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ORIGINAL PAPER

Bacteriocin production by strain *Lactobacillus delbrueckii* ssp. *bulgaricus* BB18 during continuous prefermentation of yogurt starter culture and subsequent batch coagulation of milk

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Abstract By screening for bacteriocin-producing lactic acid bacteria of 1,428 strains isolated from authentic Bulgarian dairy products, *Lb. bulgaricus* BB18 strain obtained from kefir grain was selected. Out of 11 yogurt starters containing *Lb. bulgaricus* BB18 and *S. thermophilus* strains resistant to bacteriocin secreted by *Lb. bulgaricus* BB18 a yogurt culture (*S. thermophilus* 11A + *Lb. bulgaricus* BB18) with high growth and bacteriocinogenic activity in milk was selected. Continuous (pH-stat 5.7) prefermentation processes were carried out in milk at 37°C in a 2l MBR bioreactor (MBR AG, Zurich, Switzerland) with an IMCS controller for agitation speed, temperature, dissolved oxygen, CO_2 and pH. Prefermented milk with pH 5.7 coagulated in a thermostat at 37°C until pH 4.8–4.9. *S. thermophilus* 11A and *Lb. bulgaricus* BB18 grew independently in a continuous mode at similar and sufficiently high-dilution rates ($D = 1.83\ h^{-1}$—*S. thermophilus* 11A; $D = 1.80\ h^{-1}$—*Lb. bulgaricus* BB18). The yogurt cultures developed in a stream at a high-dilution rate ($D = 2.03$–$2.28\ h^{-1}$). The progress of both processes (growth and bacteriocin production) depended on the initial ratio between the two microorganisms. The continuous prefermentation process promoted conditions for efficient fermentation and bacteriocinogenesis of the starter culture during the batch process: strong reduction of the times for bacteriocin production and coagulation of milk (to 4.5–5.0 h); high cell productivity (lactobacilli—4×10^{12} CFU ml^{-1}, streptococci—6×10^{12} CFU ml^{-1}); high productivity of bacteriocins (4,500 BU ml^{-1})—1.7 times higher than the bacteriocinogenic activity of the batch starter culture.

Keywords Bacteriocin production · Lactic acid bacteria · Continuous process · Yogurt starter culture

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Introduction

The fact that the intestinal microflora correlates closely with the host's health indicates that its balance is crucial for the host's wellness and longevity [13, 21]. Recent scientific attention has been directed towards finding ways for a positive change in the composition of intestinal microflora by stimulating the growth and/or metabolism of beneficial microflora like lactobacilli and bifidobacteria on the one hand, and by suppressing the growth of pathogens and noxious microorganisms, on the other.

The consumption of probiotic cultures or yogurt probiotic milks is a good way to exert a protective effect against pathogenic infections [9, 37, 43]. The interest in the production of fermented milks enriched with probiotic microorganisms has never been so great, and current investigations involve mainly lactic acid bacteria with specific characteristics for specific purposes [8, 37, 43].

Probiotic milks are defined as fermented milks enriched with microorganisms that are beneficial to the balance or promote the regrowth of the normal microbiota in the intestinal tract [27, 29, 33].

According to some authors, probiotic lactobacilli exert in vivo and in vitro antagonistic effect against pathogens and other harmful bacteria, which is mainly manifested by production of organic acids, competitive inhibition for adhesion sites of mucous cells, and immunomodulation [4, 37]. The synthesis of bacteriocin and bacteriocin-like substances is rarely associated with these antagonistic effects [37]. Authors accentuate, however, on the possible production of these substances in vivo, as they are assumed to be one of the antagonistic mechanisms used by bacteria of the normal microflora against colonization by harmful microorganisms [24].

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 the 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" [25]. It has been established that the target of bacteriocins is the cytoplasmic membrane of the sensitive pathogens and other bacteria [36]. Emphasis is placed on the so-called "attack" mechanisms, in which the inhibitory peptide adheres to certain targets in the cytopalsmic zone of bacteria [22, 39]. Some antibacterial peptides develop a mechanism of action close to that of the antimicrobial peptides in the host [20].

