 % (w/w) to 25% (w/w). In contrast, moisture content decreased with an increase of maltodextrin concentration. With an increase in maltodextrin concentration, the powder produced was lighter, less yellow and less reddish. Also, hygroscopicity and wettability of spray-dried powder reduced with an increase from 5% (w/w) maltodextrin concentration to 25% (w/w) maltodextrin concentration. Generally, the properties of 'Bintangor' orange powder were not affected by the increase of inlet temperature. It can be concluded that the production of 'Bintangor' orange powder is optimized at inlet temperature at 170°C with a 20% (w/w) concentration of maltodextrin.

5.References

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ANTIOXIDANT, ANTIMICROBIAL ACTIVITY OF POMEGRANATE PEEL WASTES EXTRACTED IN DIFFERENT SOLVENTS AND IDENTIFICATION OF PHENOLIC COMPOUNDS WITH HPLC-DAD

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ABSTRACT

The aim of this research was to investigate the extract of antioxidant and antimicrobial properties of pomegranate peel (PP) wastes and their effect on the *E. coli, S. aureus, S.mutans* at different concentrations of 10-100%. Total phenolic compound substance and antioxidant modification were inspected using Folin Ciocalteau and ABTS methods respectively. The antimicrobial activity was tested on the microorganisms using diffusion method and the scanning of the phenolic compounds were analyzed using HPLC-DAD and the most abundant compound was estimated to be the punicalin, gallic acid, ellagic acid and quercetin. The most common phenolic compound was detected to bepunicalin and the highest antioxidant activity was about 821.72 mmoltrolox/mg with (60%) acetone extract and the effect on the phenol increased up to 445.04 GAEq (mg GA/g) when PP extracted with ethanol, methanol and (60%)acetone. The results were evaluated and statistically analyzed according to antioxidant and antimicrobial effect of each extract on the *E. coli, S. aureus and S.mutans* which were found to be the most abundant when PP extracted with (10%-60%) acetone

1.Introduction

Punicagranatum, a member of the Punicaceae family, has been known as a healing food since ancient times, and it is grown mostly in Asia, from Iran to Himaya in the Mediterranean region (Das et al., 1999; Jafri, Aslam, Javed, & Singh, 2000; Meerts et al., 2009; Vidal et al., 2003; Viuda-Martos, Fernández-López, & Pérez-Álvarez, 2010).

Pomegranate has positive effects on health due to anti-cancer, anti-obesity, anti-diabetic and anti-ulcerogenic properties and attracts attention (Alexandre et al., 2019; Zhu & Liu, 2013) due to enhancing nutritional features in the human diet (Alexandre et al., 2019). Pomegranates may be consumed fresh and may be processed as industrial products like fruit juice, jelly, jam, and vinegar and forms significant amounts of by-product (Alexandre et al., 2019; Sood & Gupta, 2015). The by-products appear to be a source of valuable compounds (Sood & Gupta, 2015) as pomegranate peel contains high amounts of bioactive compounds and is even a source of natural antioxidants with strong biological activity content (Akhtar, Ismail, Fraternale, & Sestili, 2015). The flavonoids in pomegranate peel were shown to be rich in hydroxybenzoic acids and hydroxycinnamic acids. Pomegranate proteins with antimicrobial properties form a complex with sulfhydryl groups and were determined to show inhibitory effects against bacterial cell (Cristani et al., 2007; Goel, Puniya, Aguilar, & Singh, 2005). Synthetic and natural preservatives are used to inhibit pathogenic

organisms causing problems with food safety and to increase shelf life in the world in general. However, interest in natural preservatives has increased as synthetic preservatives cause problems in terms of health. Studies have determined that compounds in the peel sections of fruit with antimicrobial activity may prevent food degradation. Studies have shown that the extraction of active compounds in pomegranate from a variety of sections of the fruit displays variations according to the type of extraction and form of phenolic extraction (Al-Zoreky, 2009; Hegde et al., 2012; Pradeep, Manojbabu, & Palaniswamy, 2008; Tanveer et al., 2015). Many studies have been performed about pomegranate to date; however, it is important to determine the amount and type of solvent pomegranate peel wastes to provide the highest antioxidant activity amounts and best antibacterial effect.

This study determined the total phenolic compounds, antioxidant variation with Folin Ciocalteu and ABTS methods, and antioxidant compounds with phenolic compound screening using HPLC-DAD for extracts obtained using different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%) of methanol, ethanol and acetone solvents. Additionally, the aim was to determine the antibacterial effects of extracts obtained with different solvents from peel wastes on food pathogens like *E. coli, S. aureus* and *S. mutans* with the disk diffusion method.

2. Materials and methods

2.1.Materials

2.2.1. Pomegranate Samples

Pomegranate samples were purchased from a local market and constituted as research material. The pomegranates were separated from peels and membrane and granulated; the grains were pressed by a juicer and kept in a deep freezer at -18°C until the analysis. Peel pomegranate wastes were dried in vacuum oven at 55°C and dried pomegranate peels were grinded by blender and kept in a refrigerator at -40°C until used. In order to be able to evaluated the research results statistically was analyzed by using of Kruskall Wallis method (Hegde et al., 2012).

2.2.2.Methods

The extraction methods was carried out on the 30 samples of dried pomegranate peels were taken into an Erlenmeyer and extracted using of Methanol, Ethanol and Acetone (HLPC grade Merck) with dilution of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% solutions (Batta & Rangaswami, 1973). Each solvent (v:v) was prepared from one gram of sample and mixed with 20 ml of solvent then mixtures were replaced on the shaker for 24 hours at the end of that time the Erlenmeyer were removed and extracts replaced into the centrifugal tubes then centrifugated at 4000 rpm for 5 mins. At the end of this duration, filtrations were performed through filter paper then 1 to 1.5 ml of filtrates were taken into rotrary evaporater in order to remove the solvents from each sample then replaced into the Eppendorf tubes to indicate antimicrobial activity of the pomegratnate peel wastes extract and also to determine phenolic compounds by HPLC-DAD analysis (Fang, Zhang, & Wang, 2007).

2.2.3. Estimation total phenolic content (TPC)

The content of total phenolics in the extracts was determined by spectrophotometric method at 750 nm according to the Folin-Ciocalteu procedure and calculated as mg/g of gallic acid equivalents (Sellappan, Akoh, & Krewer, 2002). Principle of the method is based on a redoxreactions, in which Folin-Ciocalteu reagent of phenolic components are came down in an alkaline environment and convert into an oxidized form then total phenolic substance.

2.2.4. Antioxidant activity

The method recommended by Miller and Rice-Evans (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993; Miller & Rice-Evans, 1997; Rice-Evans, Miller, & Paganga, 1996) was used. This method is based on the principle ($ABTS^{\cdot+}$ (2,2'-azinobis-(3-etylbenzotiazoline-6-sulphonic acid)), ensuring nominal measurement is compared to antioxidative substance amount, kept by radical cation with standard amounts of Troloxs, which is a synthetic antioxidant (water-soluble vitamin E analog). The samples, separated depending on estimated antioxidant contents from all samples

for antioxidant determination, were diluted with pure water at certain rates. ABTS and 4.9 mM solvent with 14 mM concentration and $K_2S_2O_8$ (potassium persulphate) solvent were combined at the same ratio and kept waiting in a dark place and at room temperature not less than 12-16 hours. Prepared stock solution was diluted with ethanol. For each solvents in different concentrations at the 20µl, 30µl, 40µl values, antioxidant activities were determined for 3 minutes at 30 seconds intervals. Calculations were made taking account of dilution factors.

2.2.4. Determination of phenols with HPLC-DAD

After extraction for phenol analysis, blowing away solvents from separated samples by rotary evaporator analysis sample were obtained. Zorbax C Column was used. Mobile phase 2.5%, Acetic Acid/97.5% Acetonitrile, flow rate 1mL/0.5mL $^{-1}$, Lichrospher Column: C18 Nucleosil column (150x4,6mm) were adjusted as particle size is 5µm and injection volume 20µL. A scan was made at 280nm and 360nm wavelengths. Preparing 50, 60, 80,100,120, and 150ppm, standard from stock Gallic acid standard by methanol, calibration curve was drawn. For quercetin, 40, 50, 60, and 100ppmstandards of stock solvent were prepared by methanol and calibration curve was drawn. For catechin 100, 120, 150, 200, and 250ppm standards were prepared by water and calibration curve was drawn.

2.2.5. Antimicrobial activity determination

After extraction for antimicrobial activity determination, separated samples were used. 1 loop was received from the stock culture, stored at -80 °C and put into the tube, containing 9ml nutrient broth. It was left for incubation at 37°C for 24 hours. At the end of the duration, 100µl of culture, called subculture was put into the tube, containing nutrient broth and left for incubation for 14-15 hours. *Escherichia coli, Streptococcus mutans, Staphylococcus aureus* were used in the study (Dehkordi, Basti, Gandomi, Misaghi, & Rahimi, 2019). EMB and PCA were used as medium. For *S. mutans and S. aureus* PCA, *for E. coli* EMB, was used as a medium. Well diffusion method was used in the studies. Pour plate and spread plate methods were used. Since pour plate did not give result for *E. coli*, spread plate method was used. Each petri was divided into 4 equal pieces. And a well was opened into each divided part. Separated 50µl sample solvent was put into each well and left for incubation

3.Results and discussions

3.1. *Quantification of bioactive compounds*

Please complete

3.1.1. Total amount of phenolic substance

Total phenol content for ethanol, methanol and acetone in different concentrations are shown in Fig. 1A.

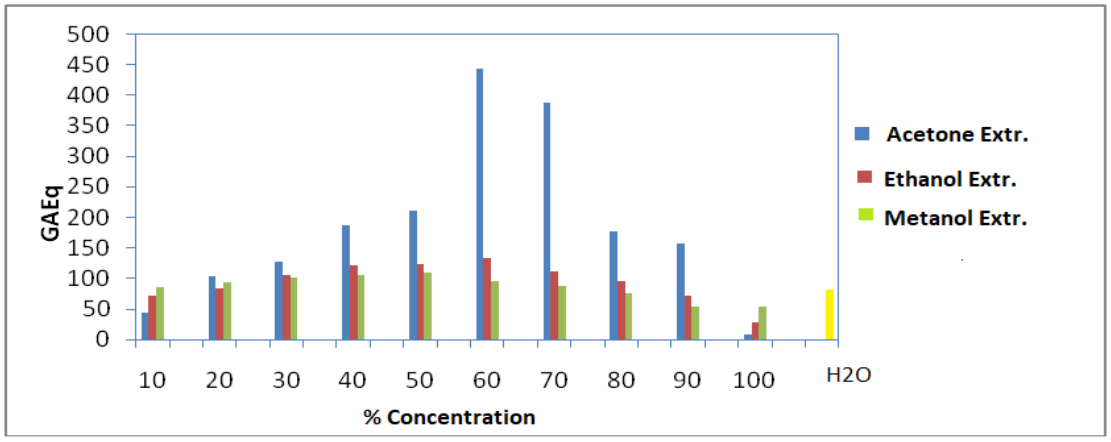


Figure1(A) Total phenol content GAEq:mg/g of gallic acid equivalent

When the results obtained from spectrometric measurements are examined, it is seen that acetone has the highest effect, making available total phenol content in all extractions and solvent types. The data from total phenol content of pomegranate applied Kruskal-Wallis test (Asymp.Sig. < 0,05) is examined, it is determined that the highest effect is on acetone and respectively on the extracts obtained from ethanol and methanol extractions. In view of the findings, which we obtained at the end of the study, while especially 60% acetone solvent 445.012GAEq (mg/g of gallic acid equivalent) affects the total phenol highly, it is determined that this is lower for ethanol and methanol. Kinetic measurements of ethanol, methanol and acetone in certain time periods are shown in Fig. 1B.

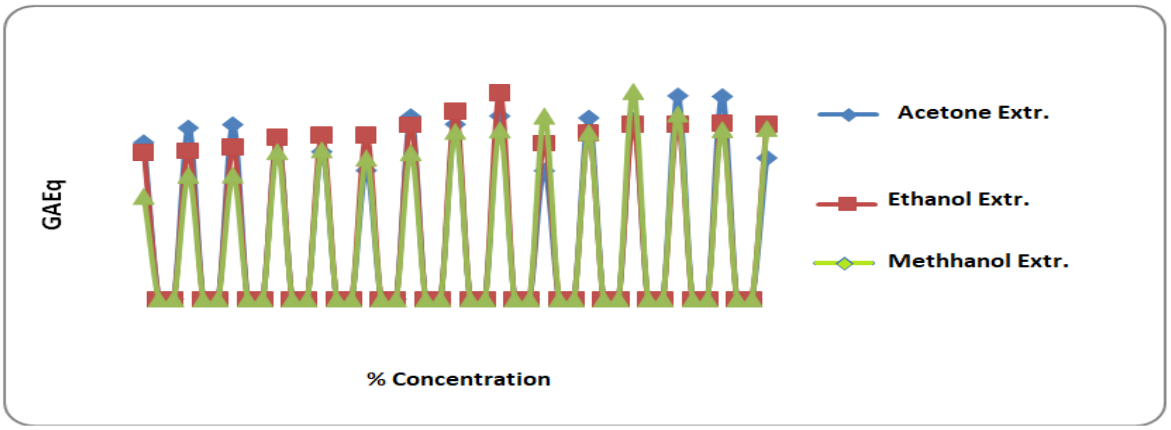


Figure 1(B). Kinetic measurements for total phenol

When the results obtained from spectrophotometric measurements are examined, it is seen that the kinetic measurement results for 3 solvents are close. In the extracts obtained from extraction against time, when total phenol effect is observed, it is seen that the extraction time does not have a significant effect on the efficiency.

3.2. Antioxidant activity

Antioxidant activity effects in ethanol, acetone and methanol extractions are shown by Figures 2A, 2B, and 2C, respectively. The results of spectrophotometric measurements demonstrated that antioxidant activities in all solvents are very high. The highest effect is measured 254228 aox in the extract obtained from 60% ethanol extraction and 143154 aox in the extract obtained from 40% methanol extraction. Meanwhile, antioxidant activities in ethanol, methanol and acetone in different concentrations are shown in Figure 3. The results demonstrated that antioxidant activity of acetone in all extractions and solvent types are the highest one. When the results obtained from the ethanol, methanol, acetone activities were considered it was seen that the aox activity given was high. In addition, it was estimated that aox activities were reduced depending on the duration of aox activity. When this data is applied to the Kruskal-Wallis test (Asymp. Sig. < 0,05) and examined, it was determined that the highest effect is on the acetone, ethanol and methanol extracts respectively. The highest efficiency is estimated as 821.72 mmol trolox/mg in the 60% acetone extraction. These results indicated that the antioxidant level of pomegranate was high and they were similar with the previous studies (Mertens-Talcott, Jilma-Stohlawetz, Rios, Hingorani, & Derendorf, 2006). In their study, Singh et al. (Singh, Chidambara Murthy, & Jayaprakasha, 2002) determined that in their extraction using ethyl acetate, methanol and water from pomegranate peels and seeds, the peels showed

83% and 81% antioxidant activity at 50 ppm with methanol extraction.

The average syneresis values of probiotic yoghurt samples were given in Table 1. On the 30th minute of the measurements, the highest syneresis rate was determined in P2 sample on the 1st day (18.50 mL), while the lowest syneresis rate was in K sample (12 mL) on the 14th and 21st 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 60th minute of the measurements, the highest syneresis rate was in P2 sample on the 1st

3.3. Antimicrobial activity determination

Antimicrobial effects for extractions of different acetone concentrations are shown in Table 1. Statistically significant different zone diameters were determined on the *E.coli, S.aureus* and *S.mutans* according to the type of extraction with different concentrations of ethanol, methanol and acetone. When antimicrobial effects for extractions of different ethanol concentrations are examined, it is determined that *E.coli* created a zone having an average 21.5 mm diameter, acetone average 20,5mm and methanol 13,5mm as a

result of the first measurement in 10% ethanol extraction. While the antibacterial effect of acetone on *E.coli* was higher than 10-40%, ethanol and methanol were found to be statistically similar at these rates. Zone diameter 14mm at 50% extraction while it was measured as 10mm at 100% extraction. When the table is examined, it is observed that while ethanol concentration increases, zone diameters gradually decrease. The zone diameter decreased due to the increase in acetone concentration as well. In methanol extraction, the zone diameter was found to be statistically similar up to 70%, and the highest zone diameter was determined at 50%. The 90% and 100% methanol extraction zone diameter was found to be higher than ethanol and acetone extraction at the same rates, and it was statistically significant. When the zone diameters were compared on the *S. aureus* pathogen bacteria, as the amount of ethanol, acetone, and methanol increased, the zone diameters decreased. The highest effect in ethanol was determined at 10-30%, in acetone 70-80% and in methanol at 10-40%. The highest antibacterial effect on *S. mutans* was determined in acetone extraction, and the lowest effect was seen in ethanol. There was no antimicrobial effect against *S. mutans* above 50% methanol extraction. In a study on the antibacterial effect of pomegranate peel on methanol extraction, it was reported that it exhibited antibacterial activity against *L. monocytogenes*, *Y. enterocolitica*, *E. coli* and *S. aureus* (Prashanth, Asha, & Amit, 2001) .It has been observed that 80% methanol extraction is an active inhibitor against pathogenic bacteria such as *L. monocytogenes, E. coli, S. aureus* and *Y. enterocolitica* in the process of extraction with water, methanol and ethyl alcohol by traditional methods (Al-Zoreky, 2009).

