

IN VITRO AND IN SITU BACTERIOCIN ACTIVITY OF LACTIC ACID BACTERIA FROM BULGARIAN DAIRY PRODUCTS AND METHODS FOR MAKING OF *LACTOBACILLUS* PROTECTIVE FERMENTED MILKS WITH BACTERIOCIN INHIBITORY SUBSTANCES

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Abstract

SIMOVA, E. D., D. M. BESHKOVA, Z. P. DIMITROV and Z. I. SIMOV, 2008. *In vitro* and *in situ* Bacteriocin activity of lactic acid bacteria from Bulgarian dairy products and methods for making of *Lactobacillus* protective fermented milks with Bacteriocin inhibitory substances. *Bulg. J. Agric. Sci.*, 14: 28-42

The studies are based on two theses: 1) Fermented milks are the shortest route and the most appropriate way to assimilate the biologically active substances of lactic acid bacteria (LAB); 2) The most efficient way to introduce bacteriocins in a milk product is by using bacteriocin-producing starter cultures. For the purpose of proving and evaluating the potential application of bacteriocin-producing probiotic LAB as protective cultures two natural milk strains (*Lactobacillus bulgaricus* BB18 and *Lactococcus lactis* BCM5) with the highest and widest spectrum of antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria selected from 1428 LAB strains isolated from authentic home-made Bulgarian fermented milk products and kefir grains were investigated for bacteriocinogenesis in milk. The strains exhibited highly efficient bacteriocinogenesis (1600 – 2000 BU ml⁻¹) and growth activity (10¹¹ – 10¹² CFU ml⁻¹) in milk. *Lb. bulgaricus* BB18 strain with the widest spectrum of bacteriocin activity against Gram (-) pathogenic bacteria (including *Helicobacter pylori*) was used for protective yogurt starter formation, in which bacteriocin yield was increased by 32% and bacteriocinogenesis was shortened by 3 h through stimulated bacteriocinogenic and growth activities. Temperature stress and *Lb. bulgaricus* BB18 growth at a lower than the optimal growth temperature (30°C) induced an increase in bacteriocin yield (3600 BU ml⁻¹) in the starter. Methods for making protective yogurt and ultrafiltrate (UF) fermentate with high concentration of the bacteriocin-inhibitory substances and viable probiotic cells were developed.

Key words: Bacteriocin activity; *Lactobacillus*; *Lactococcus*; Protective fermented milks; Inhibitory spectrum

Introduction

The fact that the gastrointestinal flora is closely related to the host's state of health indicates that its balance is of crucial importance for wellness and longevity (Hosono, 2001; Danone Nutritopics, 2004). A major priority of current scientific research worldwide, seeking to relate diet (probiotics in particular) to beneficial effects on health, is to improve the balance of intestinal microflora and intestinal transit with the aid of substances able to inhibit certain pathogens (Danone Nutritopics, 2002; Pernoud et al., 2005). Making use of antibiosis of lactic acid bacteria is the best choice for obtaining probiotics, due to their natural adaptation to the intestinal environment. The production of fermented milks, supplemented with probiotic microorganisms, has never attracted such a great interest so far, and current research involves mainly lactic acid bacteria with specific characteristics for specific purposes.

Since the 1980s, the term probiotic has been used to denote microorganisms with health benefits (Bongaerts and Severijnen, 2001; O'Sullivan et al., 2002). Probiotic milks are regarded as fermented milks enriched with microorganisms, with beneficial effect on the balance or re-growth of the normal microbiota in the intestinal tract (Saarela et al., 2000; Ouwehand et al., 2002). Yogurt is defined as fermented milk obtained by specific lactic acid fermentation, through the action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Sanders, 1994). Other lactic acid bacteria (LAB), in particular those from the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, can be combined with yogurt starters to produce fermented milk with specific desirable characteristics, such as probiotic properties (Robinson, 1991). These ingested LAB partially resist gastric acidity and bile salts and therefore pass live through the gastrointestinal tract (Marteau et al., 1997) where they may influence the metabolism and equilibrium of endogenous microflora.

The authors focus on the supposed mechanisms

through which select *Lactobacillus* and *Bifidobacterium* strains act against microbial pathogens *in vitro* and *in vivo*: competitive inhibition of epithelial and mucous adhesion of pathogens; production of antimicrobial substances; and/or stimulation of the mucous immunity; they are, however, manifested for a small number of selected strains (Blum and Schiffrin, 2003; Servin, 2004). The synthesis of bacteriocins or bacteriocin-like substances is rarely associated with these antagonistic effects (Sgouras et al., 2004). Some authors accentuate, however, on the possible production of these substances *in vivo*, and suggest they may be one of the antagonistic mechanisms used by bacteria and normal microflora for preventing the colonization by harmful microorganisms (Hudault et al., 1997). The antimicrobial substances produced by lactic acid bacteria render them more advantageous in the competition with other microorganisms (Soomro et al., 2002). Bacteriocins have been defined as "extracellularly released primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity, characterized by inclusion of at least some strains of the same species as the producer bacterium and against which the producer strain has some mechanism(s) of specific protection" (Jack et al., 1995). It has been established that the target of bacteriocins is the cytoplasmic membrane of the sensitive pathogens and other bacteria (Servin, 2004). Emphasis is placed on the so-called "attack" mechanisms, in which the inhibitory peptide adheres to certain targets in the cytoplasmic zone of bacteria (Shai, 2002). Some antibacterial peptides develop a mechanism of action close to that of antimicrobial peptides in the host (Helander and Mattila-Sandholm, 2000). It has been reported that the biological mechanism leading to anti-*Helicobacter* activity is affected by bacteriocins (Kim et al., 2003). Lactobacilli have been shown to secrete unique bacteriocins, bacterially-produced antibiotics (Vaughan et al., 2001). Bacteriocins, such as nisin, are capable of killing selective Gram-positive

bacteria (Cleveland et al., 2001). Lactobacilli could also secrete bacteriocins against *H. pylori* and other Gram-negative bacteria (Oh et al., 2002).

