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ANTIFUNGAL EFFECT OF A BACTERIOCIN OF *BACILLUS METHYLOTROPHICUS* BM47 AND ITS POTENTIAL APPLICATION AS A BIOPRESERVATIVE IN TRADITIONAL BULGARIAN YOGURT

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ABSTRACT

Bacteriocins are biologically active compounds of proteinaceous nature synthesized by a large number of microorganisms, including members of bacterial genus *Bacillus* and lactic acid bacteria (LAB). The broad antimicrobial spectrum of bacteriocins against various spoilage and pathogenic microorganisms stimulated the research efforts for their investigation and potential application in different branches of food industry as natural preservatives. The promising antimicrobial activity of bacteriocins makes them suitable for application as biopreservatives and alternatives of chemical preservatives in dairy industry for production of fermented or non-fermented milk products. Therefore, the aim of the present study was to evaluate the antifungal effect of a bacteriocin isolated from *Bacillus methylotrophicus* strain BM47 and the possibilities for its application as a potential biopreservative of traditional Bulgarian yogurt. The results demonstrated that the addition of a purified bacteriocin in dose of 1 AU/mL of milk led to significant reduction of the fungal spores and mycelial growth of the indicator microorganism *Penicillium* sp. in the yogurt, without a change of its organoleptic properties, and biochemical and microbiological parameters during the 4-week period of storage.

Keywords: Bulgarian yogurt, bacteriocins, *Bacillus methylotrophicus*, food biopreservation

INTRODUCTION

Yogurt (or yoghurt) is one of the most famous and widely spread traditional dairy products in Bulgaria, produced by bacterial fermentation of cow's, buffalo's, sheep's and goat's milk. Yogurt is known on the Balkans since ancient times, when the Thracians have obtained it from self-fermented sheep's milk. Nowadays, it takes an important role in the diet of the Bulgarians due to its excellent nutritional characteristics and beneficial effects on human health. Besides the rich chemical composition, including all natural nutritional constituents and significant amounts of calcium in a bioavailable form, yogurt possesses many therapeutic effects on lactose intolerance and some gastrointestinal disorders such as diarrhea, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), and weight control **(McKinley, 2005)**. Bulgarian yogurt it is often recommended as food for improving of the metabolism, reducing of the cholesterol and even as an anti-carcinogenic agent **(Fikiin *et al.*, 1997)**. Many recent studies have demonstrated that yogurt exhibits positive effects on the gut microbiota and is associated with a reduced risk for gastrointestinal diseases, cardiovascular diseases, type 2 diabetes, allergies, respiratory diseases, pregnancy outcomes, as well as for improving of dental and bone health **(Fisberg and Machado, 2015)**.

The traditional Bulgarian yogurt, known also as "kiselo mlyako", is a coagulated milk product, characterized by smooth texture, thick consistency and pleasant sour flavor. The uniqueness of Bulgarian yogurt is famous worldwide and it is attributed to the country's microclimate and the specific fermentation by symbiotic cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* **(Tropcheva *et al.*, 2014a)**. These starter bacteria are known to produce a wide spectrum of volatile organic aroma compounds such as acetaldehyde, diacetyl, volatile fatty acids that determine the specific flavour of Bulgarian yogurt **(Beshkova *et al.*, 1998)**. Another compound, produced by the starter microorganisms, which imparts the distinctive flavour of yogurt and its related products, is the lactic acid. Lactic acid is a major end product during the fermentation of carbohydrates by the lactic acid bacteria (LAB). The rapid fermentation process leads to improving of the organoleptic qualities of milk- and non-milk products, and also extends their shelf life by inhibition of saprophytic and pathogenic microorganisms **(Cholakov *et al.*, 2017)**.