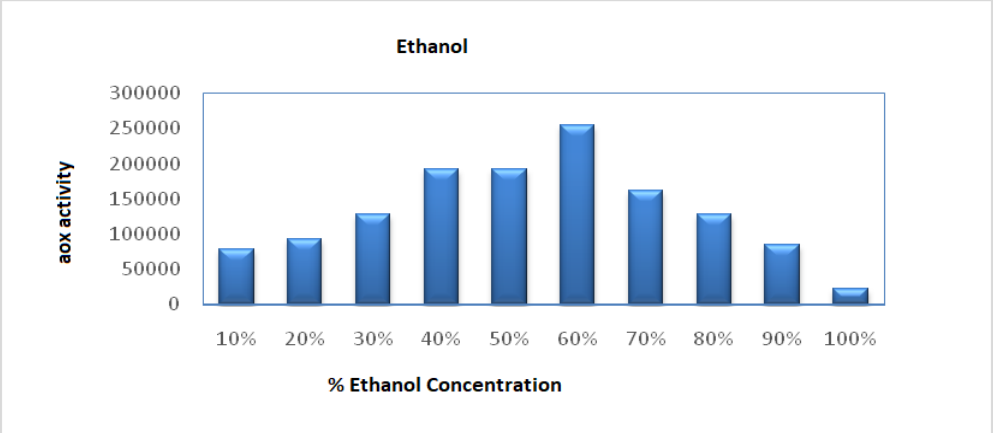
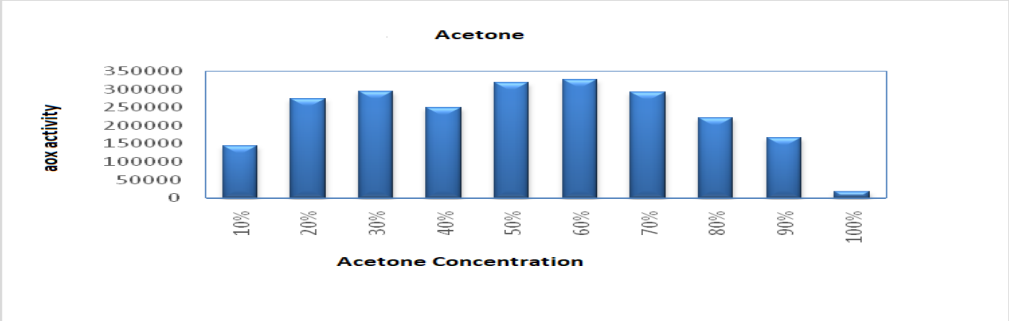


Figure 2A. Aox activity in ethanol extraction in relation to the Trolox TM standard concentration



. **Figure 2B**.Aox activity in acetone extraction in relation to the Trolox TM standard concentration

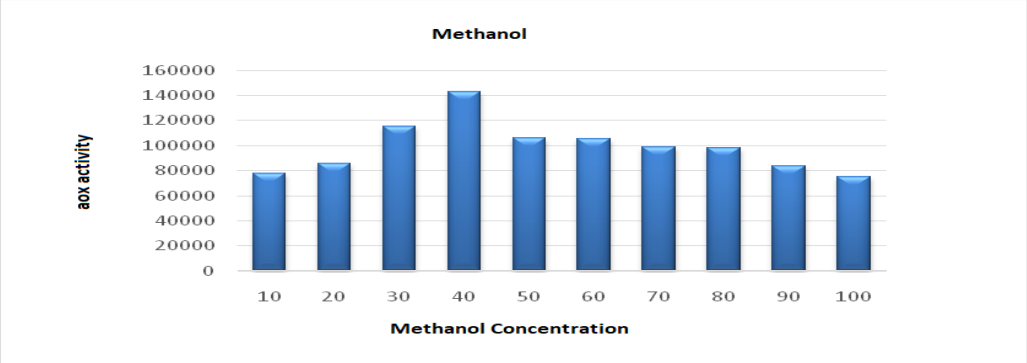


Figure 2C. Aox activity in methanol extraction in relation to the Trolox TM standard concentration

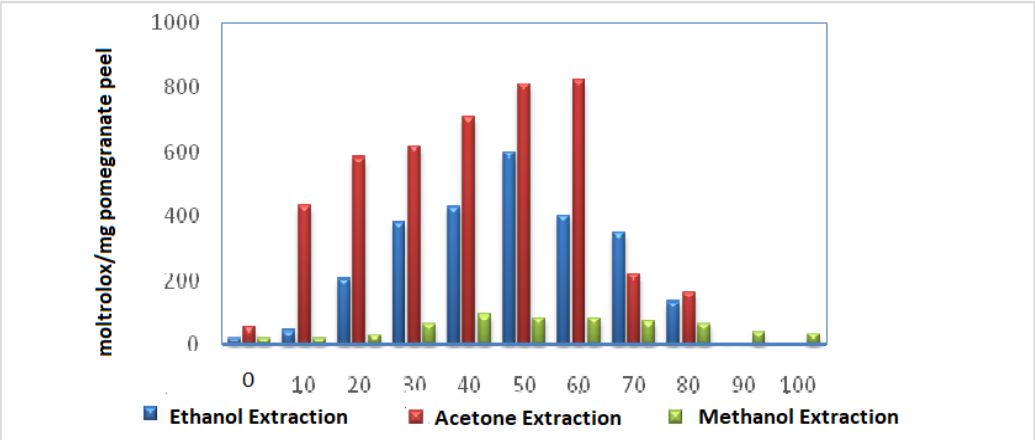


Figure 3. Aox activity in ethanol, acetone, and methanol extraction in relation to the Trolox TM standard concentration

3.4. Determination of phenols with HPLC-DAD

As shown on the Fig. 3 at 280 nm performed by us according to the results obtained from HPLC chromatogram in 50% acetone extraction, components, which cover the largest area are Punicalagin derivatives. In addition to this, gallic acid, punicalin-A and Ellagic acid derivatives follow this. Similarly the study of Nasr et.al (Nasr, Ayed, & Metche, 1996) quantitatively determined ellagic tannins and gallic and elagic acids in the peels of Tunisian pomegranate.

Table 1. Antimicrobial Effects of omegranate Peel Against Some Foodborne Bacteria in Extracts of Different Solvents (mm zone diameter)

		E.coli			*S.aureus*			*S.mutans*	
%	**Etanol**	**Aseton**	**Methanol**	**Etanol**	**Aseton**	**Methanol**	**Etanol**	**Aseton**	**Methanol**
10	21.5 ± 0.7^{Aa}	20.5 ± 0.7^{Ca}	13.5 ± 0.7^{Bb}	18.5 ± 0.7^{Ba}	14 ± 0.0^{Bb}	12.5 ± 2.1^{ABCb}	1 ± 0.0^{Bc}	17.5 ± 0.5^{Aa}	12.5 ± 2.1^{Ab}
20	19 ± 2.8^{Aab}	24 ± 0.0^{Aa}	16.5 ± 0.7^{ABb}	22.5 ± 0.7^{Aa}	14 ± 0.0^{Bb}	15 ± 0.0^{Ab}	nd	18.5 ± 0.5^{Aa}	15 ± 0.0^{Ab}
30	19 ± 2.8^{Aab}	22 ± 0.0^{BCa}	14.5 ± 0.7^{ABb}	13.5 ± 0.7^{Ca}	14 ± 0.0^{Ba}	13.5 ± 0.7^{ABCa}	nd	15 ± 3^{ABCa}	13.5 ± 0.7^{Aa}
40	15 ± 0.0^{Bb}	23 ± 1.4^{ABa}	16 ± 1.4^{ABb}	10 ± 0.0^{DEa}	14 ± 1.4^{Ba}	12.5 ± 3.5^{ABCa}	nd	10 ± 2^{CDa}	13.5 ± 2.1^{Aa}
50	18.5 ± 0.7^{Aa}	15 ± 0.0^{Db}	17.5 ± 0.7^{Aa}	12 ± 2.8^{CDa}	9.5 ± 0.7^{Da}	11 ± 1.4^{BCa}	nd	9.5 ± 1.5^{D}	nd
60	11 ± 0.0^{Cb}	16 ± 0.0^{Da}	16.5 ± 0.7^{ABa}	10 ± 0.0^{DEb}	11 ± 0.0^{Cb}	14 ± 1.4^{ABa}	nd	17.5 ± 0.5^{A}	nd
70	13 ± 1.4^{BCc}	14 ± 0.0^{Ab}	16.5 ± 0.7^{ABa}	10.5 ± 0.7^{Db}	16 ± 0.0^{Aa}	11 ± 1.4^{BCb}	nd	17.5 ± 0.5^{A}	nd
80	6.5 ± 0.7^{Dc}	14 ± 0.0^{Aa}	14 ± 1.4^{Bb}	$7,5\pm0.7^{EFc}$	17 ± 0.0^{Aa}	10.5 ± 0.7^{BCb}	nd	13.5 ± 1.5^{ABCD}	nd
90	4.5 ± 0.7^{Db}	8 ± 1.4^{Eab}	13.5 ± 3.5^{Ba}	7 ± 1.4^{Fa}	10 ± 1.4^{CDa}	11.5 ± 2.1^{ABCa}	10 ± 0.0^{A}	16 ± 3^{AB}	nd
100	5 ± 0.0^{Dc}	9 ± 1.4^{Eb}	14 ± 1.4^{Ba}	6.5 ± 0.7^{Fa}	2 ± 0.0^{Eb}	10 ± 0.0^{Ca}	10 ± 0.0^{A}	11.5 ± 0.5^{BCD}	nd

A-E: Values with different letters in the same column differ statistically significantly ($P<0.05$). a-c: Values with different letters in the same row differ statistically significantly ($P<0.05$), nd:not detected

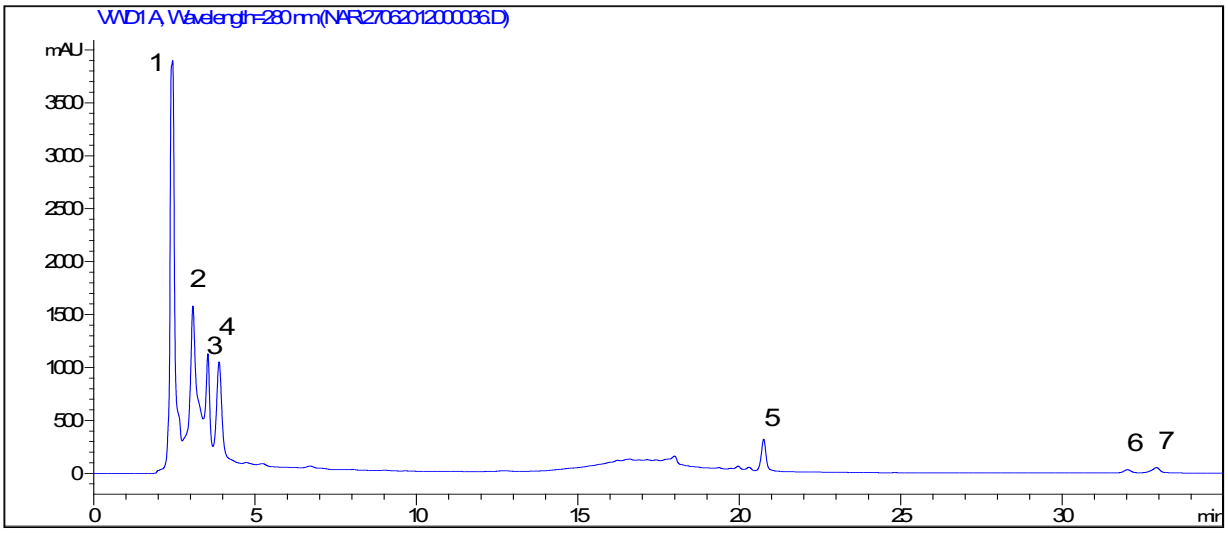


Figure 4. HPLC chromatogram of phenolic components in pomegranate peel with adding 400ppm standard gallic acid in 50% acetone extraction (1-2 Punicalagin derivatives, 3- Gallic acid, 4- Punicalin-A, 5-Ellagic acid derivatives, 6-Punicalagin-B).

4.Conclusions

This research studied the total phenolic content, antioxidant activity, antimicrobial activity and phenolic compounds from pomegranate peel using solvent extractions methods by methanol, ethanol and acetone with concentrations of 10-100% (v/v). Total phenolic contents were determined by Folin Ciocalteau method and results showed that maximum total phenol content was 445.012GAEq (mg/g of gallic acid equivalent) at 60% acetone solvent. Antioxidant activities were calculated by ABTS method and results demonstrated that highest antioxidant activity was about 821.72 mmoltrolox/mg with (60%) acetone extract. Also, HPLC-DAD analysis showed that the most abundant phenolic compounds were the punicalagin, gallic acid, ellagic acid and quercetin. The highest zone diameters against *E.coli* and *S.mutans* pathogen bacteria were determined in acetone extraction, and the highest zone diameter against *S.aureus*was determined in ethanol extraction. The antimicrobial effects of pomegranate peel extract on the *E. coli, S. aureus, S.mutans* showed that the antimicrobial effect is seen on the *E. coli* from the acetone solvent. Thus, acetone can be considered as a useful solvent for extraction of phenolic compound from pomegranate peel to approach antioxidant and antimicrobial effect of them as a preventive and therapeutic in human health.

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EFFECT OF PVC AND HDPE PACKAGING FILMS ON THE QUALITY MAINTENANCE OF GRAPE TOMATOES DURING STORAGE

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ABSTRACT

Packaging films can present a replacement for developing fresh vegetables and fruits postharvest life. The impact of polyvinyl chloride (PVC) and high-density polyethylene (HDPE) packaging films on some qualitative such as, mechanical, physical and chemical properties of grape tomatoes were examined. Packaging films were used as protective packaging on the grape tomatoes and stored at 4°C and 20°C for 40 days. The results didn't show any significant effects from statistical points in pH and total soluble solid compared with the packaging and without packaging. The packaging films significantly prevent moisture content and weight loss, preserve maximum work for break, maximum strain and also, maintain the firmness of the grape tomatoes, improve storage characteristics and its quality. The PVC and HDPE films had remarkable effects on color parameters. On the other hand the color of the packaged grape tomatoes had less brightness and products at 4°C had less redness during storage.

1. Introduction

Grape tomato (Lycopersicon esculentum Mill. `Santa') is one of the cultivars of tomato that because of its flavour, sweetness, the potential health benefits and ease of use, has gained popularity among consumers. Its substantial popularity is due at least in part to its higher sugar content compared to tomatoes, and its smaller, bite-sized shape (Simonne *et al*., 2007). Harvest maturity and postharvest conditions are a number of factors that lead to changes in sensory and nutritional quality of grape tomatoes. Grape tomatoes are harvested at level of specific (Reddish orange) color, In order to prevent postharvest losses due to softening. Maintaining the best quality of fresh product is still the biggest challenge for the food industry. The most impressive features of products include flavor, nutritional value, appearance, texture, color and microbial safety (Cantwell *et al*., 2009). Agricultural products with high durability have high commercial value, given that fruits and vegetables spoil quickly after harvest, and are degrading in the quality, so finding a solution to enhance the shelf life and quality of these products is considered. Temperature control has a positive impact on the storage of tomatoes, equally, low temperatures have a significant impact on increasing shelf life as well (Bourne, 1982). Apart from controlling the temperature of the environment, there are other influential factors, the most important of which can be packaging films (Hotchkiss, 1997).

Less processed fruits and vegetables are highly nutritious, but very perishable. Removing the skin and resize lead to change color, leakage of nutrients, weight loss, change the texture and the rapid growth of microbial and thus reduce the quality of fruits and vegetables. Various methods have been investigated to overcome these problems and increase durability and storage time of fresh fruits and vegetables (Sehat, 2012). For example, high relative humidity, low temperatures and packaging (Nadim *et al*., 2015; Sehat, 2012).

Packaging film is one of the most reliable methods that many studies are currently done about it. Storage using the packaging films leads to maintain quality and increase the shelf life of products, which slows down the chemical reactions and also reduces the growth of pathogenic microorganisms (Ozturk *et al*., 2016). Losing water in fruits and vegetables is one of the most important problems, however, losing water can be controlled using packaging films. Packaging film reduces the amount of water vapor transmission, form a physical protection around the products, and prevent water loss and decay of tissue (Debeaufort *et al*., 1998). The packaging film has many advantages compared with other methods. When the product is inside the packaging with the appropriate temperature, the film acts as a barrier to gasses, and controls microbial growth, preserves color, texture and moisture and effectively extends the shelf life of the product (Mistriotis *et al*., 2016). In the last decade, the growth conditions of temperature controlled food industries, has encouraged research groups to work on improving existing methods and the development of new innovations, mainly to the performance of high quality products, textures, flavors, with original color and nutritional value (Galetto *et al*., 2010).