The main problem with using bacteriocin-producing starters in food fermentations, according to some authors, is related to the *in situ* antimicrobial efficiency that can be adversely affected by various factors such as binding of bacteriocins to food components, inactivation by proteases, alterations in solubility and charge, alterations in the cell membrane of the attacked bacteria (Ganzle et al., 1999; Aasen et al., 2003). There are reports on *in situ* activity by lactobacteria during sourdough fermentation (Corsetti et al., 2004; Settanni et al., 2005). There are no reports on *in situ* production of inhibitory peptides in milk, especially from starter strains of lactobacteria representatives of the microflora of dairy products.

According to many authors, exogenously applied probiotics do not become implanted in the intestinal tract (Bezkorovainy, 2001; Cesena et al., 2001; Seegers, 2002; Danone Nutritopics, 2002; De Camps et al., 2003) and do not colonize it (Cesena et al., 2001; Bezkorovainy, 2001). Only temporary colonization can be achieved (presence of probiotic bacteria in the intestinal tract) in the gastrointestinal tract with exogenous probiotic bacteria (Bottazzi, 2006) upon consumption of a probiotic culture, during which time the probiotic continues to be metabolically active and to confer health benefits (Fooks and Gibson, 2002; Seegers, 2002). In relation to the above, it is reasonable to seek to increase the role of bacteriocins produced by lactic acid bacteria in their antagonism against pathogenic microorganisms, and to accept bacteriocinogenesis as a main factor of probiotic activity. Our investigations are directed towards enhancing the protective effect of lactic acid bacteria by means of: (i) production of bacteriocins with high inhibitory effect (bacteriostatic and bactericidal) against pathogenic bacteria; (ii) high concentration of active viable cells of probiotic bacteria in fermented milks aimed at temporary colonization of the probiotic dur-

ing passage along the gastrointestinal tract; (iii) expanding the probiotic potential of lactic acid bacteria, and the range of healthy fermented milks, using natural "dairy" strains of bacteria (originating from and intended for dairy products) that satisfy probioticity criteria.

The aim of this study was to: (i) investigate *in vitro* and *in situ* bacteriocin activity of selected natural milk strains isolated from authentic Bulgarian milk products and evaluate their possible utilization as starter protective cultures (individual and associated with other LAB strains); (ii) propose methods for making *lactobacillus*-protective fermented milks with bacteriocin-inhibitory activity for potential prophylactic prevention or therapeutic treatment of *H. pylori* and other pathogenic infections. Another aim of the paper was to show that the traditional microflora of Bulgarian dairy products, which does not colonize the intestinal tract, has a potential for health benefits through high concentrations of bacteriocins and live cells.

The studies are based on two proven theses: 1) fermented milks are the shortest route and the most appropriate way to assimilate the biologically active substances of lactic acid bacteria; 2) the most efficient way of incorporating bacteriocins in a milk product is the use of a bacteriocin-producing starter culture.

Materials and Methods

Strains and growth conditions

The strains *Lb. bulgaricus* BB18 and *Lc. lactis* BCM5 used in this study were selected by screening for bacteriocin activity of 1428 LAB strains isolated from traditional Bulgarian dairy products (home-made fermented milks and cheese produced from raw milk in ecological regions of Rhodopi Mountains). The LAB strains were stored in MRS lactobacilli broth (Fluka, Buchs, Switzerland) with 20% glycerol, M17 lactococci broth (Fluka, Buchs, Switzerland) or skim milk/glycerol medium at -80 °C. They were subcultured

twice in MRS/M17 broth or in 12% reconstituted skim milk (RSM) supplemented with 1% glucose (Fluka, Buchs, Switzerland) and 1% yeast extract (Fluka, Buchs, Switzerland) prior to experimental use. For the experiments, MRS broth (pH 5.4), M17 broth (pH 6.0) and 12% RSM enriched with 1% glucose and 1% yeast extract were used as growth media for inoculum. Bacteria were grown under microaerophilic conditions (10% CO₂, 80% N₂, and 10% H₂). 2% of the overnight subcultures of bacteriocin-producing strains were used and propagated for preparation of the inocula as follows: *Lb. bulgaricus* BB18 was grown in MRS broth and in 12% RSM supplemented with 1% glucose and 1% yeast extract at 37 °C for 16 h and 10 h respectively; *Lc. lactis* BCM5 – M17 broth and 12% RSM at 30 °C for 16 h.

11 yogurt starters containing bacteriocin-producing *Lb. bulgaricus* BB18 strain and 11 technological *S. thermophilus* strains resistant to bacteriocin produced by *Lb. bulgaricus* BB18 were screened for yogurt starter with high bacteriocin-producing activity in milk and highly viable cell concentration of *Lb. bulgaricus* BB18. The strain cultures were grown continuously (4 months) in association in sterilized milk (1% inoculum of each culture in a ratio *Lb. bulgaricus*: *S. thermophilus* = 1:1; incubated at 37 °C to pH 4.8 until meeting the criteria for a Bulgarian yogurt starter culture). The main criterion for selecting a starter culture including the bacteriocin-producing strain *Lb. bulgaricus* BB18 was the activity of bacteriocin production during mixed cultivation in milk.

12% RSM enriched with 1% glucose and 1% yeast extract was used as growth medium for preparation of individual and work inocula. A 1% (v/v) of each culture (11-h-old milk culture of *Lb. bulgaricus* BB18 and 6-h-old milk culture of *S. thermophilus* 11A) was propagated for 11 h and 6 h respectively at 37 °C for preparation of individual inocula. The yogurt inoculum was prepared as follows: The 12% RSM supplemented with 1% glucose and 1% yeast extract was inoculated with 1% (v/v) of individual *Lb. bulga-*

ricus BB18 inoculum and 1% (v/v) *S. thermophilus* 11A inoculum and incubated at 37 °C for 8 h.

