CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: http://chimie-biologie.ubm.ro/carpathian_journal/index.html

INHIBITION IMPACTS OF NATURAL CLINOPTILOLITE ON BIOGENIC AMINES PRODUCTION BY COMMON FOOD-BORNE PATHOGENS IN ARGININE DECARBOXYLASE BROTH

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Article history:
Received:
30 January 2016
Accepted in revised form:
30 May 2016

Keywords:
Food-borne pathogens;
Ammonia;
Agmatine;
Biogenic amines;
Clinoptilolite;
HPLC

ABSTRACT

The effect of natural clinoptilolite (CLINOPT) on ammonia (AMN) and biogenic amines (BAs) production by different food borne-pathogens (FBPs) was studied in arginine decarboxylase broth (ADB) using HPLC. All tested bacteria were found to have an ability to produce ammonia and BAs in ADB. It was demonstrated that ammonia and biogenic amine production could be significantly influenced by adding CLINOPT ($P<0.05$). Both concentrations of CLINOPT (1 and 5%) had a clear inhibition effect only on putrescine (PUT) formation by both Gram negative and positive FBPs. On the other hand, CLINOPT resulted in strong increases in biogenic amines production by *Enterococcus faecalis*, although remarkable decreases were observed for biogenic amines by *Salmonella paratyphi* A in the presence of CLINOPT. Consequently, it can be concluded that the effect of CLINOPT on AMN and BAs production varied depending on not only FBPs, but also CLINOPT concentrations used.

1. Introduction

Biogenic amines (BAs) are basic nitrogenous compounds, of low molecular weight, formed in food by the decarboxylation of amino acids or by the amination and transamination of aldehydes and ketones as a result of microbial, vegetable and animal metabolic processes (Silla-Santos, 1996). According to their chemical structure, they can be classified in three categories which are aromatic amines (e.g., histamine, tyramine, β-phenylalanine, and tryptamine), aliphatic diamines (e.g., putrescine and cadaverine), and aliphatic polyamines (e.g., agmatine, spermidine, and spermine) (Smith, 1980). Biogenic amines occur naturally in small concentrations in most living organisms, in which they have strong physiological effects and play an important role in the stabilisation of membranes, and likely also in the regulation of nucleic acid function and protein synthesis (Halász et al., 1994). However, when excessive amount of biogenic amines was ingested with food, or when the natural mechanisms for their catabolism are inhibited, they can have toxicological effects on humans' health, such as blood pressure changes, headache, nausea, respiratory disorder, cardiac palpitation and even anaphylactic shock (Kalac and Krausová, 2005).

BAs accumulation in foods requires the availability of free amino acids, the presence of microorganisms capable of producing decarboxylases, and favourable environmental conditions (such as slightly acid pH and

anaerobiosis) for bacterial growth and decarboxylase activity (Buňková et al., 2010). Microbial strains with high proteolytic enzyme activity potentially increase the risk of biogenic amines formation in food and food products by increasing the availability of free amino acids. On the other hand, many bacterial genera are involved in biogenic amine toxicity, such as *Bacillus*, *Citrobacter*, *Clostridium*, *Escherichia*, *Klebsiella*, *Photobacterium*, *Proteus*, *Pseudomonas*, *Shigella*, and the some lactic acid bacteria (Mohamed et al., 2009). It was reported that *Staphylococcus aureus* strains were histamine-producer bacteria in soy broth supplemented with 1.0% L-histidine (Chang et al., 2008). *Escherichia coli* and *Listeria monocytogenes* were shown to have the highest decarboxylase activity in tyrosine containing medium, with consequent production of tyramine (von Beutling, 1993), while *Klebsiella pneumoniae* has been reported to be the most prolific histamine producer (Emborg and Dalgaard, 2006). Middlebrooks et al. (1988) stated that *Aeromonas hydrophila* isolated from refrigerated mackerel has the ability to produce histamine, and *Enterococcus faecalis* accumulates tyramine (Bover-Cid and Holzapfel, 1999). Agmatine can be produced by a broad range of microorganisms, including *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Sakakibara and Yangisava, 2003; Griswold et al., 2006). Kuley and Özogul (2011) reported that *S. paratyphi* A had an ability to produce significant amounts of BAs in vitro conditions.

Various methods for reducing BAs accumulation in foodstuffs including storage in low temperature, appropriate packaging and the incorporation of synthetic or naturally occurring preservatives (Kuley et al., 2005; Özogul et al., 2011; Kim et al., 2011) have been used. Among the bio-preservatives studies, clinoptilolite (CLINOPT) has increasingly gained the interest of researchers and food processors. Clinoptilolite is defined as a crystalline, hydrated alumino-silicate of alkali and alkaline earth cations having an infinite three-dimensional structure. Both its physical and chemical properties to act as ion exchangers, catalysts and adsorbents have led to a wide range of industrial and agricultural applications (Mumpton, 1999). Natural CLINOPT has been previously used as effective adsorbent of ammonia and biogenic amine accumulation, particularly histamine and tyramine from the sardine fillets (Kuley et al., 2012). CLINOPT has capacity to adsorb mycotoxins and also excess moisture, ammonia and radionuclides (Djordjevic et al., 2003). Significant research efforts are being conducted regarding the use of CLINOPT for antimicrobial packaging of food products. In fact, CLINOPT is used as active antimicrobial agents when incorporated into the food contact material, which then exerts its action on the surface of the food to inhibit the growth of Gram-negative and Gram-positive bacteria, molds, and yeasts (Pehlivan et al., 2005). There is no information about the relationship between clinoptilolite and food-borne pathogenic bacteria in terms of formation of ammonia and BAs in arginine decarboxylase broth (ADB). Therefore, the purpose of the present study was to investigate the effect of natural clinoptilolite at different doses on ammonia and BAs production by common food-borne pathogens in arginine decarboxylase broth (ADB).