The controlled atmosphere and temperature are useful in delaying softening and decay of the product (Gil *et al*., 1997).

D'Aquino et al (2016) examined the effects of oriented polypropylene (OPP) packaging film on acidity, water loss, firmness, total soluble solids, vitamin C and postharvest quality of cherry tomatoes. Geeson et al (1985) examined the effect of PVC packaging film on stored tomatoes quality. Modified atmosphere packaging have been studied as an alternative method to reduce postharvest deterioration and lifetime storage of cherry tomatoes (Das, 2006). Bhowmik (1992) examined the effect of controlled atmosphere and relative humidity on quality, weight loss, titratable acidity, pH, color and firmness of green tomato. Pila (2010) studied the physicochemical changes related to the quality of tomatoes during storage. Mechanical properties, including Young's modulus and firmness and physical properties were evaluated by kabas and Ozmerzi (2008) in cherry tomatoes. Although studies on the impact of packaging and temperature on grape tomatoes have been reported in the literature, this approach has not yet been pursued for the characterization of quality and mechanical properties of grape tomato inside the PVC and HDPE packaging, in temperature controlled conditions.

The objectives of this work are studying the ability of PVC and HDPE packaging films to extend the shelf life of grape tomatoes stored at room temperature and refrigerated, compared to non-packaged product, and assessing the effects of packaging films on the quality attributes of grape tomatoes, such as surface color, weight loss, some mechanical properties, size characteristics and some chemical properties, such as total soluble solids (TSS) and titratable acidity.

2. Materials and methods

Grape tomatoes were harvested from greenhouse at Hamadan, Iran. Grape tomatoes of uniform color, shape, size and without any damage were selected to the tests. The grape tomatoes were cleaned with hand to eliminate external matters and then transferred to the laboratory and were divided into three categories: without packaging, PVC packaging, and HDPE packaging. Then these three, were stored at 4°C and 20°C. The physical, mechanical and chemical characteristics of products were analyzed every five days until the fortieth day of storage.

2.1. Color

The color of Grape tomatoes surface was determined by a colorimeter (portable colorimetric, HP-200, Guangdong, China) which presented CIE a*, b*, and L* values for each replication. b* (yellowness, b* > 0, blueness < 0), a* (redness > 0, greenness < 0), L* (lightness, black = 0, white = 100), Hue° (Hue angel, H° = tan ((b*)/(a*)), red = 0°,

yellow = 90°, green = 180°, blue = 270°) and c (Chroma, c=√((a*)^2+(b*)^2), 0 at the center of the color sphere) were quantified on each samples using a 10 degree position of the standard observer (CIE, 1978).
Total color difference was measured by equation 1, where L_0, a_0 and b_0 are control values for fresh grape tomatoes.
ΔE=√((a*-a_0)^2+(b*-b_0)^2+(L*-L_0)^2)

2.2. Weight loss

Grape tomatoes were weighed at harvest time at first, then after that weight loss during postharvest storage was measured by subtracting sample weights from their previous recorded weights and presented as percentage of weight loss compared to initial weight.

2.3. Chemical tests

2.3.1. PH

The pH measurement was made using a digital pH meter (Eco Tester PH 2 Water proof Pocket Tester, Singapore) calibrated with pH 4.0 and 7.0 buffers.

2.3.2. TSS

TSS was determined by portable digital refractometer (Model: PAL-1; Atago, Japan) with a scale of 0-53brix at room temperature (~25°c).

2.3.3. TA

For this experiment, titratable acidity (TA) was measured by solving each 5 milliliter juice of grape tomatoes in 25 ml distilled water and after that titrated to PH 8.1, according to the AOAC official method 942.15 (AOAC, 2000) using 0.1 N NAOH.
% citric acid = (Titre value (ml) ×0.1× 0.064 × 100)/(5 g of juice)

2.4. Physical properties

Digital calipers with a sensitivity of 0.01 mm was applied for determine the axial dimension of products; length, width and thickness, geometric mean diameter (Dg), sphericity (ø) and surface area (S) were calculated following equations. (3), (4) and (5) (Mohsenin, 1986):

Dg = (LWT)^(1/3)
Φ =Dg/L
S = π. Dg^2
Where T is the thickness, W is the width and L is the length and of the fruit (Figure 1).

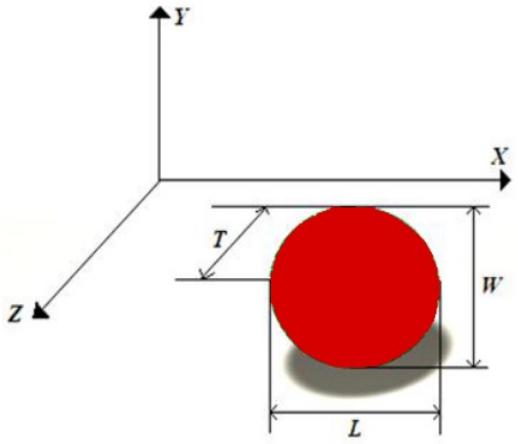

Figure 1. Three major dimensions of grape tomato

2.5. Moisture content measurement

To determine the moisture content, grape tomatoes were kept in the oven for 24 h at 70 ℃ (AOAC, 1990). These experiments were replicated thrice to obtain a reasonable average.
M.C = (M_0-M)/M_0
Where: M0 and M are initial and last (before placed in the oven) mass of product.

2.6. Determination of density and volume

Grape tomatoes volume has been determined by water displacement method (WDM) and using a graduated cylinder. The WDM is one of the most popular and simple means of measuring the volume of large objects such as fruits and vegetables (Mohsenin, 1986).
Volume (ml) = (weight of displaced water (g))/(water density (g/ml))
Product density is obtained by dividing the mass of the product to its volume.

2.7. Mechanical tests

For this experiment, mechanical features were examined using a puncture test and the grape tomatoes were pierced with a texture analyzer device (Zwick/Roell Model

BT1_FR0.5TH.D14, using Xforce HP model of load cell with capacity of 500 N, Germany) with a probe of 3.15 mm diameter (Magness–Taylor probe). Penetration speed was set at 50 mm/min and the test was finished after force fell by 30% compared to the Fmax. Five parameters were studied: firmness as the maximum puncture force Which is expressed in the form of N (Fmax), and surface area under the force diagram, As a work done to reach the maximum force which is expressed as N.mm (W), strain as the amount of maximum deformation that happen on maximum stress (ε max), modulus of elasticity was that of obtained by boussinesq techniques expressed in N/mm2 (E) (Galetto et al., 2010; Mehinagic et al., 2003).

2.8. Statistical analysis

Factors of these experiments were temperature, packaging film and storage time and these tests were described under a factorial design. ANOVA (Analysis of variance) was done on data using SPSS software (IBM SPSS Statistics 23, IBM, NY) by means of PC. The significance levels were applied as $P < 0.01$ (**) and $P < 0.05$ (*).

Duncan's multi-domain test was applied for compare the averages in this experiment. Data analysis was conducted in two groups, until twentieth day for products without packaging films and until fortieth day, for products within the packaging. The reason for this is that products without packaging film had the capability to test until the twentieth day, while products in the packaging film had the capability to do the test until the fortieth day.

3. Results and discussions

3.1. Results

3.1.1. Effect of PVC and HDPE on weight loss and moisture content

As seen from Figure 2, weight loss in grape tomatoes increased during maintenance however, the packaging films decreased rate of weight loss during maintenance. Grapes tomatoes stored without packaging increase weight loss on day 5 but reached 24.40% weight loss on day 20 along the storage at 20°C after 40 days storage. The percentages of weight loss for PVC and HDPE packaging grape tomatoes in 4°C and 20°C were 2.04%, 5.97% and 1.31%, 2.24%, respectively. Moisture content percentage in without packaging products decreased significantly during storage (Figure 2). PVC and HDPE packaging ($P < 0.05$) inhibited the decrease at storage time effectively.

At the end of the storage period, the values of without packaging moisture content in 20°C and 4°C were 66.58%, 83.71%, respectively, while the PVC and HDPE packaging After 40 days storage in 20°C and 4°C were 85.06%, 89.30% and 89.46%, 89.68%, respectively.

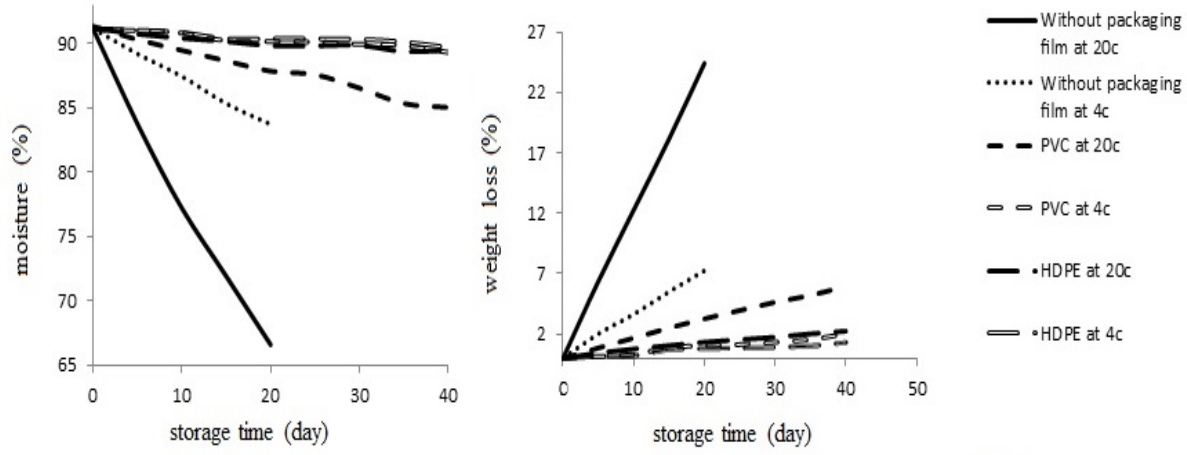

Figure 2. Effect of packaging on weight loss and moisture content in grape tomatoes during storage

3.1.2. TA, TSS and pH

Figure 3 shows the effect of packaging films and temperature on soluble solids. The results didn't show any significant differences in total soluble solid compared with the packaging and without packaging, while the results showed significant ($P < 0.05$) differences in temperature of the grape tomatoes. The levels of TA for PVC and HDPE packaging films products and without packaging film at 20°C and 4°C were 43.31%, 18.43% and 39.79%, 29.94% and 36.3%, 21.59% decrease respectively, at the end of storage time (Figure 3). This parameter for the temperatures, 4°C and 20°C had a different behavior; that is, for packaged products, reducing of TA at 20°C was 41.78% more than products that were at 20°C. For products that were inside the package until the fortieth day, significant relation ($P < 0.05$) in TA parameter between PVC and HDPE films was observed, so that, acidity of the products inside the HDPE packaging during storage was 4% less than the products in PVC films. As well temperature, storage duration and packaging significantly ($P < 0.05$) affected the TA during the experiment (Table 1 and Table 2). Also, the results showed that pH value of grape tomatoes in PVC and HDPE films at 20°C and 4°C were 7.30%, 5.63% and 9.26%, 7.37% increased, respectively during storage, while no significant differences between packaging films for pH value were observed during storage (Table 1, Table 2 and Figure 3).

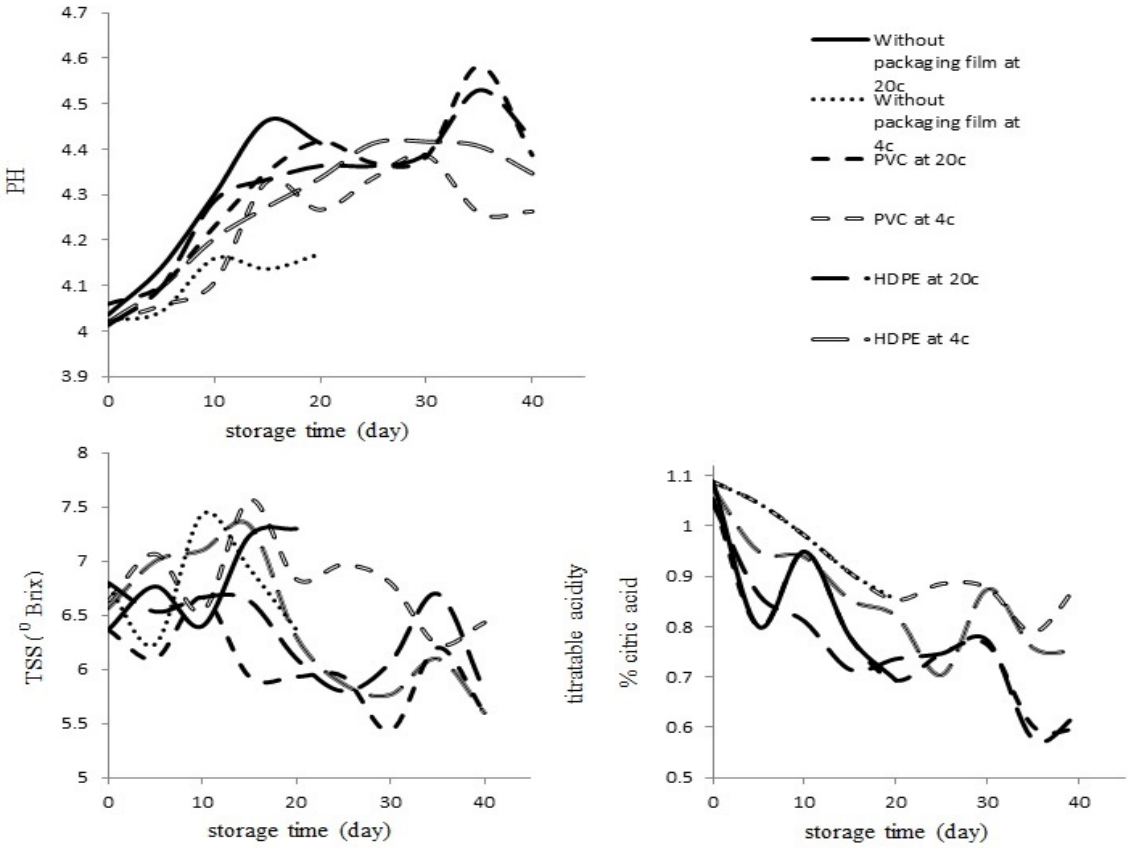

Figure 3. Effect of packaging on some chemical properties in grape tomatoes during storage

Table 1. Summary analysis of variance (mean squares) of some chemical parameters evaluated in a factorial experiment (until the day 20)

Sources of change	Degrees of freedom	PH	TSS	TA
Packaging films	2	$0.001^{n.s}$	$0.477^{n.s}$	0.013*
Temperature	1	0.185**	2.178*	0.266**
Period	4	0.320**	$0.833^{n.s}$	0.238**
Pack × Temperature	2	0.034**	1.391*	$0.001^{n.s}$
Pack × Period	8	$0.005^{n.s}$	$0.220^{n.s}$	$0.003^{n.s}$
Temperature × Period	4	0.014*	$0.231^{n.s}$	0.020**
Pack × Period × Temperature	8	$0.008^{n.s}$	0.860*	$0.005^{n.s}$
Test error	60	$0.005^{n.s}$	$0.346^{n.s}$	$0.004^{n.s}$
CV (%)		3.57	10.94	14.941

"ns" means there was no significant relationship here.
* Significant relationship between two parameters at $P < 0.05$.
** Significant relationship between two parameters at $P < 0.01$.

Table 2. Summary analysis of variance (mean squares) of some chemical parameters evaluated in a factorial experiment (until the day 40)

Sources of change	Degrees of freedom	PH	TSS	TA
Packaging films	1	$0.014^{n.s}$	$0.009^{n.s}$	0.038**
Temperature	1	0.108**	4.813**	0.389**
Period	8	0.249**	1.521**	0.169**
Pack × Temperature	1	$0.027^{n.s}$	3.203**	0.021*
Pack × Period	8	$0.004^{n.s}$	$0.312^{n.s}$	$0.004^{n.s}$
Temperature × Period	8	0.017*	0.587*	0.011**
Pack × Period × Temperature	8	$0.003^{n.s}$	$0.263^{n.s}$	$0.007^{n.s}$
Test error	72	0.008	0.276	0.004
CV (%)		3.83	11.47	17.43

"ns" means there was no significant relationship here.
* Significant relationship between two parameters at $P < 0.05$.
** Significant relationship between two parameters at $P < 0.01$.