Bacteriocin production in milk. Protective yogurt and protective UF fermentate production

2% (v/v) inoculum of each lactic strain was cultivated as follows: *Lb. bulgaricus* BB18 in MRS broth and 12% RSM at 37 °C for 24 h; *Lc. lactis* BCM5 in M17 broth and 12% RSM at 30 °C for 24 h.

Yogurt inoculum *Lb. bulgaricus* BB18 + *S. thermophilus* 11A (1:1) (2%) was cultivated in 12% RSM at 37 °C for 24 h; at 37 °C for 4 h and after cooling to 30 °C the fermentation continued at 30 °C to pH 4.8-4.9.

The parameters for yogurt and UF fermentate preparation are given in Figures 3 and 4.

The ultrafiltrate was obtained from whey (Milk Industry, Plovdiv, Bulgaria) from the manufacture of white brined cheese, and deproteinized on a Lab 38 DDS (Nakskov, Denmark), on GR61PP (Nakskov, Denmark) membranes. The ultrafiltrate was used in its native state (46.0 g lactose l⁻¹), supplemented with 1% glucose (Fluka, Buchs, Switzerland) and 0.3-0.5% amino acids from whey proteins (Ultra N.R.S. Perfect, Nutrim, Bulgaria).

Starter bacteria were cultivated in a MBR AG Ltd (Zurich, Switzerland) bioreactor (2 l) with repeated agitation, without oxygen for 24 h in experiments for bacteriocin production, and 9 h and 12 h – for protective yogurt and UF fermentate production, respectively.

Analytical methods

Culture growth, bacteriocin activity and pH were checked at every second hour.

The growth was evaluated by determination of colony-forming units (CFU) (IFD Standard 117B, 1997). The number of CFU ml⁻¹ was quantified using the plate dilution method and media for enumeration of bacteria as follows: MRS agar (Fluka, Buchs, Switzerland), Rogosa CW agar (Fluka, Buchs, Swit-

zerland) and RCPBpH5 agar (for differentiation of *lactobacillus* and *streptococcus*) (Sigma, Buchs, Switzerland) – for lactobacilli; M17 agar (Fluka, Buchs, Switzerland) and Streptococcus selective agar (Merck, Darmstadt, Germany) – for cocci.

The antimicrobial activity was detected by the well-diffusion method described by Guerra and Pastrana (2002). The bacteriocin activity was determined against the indicator strains *Listeria monocytogenes* C12 and *Lactococcus lactis* ssp. *cremoris* CRX9. The indicator strain *L. monocytogenes* C12 was obtained from section “Pathogens” (Institute of Microbiology, Bulgarian Academy of Sciences). The strain *Lc. cremoris* CRX9 belonged to the culture collection of the Laboratory of Applied Microbiology (Institute of Microbiology, Bulgarian Academy of Sciences). The indicator strains were grown as follows: *L. monocytogenes* C12 in Standard Nutrient Broth (Merck, Darmstadt, Germany) at 30 °C for 24 h; *Lc. cremoris* CRX9 in M17 broth (Fluka, Buchs, Switzerland) at 30 °C for 24 h.

After 20 h incubation at 30 °C and 37 °C of bacteriocin-producing LAB strains, the cells were removed by centrifugation (12000 x g, 15 min, 4 °C). To eliminate the inhibitory effect of lactic acid and/or H₂O₂, the culture supernatants were treated with 1N NaOH and with catalase (Sigma, Burch, Switzerland) (1 mg·ml⁻¹) (0.01 M phosphate buffer, pH 6.5) followed by filtration through a 0.22 µm pore size filter (Sartorius, Goettingen, Germany) to eliminate the possible presence of viable cells. Fifteen milliliters of appropriate culture medium (Standard Nutrient Agar or M17 agar) containing 0.7% (w/v) agar was inoculated (1%, v/v) with indicator strain at a final cellular concentration of about 10⁷ CFU ml⁻¹, poured into Petri dishes, and allowed to solidify at room temperature. Wells (10 mm in diameter) were cut into the agar and 100 µl of prepared cell free supernatant of the potential producer strains was placed into each well. Plates were refrigerated (4 °C) for 4 h to allow the radial diffusion of the compounds contained in the superna-

tant and later were incubated at the optimal growth temperature for the indicator strain for 24–48 h and the inhibition zones were measured. Activity was expressed as bacteriocin units per milliliter (BU ml⁻¹). One BU was defined as the amount of bacteriocin which inhibited growth of the indicator strain showing a clear zone of growth inhibition at the highest dilution.

Statistical analysis

All the experiments were carried out in three independent experiments and the results are shown as mean ± s.d.

Results

In previous studies 1428 LAB strains isolated from authentic home-made Bulgarian fermented product (yogurt and yogurt-type milks, Kashkaval cheese produced from raw milk in ecology regions and from kefir grains) were screened for bacteriocins against pathogenic indicator strains (Simova et al., 2006) Three LAB strains classified by means of genetic (PFGE and ARDRA) analyses as *Lb. bulgaricus* (strain BB18), *Lc. lactis* (strains BCM5 and BK15), with highest antimicrobial activity and a wide inhibitory spectrum were selected. The inhibitory substances were biologically active peptides (bacteriocins). The antagonistic effects on pathogenic microorganisms of the selected LAB strains, their high resistance to acidic conditions, bile, phenol, lizosym, and their antibiotic susceptibility were amongst the essential criteria applied for selection and guaranteed their probiotic profiles (Simova, 2007).

Preliminary studies on the inhibitory effect of bacteriocin contained in the supernatant of *Lb. bulgaricus* BB18-fermented milk and of purified bacteriocin produced by *Lb. bulgaricus* BB18 (bulgaricin BB18) showed that the bulgaricin BB18 is a bacteriostatic or bactericidal factor against *H. pylori* (results not shown) (Simova, 2007).