Although significant antimicrobial activity of the lactic acid produced at high amounts during the milk fermentation and suppressive effect of the low pH, numerous studies revealed the occurrence of foodborne pathogens, which may survive the pasteurization or to enter the pasteurized milk via contamination or experimental inoculation. The different pathogens can survive in yogurt for a various period of time, which for *Listeria monocytogenes* is limited between 9 and 15 h **(Schaack and Marth, 1988)** to >6 days **(Ikonomov and Todorov, 1967)**; up to 16 h for *Escherichia coli* O157:H7 of storage at 4-22ºC **(Bachrouri *et al.*, 2002)**; from 2 to 4 days for *Brucella* spp. **(Falenski *et al.*, 2010)**; more than one week of storage at 5°C for *Yersinia enterocolitica* **(Ahmed *et al.*, 1986)**; between 2 days at 4°C **(Pazakova *et al.*, 1997)** and 10 days of storage at room temperature (22°C) for *Staphylococcus aureus* **(Benkerroum *et al.*, 2002)**; over 43 days of storage at temperatures between 4 and 25°C for *Salmonella enterica* serovar *typhimurium* **(Álvarez-Ordóñez *et al.*, 2013)**.

The raw milk for production of yogurt is often a vector for spoilage and toxigenic microorganisms such as yeasts and fungi, which is serious problem nowadays due to the resistance of fungal spores to exposure of high temperature and low pH. The most commonly detected fungi in raw milk belong to the genera *Penicillium*, *Geotrichum*, *Aspergillus*, *Mucor* and *Fusarium* **(Quigley *et al.*, 2013)** that renders the milk unsuitable either for direct consumption or for subsequent processing. In addition, it is estimated that between 5 and 10% of the world's food production is lost due to spoilage caused by fungal contamination **(Pitt and Hocking, 2009)**.

Therefore, the aim of the present study was to determine the antifungal activity against *Penicillium* sp. of a bacteriocin isolated from *Bacillus methylotrophicus* BM47 and to evaluate the possibilities for its application as a potential biopreservative of traditional Bulgarian yogurt.

MATERIAL AND METHODS

Materials

Bacteriocin

Purified by fast protein liquid chromatography (FPLC) and lyophilized substance containing a protein of intermediate molecular size (19578 Da) was used. This bacteriocin was synthesized by *Bacillus methylotrophicus* strain BM47, isolated from natural thermal spring water in Haskovo region, Bulgaria.

Indicator microorganism

The fungus *Penicillium* sp. (our isolate) from the collection of the Department of Microbiology at University of Food Technologies, Plovdiv, Bulgaria, was selected as indicator microorganism.

Culture media

Malt extract agar (MEA)
This medium was used for cultivation of the test fungus. MEA was prepared by the following prescription: 20 g malt extract, 20 g dextrose, 6 g peptone and 15 g agar dissolved in 1L of deionized water. The final pH was corrected to 5.5 and the medium was sterilized by autoclaving at 121°C for 15 min.

Luria-Bertani glucose agar (LBG)
This medium was used for determination of MIC. LBG-agar was prepared by the following prescription: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 10 g glucose and 15 g agar dissolved in 1 L of deionized water. The final pH was adjusted to 7.5 and the medium was autoclaved at 121°C for 20 min.

Chloramphenicol glucose agar (CGA)
CGA is a selective medium for enumeration of yeasts and fungi, prepared according to the manufacturer's (Scharlab S.L., Spain) prescription: 20 g dextrose, 5 g yeast extract, 0.1 g chloramphenicol and 15 g agar dissolved in 1L of deionized water. The final pH was corrected to 6.6 and the medium was sterilized in autoclave at 121°C for 15 min.

Man, Rogosa and Sharpe agar (MRS)
MRS-agar is a selective medium for cultivation and enumeration of lactobacilli, prepared according to the manufacturer's (Merck, Germany) prescription: peptone proteose 10 g, meat extract 8 g, yeast extract 4 g, D(+)-glucose 20 g, sodium acetate 5 g, triammonium citrate 2 g, magnesium sulfate 0.2 g, manganese sulfate 0.05 g, dipotassium phosphate 2 g, polysorbate 80 - 1 g and agar 14 g dissolved in 1L of deionized water. The final pH was corrected to 6.2 and the medium was autoclaved at 121°C for 15 min.