3.1.3. Effect of PVC and HDPE films on color

Figure 4 and table 3, table 4 shows that grape tomatoes color changed significantly ($P < 0.05$) during storage. Products at 4°C and without packaging film were brighter than other products up to the twentieth day, while the products at 20°C and without film, were darker than others products up to the twentieth day. There was an effective ($P < 0.05$) reduce in L*, with increasing maintenance period until the fortieth day for other products (Table 3, 4). Packed and unpacked samples showed a significant ($P < 0.05$) decrease in a* value during storage. Application of PVC and HDPE packaging led to significantly ($P < 0.05$) low levels of a* compared with the without packaging products during the testing period. Also in the experiment, the value of a* for packaging and non-packaging at 4°C was lower than 20°C. Similarly, significant ($P < 0.05$) reduce took place in b* along the testing period (Figure 4). Based on the results, products at

20°C had lower levels of b* values compared with the others, and products in PVC packaging and without packaging, showed decrease in b* value during storage (Figure 4). The H° (Hue angle) as a function of maintenance period for PVC and HDPE, were increased 23.47%, 15.76% respectively during storage at 4°C, and for products at 20°C, were decreased 12.29% and 11.07% along the maintenance. However, the lower H° indicated more redness. Figure 4 shows an increase in the total color variation (ΔE) for every three treatments along the maintenance. The results for PVC and HDPE packaging films didn't show any effective differences in ΔE after 20 days and no significant differences were statistically observed after 40 days between PVC and HDPE films. The rate of L* of the without packaging samples were 40.47 at 20°C on the first day of storage, that decreased 12.84% and reached till 35.27% on the 20th day of maintenance, however, the rate of L* of the without packaging samples at 4°C on the first day, did not differ in the twentieth day of storage. The rate of L* for the HDPE and PVC packaging samples at 20°C and 4°C were decreased 11.87%, 11.98%, 10.68% and 9.03% respectively. The a* parameter for without packaging at 20°C and 4°C reached from 30.87 to 30.2 and 32.49 to 25.79, which has decreased 2.17%, 20.62% respectively.

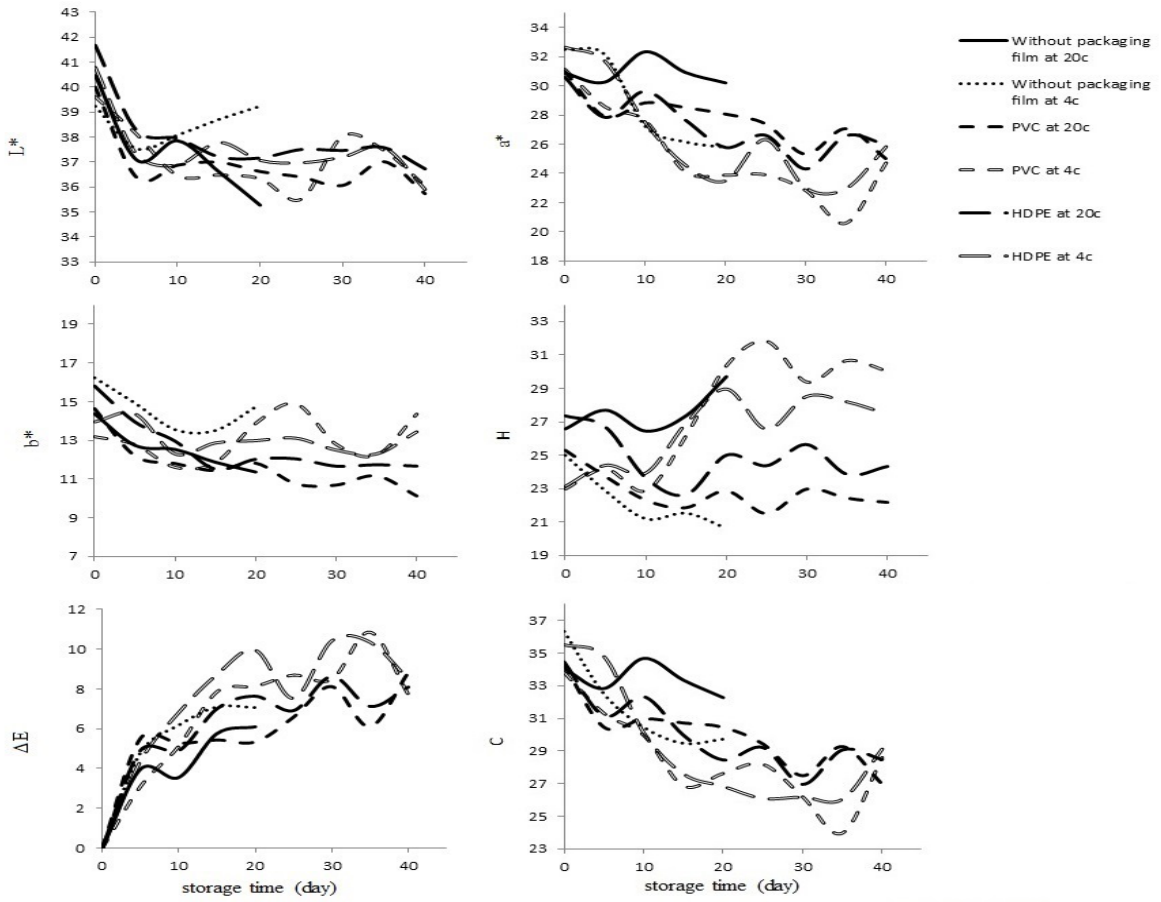

Figure 4. Effect of packaging on color in grape tomatoes during storage

Whereas this parameter is decreased about 20.61% and 19.39% respectively for the PVC packaging samples at 4°C and 20°C and decreased 15.16%, and 20.87% respectively for the HDPE packaging samples at 4°C and 20°C during storage (Figure 4 and Table 3, Table 4).

Table 3. Summary analysis of variance (mean squares) of color parameters evaluated in a factorial experiment (until the day 20)

Sources of change	Degrees of freedom	L	a*	b*	c	H	ΔE
Packaging films	2	5.515**	32.147**	8.968**	29.987**	7.754$^{n.s}$	8.530$^{n.s}$
Temperature	1	1.035$^{n.s}$	45.753**	13.642**	30.625*	152.751**	19.125**
Period	4	33.863**	80.398**	18.328**	87.873**	19.342**	156.644**
Pack × Temperature	2	4.920**	7.077$^{n.s}$	8.725**	4.368$^{n.s}$	46.706**	0.877$^{n.s}$
Pack × Period	8	1.003$^{n.s}$	3.452$^{n.s}$	0.922$^{n.s}$	4.374$^{n.s}$	2.548$^{n.s}$	1.482$^{n.s}$
Temperature × Period	4	3.095**	38.061**	4.643**	21.304**	49.323**	6.586$^{n.s}$
Pack × Period × Temperature	8	2.478**	1.914$^{n.s}$	0.725$^{n.s}$	2.608$^{n.s}$	2.225$^{n.s}$	2.008$^{n.s}$
Test error	60	0.6	4.042	1.039	4.583	3.558	2.731
CV (%)		4.27	10.99	11.88	9.92	11.99	27.29

"ns" means there was no significant relationship here.
* Significant relationship between two parameters at $P < 0.05$.
** Significant relationship between two parameters at $P < 0.01$.

Table 4. Summary analysis of variance (mean squares) of color parameters evaluated in a factorial experiment (until the day 40)

Sources of change	Degrees of freedom	L	a*	b*	c	H	ΔE
Packaging films	1	13.918**	3.579$^{n.s}$	6.770*	2.8$^{n.s}$	5.307$^{n.s}$	9.571$^{n.s}$
Temperature	1	0.357$^{n.s}$	68.513**	25.114**	37.890**	278.853**	34.039**
Period	8	19.080**	71.870**	8.248**	77.154**	17.804**	95.711**
Pack × Temperature	1	3.003*	18.089$^{n.s}$	6.011*	2.749$^{n.s}$	68.928**	0.601$^{n.s}$
Pack × Period	8	0.476$^{n.s}$	2.345$^{n.s}$	1.170$^{n.s}$	3.820$^{n.s}$	2.515$^{n.s}$	1.808$^{n.s}$
Temperature × Period	8	0.889$^{n.s}$	16.314**	6.155**	12.848*	37.576**	8.052*
Pack × Period × Temperature	8	1.091$^{n.s}$	1.449$^{n.s}$	1.108$^{n.s}$	2.026$^{n.s}$	4.475$^{n.s}$	1.556$^{n.s}$
Test error	72	0.603	4.687	1.038	5.469	2.380	3.279
CV (%)		3.94	12.37	12.03	11.4	12.16	21.34

"ns" means there was no significant relationship here.

* Significant relationship between two parameters at $P < 0.05$.

** Significant relationship between two parameters at $P < 0.01$.

3.1.4 Physical properties changes

The dimensional size features of packaged and unpackaged grape tomatoes are given in Figure 5. Based on the results, surface area, geometric mean diameter, axial dimensions, volume and sphericity were reduced along the maintenance. The thickness, length, width, geometric mean diameter, volume and surface area of product were effectively ($P < 0.05$) upper in packaging products than that of without films, while sphericity were measured effectively ($P < 0.05$) lower in without packaging than packaged ones. As well, the density of the products in the packaging decreased, and in the without packaging increased, during storage. Effective differences were seen due to packaging in the thickness, width, length, density, volume, surface area and geometric mean diameter (Table 5, Table 6).

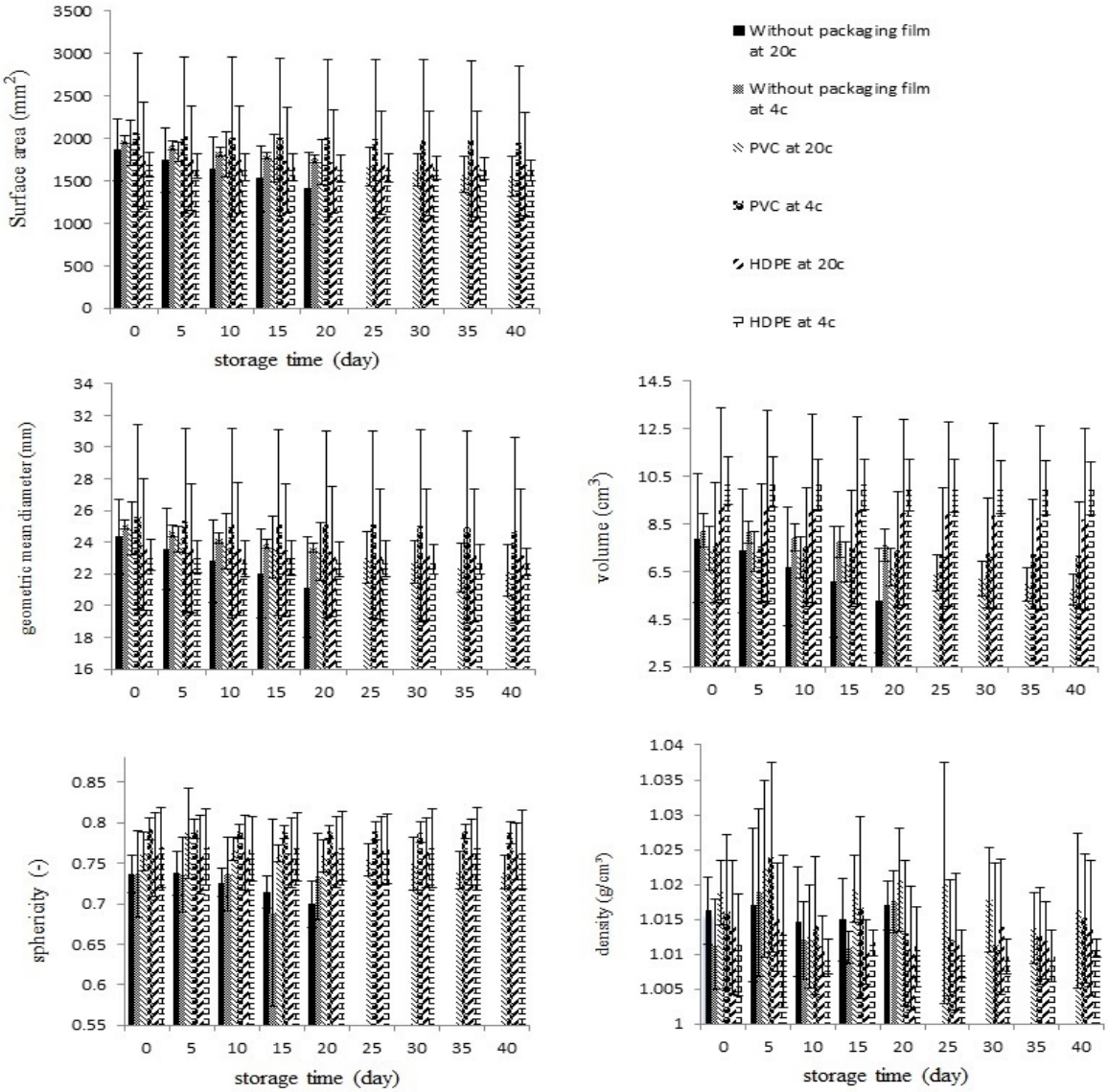

Figure 5. Effect of packaging on some physical properties in grape tomatoes during storage: Values are the means ±SE of triplicate assays. Vertical bars represent the standard errors of the means

Table 5. Summary analysis of variance (mean squares) of some physical traits evaluated in a factorial experiment (until the day 20)

Sources of change	Degrees of freedom	Length (mm)	Width (mm)	Thickness (mm)	Geometric mean diameter (mm)	Density	Surface areas (mm^2)	Sphericity	Volume (ml)
Packaging films	2	26.826*	0.055**	22.074**	15.851**	0.000**	394804.457**	0.024**	55.941**
Temperature	1	$4.515^{n.s}$	$0.004^{n.s}$	41.779**	12.766*	$5.714E\text{-}5^{n.s}$	279979.632*	0.003*	19.173**
Period	4	$4.536^{n.s}$	$0.008^{n.s}$	$4.484^{n.s}$	$4.090^{n.s}$	9.085E-5**	$82310.259^{n.s}$	$0.001^{n.s}$	$1.886^{n.s}$
Pack × Temperature	2	$13.798^{n.s}$	0.015*	10.628**	9.828*	$2.411E\text{-}6^{n.s}$	253045.254*	$0.001^{n.s}$	$1.279^{n.s}$
Pack × Period	8	$0.940^{n.s}$	$0.002^{n.s}$	$1.041^{n.s}$	$0.999^{n.s}$	$1.615E\text{-}5^{n.s}$	$23650.143^{n.s}$	$0.000^{n.s}$	$0.475^{n.s}$
Temperature × Period	4	$0.571^{n.s}$	$0.001^{n.s}$	$1.287^{n.s}$	$0.655^{n.s}$	$9.702E\text{-}6^{n.s}$	$15551.110^{n.s}$	$0.000^{n.s}$	$0.544^{n.s}$
Pack × Period × Temperature	8	$0.133^{n.s}$	$0.000^{n.s}$	$0.253^{n.s}$	$0.151^{n.s}$	$1.446E\text{-}5^{n.s}$	$1841.576^{n.s}$	$0.000^{n.s}$	$0.224^{n.s}$
Test error	60	6.363	0.004	2.032	2.714	1.814E-5	64098.348	0.000	1.471
CV (%)		7.5	7.36	8.39	7.09	0.5	14.49	4.22	20.12

"ns" means there was no significant relationship here.

* Significant relationship between two parameters at $P < 0.05$.

** Significant relationship between two parameters at $P < 0.01$.

Table 6. Summary analysis of variance (mean squares) of some physical traits evaluated in a factorial experiment (until the day 40)

Sources of change	Degrees of freedom	Length (mm)	Width (mm)	Thickness (mm)	Geometric mean diameter (mm)	Density	Surface areas (mm^2)	Sphericity	Volume (ml)
Packaging films	1	29.778$^{n.s}$	47.098**	19.270*	31.863**	0.001**	779437.149**	0.002*	173.533**
Temperature	1	0.188$^{n.s}$	1.952$^{n.s}$	47.760**	11.344$^{n.s}$	0.000**	288899.334$^{n.s}$	0.009**	24.387**
Period	8	1.601$^{n.s}$	1.304$^{n.s}$	2.142$^{n.s}$	1.629$^{n.s}$	4.901E-5*	36083.315$^{n.s}$	0.000$^{n.s}$	0.945$^{n.s}$
Pack × Temperature	1	22.313$^{n.s}$	28.541**	43.675**	32.723**	9.071E-6$^{n.s}$	860156.340**	0.005**	0.904$^{n.s}$
Pack × Period	8	0.237$^{n.s}$	0.246$^{n.s}$	0.867$^{n.s}$	0.450$^{n.s}$	1.41E-5$^{n.s}$	10200.433$^{n.s}$	0.000**	0.177$^{n.s}$
Temperature × Period	8	0.417$^{n.s}$	0.233$^{n.s}$	0.838$^{n.s}$	0.460$^{n.s}$	1.137E-5$^{n.s}$	9675.280$^{n.s}$	0.000$^{n.s}$	0.221$^{n.s}$
Pack × Period × Temperature	8	0.122$^{n.s}$	0.116$^{n.s}$	0.586$^{n.s}$	0.208$^{n.s}$	1.254E-5$^{n.s}$	4237.044$^{n.s}$	0.000$^{n.s}$	0.065$^{n.s}$
Test error	72	7.959	2.407	2.824	3.587	2.020E-5	85476.351	0.000	1.747
CV (%)		7.95	7.44	8.81	7.68	0.51	15.89	2.69	21.33

"ns" means there was no significant relationship here.

* Significant relationship between two parameters at $P < 0.05$.