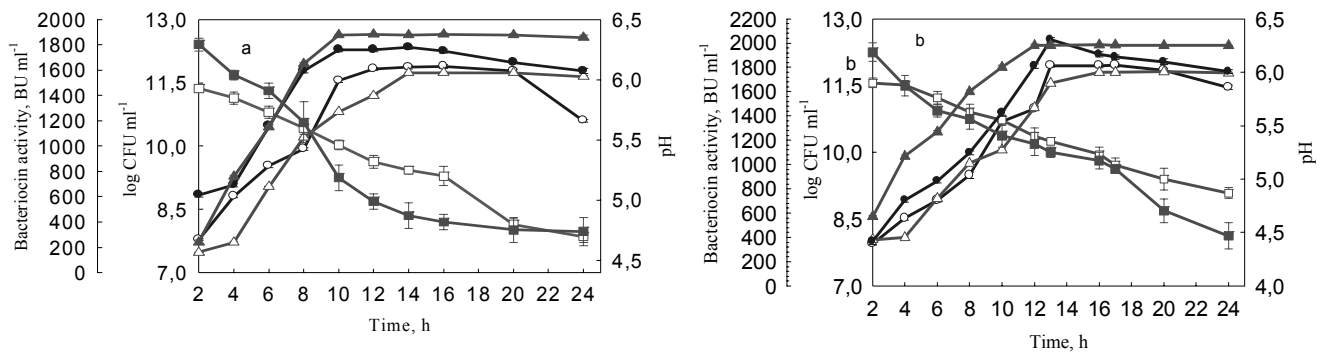


Fig. 1. Growth and bacteriocin production by *Lb. bulgaricus* BB18 in MRS medium (open symbols) and 12% reconstituted skim milk (closed symbols) at 37 °C(a), and by *Lc. lactis* BCM5 in M17 medium (open symbols) and 12% reconstituted skim milk (closed symbols) at 30 °C (b). Growth, Log CFU ml⁻¹ in MRS/M17 medium (○) and in milk (●); bacteriocin activity, BU ml⁻¹ in MRS/M17 medium (△) and in milk (▲); pH of MRS/M17 medium (□) and of milk (■). Data represent the mean±s.d. of the mean for three independent experiments

The comparison of the bacteriocinogenic characterization of the *lactobacillus* strain (*Lb. bulgaricus* BB18) and one *lactococcus* strain (*Lc. lactis* BCM5) with high inhibitory activity and wide inhibitory spectrum against Gr (+) and Gr (-) pathogenic bacteria show that bacteriocin synthesis in MRS and M17 broth occurred in parallel with cell growth in the exponential phase and started during the first hours of incubation (Figure 1 a, b). After 2 h, the recorded activity of *Lc. lactis* BCM5 was higher than that of *Lb. bulgaricus* BB18, at 380 BU ml⁻¹ and 180 BU ml⁻¹, respectively. After 12 h, bacteriocin production by *Lc. lactis* BCM5 surpassed bacteriocin production by *Lb. bulgaricus* BB18. Maximum bacteriocinogenic activity was achieved at the late log phase of growth for both producers – after 14 h of incubation for *Lb. bulgaricus* BB18 (1580 BU ml⁻¹), and 17 h of incubation for *Lc. lactis* BCM5 (1760 BU ml⁻¹). Bacteriocin production occurred in the pH range of 6.0-5.2. According to authors, the optimal pH for bacteriocin production is generally 5.5-6.0 (Chinachoti et al., 1997a). Only few bacteriocins are produced at low pH (5.0) (Börcena et al., 1998). Most probably the pH level for bacteriocin production is species- or strain-dependent.

The activity slightly decreased in the stationary phase after 18 h in the *Lb. bulgaricus* BB18 culture, and remained unchanged for 24 h in the *Lc. lactis* BCM5 culture. The decrease in the amount of bacteriocin after maximum synthesis could be due to adsorption on the cells of the producer or to degradation by specific or nonspecific proteases. The latter, however, has never been proved, although adsorption to cells typically occurs with most bacteriocins (Chinachoti et al., 1997b; Lejeune et al., 1998). Researches have observed maximum adsorption of bacteriocins to cells at pH 5.5-5.6, and a decrease at low pH (Yang and Ray, 1994). Our results, however, reveal maximum synthesis in the pH range of 6.3-5.2 without adsorption, and do not support the above authors. The reduction in the amount of bacteriocin in the late stages of the process is most probably related to the action of certain peptidases, or to the carbolytic suppression of bacteriocin production, leading to a reduction in the production rate. After 72 h, the amount of bacteriocin produced did not change.

The growth curves for the two bacteriocinogenic cultures in milk indicated active accumulation of cell mass with maximum cell concentration of *Lb. bulgaricus* BB18 2.2×10^{12} and of *Lc. lactis* BCM5 –

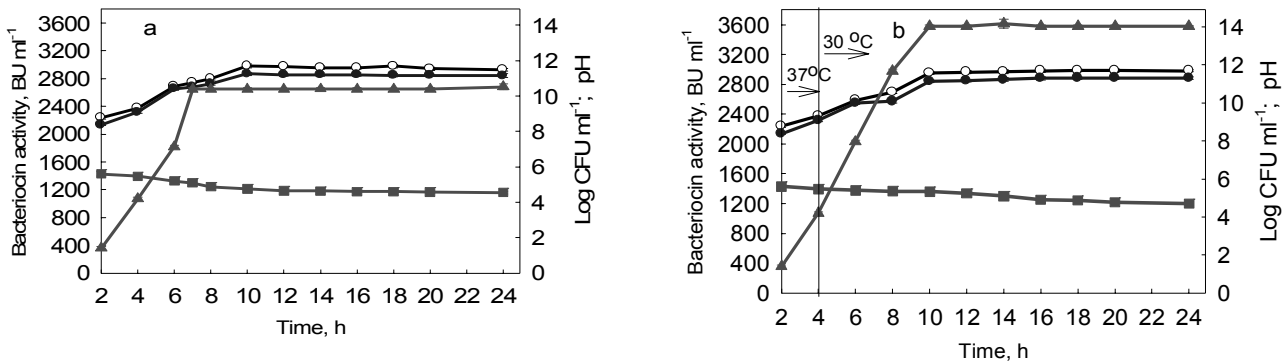


Fig. 2. Growth and bacteriocin production by *Lb. bulgaricus* BB18 grown in starter culture with *S. thermophilus* 11A at 37°C in 12% reconstituted milk (a); by *Lb. bulgaricus* BB18 grown in starter culture with *S. thermophilus* 11A, for 4 h at 37 °C, and over 4 h at 30 °C (b). Growth, Log CFU ml⁻¹ [lactobacilli (●), cocci (○)]; bacteriocin activity, BU ml⁻¹ (▲); pH of milk (■). Data represent the mean±s.d. of the mean for three independent experiments