M17 agar
M17-agar is a selective medium for cultivation and enumeration of lactic streptococci, prepared according to the manufacturer's (Merck, Germany) prescription: casein enzymic hydrolysate 2.5 g, peptic digest of animal tissue 2.5 g, papaic digest of soyaben meal 5 g, yeast extract 2.5 g, beef extract 5 g, ascorbic acid 0.5 g, magnesium sulphate 0.25 g, lactose 5 g, disodium-β-glycerophosphate 19 g and agar 10 g. The final pH was adjusted to 7.1 and the medium was sterilized by autoclaving at 121°C for 15 min.

Starter culture

Symbiotic starter culture MZ2, containing *Lactobacillus delbrueckii* subsp. *bulgaricus* LBG MZ (NBIMCC 3600) and *Streptococcus salivarius* subsp. *thermophilus* 35 (NBIMCC 3597) in ratio 1:2 was used. The starter culture MZ2 was kindly provided by Prof. Zapryana Denkova from the Department of Microbiology at University of Food Technologies, Plovdiv, Bulgaria.

Milk

Fresh, homogenized and pasteurized cow's milk with pH 6.7 and titratable acidity of 16.5°T was used. The milk was delivered by BCC Handel Ltd., town of Elena, Bulgaria.

Methods

Preparation of spore suspension

The test fungus *Penicillium* sp. was grown on MEA at 30ºC for 7 days or until sporulation. The inoculum was prepared by addition of 5 mL of sterile 0.5% NaCl into the tube. After vigorous shaking the inoculum was filtered and replaced in another tube before use. The number of fungal spores was determined using a Thoma's haemocytometer. Final spore concentration in the inoculum was adjusted to 1.6×10^7 cfu/mL then a working dilution with spore concentration of 1.6×10^5 cfu/mL was prepared.

Minimal inhibitory concentration (MIC) of purified bacteriocin (PB) and determination of the arbitrary units (AU)

MIC of the purified bacteriocin of *B. methylotrophicus* BM47 was determined by the conventional method according to **Tumbarski *et al*. (2017)**. Series of two-fold dilutions of the bacteriocin ranging from 10.0 to 0.079 mg/mL were prepared. Duplicate samples of each dilution were pipetted in quantity of 60 µL into wells cut in a preliminarily inoculated with the test microorganism LBG-agar medium. The Petri dishes were incubated at 30ºC for 48 h. The MIC value was determined as the lowest concentration of PB inhibiting completely the growth of the test microorganism around the agar well. The calculation of arbitrary units (AU) for application in milk was based on the obtained MIC value.

Experimental procedure

The traditional Bulgarian yogurt was prepared in our laboratory according to the Bulgarian State Standard (BSS 12:2010). The homogenized and pasteurized raw cow's milk was heated at 45-46°C and transferred in quantity of 100 mL into sterile plastic cups with lids. Then 1% of fresh symbiotic starter culture MZ2 (1.0×10^8 cfu/mL) was added to the cups and mixed. The cups were separated in four groups – the first group was kept as a control; the second group was treated with PB (1 AU/mL); the third group was inoculated with spore suspension of *Penicillium* sp., providing around 2.5×10^2 - 5.0×10^2 cfu/mL of milk; the fourth group was treated with PB and inoculated with spore suspension of *Penicillium* sp. (in the same amounts). For each day of the testing (1-st, 7-th, 14-th, 21-st and 28-th) separate samples were provided. All milk samples were incubated at 44°C ± 2°C for 2.5–3.5 h. After coagulation, all samples were cooled and stored at 4°C. The morphological, biochemical and microbiological changes as well as the antifungal effect of PB in yogurt were monitored once a week, for 4 weeks.

Enumeration of characteristic microorganisms

Colony-count technique for lactobacilli and lactic streptococci was implemented on MRS-agar and M17-agar respectively, at 37°C according to the Bulgarian State Standard BSS ISO 7889:2005.

Enumeration of yeasts and/or fungi in milk

Colony-count technique for yeasts and/or fungi in milk was implemented on CGA at 25°C according to the Bulgarian State Standard BSS ISO 6611:2006.