2. Materials and methods

2.1. Bacterial strains

Staphylococcus aureus (ATCC29213), *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC700603), *Enterococcus faecalis* (ATCC29212), *Pseudomonas aeruginosa* (ATCC27853), *Listeria monocytogenes* (ATCC7677) were acquired from The American Type Culture Collection (Rockville, Md., U.S.A.). *Aeromonas hydrophila* (NCIMB1135) and *Salmonella paratyphi* A (NCTC13) were obtained from National Collections of Industrial Food and Marine Bacteria (Aberdeen, UK) and National Collection of Type Cultures (London, UK), respectively.

2.2. Culture media and bacterial extraction

The production of biogenic amines by all strains used in this work was monitored using arginine decarboxylase broth (ADB). The ADB contained, in g/L of distilled water : 2 g peptone, 1 g Lab-Lemco powder (Oxoid CM0017, Hampshire, England), 5 g NaCl (Merck 1.06404. 1000, Darmstadt, Germany), 8.02 g L-arginine (Sigma, Steinheim, Germany) and 5 mg pyridoxal HCl (Sigma P9130, Steinheim, Germany). The pH was adjusted according to their optimum growth pH with 1 M KOH (Riedel-de Haen 06005, Seelze, Germany) or 6% trichloroacetic acid (Riedel-de Haen 27242, Seelze, Germany). Clinoptilolite was added to the ADB with the concentration of 1 and 5% (w/v). After that arginine decarboxylase broth (prepared with/without clinoptilolite) was pipetted in 10 mL bottles and then autoclaved at 121°C in 15 min prior to use.

Nutrient broth (Merck 1.05443.0500, Darmstadt, Germany) was used for propagation of FBP cultures. Total of 100 µL of each food-borne pathogen culture was inoculated into 10 mL of Nutrient broth. Pathogen bacterial strains were incubated at 30°C for 2 or 3 days, which after 0.5 mL of these bacterial cultures was removed and put into the ADB to allow these bacteria to produce biogenic amines.

For extraction of the bacterial strains, 5 mL of the ADB containing pathogen bacterial strains was removed to separate bottles and then 2 mL sulphosalicylic acid (6%) was added. The samples were centrifuged at 3000 ***g*** for 10 min and then filtered through a filter paper (125 mm, Schleicher & Schuell, Dassel, Germany). After that, 4 mL of bacterial supernatant from each bacterial strain was taken for derivatisation in order to analyse in high-performance liquid chromatography (HPLC). Each experiment was carried out in quadruplicate.

2.3. Chemical reagents

L-arginine and all BA standards were purchased from Sigma-Aldrich (Munich, Germany). The mobile phase consisted of acetonitrile and HPLC grade water for amine analyses.

The used BA standards were: histamine dihydrochloride, tyramine hydrochloride, typtamine hydrochloride, putrescine dihydrochloride, 2-phenylethylamine hydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, 5-hydroxytryptamine (serotonin), 3-hydroxytyramine hydrochloride (dopamine), agmatine sulphate, trimethylamine hydrochloride and ammonium chloride. The final concentration of free base for each amine was 10 mg/mL solution. A series of dilutions (0, 0.005, 0.05, 0.5 and 5 mg/mL) were prepared from the standard stock solution and used to obtain the standard curve.

2.4. Derivatisation of bacterial extraction

A stock solution was prepared by dissolving 2% benzoyl chloride in acetonitrile to enhance the reaction with amines. For derivatisation of standard amine and ammonia solutions, 100 µL was taken (4 mL for extracted bacterial cultures) from each free base standard solution (10 mg/mL). Sodium hydroxide (1 mL of 2 M) was added, followed by 1 mL of 2% benzoyl chloride (dissolved in acetonitrile) and the solution mixed on a vortex mixer for 1 min. The reaction mixture was left at room temperature for 5 min and then centrifuged for 10 min. After that, the benzoylation was stopped by adding 2 mL of saturated sodium chloride solution and the solution extracted twice with 2 mL of diethyl ether. The upper organic layer was transferred into a clean tube after mixing. Afterwards, the organic layer was evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1 mL of acetonitrile and 10 µL aliquots were injected into the HPLC.