** Significant relationship between two parameters at $P < 0.01$.

3.1.5. Mechanical properties changes

The standard errors and means of maximum work, maximum strain, modulus of elasticity and firmness of grape tomatoes as a function of maintenance period are provided in Figure 6. W max, E, ε max and Firmness were significantly different in packaged products from unpackaged ones (Table 7, Table 8 and Figure 6). In this study, the firmness of unpackaged fruits decreased in 20°C and 4°C from 2.21 to 1.52 N and 2.16 to 1.53 N, respectively, along the maintenance and for products inside the PVC and HDPE packaging in 20°C and 4°C from 2.20 to 1.56 N, 2.14 to 0.93 N and 2.16 to 1.85 N, 2.17 to 1.10 N, respectively, along the maintenance. As the figure 6 show the rate of W_{max} and E value were decreased during storage. As well the results showed that significant differences ($P < 0.05$) were statistically observed between packaging in W_{max} and E values. According to the results, ε_{max}, were increased during storage. The results showed that significant differences ($P < 0.05$) were statistically observed between packaging in ε_{max} during 20 day of storage (Figure 6).

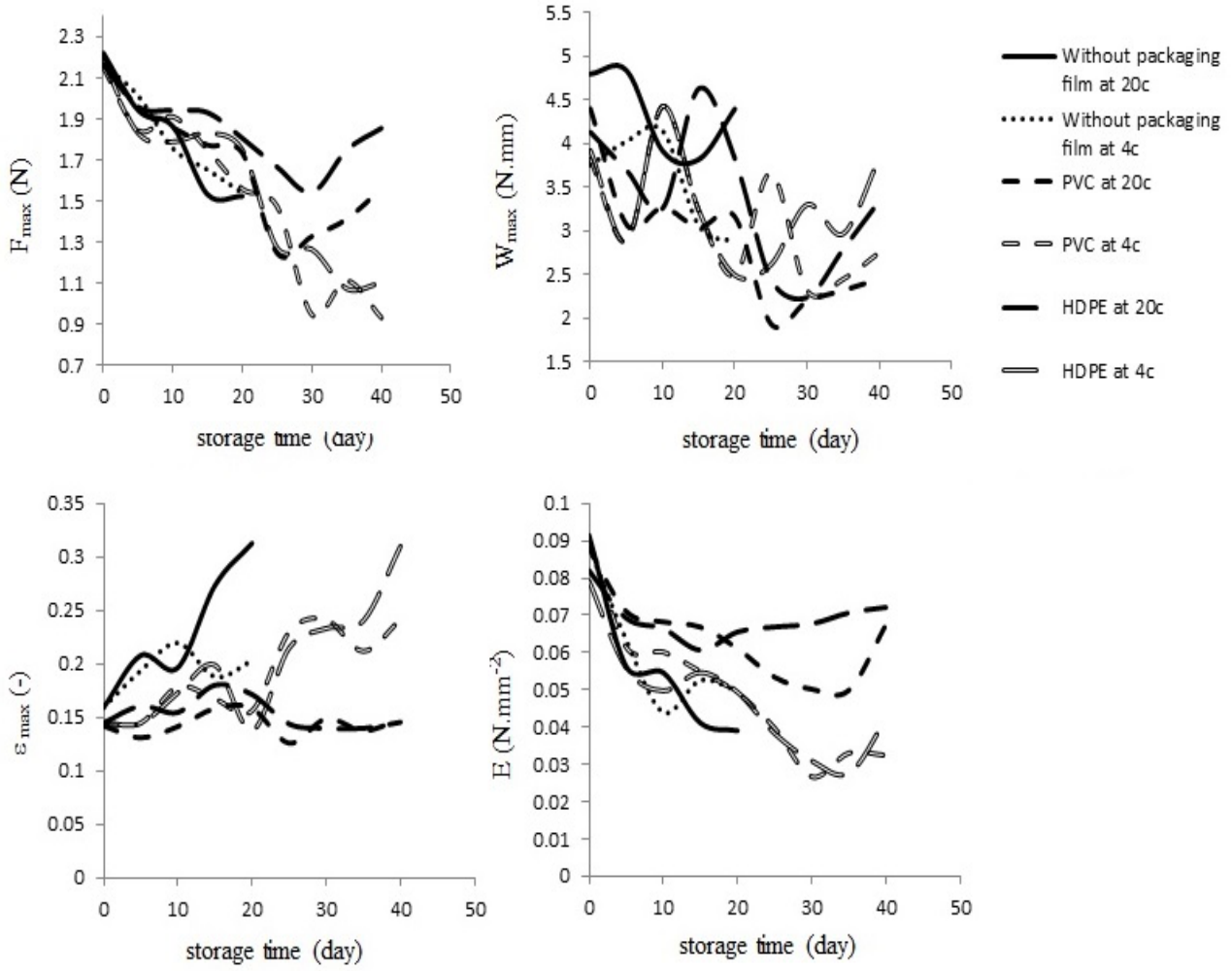

Figure 6. Effect of packaging on some mechanical properties in grape tomatoes during storage

Table 7. Summary analysis of variance (mean squares) of mechanical parameters evaluated in a factorial experiment (until the day 20)

Sources of change	Degrees of freedom	F_{max}	E	W	ε_{max}
Packaging films	2	0.78*	0.002**	1.694*	0.826**
Temperature	1	$0.35^{n.s}$	$4.946E\text{-}9^{n.s}$	2.195*	$0.25^{n.s}$
Period	4	0.850**	0.003**	1.595*	0.178**
Pack × Temperature	2	$0.022^{n.s}$	0.000**	$1.401^{n.s}$	0.099**
Pack × Period	8	0.040*	0.000**	1.533**	0.046**
Temperature × Period	4	$0.006^{n.s}$	0.000**	$0.777^{n.s}$	0.083**
Pack× Period × Temperature	8	$0.011^{n.s}$	$4.379E\text{-}5^{n.s}$	$0.179^{n.s}$	$0.024^{n.s}$
Test error	60	0.018	4.838E-5	0.539	0.013
CV (%)		12.78	23.78	22.87	24.43

"ns" means there was no significant relationship here.
* Significant relationship between two parameters at $P < 0.05$.
** Significant relationship between two parameters at $P < 0.01$

Table 8. Summary analysis of variance (mean squares) of mechanical parameters evaluated in a factorial experiment (until the day 40)

Sources of change	Degrees of freedom	F_{max}	E	W	ε_{max}
Packaging films	1	0.324**	$0.000^{n.s}$	6.226**	$0.067^{n.s}$
Temperature	1	1.249**	0.005**	$1.585^{n.s}$	1.606**
Period	8	1.274**	0.002**	3.860**	0.141**
Pack × Temperature	1	$0.120^{n.s}$	$0.000^{n.s}$	$0.008^{n.s}$	$0.007^{n.s}$
Pack × Period	8	$0.031^{n.s}$	$0.000^{n.s}$	$1.461^{n.s}$	$0.011^{n.s}$
Temperature × Period	8	0.168**	0.001**	$0.981^{n.s}$	0.220**
Pack× Period × Temperature	8	$0.43^{n.s}$	$9.165E\text{-}5^{n.s}$	$0.530^{n.s}$	$0.021^{n.s}$
Test error	72	0.37	8.24E-5	0.881	0.019
CV (%)		23.7	24.19	23.19	27.97

"ns" means there was no significant relationship here.
* Significant relationship between two parameters at $P < 0.05$.
** Significant relationship between two parameters at $P < 0.01$.

3.2. Discussions

Grape tomato is a climacteric product and storage time of that is often very short for the sake of high respiration. In this research, the use of PVC and HDPE packaging films significantly protected fresh grape tomatoes. Kader (1989) expressed that film packaging could delay the process of decay. Evelo (1996) expressed that packaged tomatoes had a significantly lower decay rate than unpackaged ones. According to the results, at the end of the maintenance period, for each treatment, weight loss slowly and linearly increased during storage. Packaging films are the useful physical protection around the products to drop humidity in packaged products for the sake of the film packaging Properties (Robertson, 2012). PVC and HDPE packaging films are as a barrier for O2, CO2, H2O, and moisture transfer, but this films have a very slight permeability to O2, CO2, H2O (Awoyale, 2016). Polypropylene packaging has been used

to cherry tomatoes for providing physical protection to gas and humidity transfer, and these findings are consistent with our results (D'Aquino et al., 2016). Choi et al. (2015) reported that modified atmosphere provided by the packaging films effectively decreased the weight loss of cherry tomatoes along the storage compared with the without packaging. Fagundes et al. (2015) expressed that bi-oriented polypropylene and low density polyethylene decreased the respiration rate while reducing weight loss and the formation of red color and use of it maintained firmness and delayed changes in sugar and organic acid contents. Tomato color is prominent feature for acceptance by Buyers; however, packaging films couldn't alter the base color of the product (kader, 2002), that in during maintenance, the product was darker and redness was less. The reduction was due to a decrease in some chemical processes (Cantwell et al., 2009).

These findings were consistent with the results of Khairi et al. (2015), that their research showed that the color of tomatoes at the end of storage was darker. based on the results, the product that are stored at different temperatures showed a significant difference in H° (hue angle) along the maintenance and samples at 4°C had more amount of H° (hue angle) degree (Khairi et al., 2015). saad et al. (2015) also observed that tomatoes showed increase in H° degree during storage as an indicator of color during 12 days of storage. kantola and helen. (2001) stated that the tomatoes packed with LDPE film initially showed an increase in a*, that is inconsistent with our research, which may be due to differences in packaging. In the present study, PVC and HDPE packaging films showed a beneficial result on controlling maintaining product size and humidity. The products inside packaging have longer shelf life than non-packaged products. According to the results, PVC and HDPE films showed a beneficial result on firmness along the maintenance, which is due to the reduction of chemical activity and thus the survival of the product (Tanada-Palmu and Grosso, 2005). Batu (1998) findings were consistent with the results of the study, according to Batu results, packaging films improve the firmness of the product during storage, also reported that the most amount of firmness obtained from packaged foods with modified atmosphere packaging Compared with the control treatment at the end of the maintenance period.

4. Conclusions

The results of this study showed that packaging films, PVC and HDPE, are effective to increase the storage time of grape tomatoes and retarded the senescence process compared to without packaging. The PVC and HDPE packaging showed useful results on moisture content, weight loss and color changes of the grape tomatoes. The packaging films have been as a physical protection around the products for humidity and gas exchange. Up to the end of the fortieth day, HDPE packaging could keep product quality better than PVC film. It was showed that the packaging delayed the softening of grape tomatoes and texture change and decreased the loss of firmness.

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OCCURANCE OF TETRACYCLINE AND OXYTETRACYCLINE RESIDUE IN HONEY SAMPLES: DEVELOPMENT OF ANALYTICAL HPLC METHOD

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ABSTRACT

Detection of tetracyclines (TCs) in honey samples is a worldwide concern for controlling of bacterial diseases in honeybees lead to drug resistance in pathogenic organisms and exerts to allergic or toxic reaction in humans. Therefore, it decided to develop of an efficient clean-up procedure for determining of tetracycline (TC) and oxytetracycline (OTC) residue in consuming honey samples using HPLC-UV methods with SPE cartridge as the clean-up procedure. Therefore, it decided to develop of an efficient method for determining of tetracycline (TC) and oxytetracycline (OTC) residue in consuming honey samples using HPLC-UV methods with SPE cartridge as the clean-up procedure. Mobile phase composed of phosphoric acid / acetonitrile (78/22, v/v) with flow rate of 1.7 ml/min and separation was done by C18 column at 25ºC. Overall, TC and OTC were detected in 100% of analyzed samples with a mean level of 0.43±0.47 and 0.7 ±0.53 ppm, respectively. Unfortunately, no maximum residues limit (MRL) has been fixed by some countries and international Committee especially EU in bee products which confirms the higher incidence of contamination and worried in honey products. Our data supposed that the current validated method is suitable for determining of TCs in honey in control laboratories and import-export inspection sites.

1. Introduction

Honey originated from the nectar of plants with different natural valuable component which are gathered, modified, stored and dehydrated by the honey bee (Wan et al. 2005). Ample of evidence indicated that residuals of antibiotic in in apiculture for treatment of bacterial disease. Tetracyclines (TCs) such as tetracycline (TC) and oxytetracyline (OTC) are commonly used to control bacterial diseases in foulbrood have been found above the regulatory standards in honey samples (Hakuta et al. 2009; Wan et al. 2005).

Existence of tetracyclines antibiotics residue in honey introduced as a central subject in monitoring of the quality and safety of honey bee (Dinkov et al. 2005; Khosrokhavar et al. 2008; Khosrokhavar et al. 2011). Consuming of honey containing with TCs resulted health implications due to drug resistance, allergic reaction, hepatotoxicity and yellow teeth (Dinkov et al. 2005; Hakuta et al. 2009).

Furthermore, the side effects of TCs in gastrointestinal disturbance is related to the selective pressure of antibiotics on human gut microflora and bacterial resistance (Tsukamoto et al. 2009; Viñas et al. 2004). Therefore, to elusion of implicated health problems associated with TCs residue in animals feed, some countries and international Committee have set maximum residue level (MRL) for TCs in honey in different range such as Japan (0.1 ppm), United Kingdom (UK) and Switzerland (0.02-0.05ppm), Codex (0.1-1.2 ppm) and FAO/WHO (0-0.03 ppm). Contamination of honey samples with TC, OTC and and the other antibiotics is a global concern studied in Chine (Chen et al. 2001), india (Solomon et al. 2006), Greece (Saridaki-Papakonstadinou et al. 2006), France (Martel et al. 2006), Brazil (Granja et al. 2009) and UK (Thompson et al. 2005).

Prevalence of antibiotics in honey samples reported that 2 out of 72 samples were contaminated with tetracycline in India (Solomon et al. 2006). Another similar study in Greece and Germany showed 29% and 21% of the honey samples had tetracycline residues, respectively (Brasse 2001; Saridaki-Papakonstadinou et al. 2006). The mean of residual tetracyclins level were higher than the European Commission permissible level (50 µg.kg-1). Nevertheless, regulatory authorities have not been fixed MRLs for TCs in bee products and practically are illegal for application in bee products (Fujita K 2008; Viñas et al. 2004). Therefore, continuous assessment of bee products with TCs residue by precise and validated method is essential for regulatory authorities and organization.

Therefore, several analyzing methods are suggested to detect of TCs residue in honey product following LLE or SPE cleanup coupled with a choice of detection methods such as HPLC with UV or fluorescence detector , liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis (Debayle et al. 2008; Jeon and Paeng 2008; Oka et al. 1987; Oka et al. 2000; Pena et al. 2005; Peres et al. 2010; Viñas et al. 2004).

Previous studies shows that clean-up of TCs were carried out by cartridge column treatments. Oka et al has used a two-dimensional clean-up and carboxylate cartridge for purifying TCs in honey sample (Oka et al. 1987; Oka et al. 2000). Pena et al. investigation show that this cartridge is not suitable in honey sample due to large variation in pH and they used the propylesulfonate cartridges for extraction of TCs in samples (Pena et al. 2005). Fujita et al. used McIlvaine buffer for extracting of TCs in honey samples with polymeric cartridge (PLS-2) and metal chelate affinity column (MCAC) and fluorescence detector (Fujita K 2008). Munstedt et al. reported the simultaneous detection of TCs in honey after SPE following extensively processing (extraction and clean up) before the analysis (Münstedt et al. 2002). In other study, trace amount of TCs antibiotics in honey determined by sulfobetaine-type polymer resin as an SPE adsorbent in HPLC analysis (Tsukamoto et al. 2009). Moreover, Carrasco-Pancorbo et al. used reversed-phase high-performance liquid chromatography coupled to ultraviolet and electrospray time-of-flight mass spectrometry on-line detection for the separation of eight TCs in honey samples (Carrasco-Pancorbo et al. 2008).

Also, a chelating agent was added to extraction buffer due to interfering effect from minerals in honey samples as same as other previous reports (Fujita K 2008; Khosrokhavar et al. 2011; Viñas et al. 2004). Therfore, it supposed that SPE cleanup and HPLC with UV-detection (HPLC-UV) are already used to determine TC and OTC residue in honey samples as the most popular and valuable method (Hakuta et al. 2009; Peres et al. 2010). We supposed that proposed routine method provides simultaneously determining of TC and OTC residue level in honey samples collected from Iran using HPLC coupled with reversed-phase SPE to examine contamination level of TCs residue in honey. The limits of detection and quantification were 0.25 µg.g-1, respectively. The recoveries varied from 74 to 91% while the relative standard deviations

(RSDs) were less than 11% under the intermediate precision conditions.

Unfortunately, no official maximum residue levels have been set for TCs residue detecting in honey products in Iran. Up to now, there is inadequate investigation on the TC and OTC residue in Iran which revealed that detectable level of OTC level in honey samples and probable a high risk of exposure with drug residues (Arabsorkhi and Sereshti 2018; Kalal et al. 2007; Mahmoudi et al. 2014). Therefore, the aim of present study was to investigate the occurrence of OTC and TC in honey in Iran.

2. Materials and methods

2.1. Materials

All reagents and solvents used for the experiments were analytical or HPLC grade. The TC and OTC standard were provided from Sigma-Aldrich. The separation was done using C18 column (RP- BONDAPAK C18, 250× 4.6 mm, particle size 5 µm, Berlin, Germany).