Determination of titratable acidity

Determination of titratable acidity was implemented according to the Bulgarian State Standard BSS 1111:1980. The titratable acidity was determined by titration of each sample with 0.1 N NaOH using phenolphthalein as an indicator until the appearance of a pale pink colour persisting over 1 min. One Torner degree (°T) corresponds to 1 mL of 0.1 N NaOH, needed for neutralisation of an equivalent amount of organic acid, contained in 100 mL of milk. The results were calculated as mean value of three consecutive experiments.

RESULTS AND DISCUSSION

After estimation of MIC of PB of *B. methylotrophicus* BM47 against the indicator microorganism *Penicillium* sp. (2.5 mg/mL), the arbitrary units (AU) for application in milk were calculated to the amount of PB in 60 µL (0.15 mg) and this dose was accepted as equivalent to 100 AU. To provide the desirable concentration of 1 AU/mL, in the experimental procedure 0.15 mg of PB was added in 100 mL of milk.
During the 4-week period of storage under refrigeration conditions (4°C), the microbiological parameters – number and ratio of lactic acid bacteria – LAB (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*), presence of yeasts and fungi, as well as the titratable acidity of four samples - control, yogurt with addition of bacteriocin (sample 1), yogurt inoculated with *Penicillium* sp. (sample 2) and yogurt with addition of bacteriocin and *Penicillium* sp. (sample 3) were monitored. The antifungal effect of the purified bacteriocin on the test microorganism *Penicillium* sp. in the artificially contaminated samples also was observed. The results are summarized in Table 1.
As seen from the results presented in Table 1, during the first day of storage, the titratable acidity values and microbiological parameters of all yogurt samples were in compliance with the BSS 12:2010. The bacteriocin did not inhibit the indicator microorganism *Penicillium* sp. in yogurt with addition of PB (sample 3) and it concentration was equal (2.3×10^2 cfu/g) to the yogurt without PB (sample 2).
Experimental results obtained on the 7-th day of monitoring period showed that *Lactobacillus bulgaricus* and *Streptococcus thermophilus* retained a high concentration of viable cells, indicating that bacteriocin did not inhibit LAB and did not affect their normal ratio (1:5), which was relatively constant until the end

of the second week. The titratable acidity of all samples increased slightly, except a control sample in which the increase in acidic degrees was most pronounced. A weak inhibition of the test microorganism *Penicillium* sp. in the yogurt with addition of bacteriocin (sample 3) was observed.

On the 14-th and 21-st days of observation period, an insignificant decrease of the viable lactobacilli and streptococci in the control yogurt sample and bacteriocin-treated sample (sample 1) was detected, which maintained a relatively high concentration of viable cells and stable ratio (1:5 – 1:6). Experimental data showed that the titratable acidity of the control yogurt sample reached 130°T on the 21-st day, while the acidity of sample with addition of bacteriocin (sample 1) remained relatively low - 110.8°T. It was found that bacteriocin of *B. methylotrophicus* BM47 inhibited effectively the indicator fungus *Penicillium* sp. and significantly reduced its concentration from 3.7×10^2 cfu/g (sample 2) to 10 cfu/g in the yogurt treated with bacteriocin (sample 3) (Fig. 1).

The results of the microbiological analyses demonstrated on the 28-th day of storage, the number of LAB decreased with 2-3 log units and their ratio changed to 1:7 with a prevalence of *S. thermophilus* due to the faster death of *Lb. bulgaricus* cells in the end of storage of fermented milk products. Titratable acidity of all samples at the end of monitoring period reached values between 130°T and 134.6°T, which was in the allowable limit of 150°T according to the BSS 12:2010. The same trend of effective inhibition of the test fungus *Penicillium* sp. in the yogurt with bacteriocin (sample 3) was observed.