3.4×10^{12} recorded after 10 and 13 h of growth, respectively (Figure 1a,b). The population dynamics of the lactobacilli and lactococci in milk were respectively about 3 and 4 times higher than those in MRS and M17 broths. Although MRS and M17 are rich synthetic media, propagation and growth of *Lb. bulgaricus* BB18 and *Lc. lactis* BCM5 were more active in milk. Both strains are typical lactic acid bacteria isolated from kefir grains. Prior studies showed that they participate in kefir formation and represent the dominant flora in the kefir grain. Perhaps it is their dairy origin that has a positive effect on the *in situ* produced bacteriocinogenic activity; the rate of bacteriocin production by both strains was higher in milk (Figure 1a,b). The maximum bacteriocinogenic activity of *Lb. bulgaricus* BB18 (1900 BU ml⁻¹) and of *Lc. lactis* BCM5 (2000 BU ml⁻¹) was obtained at the end of the exponential growth phase and at the beginning of the stationary phase, when maximum cell mass was recorded after 10 h and 13 h of incubation in milk, respectively.

A probiotic strain selected for its ability to produce bactericidal substances must not, due to that property, affect the growth and survival of the other strains in the product (Vinderola et al., 2002). In this con-

nection, *Lb. bulgaricus* BB18, active bacteriocin producer, was studied for strain compatibility with the technological strains *S. thermophilus* for the purpose of forming starters for yogurts, and investigating bacteriocinogenesis during their manufacture. *Lb. bulgaricus* BB18 produced bacteriocin whose inhibitory activity did not affect the viability of 11 strains of *S. thermophilus*. Eleven yogurt starters were formed with strain *Lb. bulgaricus* BB18 and with each of the *S. thermophilus* strains. The guiding criteria in the evaluation of the starter culture were two: 1) activity of bacteriocin production, and 2) physiological and biochemical activity of the starter culture (viable cells concentration and lactic acid production). Both activities did not always occur simultaneously. The starter culture *Lb. bulgaricus* BB18 + *S. thermophilus* 11A (1:1) produced the greatest amount of bacteriocin within the shortest period of time. *Lb. bulgaricus* BB18 + *S. thermophilus* 11A, on the other hand, met the criteria for strain compatibility despite the duration of protein gel formation (7 h) – maximum final cell concentration of lactobacilli 1.5×10^{12} CFU ml⁻¹, streptococci – 6×10^{12} CFU ml⁻¹; the yogurt had a thick coagulum, uniform cut surface and typical lactic acid flavour and aroma. Typically, milk with an active not

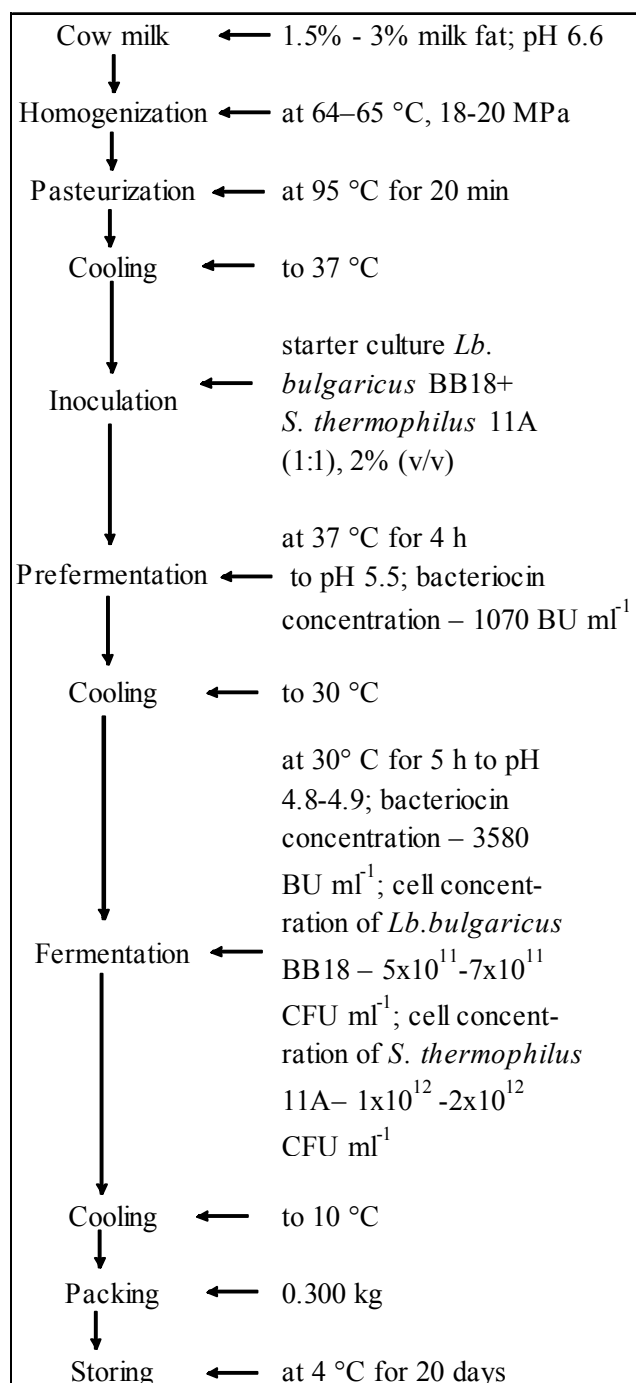


Fig. 3. Method for making of protective yogurt with bacteriocin inhibitory activity

