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GROWTH KINETICS AND BACTERIOCIN PRODUCTION BY THREE ENTEROCOCCUS STRAINS UNDER LOW-TEMPERATURE STRESS

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ABSTRACT

AIM: The production of bacteriocins under temperatures close to those that can be found during conservation of milk products had to be investigated in order to retrieve maximal advantage of these naturally occurring anti-spoilage agents. METHODS: Cultivations of the BLIS producer strains were performed at 8 ºC, and the cell number and the BLIS activities were determined at regular intervals. The growth parameters were analyzed by modeling with appropriate software. RESULTS: 1) low temperatures significantly reduce the BLIS activity of two of the strains, *Enterococcus faecium* 3587 and *Enterococcus faecalis* 3915, while in the third strain, *Enterococcus durans* M-3, it was stimulated; 2) For all strains bacteriocin activity was higher when cultivation was performed on MRS broth rather than on M-17, and 3) the three strains showed the typical growth for *Enterococci* in conditions of low temperature cultivation.
CONCLUSIONS: Temperature stress can up-regulate or down-regulate the BLIS synthesis depending on the strain.

Keywords: bacteriocin-like inhibitory substances (BLIS), lactic acid bacteria (LAB), probiotics

INTRODUCTION

Enterococci are Gram-positive *cocci* which often occur in pairs and chains and are difficult to distinguish from *Streptococci* on physical characteristics alone. Two species are common commensal organisms in the intestines of humans: *Enterococcus faecalis* and *Enterococcus faecium*. *Enterococci* are facultative anaerobic organisms.
Members of the genus *Enterococcus* are among the lactic acid bacteria (LAB) that are of importance in dairy products. Furthermore, LAB are an abundant source of diverse antibacterial substances, including organic acids, hydrogen peroxide and bacteriocins, which play significant role in food preservation. Bacteriocins are membrane-depolarizing pore-forming toxins with protein nature that are ribosomally synthesized and excreted extracellularly. They are extensively studied from both scientific and industrial point of view due to their possible usage as natural food additives for elimination of spoilage and pathogenic microflora. BLIS production is tightly connected with the growth kinetics of the producing strain which makes predictive modeling a promising field of food microbiology.
Recently, three *Enterococcus* strains isolated from various traditional Bulgarian yellow cheese starter cultures were found to produce three different bacteriocins. Two of them, *Enterococcus durans* M-3 and *Enterococcus faecium* 3587 are already described (1, [2]), while the third, *Enterococcus faecalis* 3915, is still under investigation. Their growth characteristics, bacteriocin production under low temperature conditions in particular, are subject of professed interest. The data gathered in this study are also of practical interest due to the observation that BLIS production is present under low temperatures corresponding to those of refrigerator chambers usually used for dairy product storage. In order to interpret the observed

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data, the logistic growth model was used, as it shows best concurrence with experimental values. Growth curves of the three strains under low temperatures were built and growth parameters were determined.

MATERIALS AND METHODS

Bacterial strains and media. All bacterial strains and their suppliers are enumerated in Table 1. All *Enterococcus* species were grown on M-17 broth, either agar or liquid media (Merck KGaA, Scharlau Chemie S.A.). If not mentioned otherwise, the incubation was performed at 30°C for 14-16 hours. All media were prepared as described by the suppliers.

Table 1. *Bacterial strains and suppliers*

Bacterial strain	Supplier
Enterococus durum M-3	1
Enterococcus faecium T81A	2
Enterococcus faecium 3587	1
Enterococcus faecalis 3668	1
Enterococcus faecalis 3915	1

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Growth curves, kinetics and bacteriocin production. The growth characteristics of the three producer strains were obtained by inoculating 80 µl of overnight cultures in 80 ml M-17 broth and 80 ml MRS broth. Cultures were allowed to grow at 8°C. The OD_{600} (optical density at 600 nm wavelength) of all cultures was measured every 24 hours till the 30th day by taking of aliquots. After each measurement of the OD_{600} 1 ml of a cell free culture supernatant was obtained by filtration and the bacteriocin activity was expressed in AU/ml.