Table 1 Effect of PB of *B. methylotrophicus* BM47 on some microbiological and physicochemical parameters of Bulgarian yogurt

Day	Parameter	Samples			
		Yogurt (control)	1	2	3
			Yogurt + PB	Yogurt + *Penicillium* sp.	Yogurt + PB + *Penicillium* sp.
1	**LB** (cfu/g)	1.0×10^8	1.0×10^8	n. a.	n. a.
	ST (cfu/g)	5.0×10^8	5.0×10^8	n. a.	n. a.
	Yeasts (cfu/g)	<10	<10	<10	<10
	Fungi (cfu/g)	<10	<10	2.3×10^2	2.3×10^2
	TA (°T)	90.6 ± 0.04*	90.6 ± 0.04	90.6 ± 0.04	90.6 ± 0.04
7	**LB** (cfu/g)	9.0×10^7	6.6×10^7	n. a.	n. a.
	ST (cfu/g)	4.5×10^8	3.3×10^8	n. a.	n. a.
	Yeasts (cfu/g)	<10	<10	<10	<10
	Fungi (cfu/g)	<10	<10	2.5×10^2	1.5×10^2
	TA (°T)	99.9 ± 0.05	92.2 ± 0.06	94.1 ± 0.06	93.4 ± 0.05
14	**LB** (cfu/g)	4.0×10^7	3.0×10^7	n. a.	n. a.
	ST (cfu/g)	2.0×10^8	1.5×10^8	n. a.	n. a.
	Yeasts (cfu/g)	<10	<10	<10	<10
	Fungi (cfu/g)	<10	<10	2.8×10^2	60
	TA (°T)	129.3 ± 0.06	106.7 ± 0.06	110.7 ± 0.05	108.7 ± 0.05
21	**LB** (cfu/g)	2.4×10^6	1.7×10^6	n. a.	n. a.
	ST (cfu/g)	1.5×10^7	1.0×10^7	n. a.	n. a.
	Yeasts (cfu/g)	<10	<10	<10	<10
	Fungi (cfu/g)	<10	<10	3.7×10^2	10
	TA (°T)	130.0 ± 0.1	110.8 ± 0.1	120.5 ± 0.1	126.0 ± 0.1
28	**LB** (cfu/g)	1.5×10^5	1.2×10^5	n. a.	n. a.
	ST (cfu/g)	1.0×10^6	1.0×10^6	n. a.	n. a.
	Yeasts (cfu/g)	<10	<10	<10	<10
	Fungi (cfu/g)	<10	<10	3.7×10^2	10
	TA (°T)	134.6 ± 0.1	133.3 ± 0.1	129.7 ± 0.1	130.0 ± 0.1

Legend: PB – purified bacteriocin; **LB** - *Lactobacillus bulgaricus*; **ST** - *Streptococcus thermophilus*; **TA** - titratable acidity; **n. a.** – not applicable; * - ± SD – standard deviation (n=3).

Yeasts and fungi as a side microflora or contamination in all samples until the end of storage period were not detected. It was found that bacteriocin of *B. methylotrophicus* BM47 did not affect the growth of LAB and did not change the organoleptic properties (colour, smell and consistency) of yogurt. Therefore, the results of this study revealed the potential possibilities for application of bacteriocin of *B. methylotrophicus* BM47 as an agent for a biopreservation of Bulgarian yogurt.

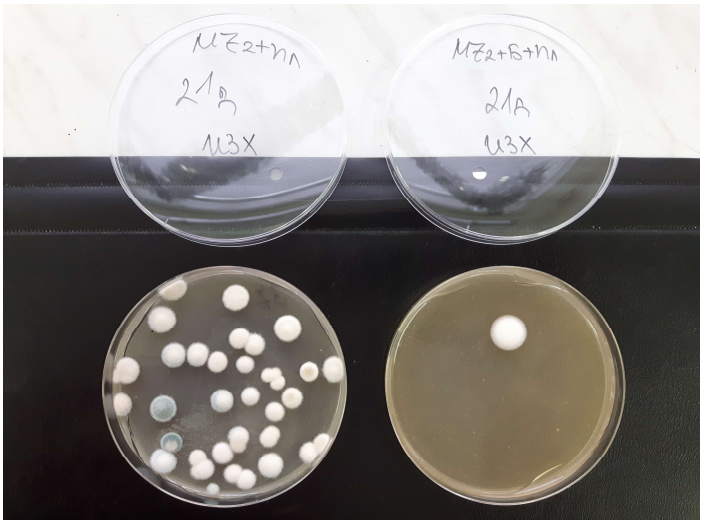

Figure 1 Antifungal effect of the PB of *B. methylotrophicus* BM47 in yogurt on the 21-st day of storage – sample inoculated with *Penicillium* sp. (left); sample inoculated with *Penicillium* sp. and addition of PB in dose 1AU/mL (right)