bacteriocinogenic starter culture takes 2.5-3 h to coagulate fully to pH 4.8-4.7 at 42°C. At 37°C (optimal

temperature for bacteriocin production by *Lb. bulgaricus* BB18) as well as at 42°C the rate of lactic acid production was relatively lower than that of the *Lb. bulgaricus* cultures used in yogurt manufacture. The result is extended milk coagulation time. The duration of acidification until full coagulation of milk with starter culture *Lb. bulgaricus* BB18+*S. thermophilus* 11A is related probably to the physiology and biochemical characteristic of the strain producer *Lb. bulgaricus* BB18. Lactic acid fermentation to pH 4.8 in the single strain culture *Lb. bulgaricus* BB18 lasted 11 h, and 8 h in the mixed culture, suggesting proto-cooperation relationships between the two bacteria in the starter culture formed for probiotic fermented milk.

The initiation of the lactic acid process with the *Lb. bulgaricus* BB18+*S. thermophilus* 11A culture was characterized by active proliferation of the lactobacilli and streptococci, and after a 2-h growth the latter dominated over *Lb. bulgaricus* BB18 by one order of magnitude. Bacteriocin production in milk by *Lb. bulgaricus* BB18 associated with *S. thermophilus* 11A took place in the exponential growth phase and terminated at the end of that phase. Bacteriocin synthesis surpassed cell growth. Bacteriocin synthesis stopped after 7 h of cultivation, however a slight increase in the lactobacillus and streptococcus cell concentrations was observed up to the 12th h. Active bacteriocin production in milk by *Lb. bulgaricus* BB18 associated with *S. thermophilus* 11A occurred in the pH range of 6.0–5.0. Further cultivation with a decline in the pH level of the culture did not result in bacteriocin production. There was no production at pH below 5.0. Therefore, pH of the culture medium is an important factor for bacteriocin synthesis. Milk acidification, a result from the lactic acid production up to pH levels below 5.0 (4.75 - 4.80), after bacteriocinogenic maximum, proceeds until the formation of the protein gel and lactic acid flavour and aroma typical of probiotic yogurt. The maximum bacteriocinogenic activity produced by *Lb. bulgaricus* BB18 during associated cultivation with *S.*

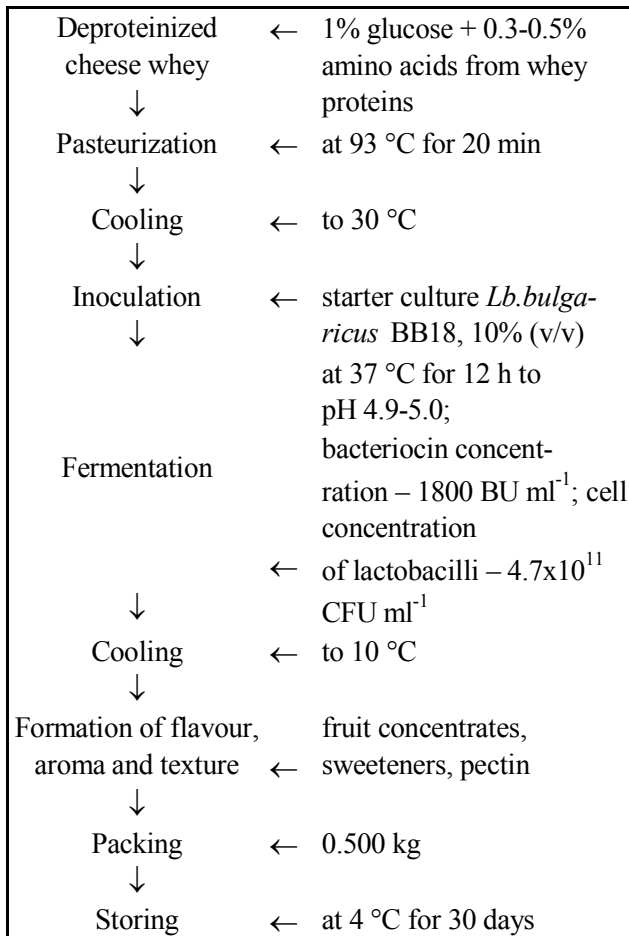


Fig. 4. Method for making of *Lactobacillus bulgaricus* protective fermentate with bacteriocin inhibitory activity

thermophilus 11A was the highest (2700 BU ml⁻¹). The bacteriocin-synthesizing activity in the mixed yogurt culture was 1.4 times higher than the maximum activity of the single strain *Lb. bulgaricus* BB18 in milk, and 1.7 times higher than that in MRS. *Lb. bulgaricus* BB18 increased bacteriocin synthesis activity during associated growth in the starter culture *S. thermophilus* 11A+*Lb. bulgaricus* BB18 by about 32% as compared to the single strain culture *Lb. bulgaricus* BB18; grown in association with *S. thermophilus* 11A, *Lb. bulgaricus* BB18 produced maximum amount of bacteriocin within a shorter pe-

riod of time – 7 h faster than the single strain culture in MRS medium, 3 h faster than the single strain culture in milk (Figure 2a,b). Once bacteriogenesis in milk had ceased after 7 h, the cell concentration continued to increase until 10 h. Despite the increase in the bacterial populations after 7 h, when maximum bacteriocin production was recorded, bacteriocin synthesis stopped. The most likely reason for that is the pH drop after 6 h of cultivation to levels below 5.0. To avoid an increase in the lactic acid concentration in the fermented milk after 6 h, and to prolong the bacteriocinogenic activity of *Lb. bulgaricus* BB18, after 4 h of cultivation of the starter culture at 37 °C the temperature was lowered to 30 °C (Figure 2b). Thus the time for milk coagulation was extended to coincide with the time of maximum bacteriocin accumulation. Under these conditions bacteriocin production activity was stimulated and maximum bacteriocin activity was obtained after 9 h of cultivation – 3600 BU ml⁻¹, which is one very high activity, by far exceeding the activities reported on bacteriocin by strains of lactic acid bacteria.