Many authors hold the view that exogenously applied probiotics do not become implanted in the intestinal tract [5, 10, 12, 14, 17 35] and do not colonize it [5, 10]. Only temporary colonization can be achieved in the intestinal tract with exogenous probiotic bacteria [8] with consumption of a probiotic culture, during which time the probiotic continues to be metabolically active and to confer heath benefits [18, 35]. In relation to the above, it seemed 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 probioticity. Our investigations are directed towards enhancing the protective effect of lactic acid bacteria by means of: (1) production of bacteriocins with high-inhibitory effect (bacteriostatic and bactericidal) against pathogenic bacteria; (2) high concentration of active viable cells of probiotic bacteria in fermented milks aimed at temporary colonization of the probiotic during passage along the gastrointestinal tract; (3) expanding the probiotic potential of the 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.

There are no reports on production of bacteriocins in milk by *Lb. bulgaricus* strains and especially by a yogurt starter culture. There is also no data available on bacteriogenesis in milk for a continuous prefermentation yogurt culture. Making use of antibiosis of lactic acid bacteria is the best choice for obtaining probiotics.

The aim of this study was to: (1) investigate bacteriocin production during a continuous pH-stat prefermentation process with subsequent batch fermentation in milk by a probiotic strain *Lb. bulgaricus* BB18 isolated from kefir grains; (2) increase bacteriocin production and viable cell concentration of a yogurt-protective starter culture *S. thermophilus* 13a + *Lb. bulgaricus* BB18 in continuous prefermentation and subsequent batch fermentation until milk coagulation.

Materials and methods

Strains and growth condition

Lb. bulgaricus BB18 strain was selected by screening for bacteriocin activity 1428 LAB strains isolated from traditional Bulgarian dairy products (home-made fermented milks and cheese produced from raw milk in ecological regions of the Rhodopi Mountains) and from kefir grains [40]. Eleven yogurt starters containing bacteriocin-producing *Lb. bulgaricus* BB18 strain and 11 *S. thermophilus* strains resistant to bacteriocin produced by *Lb. bulgaricus* BB18 were formed and screened for high bacteriocin production in milk. The starter culture *Lb. bulgaricus* BB18+*S. thermophilus* 11A was selected on the basis of its bacteriocin activity and viable cell concentration. Two strains *Lb. bulgaricus* BB18 and *S. thermophilus* 11A and a yogurt culture were stored at −80°C in skim milk/glycerol medium and subcultured twice in 12% reconstituted skim milk (RSM) supplemented with 1% glucose and 1% yeast extract prior to experimental use. For experiments 12% RSM enriched with 1% glucose and 1% yeast extract was used as growth medium for preparation of individual and work inocula. 1% (v/v) of each culture (11-h-old milk culture of *Lb. bulgaricus* BB18 and a 6-h-old milk culture of *S. thermophilus* 11A) was propagated for 11 and 6 h, respectively, at 37°C for preparation of individual inocula. The yogurt inocula were prepared as follows: Inoculum A—A 1% (v/v) individual inoculum of each bacterial yogurt strain was inoculated in 12% RSM supplemented with 1% glucose and 1% yeast extract and incubated at 37°C for 8 h; Inoculum B—the 12% RSM with 1% glucose and 1% yeast extract was inoculated with 3% (v/v) individual *Lb. bulgaricus* BB18 inoculum and 1% *S. thermophilus* 11A inoculum and incubated at 37°C for 6 h.

Bacteriocin production in milk. Yogurt preparation with protective functions

A 2% (v/v) individual bacterial inoculum of *Lb. bulgaricus* BB18 or *S. thermophilus* 11A, and yogurt inoculum A and inoculum B were cultivated at 37°C in 12% (RSM) in

continuous pH-stat 5.7 pre-fermentation process carried out in a 2 l MBR bioreactor (MBR AG, Zurich, Switzerland). The bioreactor was equipped with system control circuits for agitation speed, temperature, dissolved oxygen, CO_2 and pH. The dissolved oxygen concentration was measured by the oxygen-circuit of the control system consisting of a steam-sterilizable Clark-type polarographic electrode (Ingold, Switzerland). The system was calibrated in distilled water. The obtained data were expressed as % of oxygen saturation of water at the respective temperature.