2.2.1. Samples

The analysis was performed on 31 samples which were bought from local supermarkets. Samples were collected by trained personnel from various sales in different geography zones in Tehran, Iran and then stored at 4ºc before analysis.

2.2.2. Apparatus:

TCs analysis were done by HPLC (Dionex, München, Germany) equipped with a Dionex P680 Pump (Dionex) and UV detector (Dionex UVD 170U/340U) operated at wavelength of 268 nm. The analytical separation column was performed by C18 column (250×4.6 mm, ACE C18-A3681, Dionex).The Chromeleon (version 6.60, Dionex) software was used to acquisition of data.

2.2.3. Calibration of HPLC Standard preparation

The mixed stock standard solutions of TC and OTC (1000 $\mu g.ml^{-1}$) were prepared weekly composed of 100 mg TC and 100 mg OTC in 100 ml methanol. Working standard solutions (0.25-4 $\mu g.ml^{-1}$) were prepared daily before using by diluting the stock solutions with deionized water. All standard solutions wrapped with aluminum foil to prevention of TCs decomposition after light exposure (Anderson et al. 2005). The standard curve was built using five point against the AUC with higher regression coefficient (r^2>0.998).

2.2.4. Mobile phase preparation

A phosphate buffer solution (pH=2.3) was prepared by dissolving 1.35 ml orthophosphoric acid in 900 ml of water, adjusting to pH 2.3 with drop wise addition of 0.1 $mol.L^{-1}$ potassium hydroxide and finally making up to 1liter with water. The optimum analysis condition obtained using phosphate buffer: acetonitrile 78:22, v/v in isocratic elution program with a flow rate of 1.7 ml/min.

2.2.5. Extraction procedure:

Briefly, one gram of honey samples were mixed by 5 ml of 0.1 M Na_2-EDTA solution (pH=4) and centrifuged in 8000 rpm for 20 min. The extract was passed through SPE cleanup column following preconditioning. The column was washed with 5 ml ethyl acetate and was filtered by 0.45 µm membrane filter and then dried by gentle stream of nitrogen. Finally, the residue was reconstituted with mobile phase and 50 µl of the solvent was injected into the HPLC system (Fig.1).

2.2.6. Method Validation:

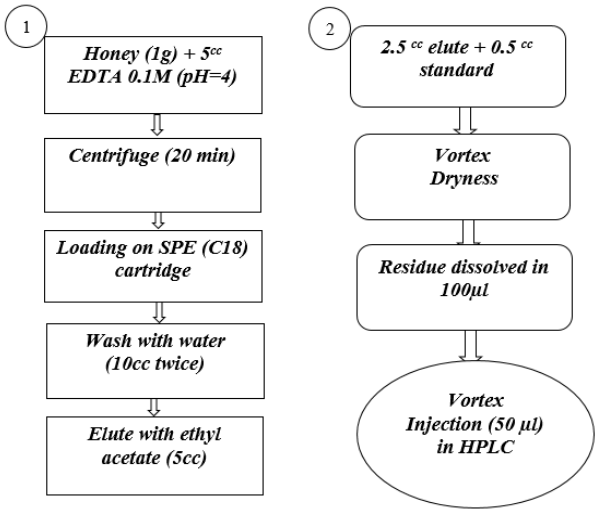
The reliability of analytical method such as extraction and cleanup efficacy was verified by evaluation of accuracy, precision and recovery by spiking at three concentrations of TC and OTC (0.5, 1 and 4 $\mu g.ml^{-1}$) by standard addition method in fortified or spiked sample. Repeatability (intra-day) and reproducibility (inter-day) was determined by three-replicated samples spiked with TC and OTC on the same day and three other different days.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated for a signal-to-noise ratio of 3 and 10, respectively with precision within ±20%. The method linearity was reported as x (TC or OTC concentration in $\mu g.g^{-1}$) and y (Peak area of OTC or TC) with the regression coefficient (r^2) of 0.99.

2.3. Statistical Analysis

Data analysis was performed using the Excel 2007 software. $p<0.05$ was considered as statistically significant in the present study.

Fig.1.Extraction procedures of honey samples



3. Results and discussions

TCs are used against bacteria to prevent two of the main enemies of the honeybee, *streptococcus pluton* in Europe and *Bacillus Larvae* in America, which cause food born disease; beekeepers commonly apply the wide-spectrum antibiotics. Although, the residue of TCs in honey can leads to possible toxic or allergic reaction, but in many countries, no maximum residue limits (MRL) are established for TCs in honey (Pastor-Navarro et al. 2007). Besides, there are no data level of TC and OTC residue contamination in honey of Iran and many countries. Therefore, it decided to evaluate the contamination level of TCs (TC and OTC) in honeys using validated and routinely HPLC-UV method in honey samples in present study.

3.1. Analytical method performance

The average recovery experiments were performed at desired levels at ranged 74.4% to 92.4% for TC and 75.5% to 91.5% for OTC are shown in Table 1 and Table 2, to determine of accuracy in proposed method which is in agreement with the European Union regulation (2002/657/EC) (Comission 2002).

The average of recovery results confirmed the accuracy of the method. The results of precision and accuracy are presented in Table 1. Our data in precision showed that less than 11% percentage of correlation variation in three spiked levels within an acceptable range with EC recommendation (Comission 2002) similar to Giannetti et al.,2010 (Giannetti et al. 2010).

Table 1. Inter and intra-assay recoveries of TC from honey sample

Conc. TC (ppm)	Intraday	Interday
	Mean Recovery(%) ± SD	Mean Recovery(%) ± SD
0.5	80.8±6.4	74.4±3.1
1	87.9±2.1	84.4±2.9
4	92.4±5.9	86.3±1.4

Table 2. Inter and intra-assay recoveries of OTC from honey sample

Conc. OTC (ppm)	Intraday	Interday
	Mean Recovery(%) ± SD	Mean Recovery(%) ± SD
0.5	77.7±10.9	75.5±11.4
1	90.9±6.1	79.0±9.1
4	91.5±4.1	78.1±3.8

The HPLC technique with an SPE cleanup is a routine and reliable method which used in the current study to determine simultaneous of TC and OTC level in honey samples. In sample preparation stage, we used only 1g of honey samples to reduce the solvents usage. Calibration curves were obtained from five spiked samples ranged from 0.25-4 µg.ml^{-1} for TC and OTC. The linearity of calibration curve was plotted TC and OTC concentration versus AUC. Least squares linear regression analysis gave a correlation coefficient of r^2 = 0.997 with calibration curve of [Y=1.944x+0.387] for OTC and correlation coefficient of r^2 = 0.999 and a calibration curve of [Y=4.28x +0.652] for TC was concluded. RSD for each concentration in the standard solutions was lower than 3%. The LOD and LOQ of chromatographic condition were obtained for TC and OTC equal to 0.03 and 0.1 µg.ml^{-1} for TC and OTC, respectively. Although the obtained LOQ amount is similar to the LOQ which reported previous study. The mean retention time of OTC and TC in the standard and spiked samples were 4.3 and 5.1 minutes. Although the obtained retention time is present investigation is less than the previous reports in suggested line.

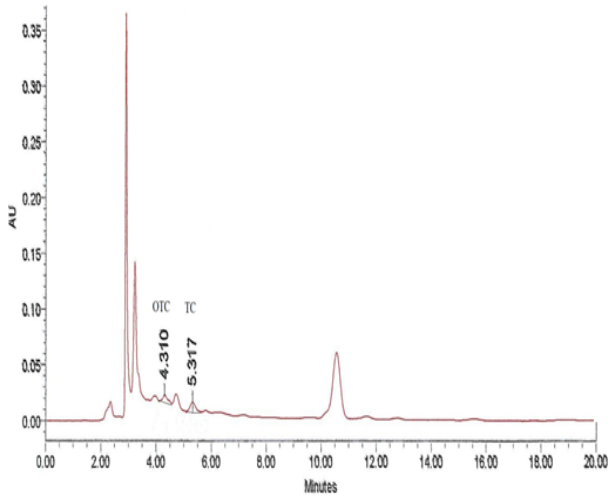


Fig.2.Chromatogram of a honey spiked with 0.5 ppm of OTC and TC

Also, specificity of method was evaluated with respect to standard addition by extracting and analyzing fortified samples. The chromatograms recorded at 268 nm were free of interfering peaks (Figure.2). Buffering at pH = 2.3 with Na_2-EDTA and orthophosphoric acid caused a sharp peak for TC and OTC. The stability of the column was evaluated by calculating the retention time of a standard solution of TC and OTC every sample injections (after washing with the eluent for 20 min). The stability of TC and OTC was evaluated both in spiked honey matrix and in standard solution under various storage conditions. These samples were stable at -20ºC even after a 40-day storage. The selectivity of methods was determined by no significant interfering peaks in the chromatograms with other compounds.

3.2. Incidence of TC and OTC in the honey samples

Honey as a popular food is used with most of the people, all over the world. Results of the studied commercial honey samples are presented in Table 3. According to the results, the amount of TC and OTC residue in 31 real samples were 0.43±0.47 µg.g^{-1} and 0.7 ±0.53 µg.g^{-1} in honey samples, respectively. Our data showed that TC and OTC were detected in all of the analyzed honey samples (100%) which has been indicated in Table.3. Furthermore, the minimum and maximum level of TC and OTC in real samples were observed between 0.03-2.27 and 0.02-1.95 µg.g^{-1}, respectively. The data showed that OTC content in all samples were higher than the maximum permissible levels established by codex and Japan. But, 80.6% of honey samples was higher than the maximum permissible levels (Table 3).

The amount of TC, OTC and same antibiotic contaminations in homey samples were reported by the other studies. Solomon reported that 14%, 28%, 29.2% of honey samples in 2006, 2007, 2009 were contaminated with tetracycline and same antibiotic more than the prescribed limit (Solomon et al. 2006). In Greece, 29% of the honey samples had tetracycline-derived residues 251 which were analyzed by HPLC (Saridaki-Papakonstadinou et al. 2006). The results of Martel et al., in France revealed that tetracycline residues were detected in honey after a treatment in hives, indicating their

permanence and spreading into the hive (Martel et al. 2006).

Other similar study in UK showed that the higher level of OTC residue levels (3.7 mg/kg) in honey after treatment of honeybee colonies after eight weeks (Thompson et al. 2005). Chen et al., were successfully separated TC, OTC, and the other antibiotics in honey samples collected in china by high performance capillary electrophoresis (Chen et al. 2001).

Table 3. TC and OTC level in real honey samples (n=31)

Sample NO.	TC (ppm)	Higher than MRL in			OTC (ppm)	Higher than MRL in		
		Japan (0.1 ppm)	UK (≥0.02ppm)	Codex (≥0.1ppm)		Japan (0.1 ppm)	UK (≥0.02ppm)	Codex (≥0.1ppm)
1	0.28	Y	Y	Y	0.39	Y	Y	Y
2	0.07	N	Y	N	0.98	Y	Y	Y
3	0.22	Y	Y	Y	0.60	Y	Y	Y
4	0.08	N	Y	N	0.02	Y	Y	Y
5	0.22	Y	Y	Y	0.30	Y	Y	Y
6	0.40	Y	Y	Y	0.13	Y	Y	Y
7	0.23	Y	Y	Y	0.56	Y	Y	Y
8	0.27	Y	Y	Y	0.12	Y	Y	Y
9	1.07	Y	Y	Y	0.60	Y	Y	Y
10	0.30	Y	Y	Y	1.40	Y	Y	Y
11	0.10	Y	Y	Y	0.35	Y	Y	Y
12	0.43	Y	Y	Y	0.38	Y	Y	Y
13	0.44	Y	Y	Y	0.25	Y	Y	Y
14	0.05	N	Y	N	0.78	Y	Y	Y
15	0.32	Y	Y	Y	0.43	Y	Y	Y
16	0.91	Y	Y	Y	0.60	Y	Y	Y
17	0.36	Y	Y	Y	0.29	Y	Y	Y
18	0.07	N	Y	N	0.65	Y	Y	Y
19	0.03	N	Y	N	0.03	Y	Y	Y
20	1.35	Y	Y	Y	0.76	Y	Y	Y
21	0.40	Y	Y	Y	1.37	Y	Y	Y
22	0.28	Y	Y	Y	1.95	Y	Y	Y
23	0.12	Y	Y	Y	0.77	Y	Y	Y
24	0.36	Y	Y	Y	0.25	Y	Y	Y
25	0.04	N	Y	N	1.60	Y	Y	Y
26	1.07	Y	Y	Y	1.92	Y	Y	Y
27	0.51	Y	Y	Y	0.66	Y	Y	Y
28	0.26	Y	Y	Y	1.08	Y	Y	Y
29	2.27	Y	Y	Y	0.83	Y	Y	Y
30	0.33	Y	Y	Y	1.29	Y	Y	Y
31	0.36	Y	Y	Y	0.23	Y	Y	Y

4. Conclusions

Our present study investigated that TC and OTC residue level in honey samples available in the Iranian market. The recent study employed the HPLC method to simultaneous analysis of TC and OTC level in honey product. An optimization step was employed for the simple and SPE extraction procedure to decrease the amount of used solvents, samples, and the time consumed in the extraction stage, and reliable method to determine TC and OTC residue level in honey samples. A total of 31 samples in current study were examined and all selected samples had mean concentration of TC and OTC

higher than MRL, suggesting the serious health problems in Iranian honey samples. Although, the preliminary work in the first step by antibiotic kits and separation of positive samples (31 sample). Finally, 31 positive samples were identified to investigate the residue level of TC and OTC in honey samples by the modified method. It seems that surveillance programs are required to monitor TC and OTC level in honey samples and their gradients in Iran. Therefore, it suggested application of this method in control laboratories and import-export inspection sites as alternative methods. It is necessary to comprehensively investigate the presence of antibiotics in food products. In this regard, it is essential to establish and enforce strict rules to minimize the contamination to the least possible amount and to prevent its adverse effects. Moreover, it is needed to properly assess other sources and to compare with other methods for stimulation of OTC and TC in food stuff such as milk, honey and so on.

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ISOLATION AND IDENTIFICATION OF NEW YEAST STRAINS FROM BEE BREAD

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ABSTRACT

Bee bread is a preserved bee pollen, which is a mixture of plant pollen, nectar, secretions from the digestive system of bees, and a layer of honey. It has a very high nutritional value. Bee bread can be used as dietary supplements due to their rich protein content and the presence of essential amino acids, fatty acids, mineral salts, and vitamins. This work carried out the isolation and identification of yeast strains from bee bread. The obtained strains displayed the phenotypic characteristics of *Rhodotorula* yeast. The DNA electrophoretic analysis showed a band size of 640 bp. Sequencing analysis of the internal transcribed spacer regions of the 5.8S rRNA gene confirmed the presence of the yeast *Rhodotorula mucilaginosa* (MK1).

1. Introduction

One of the least known and studied bee products is bee bread (Khalifa *et al*., 2020). During winter and early spring, this product is the only source of food for the bee colony, following honey (Babarinde *et al*., 2018). It is formed from pollen and provides protein, lipids, vitamins, and minerals for bees. Water constitutes the largest part of the mass of bee feathers, which is present in the amount of 20–30% of the mass of the fresh product. Proteins (up to 30%) constitute the second ingredient with the highest content in the bee bread. Carbohydrates are present at an amount of 13–55% in the bee bread, with most of them being simple sugars—mainly glucose and fructose (Kieliszek *et al*., 2018). Fatty acids and vitamins are considered as other significant ingredients. The rich composition of bee bread contributes to its nutritional and health-promoting properties. The presence of various ingredients, characteristic of natural origin, indicates that bee bread can be successfully used as a supplement and a dietary component (Tomás *et al*., 2017).

The use of bee bread and its derivatives poses some health risks. The unprocessed bee bread, obtained from the natural environment, may contain microbiological contaminants (Janashia *et al*., 2018). Insects and various microorganisms are a threat to beekeeping related to the acquisition and processing of bee bugs. The very transformation of pollen into

bee bread occurs under the influence of biochemical and metabolic processes carried out by bacteria and yeast in the product. Lactic acid bacteria *Lactobacillus* and *Bifidobacterium* are the dominant microflora in bee bread, while yeasts (*Candida*, *Saccharomyces*, *Cryptococcus*, *Zygosaccharomyces*) and molds (including *Penicillium*, *Rhizopus*, *Trichothecium*) (Gilliam *et al*., 1989; Nogueira *et al*., 2012) constitute the second significant group of microorganisms. The results of various studies suggest that the microorganisms may be derived partly from pollen and partly from the bees (Gilliam, 1997; de Arruda *et al*., 2017).

This study aimed to identify and characterize new yeast strains isolated from bee bread using classical microbiology and molecular methods such as polymerase chain reaction (PCR).

2. Materials and methods

2.1. Materials

2.1.1. Bee bread

A natural product originating from bees—bee bread—was used as the biological material. The study used bee brood obtained from Lubelskie Voivodeship (Poland). Bee bread was crushed under sterile conditions from honeycombs (Fig. 1).

