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ANTIMICROBIAL EFFECT OF SELECTED LACTIC ACID BACTERIA AGAINST MICROORGANISMS WITH DECARBOXYLASE ACTIVITY

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ABSTRACT

The main purpose of this study was to evaluate the antimicrobial activity of twenty-one bacteriocinogenic lactic acid bacteria (12 strains of *Lactococcus lactis* subsp. *lactis*, 4 strains of *Lactobacillus gasseri*, 3 strains of *Lb. helveticus* and 2 strains of *Lb. acidophilus*, LAB) against 28 *Staphylococcus* and 33 *Enterococcus* strains able to produce tyramine, putrescine, 2-phenylethylamine and cadaverine. The antimicrobial activity of cell-free supernatants (CFS) from tested LAB was examined by an agar-well diffusion assay. Nine out of twenty-one strains (33%) showed the inhibitory effect on tested enterococci and staphylococci, namely 9 strains of *Lactococcus lactis* subsp. *lactis*. The diameters of inhibition zones ranged between 7 mm and 14 mm. The biggest diameter of 14 mm inhibition was obtained with the CFS's from strains CCDM 670 and CCDM 731 on *Enterococcus* sp. E16 and E28. The cell-free supernatants from *Lactococcus lactis* subsp. *lactis* CCDM 71 and from *Lactococcus lactis* subsp. lactis CCDM 731 displayed the broadest antibacterial activity (52% inhibition of all tested strains). On the other hand, the cell-free supernatants from the screened *Lactobacillus* strains did not show any inhibitory effect on the tested *Staphylococcus* and *Enterococcus* strains. Nowadays, the great attention is given to the antibacterial substances produced by lactic acid bacteria. With the ability to produce a variety of metabolites displaying inhibitory effect, the LAB have great potential in biopreservation of food.

Keywords: biogenic amines; lactic acid bacteria; bacteriocins; nisin; *Enterococcus* spp.; *Staphylococcus* spp.

INTRODUCTION

Since ancient time, wild microorganisms naturally present in raw material has been played important role in food preservation **(Galvéz et al., 2007)**. The lactic acid bacteria (LAB) have been used for centuries in the fermentation of food, not only for organoleptic properties **(McAuliffe et al., 2001)**, but also as a natural competitor to other microorganisms that share the same niche **(Reis et al., 2012)**. The antimicrobial activity of LAB is due to the production of metabolites such as organic acids (lactic and acetic acid), ethanol, diacetyl, hydrogen peroxide and carbon dioxide **(Šušković et al., 2010; Reis et al., 2012; Cizeikiene et al., 2013)**. In addition, some strains are able to synthesize antimicrobial peptides known as bacteriocins and bacteriocin-like inhibitory substances **(BLIS; Cleveland et al., 2001; Cizeikiene et al., 2013)**.

The bacteriocins produced by LAB are cationic amphiphilic molecules containing 20 to 60 amino acid residues **(Chen and Hoover, 2003)**. These bacteriocins are thermostable and retained its activity in a wide range of pH values. Moreover, they are colorless, odorless, tasteless and they are easily digestible in the digestive tract and thus, they do not affect the composition of the intestinal microflora **(Perez et al. 2014)**. Nowadays, nisin is the only bacteriocin of gram-positive bacteria, which is approved by the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA) for use as a food preservative **(EFSA, 2006)**. Nisin is a low-molecular-weight polypeptide (34 amino acids) with a pentacyclic structure containing one lanthionine and four β-methyllanthionine residues **(Ross et al., 2002)**. It is produced by Lactococcus lactis subsp. lactis, commonly found in milk and dairy products **(Favaro et al., 2015)**. Therefore, it is non-toxic to humans and food containing nisin, can carry the label "preserved in a natural way" **(Cleveland et al., 2001)**.

Biogenic amines (BA) are nitrogenous substances naturally occurring in living organisms, where they play an important role in many physiological processes **(Silla Santos, 1996; Shalaby, 1996)**. On the other hand, their excessive intake due to the consumption of BA rich food may pose a potential health risk to the consumers **(Gardini et al., 2016)**. The intake of BA can induce several digestive, circulatory and respiratory symptoms **(Ladero et al., 2010)**.