2.5. Analytical method

Biogenic amines analysis was done using the method of Özogul (2004) and measured in milligram amines per litre broth. The confirmation of biogenic amines production was accomplished using a rapid HPLC method

with a reversed phase column by using a gradient elution program. The same analytic method was used for ammonia and trimethylamine separation.

2.6. HPLC apparatus and column

The apparatus for HPLC was a Shimadzu (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A diode array detector and two binary gradient pumps (Shimadzu LC-10AT), auto sampler (SIL 20AC), column oven (CTO-20AC), and a communication bus module (CBM-20A) with valve unit FCV-11AL. For the biogenic amine analyses, the column was Columbus C18, 150 × 4.6 mm (Phenomenex, Macclesfield, Cheshire, UK).

2.7. Statistical analysis

All statistical analyses were performed using the Statistical software, SPSS Version 15.0 for windows (SPSS Inc., Chicago, IL, USA). Contents of biogenic amines in different samples were expressed as mean values accompanied by the standard deviation of means (mean of at least four determinations for each sample). The Duncan's test was used for mean comparison when a significant variation was found by the ANOVA test. The significance of results was at $P<0.05$.

3. Results and discussions

3.1. The effect of clinoptilolite on biogenic amines production by Gram negative FBPs

The effect of clinoptilolite (CLINOPT) on ammonia (AMN) and biogenic amines production by Gram negative FBPs in ADB is shown in Table 1 and Figure 1.

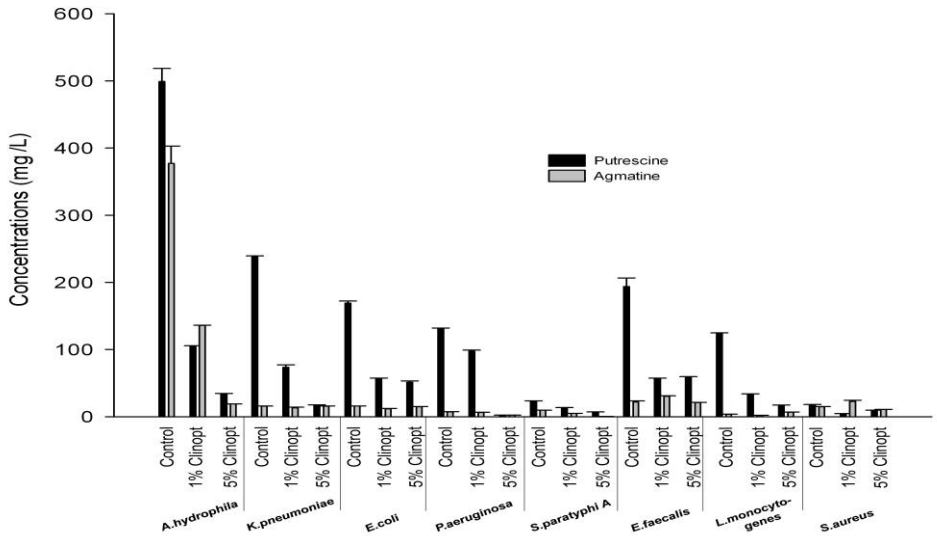

Figure 1. Putrescine and agmatine production by food-borne pathogens in the presence or absence of clinoptilolite (CLINOPT) at different doses in arginine decarboxylase broth

AMN production by Gram negative FBPs was very high (>1250.46 mg/L), indicating arginine degradation into AMN through arginine. L-arginine was present in the broth at concentration high (8020 mg/L) enough to produce that level of AMN. Significant reduction ($P<0.05$) on AMN production by *K. pneumoniae*, *P. aeruginosa* and *S. paratyphi* A was observed in the presence of CLINOPT. Others authors have already reported that CLINOPT had capacity to adsorb mycotoxins and also excess moisture, ammonia and radionuclide (Djordjević et al., 2003). We also observed that the removal efficiency of

ammonia by both CLINOPT concentration (1 and 5%) increases with increasing amount of CLINOPT. This effect can be attributed to an increased surface area and number of adsorption sites of CLINOPT (Nemr et al., 2009). However, in this study, the presence of CLINOPT in the ADB resulted in significantly higher AMN production by *A. hydrophila* and *E. coli*. This might be due to the accumulation of higher ammonia in ADB by these bacteria in the presence of CLINOPT, and the decrease of ammonium adsorption by the CLINOPT, which reached the saturation point (Wu et al., 2008).

Table 1. Ammonia and biogenic amines production by gram negative foodborne pathogens treated with clinoptilolite at different doses in ADB.