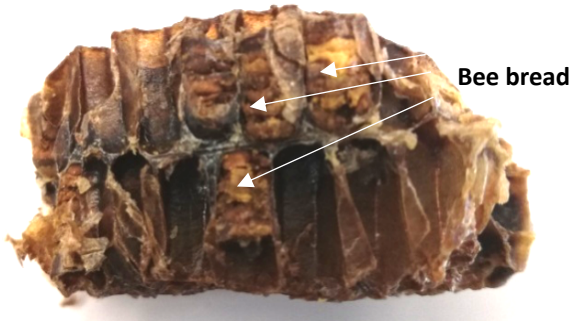

Figure 1. Bee bread in honeycomb.

2.2. Methods

2.2.1. Culture Conditions

YPD (Yeast Extract–Peptone–Dextrose) liquid medium was used to multiply yeasts from the biological material (bee bread) and isolate them. After adding 3 grains of bee bread to the liquid medium, the mixture was homogenized by vortexing (1 min). Multiplication of the yeast microflora was allowed to occur at 28°C for 48 h. Approximately 30 inoculations were performed.

After 48 h, a reduction inoculation of the liquid medium was performed on solid YPD (Petri dishes). Cultures were developed at 28°C for 48 h, and those representing each type of colony were isolated and purified by repeated streaking on YPD agar. The obtained pure yeast colonies were used as the basic material for further studies and stored at 4°C on YPD slants with 2% agar.

2.2.2. Microscopic observations

The isolated yeast was observed under the OPTA-VIEW optical microscope with Vision 7 software (Poland).

2.2.3. DNA isolation from yeast cells

DNA was isolated from yeast cells according to a modified method of Bzducha-Wróbel *et al*. (2013). Briefly, liquid yeast cultures were centrifuged (10 min, 5000 rpm), then washed twice in sterile water, and centrifuged again. The obtained pellet was suspended in 0.1 mL of lysis buffer (200 mM Tris–HCl, pH 7.5; 0.5% SDS; 30 mM EDTA) and incubated at 70°C for 15 min.

Furthermore, 0.1 mL of a 2.5 M potassium acetate solution was added and incubated at –20°C for 20 min. Subsequently, 0.3 mL of phenol:chloroform:isoamyl alcohol mixture (25:24:1) was added and vortexed for 1 min, and the sample was centrifuged once again (10 min, 13,000 rpm).

The top layer was gently pipetted from the sample and transferred to new Eppendorf tubes. 2-Propanol was added to the mixture and centrifuged (20 min; 13,000 rpm). Subsequently, the supernatant was decanted, and the remaining pellet was rinsed with 70%

ethanol and centrifuged again (20 min, 13,000 rpm). After centrifugation, ethanol was decanted, and the isolated DNA was suspended in 0.03 mL of sterile nuclease-free water. The genetic material was stored at 4°C for further analysis.

2.2.4. Yeast identification using API tests

The precise identification system API 20 C AUX (bioMérieux, France) was used for the identification of the obtained yeast strains, in accordance with the manufacturer's instructions.

2.2.5. DNA amplification and agarose gel electrophoresis

Specific primers ITS1 (5′-CGGGATCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-CGGGATCCTCCGCTTATTGATATG-3′) (a concentration of 20 pmol each) were used in the PCR process to amplify internally transcribed gene regions (ITS) and 5.8S rDNA. $MgCl_2$ (1.5 mM/L), 0.25 mmol/L dNTP, 0.5 U Taq polymerase (Fermentas, Lithuania) were added to the template DNA at a concentration of 300 ng/μL (3 μL).

The amplification reaction was carried out in a Mastercycler gradient thermocycler (Eppendorf, Germany) according to the following program: predenaturation at 94°C for 120 s, followed by 34 cycles: at 94°C for 30 s, at 53°C for 30 s, at 72°C for 60 s, and at 72°C for 30 s.

The obtained PCR products were separated by using 1.5% agarose gel added with Midori Green DNA Stain reagent (2 μL) in 1× TAE buffer (40 mM Tris–HCl, 20 mM acetic acid, and 1 mM disodium edetate). The GeneRuler 1 kb DNA Ladder molecular weight marker (ready-to-use; Thermo Fisher Scientific, USA) was used to determine the size of the resulting bands.

The parameters of the electrophoresis process were 70 mA and 120 V. During visualization, Quantity One 4.2.1 program (Bio-Rad, Poland) was used to read the molecular masses of the amplified DNA.

2.2.6. Sequencing of genetic material

The PCR products were sequenced at Genomed S.A. (Warsaw, Poland) by enzymatic synthesis (Sanger's dideoxy). The yeasts were identified by sequence comparison with the Genbank® genetic sequence database, NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), using the BLAST sequence analysis tool.

3. Results and discussion

3.1. Macro- and microscopic evaluation of yeast isolated from bee bread

It is highly necessary to conduct a microbiological analysis on bee products in order to ensure consumer safety and for the development of new technologies. Numerous scientific publications (DeGrandi-Hoffman *et al*., 2013; Kaplan *et al*., 2016; Kieliszek *et al*., 2018) confirm the variability of the contaminating microflora depending on the origin of the bee bread (botanical and geographical).

The present study found only one strain of yeast belonging to the genus *Rhodotorula*. A uniform growth pattern of both yeast strains was noted on the YPG solid medium after 2 days of cultivation at 28°C. The colonies exhibited pink-salmon color with a dull surface and a compact structure (Fig. 2A). The yeast cells were spherical and ovoid in shape with a vacuole located at the center of the cell. *Rhodotorula* MK1 yeasts had an average length of 3.50 ± 0.11 μm and a width of 3.07 ± 0.09 μm (Fig. 2B).

Owing to the diversity and proven ability to produce potential metabolites, *Rhodotorula* yeast is used for producing many commercially bioactive compounds and natural pigments, such as carotenoids (Kot *et al*., 2016). The identified *Rhodotorula* strains were found to belong to red yeast as they were characterized by a pink-colored colony. A typical feature of this type of microorganism is the production of carotenoids. These metabolites are naturally occurring lipid-soluble pigments (Ram *et al*., 2020) and can exhibit yellow, orange, and red colors (Yoo *et al*., 2016).

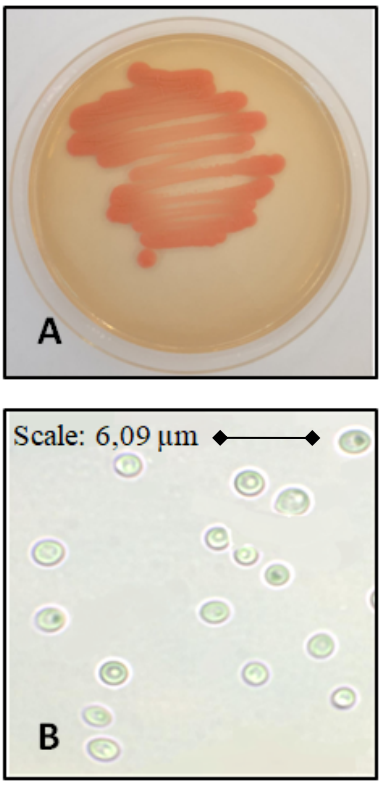

Figure 2. Yeast biomass on YPD agar medium (**A**); Exemplary microscopic photographs of *Rhodotorula* (**B**).

Since the beginning of the 21st century, the diversity of microorganisms (including yeasts) in bees and their food sources has been increasingly studied. The work of Thai scientists (Saksinchai *et al*., 2012) confirmed the validity of yeast studies in bee products. The authors managed to isolate as many as 186 yeast strains. Based on the morphological and physiological characteristics, 55 representative strains were identified (including *Zygosaccharomyces siamensis*).

Bee bread is a source of various species of yeast used in the production of mead. The study conducted by Silva *et al.* (2020) showed that bee products can contain the microorganisms used for the fermentation of alcoholic beverages. The following yeast species were isolated during the research: *Papiliotrema flavescens*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, and *Starmerella meliponinorum*. Similar dependencies were reported by Čadež *et al*. (2015). The authors isolated five new strains of *Zygosaccharomyces* from Hungarian bee bread. It is noteworthy that the obtained microorganisms cannot grow on ordinary yeast nutrients; however, they develop well on media with a high content of sugar. Research by Brysch-Herzberg *et al*. (2019) showed the presence of *Schizosaccharomyces* strains of osmophilic yeast in bee bread (originating from Germany).

3.2. Strain identification (API 20C)

After conducting the API 20 C AUX test, the obtained results allow the conclusion that the first identified strain was R. mucilaginosa MK1. The tested strain was able to metabolize i.a. glucose, arabinose, sucrose, galactose (Table 1).

It is noteworthy that *Rhodotorula* yeast is widely used in industries owing to its potential to produce several interesting compounds such as carotenoids and lipids in significant amounts (Kot *et al*., 2019). From their research, Prabhu *et al*. (2019) found that the strain of *R. mucilaginosa* belongs to the class of oily microorganisms, because, when grown in a bioreactor, it accumulates lipids up to 25% of dry substance.

The outstanding biotechnological properties of the strain *R. mucilaginosa* are widely mentioned in the scientific literature. The yeast species *R. mucilaginosa* is considered as a valuable source of carotenoids and has a potential commercial value. However, the low carotenoid production limits its commercial use (Wang *et al*., 2017).

Further research aimed at determining the biochemical properties of the isolated strain of bee yeast is required. In this study, molecular identification of the isolated yeast species was performed for a more detailed analysis.

Table 1. Biochemical results for the yeast strain.

Sugars	*Rhodotorula* MK1
Glucose	+
Glycerol	–
Calcium 2-keto-gluconate	–
Arabinose	+
Xylose	+
Adonitol	+
Xylitol	+
Galactose	+
Inositol	–
Sorbitol	+
Methyl-αD-glucopyranoside	–
N-acetyl-glucosamine	–
Cellobiose	–
Lactose	–
Maltose	+
Saccharose	+
Trehalose	+
Melezitose	+
Rafinose	+

3.3. Molecular identification of yeast

The DNA amplification products with primers ITS1 and ITS4 were analyzed to confirm that a given strain of yeast isolated from bee belongs to the *Rhodotorula* genus. The PCR products separated by agarose gel electrophoresis are shown in Fig. 3.

A band with a mass of about 640 bp was obtained from the analyses. By comparing the results with the current scientific literature, it can be concluded that the mass of the PCR product bands is characteristic of the species *R. mucilaginosa*. Esteve-Zarzoso *et al.* (1999) determined the band weight of various yeast strains of *Rhodotorula* genus: *R. acuta*, 675 bp; *R. minute*, 660 bp; and *R. mucilaginosa* 640 bp. The obtained yeast strain was deposited in the Culture Collection of the Department of Food Biotechnology and Microbiology of the Warsaw University of Life Sciences (SGGW) as MK1. In addition, the information of the obtained yeast was documented in a file with GenBank (NCBI) under number LC527461.1. BLAST analysis showed 100% identity, including the following sequences: MT465994.1, MK805510.1, and MT328138.1.

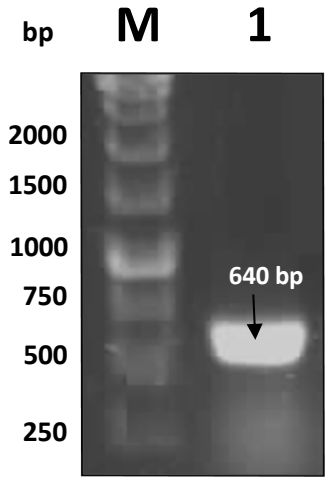

Figure 3. PCR gel electrophoresis of yeast from the bee bread (M—marker, 1—*Rhodotorula* MK1).

Of note, various scientific publications state that *R. mucilaginosa* yeast was isolated not only from fermented milk but also from various extreme ecosystems, for example, sea and glacial shores, products of plant origin, and industrial areas polluted with hydrocarbons (Deligios *et al.*, 2015). Observations by Tartor *et al.* (2018) confirmed our results showing that the DNA amplification product of the yeast *R. mucilaginosa* isolated from diseased freshwater fish was approximately 640 bp.

In conclusion, the yeasts isolated from different natural environments exhibit outstanding biochemical features that can be applied in various branches of the biotechnology industry.

4. Conclusions

The results confirm the need for research on the microflora contaminating bees. In our attempts to determine the presence of yeast in bee bread, we isolated the strain *Rhodotorula* MK1. In the future, particular attention will be paid to the determination of new strains of

microorganisms in other bee products (e.g., propolis) and their biochemical characteristics.

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EVALUATION OF *IN VITRO* PROTEIN DIGESTIBILITY OF *Moringa oleifera* LEAVES WITH VARIOUS DOMESTIC COOKING

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ABSTRACT

Moringa oleifera is gaining attention for having high protein content with balanced amino acid composition. However, as in other plant-based protein, its protein digestibility becomes of concern presumably due to the presence of antinutritional compounds such as tannins, phytic acid, and saponins. In this study, the effect of domestic cooking (blanching, steaming, boiling, and sautéing) to protein content, protein digestibility, and antinutritional compounds of Moringa leaf powder was investigated. Analysis revealed that the Moringa leaf powder contained 33.12% protein (with 90.52% pepsin digestibility), 41.97% carbohydrates, 7.56% fat, 9.77% ash, and 33.88% dietary fiber. The protein content and pepsin digestibility (>84%) were notably high and were comparable to those of other plant-based protein sources, such as soybean and peas. Protein content tend to increase with domestic cooking. The treatments applied did not manage to reduce phytic acid and saponins. Blanching and boiling significantly reduced the tannin content while, blanching and sautéing increased the saponin content. The pepsin digestibility remained the same despite of reduction in tannins. Correlation study showed that among the three antinutritional compounds, it was saponin which adversely affect the pepsin digestibility of Moringa leaf powder (r=-0.463). Overall, boiling seemed to be the best method of cooking for Moringa leaves in term of protein content and pepsin digestibility.

1.Introduction

Moringa oleifera, best known as "kelor" in Indonesia, is often referred as one of the most potential commodities to combat malnutrition due to its rich and balance nutrient composition (Moyo & Masika, 2011; Mune Mune, Nyobe, Bassogog, & Minka, 2016; Teixeira, Carvalho, Neves, Silva, & Arantres-Pereira, 2014; Titi, Harijono, Estiasih, & Endang, 2013). The plant is indigenous in South Asia, but has been widely distributed in many tropical and subtropical countries, including Indonesia (Moyo & Masika, 2011). *Moringa oleifera* is a perennial foliaged tree that can grow around 7-11 meters tall. The tree is widely cultivated for its functionality and high adaptability to dry condition.

Almost all parts of the tree can be used for food, traditional medicines, and for industrial purposes. As food, the leaves are probably the most utilized part among others. The protein content in Moringa leaves is found to be comparable to that of legumes, such as soy beans and black beans (Mune Mune et al., 2016). Additionally, it is reported that 19 out of 20 essential amino acids are present in Moringa

leaves and are in balanced proportion (Anwar, Latif, Ashraf, & Gilani, 2007; Johnson, 2005). In Africa region, Moringa leaves are recommended for breast-feeding mothers and children to help meeting their iron and protein needs. In Indonesia, Moringa leaves are consumed as nutritive vegetables, to boost breast milk production and to cure anemia (Sallau, Mada, Ibrahim, & Ibrahim, 2012; Titi, Harijono, Estiasih, & Endang, 2013).

It is known that protein quality depends on both amino acid composition and protein digestibility. Many studies reported that plant protein tend to have lower digestibility compared to animal protein, presumably due to the presence of antinutritional compounds. Moringa and other plants alike contain several antinutritional compounds such as tannins, phytic acid, and saponins (Sallau et al., 2012). These components may form complex with proteins, enzymes, or minerals and hamper their digestion (Liener, 2003). In the case of malnourished patients or when this type of foodstuffs become the main source of protein, this condition may greatly affect the health of its consumers.

Processing methods such as boiling, soaking, heating, steaming, and fermentation have been reportedly reduce the antinutritional compunds in food stuff (Fabbri & Crosby, 2016; Hefnawy, 2011; Titi, et al., 2013; Yang, Tsou, & Lee, 2002). However, report on the effect of such processes on the antinutritional compunds of Moringa as well as the correlation on its digestibility is still limited. Hence, this study aimed to evaluate the effect of domestic processing on protein content, antinutritional compounds, and the *in vitro* protein digestibility of Moringa leaves.

2.Materials and methods

2.1.Sample collection and preparation

Moringa leaves were purchased from local farmer in Tangerang Selatan, Banten, Indonesia. Fresh leaves were packed in plastic container to the laboratory.

Fresh Moringa leaves were washed and subjected to 4 (four) treatments; blanching, steaming, boiling, and sautéing, with raw/ untreated leaves as control. For blanching, Moringa leaves were blanched in boiling water for 5 minutes followed by immediate cooling in cold water. For steaming, Moringa leaves were steamed for 5 minutes. For boiling, Moringa leaves were immersed in boiling water for 15 minutes. For sautéing, Moringa leaves were sautéed in preheated cooking oil (temperature of 180^{o}C) for 5 minutes. The excess oil was then removed with hexane. The treated and raw (control) Moringa leaves were drained and dried in oven at temperature of 45^{o}C. Dried leaves were then milled into powder and were subjected to analyses.