In food, they are generally formed by microorganisms demonstrating decarboxylase activity from amino acids **(Silla Santos, 1996; Shalaby, 1996, Ladero, 2010; Buňková et al., 2010)**. Therefore, microorganisms naturally present in raw materials, introduced throughout the processing or added as starter culture can critically influence BA production during the manufacture of fermented products **(Bover-Cid et al., 2001)**. Enterococci and coagulase-negative staphylococci are commonly present in fermented dairy and meat products, where they can produce a large amount of BA **(Martuscelli et al., 2000; Pleva et al., 2012; Buňková et al., 2012)**.

Most research works have focused on the isolation and characterisation of LAB with amino-oxidase activity **(Dapkevicius et al., 2000; Fadda et al., 2001; García-Ruiz et al., 2011; Capozzi et al., 2012; Callejón et al., 2014)**. However, studies on inhibiting effect of bacteriocin-producing LAB on decarboxylase-positive bacteria are still lacking. Therefore, the main goal of this work was to evaluate the antimicrobial activity of cell-free supernatant from selected lactic acid bacteria against *Enterococcus* and *Staphylococcus* strains with decarboxylase activity.

MATERIAL AND METHODOLOGY

Tested microorganisms

The bacteriocin-producing lactic acid bacteria (12 strains of *Lactococcus lactis* subsp. *lactis*, 4 strains of *Lactobacillus gasseri*, 3 strains of Lb. *helveticus* and 2 strains of Lb. *acidophilus*) were obtained from Cultures Collection of Dairy Microorganisms Laktoflora® (CCDM; Czech Republic). The antimicrobial activity of the investigated cultures was tested against 28 *Staphylococcus* strains (8 strains of *S. warneri*, 4 strains of *S. haemolyticus*, 4 strains of *S. succinus*, 4 strains of *S. hominis*, 4 strain of *S. epidermidis*, 2 strains of *S. pasteuri* and 2 strains of *S. vitulinus*) and 33 *Enterococcus* strains (14 strains of *E. faecium*, 7 strains of *Enterococcus* sp., 6 strains of *E. durans*, 4 strains of *E. hirae* and 2 strains of *E. faecalis*) able to produce tyramine, putrescine, 2-phenylethylamine and cadaverine. Growth condition and origin of these isolates are displayd in Table 1. The strains were isolated from dairy and meat products (raw milk, cheese, pheasant meat, poultry and fish) in the Department of Environmental Protection Engineering of Faculty of Technology Tomas Bata University in Zlín. Some of these strains (isolated from pheasant meat) were described in previous study **(Buňková et al., 2016)**.

Preparation of cell-free supernatants from LAB

The tested *Lactococcus* strains were cultivated in 10 mL M17 (Oxiod, United Kingdom) broth (1%, v/v) under aerobic condition at 30 °C for 72 h. The tested *Lactobacillus* strains were cultivated in 10 ml MRS (De Man, Rogosa and Sharpe; Merck, USA) broth (1%, v/v) under anaerobic condition at 37 °C for 72 h. After 72h cultivation, the cells were harvested by centrifugation at 10 000 x g for 15 min. The obtained cell-free supernatant (CFS) was adjusted to pH 6.0 ±0.2 with 10% NaOH in order to eliminate the inhibition effect due to organic acids (low pH) and was filtered through a membrane filter (0.22 μm pore size).

Agar-well diffusion test

The antimicrobial activity of selected lactic acid bacteria was tested by an agar well diffusion assay. The tested bacteria with decarboxylase activity were incubated in nutrient medium at appropriate temperature according Table 1 for 24 h. After incubation, the overnight cultures were serially diluted in 0.85% NaCl solution. Fraction (1 mL) of the dilution 10^{-2} was plated in triplicate to a Petri dish and 20 mL of an appropriate medium (Table 1; HiMedia, India) was poured. A culture supernatant

Table 2 Tested strains with decyrboxylase activity (AE – aerobic, MHB/A – Mueller-Hinton Broth/ Agar).