Bacterial cell number/OD_{600} plotting. In order to find the total cell number corresponding to 1 OD_{600} unit a 1% inoculated 15 ml culture of one arbitrary chosen strain was allowed to develop at 37 ºC, and the OD_{600} was measured several times in Ultrospec 1000E spectrophotometer (GE Healthcare) after vigorous vortexing for 2-3 seconds. After each measurement the total cell number in the probes was estimated by the 10-fold serial dilutions method.

Software used for mathematical modeling. For fitting the data with nonlinear regression Sigma Plot 9.0 was used (Systat Software Inc). Equation solving and finding of the derivatives needed for the growth parameter determination were performed with MatLab 6.5 (Math Works).

Determination of bacteriocin titers. The titers of the produced bacteriocin were quantified by two-fold serial dilutions of the supernatant probes or precipitates. Aliquots of 100 µl were placed in wells seeded with the bioassay strain. The antimicrobial activity was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in activity units per ml (AU/ml) (3).

Determination of bacteriocin activity. A well diffusion assay procedure was used. Pre-cooled at 30°C 0,7% agar media were contaminated with the tested bacterial strains, laid on Petri dishes and allowed to solidify. After 20-30 min the desired number of wells with diameter of 5 mm was made with a sterile hollow ([4]). Aliquots of 100 µl from each bacteriocin probe were placed into the wells of the plates seeded with the bioassay strain. After 12-18 hours of incubation at optimal for the tested strain temperature, clear zones of inhibition appeared where the strain was sensitive.

RESULTS AND DISCUSSION

Models are used to describe the behavior of microorganisms under different conditions such as temperature, which is a very important variable for bacteriocin synthesis.

Low temperatures are of particular importance in view of the usual storage of measured and modeled. Bacterial growth often shows a phase in which the specific growth rate starts at a value of zero and then accelerates to a maximal value for a certain period of time. In addition, growth curves contain a final phase in which the rate decreases and finally reaches zero.

Proposed is a logistic regression model which enables one to model the boundary between growth and no growth for bacterial strains in the presence of one or more growth controlling factors such as temperature. The modeling was performed as previously described ([5]).

Table 2 represents the statistical parameters describing the arbitrary chosen *Enterococcus faecalis* 3915 probe growth at different temperatures as an example. The statistic parameters such as the correlation coefficient (R), the coefficient of determination (R^2), and adjusted R^2 are represented. These parameters show how well the logistic model describes the data observed. The calculated values close to 1 show that good fittings with the experimental growth data were obtained.

dairy products in chilling conditions. In order to build such models, growth has to be The model showed no difficulties for predicting the values in the log phase, the only deviations being found at the beginning of the lag phase.

In LAB, pH and temperature have been found to influence bacteriocin production (6, 7, 8), and De Vuyst et al. (9) suggested that amylovorin synthesis was enhanced by stress. However, the mechanisms by which bacteriocin production is regulated by environmental factors have not been studied. As it can be seen from Figure 1, low temperature stress can influence bacteriocin production in opposite directions. If the BLIS titers at the beginning of the stationary phase of development are considered, for *Enterococcus durans* M-3 bacteriocin synthesis was increased, while for *Enterococcus faecium* 3587 and *Enterococcus faecalis* 3915 it was decreased (Table 3). It was also observed that the cultivation medium, too, has impact on bacteriocin production, the last being generally higher when cultivation was performed on MRS broth.

Table 2. *Statistical parameters describing Enterococcus faecalis 3915 growth at different temperatures as an example*

Temperature of incubation	Maximal growth rates (μ_m)	R	R^2	Adjusted R^2
8 ºC (M17)	0,037623	0,9999	0,9998	0,9997
8 ºC (MRS)	0,036627	0,9999	0,9999	0,9998
25 ºC	0,666727	0,9997	0,9995	0,9989
30 ºC	0,858135	0,9997	0,9993	0,9986
33 ºC	0,944936	1	0,9999	0,9998
37 ºC	1,170793	0,9998	0,9997	0,9993
42 ºC	1,271383	1	0,9999	0,9999

Table 3. *Bacteriocin titers at the beginning of the stationary phase*

Bacteriocin Producer Strain	Bacteriocin titer at the beginning of the stationary phase [AU/ml]			
	37 ºC		8 ºC	
	M-17 broth	MRS broth	M-17 broth	MRS broth
Enterococcus durans M-3	1600	1600	2560	5120
Enterococcus faecium 3587	40	80	5	20
Enterococcus faecalis 3915	40	40	10	0