Microorganism	Clinoptilolite [g L^{-1}]	AMN	PUT	CAD	SPD	TRPT	PHEN	SPN
		[mg L^{-1}]						
Aeromonas hydrophila	0	1250.46 ± 24.43 ^{b}	499.16±19.51^{a}	4.55±0.09^{b}	27.10±0.15^{a}	0.00±0.00^{b}	23.81±0.91^{a}	0.00±0.00^{b}
	10	1523.46±106.98^{a}	105.33±0.47^{b}	47.74±0.33^{a}	2.70±0.01^{b}	3.96±0.22^{a}	6.78±0.36^{c}	0.00±0.00^{b}
	50	1671.89±25.11^{a}	34.10±0.56^{c}	0.36±0.01^{c}	0.00±0.00^{c}	0.00±0.00^{b}	11.87±0.59^{b}	1.28±0.12^{a}
Klebsiella pneumoniae	0	1454.16±30.81^{a}	237.80±1.61^{a}	1.64±0.01^{b}	17.88±0.20^{b}	0.00±0.00^{b}	24.78±0.66^{a}	0.00±0.00^{b}
	10	1089.63±22.67^{b}	73.57±3.49^{b}	42.90±1.02^{a}	45.77±0.30^{a}	0.00±0.00^{b}	13.12±0.10^{b}	0.00±0.00^{b}
	50	713.17±0.24^{c}	17.33±0.24^{c}	0.66±0.01^{b}	0.00±0.00^{c}	1.55±0.07^{a}	11.24±0.09^{c}	67.96±2.77^{a}
Escherichia coli	0	1293.41±65.45^{c}	169.13±2.99^{a}	13.15±0.48^{a}	6.36±0.55^{b}	0.00±0.00^{b}	22.58±0.73^{b}	0.00±0.00^{c}
	10	1928.76±15.27^{b}	56.78±0.49^{b}	4.75±0.21^{b}	39.32±0.22^{a}	0.00±0.00^{b}	18.82±1.07^{c}	17.24±0.08^{b}
	50	2454.04±30.33^{a}	51.18±2.09^{b}	1.27±0.04^{c}	1.29±0.06^{c}	6.58±0.27^{a}	25.30±0.38^{a}	40.45±4.39^{a}
Pseudomonas aeruginosa	0	1861.23±53.50^{a}	131.85±0.21^{a}	0.67±0.06^{a}	8.55±0.78^{b}	0.00±0.00^{b}	7.44±0.31^{b}	1.69±0.02^{b}
	10	1488.35±34.95^{b}	97.45±1.67^{b}	0.57±0.05^{a}	24.09±0.01^{a}	11.72±0.11^{a}	10.31±0.20^{a}	13.59±0.13^{a}
	50	506.57±42.31^{c}	2.18±0.05^{c}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}	3.04±0.18^{c}	0.00±0.00^{c}
Salmonella paratyphi A	0	2074.84±57.08^{a}	23.47±0.18^{a}	0.93±0.01^{a}	2.63±0.04^{a}	0.00±0.00	9.51±0.23^{a}	43.30±0.05^{a}
	10	1028.57±14.09^{b}	13.67±0.03^{b}	0.35±0.00^{b}	0.00±0.00^{b}	0.00±0.00	2.95±0.02^{c}	0.81±0.01^{b}
	50	937.82±8.53^{b}	7.03±0.04^{c}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00	4.10±0.03b	0.00±0.00^{c}

AMN: ammonia; PUT: putrescine; CAD: cadaverine; SPD: spermidine; TRPT: tryptamine; PHEN: 2-Phenyl-ethylamine; SPN: spermine. Values are expressed as mean ± standard deviation, n = 4. a–c: indicate significant differences ($P<0.05$) in a row among groups. ADB: Arginine Decarboxylase Broth.

Table 1. Ammonia and biogenic amines production by gram negative foodborne pathogens treated with clinoptilolite at different doses in ADB (Continued).

Microorganism	Clinoptilolite [g L^{-1}]	HIS	SER	TYR	TMA	DOP	AGM
		[mg L^{-1}]					
Aeromonas hydrophila	0	1.40±0.09^{b}	120.79±10.69^{a}	17.00±0.14^{a}	3.86±0.02^{a}	435.31±24.48^{a}	377.25±25.76^{a}
	10	4.10±0.39^{a}	1.10±0.04^{b}	0.91±0.01^{c}	0.37±0.03^{b}	38.32±0.06^{b}	135.68±0.50^{b}
	50	3.74±0.02^{a}	0.93±0.01^{b}	4.66±0.14^{b}	0.41±0.00^{b}	40.23±0.03^{b}	18.88±0.05^{c}
Klebsiella pneumoniae	0	0.76±0.03^{b}	4.40±0.38^{b}	12.42±0.41^{a}	1.11±0.06^{a}	53.53±0.37^{b}	15.72±0.18^{a}
	10	1.35±0.12^{a}	18.71±0.45^{a}	1.45±0.05^{c}	0.16±0.01^{b}	106.64±0.55^{a}	13.00±1.07^{b}
	50	1.59±0.05^{a}	1.36±0.03^{c}	3.63±0.20^{b}	0.11±0.01^{b}	42.59±2.95^{c}	15.63±0.15^{a}
Escherichia coli	0	4.40±0.26^{b}	53.68±0.94^{a}	10.81±0.14^{c}	30.29±0.32^{c}	90.11±1.46^{c}	15.83±0.14^{a}
	10	5.74±0.21^{a}	24.63±0.81^{c}	17.34±0.43^{b}	158.61±0.36^{b}	164.17±1.50^{b}	11.64±0.50^{b}
	50	4.18±0.06^{b}	43.48±0.67^{b}	29.15±0.43^{a}	178.34±0.27^{a}	360.99±9.72^{a}	14.79±0.36^{a}
Pseudomonas aeruginosa	0	1.76±0.10^{a}	32.75±0.07^{b}	1.49±0.01^{b}	1.08±0.06^{b}	62.69±1.35^{b}	7.06±0.54^{a}
	10	1.59±0.10^{a}	111.13±1.35^{a}	8.30±0.10^{a}	1.99±0.10^{a}	120.74±0.21^{a}	6.35±0.34^{a}
	50	0.00±0.00^{b}	0.15±0.00^{c}	1.14±0.05^{c}	0.14±0.00^{c}	10.03±0.34^{c}	2.46±0.08^{b}
Salmonella paratyphi A	0	1.65±0.02^{a}	31.72±1.16^{a}	5.62±0.35^{a}	2.38±0.52^{a}	60.62±0.31^{a}	9.61±0.18^{a}
	10	0.29±0.41^{b}	1.25±0.08^{b}	2.03±0.16^{b}	0.37±0.02^{b}	9.46±(0.81) ^{c}	4.87±0.16^{b}
	50	0.34±0.00^{b}	1.83±0.05^{b}	1.54±0.13^{b}	0.13±0.01^{b}	36.42±1.13^{b}	0.47±0.03^{c}