Microorganisms with decarboxylase activity		Growth condition	Origin
Strain	**Name**		
B: 151, 152, 153,154,160, 169	*Enterococcus durans*	30 C, AE, M-17	Pheasant meat
B: 131, 142	*Enterococcus faecalis*	30 C, AE, M-17	
B:129, 133, 143, 144, 145	*Enterococcus faecium*	30 C, AE, M-17	
B: 76, 122, 124, 147	*Enterococcus hirae*	30 C, AE, M-17	
B: 29	*Staphylococcus warneri*	37 C, AE, MHB/A	
B: 40,136, 137	*Staphylococcus epidermis*	37 C, AE, MHB/A	
B: 47, 77, 80, 89	*Staphylococcus succinus*	37 C, AE, MHB/A	
B: 81, 82	*Staphylococcus vitulinus*	37 C, AE, MHB/A	
B: 138	*Staphylococcus hominis*	37 C, AE, MHB/A	
E: 2, 5, 8, 11, 13, 14,17, 25, 27	*Enterococcus faecium*	30 C, AE, M-17	Raw milk, cheese
E: 15, 16, 18, 21, 26, 28, 30	*Enterococcus* sp.	30 C, AE, M-17	
S: 1, 2, 3, 13, 15,16,17	*Staphylococcus warneri*	37 C, AE, MHB/A	Fish and poultry
S: 4, 14	*Staphylococcus pasteuri*	37 C, AE, MHB/A	
S: 5, 6, 7	*Staphylococcus hominis*	37 C, AE, MHB/A	
S: 8	*Staphylococcus epidermidis*	37 C, AE, MHB/A	
S: 9, 10, 11, 12	*Staphylococcus haemolyticus*	37 C, AE, MHB/A	

(100 μL) was added to each well (6 mm in diameter) punched in the cooled agar plates and incubated for 24 – 48 h at the optimal growth temperature for inhibited bacteria. The antimicrobial activities of LAB were determined by measuring the inhibition zones (mm).

Statistical analysis

The obtained experimental data were analysed using a Statistical software Unistat 6.5 (Unistat, London, UK). The significance level of all statistical tests was set at $p < 0.05$.

RESULTS AND DISCUSSION

Inhibition effect of CFS on tested *Enterococcus* strains

Enterococci are known as ubiquitous bacteria and based on their association with the gastrointestinal tract, they often occur in foods of animal origin **(Franz et al., 2011)**. *Enterococci*; due to their salt and pH tolerance, as well as their ability to grow over a wide range of temperature; can survive to the fermentation process and can be found in fermented foods such as sausages and cheeses **(Bargossi et al., 2015)**. In these product, they can additionally produce a relevant amount of biogenic amines, especially tyramine **(Suzzi and Gardini, 2003; Ladero et al., 2012; Jimenéz et al., 2013)**.

In present work, twenty-one lactic acid bacteria able to produce nisin and bacteriocin like inhibitory substances (BLIS) were screened for their antimicrobial effect on 33 Enterococcus strains with decarboxylase activity. The data obtained from this experiment are demonstrated in Table 2. As can be seen in this table, out of 21 screened LAB strains, 7 strains (33%) showed the inhibitory effect on tested enterococci, namely *Lactococcus lactis subsp. lactis* CCDM 71, CCDM 670, CCDM 686, CCDM 689, CCDM 695 and CCDM 698 and CCDM 731. The diameters of inhibition zones ranged between 7 mm and 14 mm (including diameter of well). The biggest diameter of 14 mm inhibition was obtained with the CFS's from strains CCDM 670 and 731 on strains E16 and E28 isolated from raw milk. The broadest antibacterial activity displayed CFS from *Lactococcus lactis subsp. lactis* CCDM 71 (85% inhibition of all tested strains) followed by CFS from *Lactococcus lactis subsp. lactis* CCDM 731 (82% inhibition) and *Lactococcus lactis subsp. lactis* CCDM 670 (82% inhibition). Similar study was carried out by **Şanlibaba et al. (2009)** who studied the antimicrobial effect of *Lactococcus lactis subsp. lactis* LL27 isolated from Turkish raw milk. The CFS of this strain was found to show the inhibitory activity at different levels to 17 out of 23 indicator bacteria, namely, 9 strains of *L. lactic subsp. lactis*, 2 strains of *Enterococcus faecalis*, 1 strain of *Lactobacillus sakei*, 1 strain of *Lactobacillus plantarum*, 1 strain of *Pediococcus pentosaceus*, 1 strain of *Listeria innocua*, 1 strain of *Staphylococcus carnosus* and 1 strain of *Bacillus cereus*.