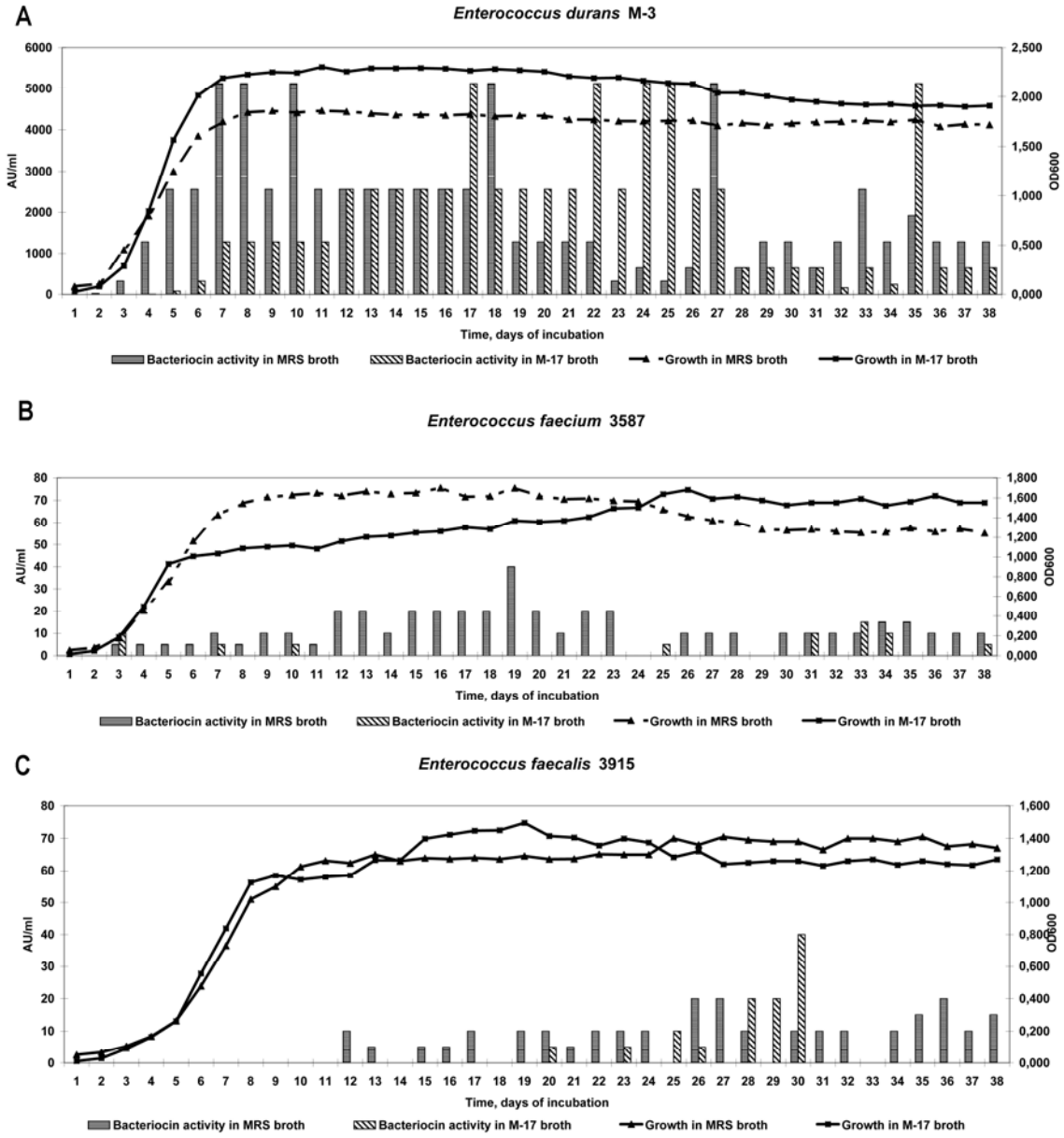

Figure 1. Growth and bacteriocin production at 8 ºC. A – *Enterococcus durans* M-3; B – *Enterococcus faecium* 3587; C – *Enterococcus faecalis* 3915. Growth is monitored by the OD_{600} extinction while BLIS activity is expressed in arbitrary unites per milliliter [AU/ml].

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