The obtained results prove the important role of temperature as a leading factor in bacteriocinogenesis, and evidence of the fact that temperature stress from higher to lower than the optimum growth temperature can enhance bacteriocin yield. A decrease in the temperature results in lower growth rates, which, according to some authors, increases bacteriocin production (Moretto et al., 2000). At 30 °C pH levels remained above 5.0 (5.5-5.35) for a longer period of time, which suggests that the pH level is of crucial importance for bacteriocin synthesis. The prolongation of bacteriocinogenesis by 2 h at 30 °C increased bacteriocin yield by *Lb. bulgaricus* BB18 by 45% compared to the single strain culture in milk, and by 30% compared to the a starter culture at 37 °C. The lactobacillus and streptococcus cell concentrations increased for 9 h and reached levels higher than the population levels of milk fermented for 7 h at 37 °C. Bacteriocin synthesis at 30 °C occurred in the exponential growth phase and

reached its maximum at the end of that phase. The present results were used as a basis for developing technologies (with single strain culture *Lb. bulgaricus* BB18 or associated with *S. thermophilus* 11A) for production of protective probiotic yogurt (Figure 3) and probiotic UF fermentate (Figure 4) containing high concentrations of viable cells and bacteriocin.

Discussion

For the purpose of proving and evaluating the potential application of bacteriocin-synthesizing lactobacteria as probiotic cultures with protective functions, two LAB strains (*Lb. bulgaricus* BB18 and *Lc. lactis* BCM5) with the highest and widest spectrum of antimicrobial activity were investigated for bacteriocinogenesis in milk. The strains were selected on the basis of screening for bacteriocinogenic activity of the 1428 LAB strains, isolated from authentic home-made Bulgarian yogurt, yogurt-like milks, kefir grains and well matured batches of Kashkaval cheese and white brine cheese, produced from raw milk in ecological regions of the Rhodopi Mountains. *In situ* bacteriocin production by the lactobacteria strains was influenced by the growth conditions. This was evidenced by the occurrence of bacteriocinogenesis in MRS/M17 broth media and in milk with individual starters. Many bacterial processes such as synthesis of extracellular proteins and natural competition are dependent on the growth phase. There are reports on the influence of the growth phase on bacteriocin production (Cheigh and Pyun, 2005; Collado et al., 2005). It was proved that bacteriocin production by the four lactobacilli and *Lc. lactis* BCM5 is related to growth, indicating kinetics of primary metabolism: 1. The activity of bacteriocin production by the five strains was strongly expressed from the onset of growth (after 2 h of incubation) indicating that bacteriocins are primary metabolites; 2. Bacteriocin production significantly increased in the exponential growth phase; 3. The highest bacteriocin activity was observed at the

end of the exponential growth phase, in which maximum cell mass was recorded; 4. Bacteriocin production stopped completely when the cells entered the stationary phase. Maximum bacteriocin-synthesizing activity was reached after 10-13 h of growth in milk.

The characteristics of the bacteriocinogenic strains in growth conditions and bacteriocin production were studied in relation to their potential application as protective fermented cultures (fermented milks). The probiotic fermented milks formed with *Lactobacillus* and *Lactococcus* strains correlate with our requirement: obtain the highest possible amount of live lactobacteria and the highest level of bacteriocin production under optimal growth conditions and bacteriocin synthesis. In order to evaluate the ability of the *Lactobacillus* and *Lactococcus* strains to produce bacteriocins in their natural medium – milk, as well as during associated growth with other lactobacteria, the strains were first grown in milk as individual starters. *Lb. bulgaricus* BB18 and *Lc. lactis* BCM5 isolated from kefir grains and from Kashkaval revealed high *in situ* bacteriocinogenic activity (1900 BU ml⁻¹ and 2000 BU ml⁻¹) in milk acquired for the shortest period of time (10 h and 13 h) and high concentration of probiotic live cells (2.2x10¹² CFU ml⁻¹ and 3.4x10¹² CFU ml⁻¹). These bacteriocinogenic characteristics of both probiotic strains in milk significantly exceed reported data for LAB strains in synthetic media (Vaughan et al., 2001; Corsetti et al., 2004). At 4 h, about 40% of the total bacteriocin concentration in milk produced by *Lb. bulgaricus* BB18, and about 50% produced by *Lc. lactis* BCM5 were synthesized. The duration of bacteriocinogenesis for maximum *in situ* bacteriocin-synthesizing activity by *Lb. bulgaricus* BB18 was 4 h shorter than bacteriocinogenesis in MRS, and 4 h than *Lc. lactis* BCM5 in M17. *Lb. bulgaricus* BB18 and *Lc. lactis* BCM5 maintained maximum production activity for 168 h. Starting from pH 6.5, both individual cultures fermented milk until pH 4.85 (*Lb. bulgaricus* BB18), and pH 4.90 (*Lc. lactis* BCM5) for 12 h and 14 h of incubation followed by refrigera-

tion at 4⁰ C, and a final cell concentration at 24 h of 8x10¹¹ CFU ml⁻¹ (*Lb. bulgaricus* BB18) and 2x10¹² CFU ml⁻¹ (*Lc. lactis* BCM5).