HIS: histamine; SER: serotonin; TYR: tyramine; TMA: trimethylamine; DOP: dopamine; AGM: agmatine.
Values are expressed as mean ± standard deviation, n = 4. a–c: indicate significant differences ($P<0.05$) in a row among groups. ADB: Arginine Decarboxylase Broth.

The main amines formed by Gram negative FBPs were putrescine (PUT), followed by dopamine (DOP). The used Gram negative FBPs were seemed to convert agmatine (AGM) into PUT. It has been reported that PUT can be formed from L-arginine by arginine decarboxylase (ADC) via AGM, which is converted directly to PUT by the enzyme

agmatinase, while AGM is first hydrolysed by AGM deiminase into N-carbamoylputrescine and AMN, and PUT is formed by removal of the ureido group from N carbamoylputrescine by the enzyme N-carbamoylputrescine amidohydrolase (Wunderlichová et al., 2012). The addition of CLINOPT significantly inhibited PUT production by Gram negative bacteria ($P<0.05$) (Fig. 1). Treated group with 5% CLINOPT was more effective to suppress PUT formation than treated group with 1% CLINOPT. The CLINOPT concentration was found to have a significant effect on the formation of PUT.

In the present study, *E. coli* produced considerably higher cadaverine (CAD) (13.15 mg/L) than the other tested bacteria. Similar findings are reported also by other researchers (Durlu-Özkaya et al., 2001), who also found that *E. coli* EC03 isolated from meat products was the most important CAD producer with a production level of 454.8 mg/L in brain-heart infusion medium. The effectiveness of dose used was strain-dependent. The addition of CLINOPT had an inhibitor effect on CAD production by *E. coli*, *P. aeruginosa* and *S. paratyphi* A. However, the use of CLINOPT at dose of 1% resulted in significant increases in CAD accumulation by *A. hydrophila* and *K. pneumoniae*, while CLINOPT at dose of 5% suppressed CAD production by Gram negative bacteria. Spermidine (SPD) production ranged from 2.63 mg/L for *S. paratyphi* A to 27.10 mg/L for *A. hydrophila*. The effect of CLINOPT on SPD production was dependent on bacterial strains. The use of CLINOPT significantly suppressed SPD accumulation by *A. hydrophila* and *S. paratyphi* A, although CLINOPT addition stimulated SPD production by *K. pneumoniae*, *E. coli* and *P. aeruginosa*.

Tryptamine (TRPT) was not produced in the absence of CLINOPT by Gram negative bacteria. The use of CLINOPT significantly stimulated TRPT production by *A. hydrophila*, *K. pneumoniae*, *E. coli* and *P. aeruginosa* whilst *S. paratyphi* A had no ability to produce TRPT in ADB. Production of 2-Phenyl-ethylamine (PHEN) by *E. coli* was low (22.58 mg/L). Durlu-Özkaya et al. (2001) reported production of small amounts of PHEN from *E. coli* (<30 mg/L). CLINOPT decreased PHEN accumulation by Gram negative FBPs, except for *E. coli* and *P. aeruginosa*. Spermine (SPN) was produced only by *S. paratyphi* A and *P. aeruginosa*, while *A. hydrophila*, *K. pneumoniae* and *E. coli* had not an ability to produce SPN. However, Özogul and Özogul (2007) found that *K. pneumoniae* (673) accumulated more than 32 mg/L SPN in ADB, formed from PUT and SPD. The CLINOPT application increased SPN production by Gram negative FBPs except for *S. paratyphi* A.