Dissolved carbon dioxide partial pressure was measured with an additional circuit consisting of a steam-sterilizable potentiometric CO_2 electrode (Ingold) and CO_2 accelerator, type 525 (Ingold). The system was calibrated by the method described by Spinnler et al. [41].

The dilution rate was calculated with the formula $D = F/V_R$, where F is the volumetric flow rate of the outgoing milk. During the process the flow rate was measured at 30-minute intervals as the quantity of fermented milk passing through the bioreactor per 10 min.

The sterilized 12% RSM prefermented milk from the bioreactor and inocula was transported by peristaltic pumps, types PPU and 502 s50, Watson—Marlow Ltd., England.

The continuous prefermentation process occurred at 37°C and pH-stat 5.7. The initial concentration of oxygen dissolved in milk for all experiments was 25%; during the continuous cultivation oxygen was not supplied. The prefermented milks, packed in 200-ml packages, were placed for coagulation in a thermostat at 37°C until pH 4.8–4.9.

Analytical methods

Growth of the yogurt bacteria, bacteriocin activity, pH and lactic acid were checked on an hourly basis.

The growth was evaluated by determination of colony-forming units (CFU) [23]. The number of CFU ml^{-1} was quantified using the plate dilution method, and media for the enumeration of bacteria were as follows: MRS agar (Fluka, Buchs, Switzerland) and RCPBpH5 agar (Sigma, Buchs, Switzerland) for *Lb. bulgaricus*; M17 agar (Fluka, Buchs, Switzerland) and Streptococcus selective agar (Merck, Darmstadt, Germany) for *S. thermophilus*.

The antimicrobial activity was detected by the well-diffusion method described by Guerra and Pastrana [19]. 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 of incubation at 30°C and 37°C of bacteriocin-producing LAB strains, the cells were removed by centrifugation (12,000*g*, 15 min, 4°C). To eliminate the inhibitory effect of lactic acid and/or H_2O_2, the culture supernatants were treated with 1 N 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 10^7 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 supernatant 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.

Lactic acid was determined by the enzymatic method as described by Boehringer Mannheim [7].

Morphological control on the microbial cells was performed with a microscope Laboral 4 (Carl Zeiss Jena, Germany) with 1,600× magnification. For an estimation of *S. thermophilus—Lb. bulgaricus* ratio in the yogurt culture, direct microscopic examination was used, previously staining slide preparation with methylene blue according to Breed's method.

Statistical analysis

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

Results and discussion

Our previous investigations showed how by screening for bacteriocin-producing LAB of 1428 LAB strains, isolated from authentic Bulgarian dairy products and from kefir grains, seven strains with the highest and wide-spectrum antimicrobial activity against Gr (+) and Gr (−) pathogenic microorganisms were selected [40]. One of these LAB

strains, *Lb. bulgaricus* BB18, isolated from kefir grains, possesses a high-inhibitory effect against Gr (+) and Gr (−) pathogenic microorganisms (*Listeria monocytogenes, Corynebacterium difterium, Clostriduim difficile, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Helicobacter pylori, Salmonella typhimurium, Candida albicans*) [40]. For the first time it was demonstrated that antimicrobial compounds of proteinaceous nature secreted by *Lb. bulgaricus* BB18 (bulgaricin BB18) inhibit the growth of clinical isolates of *H. pylori*; this bacteriocin is bacteriostatic or bactericidal factor against *H. pylori* [40].

High-bacterionogenic activity of *Lb. bulgaricus* BB18 during batch cultivation in milk was shown [40]. To enhance the bacteriocinogenic productivity of *Lb. bulgaricus* BB18 in milk, the strain was associated in yogurt cultures with 11 technological *S. thermophilus* strains isolated from home-made fermented milk resistant to bulgaricin BB18. After cultivation of 11 yogurt cultures in milk for 1 year a yogurt culture *Lb. bulgaricus* BB18 + *S. thermophilus* 11A demonstrating stable symbiotic relationships between the two yogurt bacteria, high-growth activity (lactobacilli—1.1×10^{12} CFU ml^{-1} and streptococci—2.4×10^{12} CFU ml^{-1} and bacteriocinogenic activity (2,600 BU ml^{-1}) was selected (results not shown).