Focus is placed on the potential usage of lantibiotics produced by *Lc. lactis* (lacticin 3117, 481; lactococcin ABM) during cheese fermentation for control on the growth of non-starter bacteria (O'Sullivan et al., 2002, Morgan et al., 2002). It is reported that the usage of lacticin 481-producing culture (*Lc. lactis* DPC5552) as a starter component leads to a severalfold log reduction in the growth of non-starter lactobacteria in cheese (O'Sullivan et al., 2002). It has been proved that there is a bacteriolytic effect of bacteriocin produced by *Lc. lactis* on cells of lactic acid bacteria, resulting in increased release of intracellular enzymes and accelerated ripening of Cheddar cheese (Morgan et al., 2002; Martines-Cuesta et al., 2001).

The results show that the selected strains of lactobacilli and lactococci with high bacteriocinogenic activity can be successfully used in formation of probiotic milks containing high bacteriocin and viable cells concentrations. The results are indicative because they provide evidence that continuous growth does not always lead to an increased level of bacteriocin activity, and that not all bacteriocins are influenced by the lower pH. Bacteriocin production stopped when pH dropped below 5.0 during the lactic acid process.

The growth of the bacteriocin strain-producers at an optimal temperature generally expressed itself in optimal bacteriocin production (Dada et al., 1993; Chinachoti et al., 1997a; Lejeune et al., 1998). However, the exercised temperature stress and *Lb. bulgaricus* BB18 growth at a temperature lower than the optimal (after 4 h of growth of *Lb. bulgaricus* BB18 at 37⁰ C the growth temperature was lowered to 30⁰ C) induced an increase in the bacteriocin yield. The decrease in the growth temperature correlated with a slow pH reduction in the starter culture, longer maintenance of pH levels of 5.5-5.35 in the starter culture, which is of crucial importance for *Lb. bulgaricus* BB18 bacteriocinogenesis. As a result, the bacteriocin yield

of *Lb. bulgaricus* BB18 grown in a starter culture with *S. thermophilus* 11A was 30% higher than that of a starter culture grown at 37⁰ C, and a high bacteriocinogenic activity - 3600 BU ml⁻¹ - was thus obtained. The established highly efficient bacteriocin production by *Lb. bulgaricus* BB18 during co-cultivation in milk with the bacteriocin-insensitive technological strain *S. thermophilus* 11A - 30% higher level of bacteriocin production, high level of viable cells (10¹² CFU ml⁻¹ - *S. thermophilus* 11A and 8x10¹¹ CFU ml⁻¹ - *Lb. bulgaricus* BB18) and the reduction of bacteriocinogenesis time by 3 h are evidence of the mutual stimulation of the cell growth, and the stimulation of bacteriocin and lactic acid synthesis. The established high stability of bacteriocins produced in milk at 72 h at the growth temperatures and during 20 days of storage at 4⁰ C is an important property related to the probiotic properties of fermented milk. It is suitable for long-term consumption (20 days), and storage at 4⁰ C preserves its bacteriocinogenic activity and cell concentration. Bacteriocin production during growth in milk of the typical dairy LAB strains has the following characteristics which are important for the efficient production of a high technological protective fermented milks with bacteriocin-inhibitory substances: 1) Bacteriocin production occurs in the exponential growth phase and finishes at the end of that phase (before the cultures enter the stationary phase) in the pH range of 6.2-5.2; 2) It is a stable process with high activity of bacteriocin production and high final bacteriocin concentration; 3) Bacteriocin production takes place in simultaneously with an active growth process, yielding a high final concentration of viable probiotic cells; 4) Bacteriocinogenesis occurs in parallel with the production of lactic acid by the strains of lactobacteria; 5) Stability of bacteriocin activity during production, milk coagulation and 20 days of storage of the fermented milk at 4⁰ C; 6) Effective bacteriocinogenesis occurs at temperatures lower than the optimal growth temperatures for thermophilic lactobacilli.

A lower activity of lactic acid production during fermentation, i.e. extended lactic acid production characterized the strain-producers of bacteriocins as compared to typical starter strains for fermented milks. Our previous studies proved active accumulation of lactic acid during fermentation with a selected starter culture *Lb. bulgaricus* 2-11+ *S. thermophilus* 13a, and full coagulation of milk for 2 h (pH 4.7) (Beshkova et al., 1998a). While lactic acid production is an important technological property of the starter culture in the manufacture of yogurt and yogurt-like milks, with antimicrobial activity being of secondary importance, the contrary is true for a given bacteriocin strain that can be used as a protective culture individually or as a co-culture. This fact was noted by some researchers for other food fermentations (Messens and De Vuyst, 2002; Foulquie-Moreno et al., 2005) and supported by our investigations of fermentation+ bacteriocin production in milk, where priority is given to bacteriocinogenesis over lactic acid fermentation.

These results are significant as they point to the fact that through protosymbiosis it is possible to enhance the bacteriocinogenic activity of the strain-producer in a yogurt starter culture. The obtained results support our previous studies providing proof for stimulated growth activity and metabolism in yogurt starter cultures (Beshkova et al., 1998a, 1998b; Frengova et al., 2000). So far no studies evaluating *in situ* bacteriocin production by starter lactobacteria in milk have been reported. There are reports on bacteriocin-like substances and their characteristics in conditions for sourdough formation and bread making (Corsetti et al., 2004; Settanni et al., 2005). This study is the first to demonstrate possible production of bacteriocin by *Lb. bulgaricus* BB18 strain with highly bacteriocinogenic activity in milk. As the result of the positive effects of the mutual metabolism between *Lb. bulgaricus* BB18 and *S. thermophilus* 11A, we obtained a yogurt protective culture with high inhibitory activity against a wide range of Gr-positive and Gr-negative pathogenic microorganisms.

Methods for making *Lactobacillus bulgaricus* protective cultures with bacteriocin-inhibitory substances and high concentration of viable probiotic cells – protective yogurt and UF fermentate were developed (Figures 3 and 4). There were obtained novel antimicrobial agents for fermented milks with protective functions ensuring health benefits. The high bacteriocin and cell-probiotic concentration in the fermented milk can exert efficient bacteriostatic or bactericidal effect on the pathogenic bacteria in the gastrointestinal tract during milk intake.

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