Enan et al. (2013) also reported the antibacterial activities of bacteriocinogenic strain *L. lactis subsp. lactis* Z11 isolated from Zabady (Arabian yoghurt). The inhibitory activity of cell-free supernatant of this strain inhibited other strains of lactic acid bacteria and some food-borne pathogens including *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*.

In this study, the cell-free supernatants from the tested

Table 2 Antimicrobial activity of selected LAB strains against *Enterococcus* strains.

Strains	Inhibition effect of selected lactic acid bacteria*						
	CCDM 71	CCDM 670	CCDM 686	CCDM 689	CCDM 695	CCDM 698	CCDM 731
E2	++	+	++	++	+	++	++
E5	++	++	++	++	++	++	++
E8	++	++	++	++	++	++	++
E11	++	++	++	++	++	++	++
E13	+	+	++	++	++	+	+
E14	++	++	++	++	++	++	++
E15	++	++	++	++	+	++	++
E16	++	+++	++	++	++	++	++
E17	+	+	+	++	++	+	+
E18	++	++	+	++	++	++	++
E21	++	++	++	++	+	++	++
E25	++	++	++	++	++	++	++
E26	++	++	++	+	++	+	+
E27	+	+	+	+	+	+	+
E28	+	++	++	++	++	++	+++
E30	++	+	+	+	++	+	+
B76	+	+	-	-	-	+	+
B122	++	++	++	+	+	+	++
B129	++	++	++	+	+	+	++
B131	++	++	++	++	+	+	++
B133	++	++	++	+	+	+	+
B142	+	+	-	-	-	-	+
B143	+	-	+	-	-	-	-
B144	+	+	+	+	+	+	+
B147	+	+	-	-	-	-	+
B152	+	+	+	+	+	+	+
B154	+	+	+	+	+	+	+
B160	+	++	++	+	++	++	++

Note:*(-) no inhibition; (+) 7 – 10 mm inhibition zone; (++) 11 – 13 mm inhibition zone; (+++) 14 ≤mm inhibition zone.

Table 3 Antimicrobial activity of selected LAB strains against *Staphylococcus* strains.

Strains	Inhibition effect of selected lactic acid bacteria*								
	CCDM 71	CCDM 414	CCDM 418	CCDM 670	CCDM 686	CCDM 689	CCDM 695	CCDM 698	CCDM 731
B40	+	+	+	+	+	-	+	-	+
B138	+	+	+	+	+	-	-	-	+
S1	+	+	+	-	+	-	-	-	+
S4	-	+	+	-	-	-	-	-	-
S6	-	+	+	-	+	+	+	+	+
S7	-	+	+	-	-	-	-	-	-
S8	+	+	+	+	+	+	+	+	+
S9	-	++	+	-	-	-	-	-	-
S11	-	+	+	-	-	-	-	-	-
S12	-	++	+	-	-	-	-	-	-
S14	-	+	-	-	-	-	-	-	-
S17	-	+	-	-	-	-	-	-	-

Note: *(-) no inhibition; (+) 7–10 mm inhibition zone; (++) 11–13 mm inhibition zone; (+++) 14 ≤mm inhibition zone.

Lactobacillus strains did not show any inhibitory effect on the tested *Enterococcus* strains. This result is in disagreement with the results of study carried out by **Xie et al. (2016)**. This study aimed to investigate the inhibitory effects of cell-free supernatant from *Lactobacillus plantarum* on four amine-positive bacteria, namely, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterococcus faecium* and *Enterococcus faecalis*. Results showed that CFS from *L. plantarum* significantly reduced the cell growth and diamine production of all tested bacteria.

Study provided by **Cizeikiene et al. (2013)** also observed that CFS from BLIS-producer *Lactobacillus sakei* KTU05-6 isolated from spontaneous Lithuanian rye sourdoughs showed wide-ranging antimicrobial activities against gram-positive and gram-negative strains.