The significant antifungal activity of the bacteriocin of *B. methylotrophicus* BM47 was demonstrated in our previous study, in which besides on *Penicillium* sp. this bacteriocin showed inhibitory effect on fungi from the genera *Aspergillus* sp. and *Fusarium* sp. with the same values of MIC – 2.5 mg/mL, and revealed promising potential as biopreservative of raw cow's milk **(Tumbarski *et al.*, 2018)**.

Filamentous fungi are among the main spoilage microorganisms of different stored foods such as fermented dairy products (cheese and yogurt), bread, stored crops and animal food. Presently, the contamination of food with various fungal species is a serious problem due to three main reasons: a) alteration of the texture profile and external aspect of affected products; b) production of mycotoxins, conidia, mycelia and spores during fungal growth, which is related to some health risks such as allergic conditions; c) significant economic loses for the food industry. These problems could be overcome by adoption of different advanced approaches that prevent the growth of spoilage microorganisms and extend the shelf-life of milk products. The shelf-life of various cultured milk products could be enhanced by application of a single or a combination of the following biopreservation techniques: a) use of suitable starter cultures containing lactobacilli with proven antifungal activity - *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus coryniformis* subsp. *coryniformis*, *Lactobacillus sanfrancisensis*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus lactis* subsp. *cremoris* **(Tropcheva *et al.*, 2014b; Salas *et al.*, 2017)**; b) application of natural antimicrobial compounds such as bacteriocins (nisin) or bacteriocin-like substances (BLIS) from various bacterial species/strains (MicrogardTM, Lacticin 3147, Leucocin F10 and pediocin AcH); c) use of non-toxic and generally recognised as safe (GRAS) antifungal antibiotics obtained from different microorganisms such as Natamycin from *Streptomyces natelensis* **(Sarkar, 2006; Hasan *et al.*, 2008).**

Some recent studies concerning the bioprotection of food described the possibilities for application of bacteriocins against different spoilage microorganisms and their effectiveness as biopreservatives of yogurt. **Misirlilar *et***

***al.* (2012)** reported the inhibitory effect of nisin (produced by *Lactococcus lactis* subsp. *lactis*) on undesirable yeasts and fungi, and its application as GRAS agent for extension of shelf-life without changes of the sensory properties of yogurt. The application of nisin against spoilage microorganisms in fermented dairy products is exploited also by other authors. **Gupta and Prasad (1989)** found that nisin at a concentration of 25 RU/g enhanced the shelf-life of stirred yogurt from 5–10 days at 7°C without significant change in its sensory properties such as flavor, body, texture and consistency. Nisin and nisin producing microorganisms were also found effective in biopreservation of *dahi* (traditional fermented milk product typical for South Asian countries) during storage at 25°C **(Rajmohan and Prasad, 1995)** and addition of nisin in *dahi* at a dose of 15 RU/g retained all its desirable characteristics up to 35 days at 15±1°C **(Kumar *et al.*, 1998)**.

CONCLUSION

This is the first report performing the application of a bacteriocin synthesized by *B. methylotrophicus* BM47 as an antifungal agent in biopreservation of traditional Bulgarian yogurt. The addition of bacteriocin in milk for production of yogurt led to effective inhibition and reduction of the number of test microorganism *Penicillium* sp. in experimentally contaminated yogurt samples during four weeks of storage at 4°C, without alteration of their organoleptic characteristics. Therefore, the bacteriocin isolated from *B. methylotrophicus* BM47 could be considered as a potential agent for biopreservation of Bulgarian yogurt and suitable for application in the dairy industry against spoilage microorganisms of fungal origin.

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The authors declare that no conflict of interest exists.

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