Histamine (HIS) production by *K. pneumoniae* was 0.76 mg/L, which is lower than that produced by the other tested Gram-negative bacteria. Low production of HIS by *K. pneumoniae* may be attributed to HIS degradation by diamine oxidase (DAO) (Dapkevicius, 2000), which has been detected in several types of bacteria including *Klebsiella* spp. (Ienistea, 1971). CLINOPT had no effect on HIS production by *E. coli*, whereas significant reduction on HIS production by *P. aeruginosa* and *S. paratyphi* A was observed in the presence of CLINOPT, but the CLINOPT application increased HIS production by *A. hydrophila* and *K. pneumoniae*. Serotonin (SER) production was the highest for *A. hydrophila* (120.79 mg/L). Among the Gram negative FBPs, *K. pneumoniae* was characterized as the lowest SER producer, followed by *S. paratyphi* A and *P. aeruginosa*. Both of CLINOPT doses used reduced SER accumulation by *A. hydrophila*, *E. coli* and *S. paratyphi* A, whereas significant increases were observed for SER production by *K. pneumoniae* and *P. aeruginosa* at dose of 1% CLINOPT, but CLINOPT at dose of 5% suppressed SER production by Gram negative FBPs.

Tyramine (TYR) was generally formed at low levels (1.49–17 mg/L). *P. aeruginosa* synthesized lower amounts of TYR (<2 mg/L) than the other tested bacteria. However, it was found that *Pseudomona*s spp. is usually TYR producer bacteria (Georgnaras et al., 1995; Silla-

Santos, 1996). The use of CLINOPT significantly suppressed TYR accumulation by *A. hydrophila*, *K. pneumoniae* and *S. paratyphi* A, although CLINOPT addition stimulated TYR production by *E. coli* and *P. aeruginosa*. There were significant differences in trimethylamine (TMA) content among the control and the treated groups ($P<0.05$). The addition of CLINOPT in the ADB reduced TMA accumulation by *A. hydrophila*, *K. pneumoniae* and *S. paratyphi* A. However, the presence of CLINOPT in the ADB resulted in significantly higher TMA production by *E. coli*, but CLINOPT at dose of 5% suppressed TMA accumulation by *P. aeruginosa*.

Dopamine (DOP) production by *A. hydrophila* was 435.31 mg/L, whereas the other Gram negative bacteria produced lower than 91 mg/L. The addition of CLINOPT had an inhibitor effect on DOP production by *A. hydrophila* and *S. paratyphi* A, whilst significant increases on DOP production by *K. pneumoniae*, *E. coli* and *P. aeruginosa* was found in the presence of 1% or 5% CLINOPT in the medium, but CLINOPT at dose of 5% suppressed DOP production by *P. aeruginosa*. AGM is produced directly from arginine by the reaction of arginine decarboxylase that had been secreted from lactic acid bacteria and nitric acid-reducing bacteria (Umezu et al., 1977; Inaba et al., 2004). In this work, there were significant differences between *A. hydrophila* and the other Gram negative bacteria in term of AGM production (Fig. 1). *A. hydrophila* produced considerably higher AGM (377.25 mg/L) than the other tested bacteria. *E. coli* produced low amount of AGM (15.83 mg/L). Low in vitro production of AGM (7.20 ± 0.81 mg/L) by *E. coli* was also reported by Buňková et al. (2009). CLINOPT had no effect on AGM production by *K. pneumoniae* and *E. coli*, whereas significant reduction on AGM production by *A. hydrophila*, *P. aeruginosa* and *S. paratyphi* A was observed in the presence of CLINOPT.

3.2. The effect of clinoptilolite on biogenic amines production by Gram positive FBPs

The influence of CLINOPT on AMN and biogenic amines production by Gram positive FBPs in ADB was given in Table 2. In this study, stronger AMN accumulation in ADB was observed for *L. monocytogenes* (1532.26 mg/L), *E. faecalis* (1122.27 mg/L) and *S. aureus* (896.86 mg/L). The respective AMN production by *L. monocytogenes*, *E. faecalis* and *S. aureus* were found as 1552.22, 2321.43 and 2620.19 mg/L in ADB (Özogul, 2011), whilst Gökdogan et al. (2012) found lower AMN production in histidine decarboxylase broth (52.29, 125.49, 78.89 vs. mg/L). CLINOPT inhibited significantly ($P<0.05$) AMN accumulation by *S. aureus*, although the use of CLINOPT at dose of 1% resulted in higher AMN production by *L. monocytogenes* and *E. faecalis*.

All strains produced various amounts of PUT (Fig. 1). Highest amount of PUT was accumulated by *E. faecalis* (193.79 mg/L), while the lowest amount of that was produced by *S. aureus* (18.12 mg/L). Similar findings are reported also by other researchers (Ladero et al., 2012; Llácer et al., 2007), who found that *E. faecalis* has been identified as PUT producer. They also found that *E. faecalis*, independent of their origin, produced PUT from AGM indicating that PUT production is a general, species-level characteristic of *E. faecalis*. The use of CLINOPT resulted in a lower PUT accumulation by Gram positive FBPs. CAD production by Gram positive bacteria was below 5 mg/L (Table 2).

Table 2. Ammonia and biogenic amines production by gram positive foodborne pathogens treated with clinoptilolite at different doses in ADB.