2.2.Analysis of Chemical Composition

The percentages of protein, lipids, ash, moisture, and dietary fiber were determined by standard methods of the AOAC International (2012). Carbohydrates were calculated by difference. Iron content was determined by Atomic Absorption Spectrometry (AAS).

2.3.Analysis of Antinutritional Compounds

Tannins were determined by spectrophotometry at 725 nm as described in Makkar, Blummel, Borowy, and Bekker (1993). Total tannins were calculated as the difference of total phenols prior to and after tannin removal from the sample extract using polyvinylpyrrolidone. Phytic acid content was determined by spectrophotometry as described in Haug and Lantzsch (1983). Samples were extracted with HNO_3 and reacted with $FeNH_4(SO_4)_2$. Following centrifugation, the filtrate was then reacted with NH_4CNS and its absorbance was measured at 465 nm. Saponin was determined by spectrophotometry at 544 nm as described in Hiai, Oura, and Nakajima (1976). Methanol-extract of the sample was reacted with vanillin solution and 72% H_2SO_4 followed by measurement of absorbance at 465 nm.

2.4.Analysis of Pepsin Digestibility

Protein digestibility was analysed based on their susceptibility to pepsin (Association of

Official Analytical Chemists [AOAC], 2012). Defatted samples were digested with warm solution of pepsin for 16 hours under constant agitation. The insoluble residues obtained were then washed, dried, and analysed for its remaining protein content.

2.5.Statistical analysis

All analyses were performed at least in duplicate and results were subjected to analysis of variance (ANOVA). Significant difference between means were determined by Duncan test at 5% significance level. Data were expressed as mean ± SD.

3.Results and discussions

3.1.Chemical composition

Chemical composition of Moringa leaf powder (control) is presented in Table 1. Moringa leaf powder was found to contain 41.97% carbohydrates, 33.12% protein, 7.56% lipid, 7.02% moisture, 9.77% ash, 33.88% dietary fiber, and 7.8 ppm iron. Protein was the second major macronutrient in Moringa leaf powder after carbohydrates.

Table 1. Macronutrients, fiber, and iron of Moringa leaf powder and common legumes

Commodity	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)	Dietary fiber (%)	References
Moringa leaf powder	33.12±0.66	41.97±0.32	7.56±0.17	9.77±0.00	33.88±0.29	
Soybean	37.81	31.92	20.65	4.46	9.6	USDA (2019)
Chickpea	23.7±1.1	61.1±1.8	4.8±0.1	2.2±0.0	14.8±0.4	Sreerama (2012)
Cowpea	24.1±0.9	63.3±1.2	2.3±0.0	2.9±0.0	14.1±0.3	Sreerama (2012)
Horse gram	22.5±1.0	66.6±2.1	1.4±0.0	2.7±0.0	16.3±0.5	Sreerama (2012)

Out of all its nutritional components, it is mainly the protein content that becomes of attention. Lower protein values of Moringa leaves were reported by Mune Mune et al. (2016), Teixeira et al. (2014), Moyo and Masika (2011), and Olabode, Akanbe, Olunlade, and Adeola. (2015), which were 18.63%, 28.65%, 30.29%, and 31.33% respectively. This difference could be attributed to difference of cultivar and/or environmental condition.

Compared to that of soybean, the protein content of Moringa leaf powder was still lower (Table 1). Soybean, which currently is the main plant-based protein source in Indonesia, contain considerably high protein content at 37.81% (United States Department of Agriculture (USDA), 2019). However, protein content of Moringa leaf powder was still higher than that of the alternative commodities such as chickpeas (23.7%), cowpeas (24.1%), and horse gram (22.5%) (Sreerama, Sashikala, Pratape, & Singh, 2012). This level of protein suggests the potential of Moringa as alternative protein source to animal protein, together with soybean and other common legumes.

3.2.Effect of cooking on antinutritional compounds

Table 2 displayed the tannin, phytic acid, and saponin content of Moringa leaf powder. Untreated Moringa leaf powder (control) recorded 0.60% tannins, 2.23% phytic acid, and 8.72% saponins. The level of tannins was slightly higher than the 0.31% of condensed tannins by Moyo and masika (2011) but lower than the 2.06% of total tannins reported by Teixeira et al. (2014). The level of phytic acid and saponins in this study were significantly higher than those found by Devisetti et al. (Devisetti, Sreerama, & Bhattacharya, 2016) at

0.35% and 1.6% respectively, but more similar to the finding by Makkar and Bekker (1996), which were 3.1% and 5% for phytic acid and saponins.

Table 2. Protein, tannin, phytic acid, saponin content, and pepsin digestibility of Moringa leaf powder with different domestic cooking

Treatment	Protein (g/100 g)	Tannin (g/100 g)	Phytic acid (g/100 g)	Saponin (g/100 g)	Pepsin digestibility (%)
Control	33.12±0.66^{a}	0.60±0.27^{a}	2.23±0.12^{a}	8.72±0.29^{a}	90.52±1.58
Blanching	35.10±0.75^{b}	0.12±0.06^{b}	2.35±0.55^{ab}	11.96±0.30^{b}	84.48±4.98
Steaming	31.48±0.52^{c}	0.56±0.34^{a}	2.33±0.95^{ab}	8.36±0.16^{a}	89.04±6.13
Boiling	34.83±0.69^{b}	0.22±0.07^{b}	2.42±0.14^{b}	8.45±0.56^{a}	91.02±10.62
Sautéing	33.37±0.27^{a}	0.41±0.06^{ab}	2.19±0.13^{a}	12.46±1.05^{b}	88.14±5.57

Note: Means in the same column with different letters (a–c) are significantly ($p < 0.05$) different

A general reduction in tannin content was observed upon cooking, with blanched and boiled leaves showed significantly the greatest reduction (Table 2). Tannins were recorded at 0.56% and 0.41% in steamed and sautéed samples, which were not significantly different ($p>0.05$) from that the 0.60% in control. In blanched and boiled samples, tannins were found at 0.12% and 0.22%, which were significantly lower ($p<0.05$) than that of the control. This could be attributed to tannin's properties being heat sensitive and water soluble (Liener, 2003). During blanching and boiling, Moringa leaves were immersed in boiling water. Part of tannins would likely be degraded and leach into the water, causing significant reduction in tannins. This finding was in agreement with previous studies which also demonstrated the decreased in tannins with boiling treatment in yellow field peas (Ma, Boye, & Hu, 2017), lentils (Hefnawy, 2011), and various types of beans and peas (Habiba, 2002; Wang, Hatcher, Tyler, Toews, & Gawalko, 2010). During sautéing and steaming, there was no direct contact of Moringa leaves with liquid water. It suggested that the degradation of tannins in these two treatments was solely due to the heat but was simply not able to cause a significant reduction compared to control.

There was no reduction in phytic acid observed with given treatments in this study. This result was in agreement with Wang, et al. (2010) who also reported no significant changes in phytic acid content of beans and chickpeas upon cooking (combination of soaking and boiling). Phytic acid is relatively heat stable but can be broken down hydrolytically by enzymes or by heat in combination with acid (Konietzny & Greiner, 2003; Liener, 2003). Prolonged soaking, germination, as well as fermentation may increase the exposure of phytic acid to endogenous or microbial phytase, which in turn reduce the phytic acid or phytate content (Gilani, Cockell, & Sepher, 2005). Soaking may activate endogenous phytase (Margier et al., 2018). Germination significantly increased phytase activity presumably via *de novo* synthesis in cereal grains (Azeke, Egielewa, Eigbogbo, & Ihimire, 2011). But, none of the three treatments were performed in this study. Meanwhile, heat treatment alone was reported to be ineffective to reduce phytate content (Liener, 2003). This explains why the phytic acid contents of the samples remained similar in this study. However, different results were reported by Sallau et al. (2012) who observed significant phytic acid reduction in Moringa leaves with boiling, simmering, and blanching treatment.

There was also no reduction in saponins observed with given treatments in this study. The blanched and sautéed samples showed significantly higher level of saponins (11.96% and 12.46%) compared to that of control, steamed, and boiled samples (8.72%, 8.36%, and 8.45% respectively). Study by Duhan et al. (2001) demonstrated that the level of saponins in pigeon pea cultivars decreased with cooking.

However, saponins are reported to be stable to heating and that their biological activity does not decrease with normal cooking (Savage, 2003). This is the reason why there is no reduction in saponin level observed with various heat treatments applied. It is not clear why the level of saponins was significantly higher in the blanched and sautéed samples.

The effect of domestic cooking on dietary fiber was not analyzed in this study. However, it is expected that the level would not greatly change as previous studies have demonstrated that total dietary fiber was not affected by heating and drying in Moringa leaves (Devisetti et al., 2016) and by boiling, roasting, and pressure cooking in pearl millet (Pushparaj & Urooj, 2011).

3.3. Effect of cooking on protein content and pepsin digestibility

The protein content and pepsin digestibility of Moringa leaf powder with different domestic cooking are presented in Table 2. In addition to high protein content, Moringa leaf powder showed high pepsin digestibility (>84%). Becker (as cited in Teixeira et al., 2014) assessed fodder of fresh Moringa leaves and observed more similar value of *in vitro* protein digestibility (79%).

The protein content of Moringa leaf powder were 33.12%, 35.10%, 31.48%, 34.83%, and 33.37% for control, blanched, steamed, boiled, and sautéed leaves respectively. Generally, it seemed that heat treatment applied resulted in increased protein content of Moringa leaf powder, except for steaming. This finding was in agreement with study by Kaushik et al. (2010) and Wang et al. (2010) which reported increased in protein after domestic cooking (boiling) in various beans and chickpeas. It was said that the increase in protein was presumably due to the loss of soluble solid during cooking, hence increase the proportion of protein. However, other studies found no significant changes in protein content upon blanching and steaming of Moringa leaves (Titi, Harijono, Estiasih, & Sriwahyuni, 2013) and upon boiling in lentils (Hefnawy, 2011).

The pepsin digestibility in all treatments were not significantly different ($p>0.05$). This finding was in agreement with Titi et al. (2013) which reported the same protein digestibility in control, blanched and steamed Moringa leaves with *in vitro* multienzyme assay.

Table 3. Protein digestibility of Moringa leaf powder and other protein sources

Commodity	Protein Digestibility (%)	References
Moringa leaf powder (control) [a]	90.52±1.58	
Raw yellow pea flour [b]	83.99±1.15	Ma et al. (2017)
Peas (*Pisum sativum*) [b]	73.5±1.3	Habiba (2002)
Soybean (raw) [c]	58	Gilani et al. (2012)
Soybean (boiled) [c]	93	Gilani et al. (2012)
Soybean meal [b]	50 – 60	Bai et al. (2016)

[a]based on *in vitro* pepsin digestibility
[b]based on *in vitro* multienzyme digestibility
[c]based on true faecal digestibility (in rat)

Table 3 showed the pepsin digestibility of Moringa in comparison to that of other plant-based protein. The limitation in this study was that the protein digestibility was measured only based on protein susceptibility to pepsin with prolonged digestion time. This method is more suitable to assess protein quality for feed. The values of pepsin digestibility in this study (>84%) were more than double of that found by Mune Mune et al. (2016), which was 41.11%. This difference could be due to the difference in cultivar and/or method of analysis since the digestion time with pepsin in this study (16 hours) was considerably longer than in Mune

Mune's (3 hours). The long duration of enzymatic digestion may increase the measured *in vitro* protein digestibility (Bai, Qin, Sun, & Long, 2016).

With the same digestion time, Bai et al. (2016) found that soybean meal only showed 50 – 60% of protein digestibility with pepsin-pancreatin assay. This value was much lower than the pepsin digestibility of raw Moringa leaf powder (control) (90.52%). Pepsin and pancreatin work complementarily since both enzymes have different specificity. Pepsin preferentially hydrolyzes peptide bond where amino group of aromatic amino acid is located. Pancreatin preferentially hydrolyze peptide bond where carboxylic group of aromatic and basic amino acid are located and the peptide bond where the amino group of aromatic amino acid is located (Mune Mune et al., 2016). It has been demonstrated that further digestion with pancreatin (trypsin, chymotrypsin, chymosin) following that with pepsin increased the protein digestibility (Mune Mune et al., 2016). This information suggested that the use of pepsin-pancreatin in combination with long digestion time should have resulted in high protein digestibility. The fact that soybean meal recorded much lower protein digestibility than Moringa with such condition further support Moringa as alternative protein source to soybean. In addition to that, there is possibility that the multienzyme *in vitro* protein digestibility of Moringa leaf powder would be even higher than that of soybean meal.

3.4.Protein digestibility and antinutritional compounds

A correlation study was carried out between pepsin digestibility with tannins, phytic acid, and saponins using Pearson analysis (Table 4). In this study, pepsin digestibility was almost uncorrelated with tannins (r=0.088), which was unexpected because previous studies often demonstrated how tannins adversely affect protein digestibility (Gilani et al., 2005; Gilani, Xiao, & Cockell, 2012; Ma et al., 2017). Tannin is known as one of major antinutritional compound for protein. Tannins can bind and precipitate proteins including enzymes, reducing the digestibility and amino acid bioavailability or the activity of the enzymes (Liener, 2003). However, in opposite to that, Pushparaj and Urooj (2011) reported positive correlation between protein digestibility and tannins in pearl millet, indicating that other factor might be responsible for the low protein digestibility.

Table 4. Association of pepsin digestibility with tannins, phytic acid, and saponins of Moringa leaf powder

Dependent variable	Independent variables	Correlation coefficient
Pepsin digestibility	Tannins	0.088
	Phytic acid	0.135
	Saponins	-0.463

A weak positive correlation was observed between pepsin digesitibility and phytic acid (r=0.135), but the value was not significant (p>0.05). Phytic acid or its salt, phytate, is known to chelate cations such as Ca, Mg, Zn, and Fe and interfering with their bioavailability. The antinutritional effect of phytic acid to protein is mainly due to their direct binding to protein (enzyme or substrate) and indirect binding by chelating the mineral cofactors (Gilani et al., 2012). Phytate can form complexes with proteins at both acidic and alkaline pH. Binding of phytic acid and minerals that act as enzyme cofactors will lower the activity of the digestive enzymes, forming insoluble complexes that cannot be absorbed by human intestines (Bessada, Barreira, & Oliveira, 2019). Meanwhile, formation of protein-phytate complex may alter protein structure that in turn can reduce its enzymatic activity, solubility, and susceptibility to proteolytic enzymes (Konietzny & Greiner, 2003). Addition of phytase was

reported to increase the apparent ileal digestibility of nitrogen and amino acids (Gilani et al., 2005).

Among other antinutritional compounds measured, saponin was present at the highest concentration and was the only variable which showed negative correlation with pepsin digestibility (r=-0.463), although it was not significant (p>0.05). Saponin was better known to interfere with the absorption of dietary lipids, cholesterol, and bile acid (Margier et al., 2018). However, it was also reported that saponins may reduce protein digestibility by forming less digestible saponin-protein complexes. Previous study showed that saponins adversely affect the hydrolysis of soybean protein by chymotrypsin and the digestibility of bovine serum albumin (Francis, Kerem, Makkar, & Becker, 2002).

The correlation study suggested that saponins was more detrimental to protein digestibility of Moringa leaves than tannins and phytic acid. However, other factor such as dietary fiber and/or molecular structure of the protein may play a role in affecting protein digestibility. Dietary fiber refers to edible fraction of plants that are resistant to digestive enzymes. A reduction in protein digestibility may be due to dietary fiber binding with proteins or acting as physical barrier to proteolytic enzymes (Duodu, Taylor, Belton, & Hamaker, 2003; Mongeau, Sarwar, Peace, & Brassard, 1989). Those studies showed that additional and removal of fiber-rich rich components resulted in reduced and improved protein digestibility respectively. Meanwhile, Bai et al. (2016) demonstrated that the percentage of β-sheet structures of protein was inversely correlated to protein digestibility since β-sheet structures contained high number of hydrogen bond that may hinder protease activity. However, neither the dietary fiber nor the molecular structure of Moringa protein and their effect to its protein digestibility were not analyzed in this study.

In general, this study suggested Moringa leaves as alternative protein source due to its relatively high protein content and digestibility especially when compared to other plant commodities. Further analysis on protein structure of Moringa leaves, dietary fiber, and their effect on protein digestibility determined with more proper method using Digestible Indispensable Amino Acid Score (DIAAS) are recommended for future studies.

4.Conclusions

Moringa oleifera leaves contains high level of protein with considerably high pepsin digestibility. Blanching and boiling increased the protein content but steaming reduced it. The antinutritional compounds reacted differently towards the domestic cooking applied. Steaming and boiling managed to significantly reduce the tannin content. But, phytic acid failed to decrease upon treatments. Instead, blanching and sautéing increased the saponin content. Regardless changes in antinutritional compounds, the domestic cooking applied did not significantly affect the pepsin digestibility. Correlation study showed that among the three antinutritional compounds, it was saponins which adversely affect the pepsin digestibility of Moringa leaf powder. Based on the protein content and pepsin digestibility, this study suggested boiling as the best domestic cooking for Moringa leaves in comparison to blanching, steaming, and sautéing.

5.References

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