Among *Enterococcus* genus, *Enterococcus faecium* and *Enterococcus faecalis* are the main causative agents for serious relevant nosocomial infections such as urinary tract infections, endocarditis, bacteremia, intra-abdominal and intra-pelvic abscesses **(Bhardwaj et al., 2013)**. In our study, the growth of *E. faecium* (E2, E5, E8, E11, E13, E14, E17, E25, E27, B129, B133 and B144) and the growth of *E. faecalis* B131 were effectively inhibited by all 7 strains displayed in Table 2. Among them, the strain *Lactococcus lactis subsp. lactis* CCDM 71 demonstrated a great inhibitory effect against 13 out of 14 strains of *E. faecium* and 2 out of 2 strains of *E. faecalis*.

In accordance with obtained results it can be concluded that strains isolated from raw milk were more sensitive to the antimicrobial metabolites produced by tested LAB than strains isolated from pheasant meat. The most resistant strain was *E. durans* (B151, B153 and B169).

Inhibition effect of CFS on tested *Staphylococcus* strains

The antimicrobial activity of tested lactic acid bacteria against the *Staphylococcus* strains is demonstrated in Table 3. As can be seen in this table, nine strains of *Lactococcus lactis subsp. lactis* (CCDM 71, CCDM 414, CCDM 418, CCDM 670, CCDM 686, CCDM 689, CCDM 695, CCDM 698 and CCDM 731) produced an inhibition zone against one or more *Staphylococcus* strains. The diameters of inhibition zones ranged between 7 mm and 12 mm (including diameter of well). The largest inhibition zone of 12 mm in diameter was obtained with the cell-free supernatant from strain *Lactococcus lactis subsp. lactis* CCDM 414 on *S. haemolyticus* S9, isolate from fish. Moreover, the CFS from this strain displayed the broadest antibacterial activity against tested staphylococci including 2 strains of *S. epidermis*, 2 strains of *S. warneri*, 2 strains of *S. pasteuri*, 3 strains of *S. hominis* and 3 strains of *S. haemolyticus* (43% inhibition of all tested strains). Similar antibacterial spectrum showed also strain *L. lactis subsp. lactis* CCDM 418. Ten out of 28 strains were inhibited by this strain. The most sensitive strain was *S. epidermis* S8 inhibited by all 9 *Lactococcus* strains.

The antimicrobial activities of 5 *Lactococcus* strains (*L. garviae* K2, *L. piscium* SU4, *L. lactis subsp. cremoris* E22, *L. lactis subsp. hordinae* E91 and *L. plantarum* L7) against spoilage and pathogenic organisms were also studied by **Olaoye (2016)**. The CFS of all 5 strains demonstrated an inhibition effect on growth of *Staphylococcus aureus*. The diameters of inhibition zones ranged between 1.5 mm and 2.5 mm. Also **Lee et al. (2013)** reported an inhibitory effect of *Lactococcus lactis* KU24 isolated from kimchi against methicillin-resistant *S. aureus* in their study. Same inhibitory effect against methicillin-resistant *S. aureus* was also displayd by *Lactobacillus acidophilus* and *Lactobacillus casei* **(Karska-Wysocki et al., 2010)**. In present work, the cell-free supernatants from the screened lactobacilli did not show any inhibitory effect on the tested *Staphylococcus* strains.

In accordance with obtained results it can be concluded that strains isolated from fish were more sensitive to the antimicrobial metabolites produced by *Lactococcus* strains than isolates from pheasant meat and poultry. The most resistant strains were *S. warneri* (B29), *S. vitulinus* (B81 and B82) and *S. succinus* (B47, B77, B80 and B89).

CONCLUSION

The consumption of food containing large amounts of biogenic amines is potential health risk for some consumers. Therefore, a great effort is arising to prevent the formation and accumulation of these substances in foodstuffs, especially in fermented foods, where is their occurrence most common. The addition of selected starter cultures is one of the main tools able to prevent the

formation of high levels of BA in fermented meat and dairy products. According to the results presented above, it can be concluded that nine out of twenty-one *Lactococcus* strains demonstrated antimicrobial effect against tested *Enterococcus* and *Staphylococcus* strains. The use of bioprotective cultures producing bacteriocins or other antimicrobial substances needs greater attention due to their not fully explored potential in this field **(Gardini et al., 2016)**.

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