Microorganism	Clinoptilolite [g L^{-1}]	AMN	PUT	CAD	SPD	TRPT	PHEN	SPN
		[mg L^{-1}]						
Enterococcus faecalis	0	1122.27±19.77^{c}	193.79±12.67^{a}	4.43±0.09^{a}	9.41±0.01^{b}	0.00±0.00^{b}	7.03±0.61^{b}	0.58±0.04^{c}
	10	1706.37±2.49^{a}	57.25±0.06^{b}	3.37±0.18^{b}	28.29±0.03^{a}	7.30±0.30^{a}	10.45±0.13^{a}	18.94±1.33^{a}
	50	1409.37±30.13^{b}	58.14±1.38^{b}	2.88±0.16^{c}	2.90±0.29^{c}	0.32±0.02^{b}	11.57±0.42^{a}	6.41±0.13^{b}
Listeria monocytogenes	0	1532.26±7.90^{b}	124.40±0.29^{a}	2.61±0.16^{b}	12.61±0.16^{a}	3.22±0.11^{a}	10.89±0.21^{b}	86.04±4.30^{a}
	10	1666.34±55.02^{a}	33.76±0.16^{b}	0.41±0.02^{c}	6.86±0.37^{b}	0.00±0.00^{b}	6.90±0.22^{c}	2.78±0.12^{b}
	50	783.18±38.18^{c}	16.05±1.32^{c}	22.61±0.05^{a}	1.89±0.01^{c}	0.17±0.01^{b}	13.86±0.08^{a}	2.01±0.01^{b}
Staphylococcus aureus	0	896.86±14.06^{a}	18.12±0.14^{a}	2.51±0.03^{a}	9.04±0.00^{a}	0.00±0.00	2.68±0.10^{b}	0.00±0.00^{a}
	10	568.46±1.90^{b}	4.41±0.03^{c}	2.31±0.02^{b}	1.32±0.03^{c}	0.00±0.00	3.61±0.07^{a}	0.52±0.02^{a}
	50	511.74±1.26^{c}	9.39±0.20^{b}	0.85±0.01^{c}	4.80±0.28^{b}	0.00±0.00	1.83±0.14^{c}	0.59±0.83^{a}

AMN: ammonia; PUT: putrescine; CAD: cadaverine; SPD: spermidine; TRPT: tryptamine; PHEN: 2-Phenyl-ethylamine; SPN: spermine. Values are expressed as mean ± standard deviation, n = 4. a–c: indicate significant differences (P<0.05) in a row among groups. ADB: Arginine Decarboxylase Broth.

Table 2. Ammonia and biogenic amines production by gram positive foodborne pathogens treated with clinoptilolite at different doses in ADB (Continued).

Microorganism	Clinoptilolite [g L^{-1}]	HIS	SER	TYR	TMA	DOP	AGM
		[mg L^{-1}]					
Enterococcus faecalis	0	0.44±0.02^{b}	13.94±0.09^{c}	7.30±0.34^{c}	0.15±0.01^{c}	16.93±0.21^{c}	21.77±1.65^{b}
	10	12.32±0.18^{a}	85.65±3.40^{a}	15.62±0.17^{a}	536.06±7.91^{a}	382.33±10.44^{a}	30.32±0.70^{a}
	50	1.70±1.46^{b}	45.58±2.38^{b}	10.76±0.30^{b}	28.93±0.06^{b}	55.68±1.55^{b}	21.14±0.03^{b}
Listeria monocytogenes	0	1.49±0.12^{c}	114.69±4.44^{a}	12.22±0.20^{b}	160.39±0.41^{a}	232.74±0.81^{a}	3.54±0.33^{b}
	10	15.06±0.09^{a}	2.43±0.11^{b}	13.84±0.59^{a}	0.36±0.02^{b}	124.46±0.15^{b}	1.77±0.14^{c}
	50	3.80±0.29^{b}	2.29±0.04^{b}	6.27±0.13^{c}	0.54±0.01^{b}	8.34±0.08^{c}	6.49±0.38^{a}
Staphylococcus aureus	0	0.00±0.00^{b}	5.09±0.01^{a}	2.77±0.13^{b}	4.87±0.25^{a}	85.71±5.83^{a}	14.58±0.37^{b}
	10	0.57±0.03^{a}	1.34±0.05^{b}	3.27±0.08^{a}	0.08±0.02^{c}	8.09±0.02^{b}	22.54±1.92^{a}
	50	0.00±0.00^{b}	0.95±0.07^{c}	1.93±0.02^{c}	2.06±0.07^{b}	17.96±0.64^{b}	10.68±0.32^{c}

HIS: histamine; SER: serotonin; TYR: tyramine; TMA: trimethylamine; DOP: dopamine; AGM: agmatine.
Values are expressed as mean ± standard deviation, n = 4. a–c: indicate significant differences (P<0.05) in a row among groups. ADB: Arginine Decarboxylase Broth.

The effect of CLINOPT on CAD production was also strain-dependent. CLINOPT had generally significant effect on reducing CAD accumulation by *E. faecalis* and *S. aureus*, although application of CLINOPT at dose of 5% resulted in higher CAD accumulation by *L. monocytogenes*.

The production of SPD by Gram negative FBPs was above the 9 mg/L. *L. monocytogenes* produced medium amounts of SPD (12.61 mg/L). However, Geornaras et al. (1995) found that *Listeria* strains had not an ability to produce BAs. The use of 1% CLINOPT caused significant reduction on SPD formation by *L. monocytogenes* and *S. aureus*, whereas significant increases in this amine were observed with 1% CLINOPT by *E. faecalis*. TRPT production by *L. monocytogenes* was suppressed by use of CLINOPT, but *S. aureus* did not form TRPT. The presence of 1% CLINOPT in broth resulted seven-fold higher TRPT production by *E. faecalis*, whereas *E. faecalis* did not have the ability to produce TRPT in the absence of CLINOPT in ADB. There was a stimulation of SPN for *E. faecalis* by the use of 1% or 5% CLINOPT while CLINOPT suppressed SPN accumulation by *L. monocytogenes*.

HIS production by Gram-positive FBPs was below 2 mg/L whilst *S. aureus* had no ability to produce HIS in ADB. Chang et al. (2008) reported that *S. aureus* isolated from swordfish fillets implicated in a food poisoning was weak histamine-former and produced only between 12.7 ppm and 33.0 ppm of histamine in trypticase soy broth supplemented with 1.0% L-histidine, while, Komprda et al. (2010) identified *E. faecalis* as the most prolific HIS producer within *Enterococcus* isolates from dry fermented sausages, which was not in

agreement with our own experimental results. PHE may result from phenylalanine decarboxylation by various species of *Enterococci*, *Cocci* Gram positive and *Lactobacillus* (Ansonera et al., 2002; Bover-Cid et al., 2001). In this study, we observed that a higher PHEN production was related to a higher increase in TYR. These results are in agreement with a study reporting that PHEN generally occurs when a high amount of TYR is present because microorganisms also have moderate capacity to decarboxylate phenylalanine (Joosten, 1987). The use of CLINOPT also showed stimulation effect on PHEN and HIS production by Gram positive bacteria. Similar results were reported by Gökdogan et al. (2012), who found that PHEN and HIS production was stimulated for Gram positive FBPs by the use of 1% or 5% CLINOPT concentrations.

CLINOPT showed a stronger inhibitor effect on SER formation by *L. monocytogenes*, although CLINOPT addition stimulated SER production by *E. faecalis*. TYR production by Gram positive FBPs was ranged from 2 to 13 mg/L. TYR formation by *E. faecalis* was above the 7 mg/L. Özogul (2011) found little amount of TYR production (2.52 mg/L) by Gram positive bacteria in histidine decarboxylase broth. However, Pircher et al. (2007) reported that *E. faecalis* formed significant concentrations of TYR (100 to 1000 mg/L) in dry fermented sausages. Engesser et al. (1990) also reported that TYR was produced in highest concentration by *E. faecalis* under experimental conditions. The highest TMA production was obtained by *L. monocytogenes* (160.39 mg/L), followed by *S. aureus* (4.87 mg/L) and lowest with *E. faecalis* (0.15 mg/L). By the presence of CLINOPT in ADB, significant reduction in TMA accumulation was observed by *L. monocytogenes* and *S. aureus*, though the use of CLINOPT increased TMA formation by *E. faecalis*.

The highest DOP accumulation was found for *L. monocytogenes* (232.74 mg/L), which is not in agreement with result of Özogul (2011) reporting that the *E. faecalis* produced significantly higher DOP than *L. monocytogenes* and *S. aureus*. Significant reductions by CLINOPT application were also observed for DOP by *L. monocytogenes* and *S. aureus*. However, the presence of 1% CLINOPT in the ADB resulted in significantly higher DOP production by *E. faecalis*. Gram positive FBPs showed a low AGM production in ADB (Fig. 1). Özogul and Özogul (2007) found that the highest AGM production by *K. pneumoniae* and *E. faecalis* were in lysine decarboxylase broth and in tyrosine decarboxylase broth, respectively. They demonstrated that bacteria could vary in their relative importance as biogenic amine producers in different decarboxylase broths. Others authors reported that the availability of precursors is not a fundamental problem in BAs accumulation, and the presence of specific amino acids may not be indicative of specific peptidase activities but represents the results of an equilibrium between amino acid liberation and metabolization (Lanciotti et al., 2007). AGM production was stimulated for *E. faecalis* and *S. aureus* by the use of 1% CLINOPT, and for *L. monocytogenes* use of 5% CLINOPT dose.

4. Conclusions

All tested bacteria had an ability to produce ammonia and biogenic amines in ADB. The study results showed that CLINOPT had a clear inhibition effect only on PUT accumulation in ADB by both Gram negative and positive FBPs. On the other hand, the results demonstrate significant variations on biogenic amine production in the presence of CLINOPT. These results confirm that the effect of CLINOPT on AMN and biogenic amines was strain dependent and differed for specific amine. More in-depth research is needed to determine how CLINOPT affects decarboxylase activity in food products and how this can ultimately affect the formation of biogenic amines both quantitatively and qualitatively.

5. References

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