Since bacteriocin production is growth-associated it can be expected that bacteriocin production rates should improve in continuous fermentation, where high-growth rates can be maintained [31]. A linear relationship is observed between the dilution rate [26] and the specific enterocin 1146 production rate [30] for divercin [6]. According to some authors the efficiency of the continuous process of cultivation of LAB can be boosted through associated growth of cultures with close growth rates [16]. They show that the associated growth of *Lb. bulgaricus* and *S. thermophilus* in a continuous prefermentation process and subsequent batch fermentation until full coagulation of milk is one of the most efficient methods proving the biosynthesizing potential of the cultures and the relationships between them.

Bacteriocin production in milk during continuous cultivation of starter culture *Lb. bulgaricus* BB18+*S. thermophilus* 11A in a pH-stat prefermentation process was carried out under selected optimal conditions based on results from earlier investigations. The prefermentation temperature was 37°C despite the fact that in a prefermentation process at 42°C followed by batch fermentation for milk coagulation the final concentration of lactobacilli and streptococci was 1.5–2.0 times higher than at 37°C. At 37°C the bacteriocinogenic activity was about 2.5 times higher in the mixed culture during continuous prefermentation and subsequent batch fermentation. The highest process characteristics were recorded with a pH-stat regimen of the continuous prefermentation process pH 5.5; nevertheless, for obtaining a protective probiotic culture *Lb. bulgaricus* BB18+*S. thermophilus* 11A a pH-stat value of pH 5.7 was selected. This value most thoroughly satisfied the requirements of the process. It is the nearest to pH 5.5, at which value the process was characterized by the highest microbial and metabolic productivity, being at the same time well distanced from it to avoid the first signs of coagulation of milk throughout the whole processing. Analysis of results of preliminary studies was the basis on which the most appropriate initial oxygen concentration (20–25%) for initiation of the associated growth of the thermophilic lactobacilli and streptococci was determined. Earlier studies showed that the resistance of the initial microbial population during continuous cultivation of yogurt starter cultures depends not only on the relative similarity of the specific growth rates of both cultures, but also on their mutual influence during the process of associated growth [1]. It was proved that the ratio between the two types of lactobacteria, *S. thermophilus* 13a and *Lb. bulgaricus* 2–11, in the inoculum, and the ratio alteration during the fermentation process was essential to the outcome of the fermentation process and the formation of a starter culture or yogurt of high-biological value. Bacteriocin production is growth-related, so the rate of bacteriocin production could be expected to improve during continuous fermentation, in which it is possible to maintain high-growth rates, for the realization of which the relationships between the two thermophilic species, *S. thermophilus* and *Lb. bulgaricus*, are of primary importance. In the mixed culture, made up of the two species, the protocooperation is manifested exclusively in relation to acidification [32], the final cell concentration [3], and the specific growth rate [15]. It needs to be established whether under conditions of continuous prefermentation and subsequent batch fermentation bacteriocin production is influenced by protocooperation in the starter culture.

In order to determine to what extent each strain participated in the continuous process, *S. thermophilus* 11A and *Lb. bulgaricus* BB18 were cultivated individually in a stream. The most important parameters of the state of the processes of prefermentation and batch fermentation for milk coagulation are shown in Figs. 1 and 2. The streptococci produced about three times more CO_2 than the amount contained in the milk. The partial pressure (pCO_2), CO_2 concentration, respectively, increased exponentially and continued to increase during the second phase (stationary regimen of the continuous process) correlated with their metabolism. In the culture medium of *Lb. bulgaricus* BB18, an insignificant amount of CO_2 (3 kPa) was recorded, which was commensurate with the CO_2 content in the raw milk. The lactobacilli did not produce CO_2. The level of CO_2 produced by *S. thermophilus* 11A was not high (13 kPa); however for some authors it was sufficient [15] for stimulation of *Lb. bulgaricus*, i.e. for initial

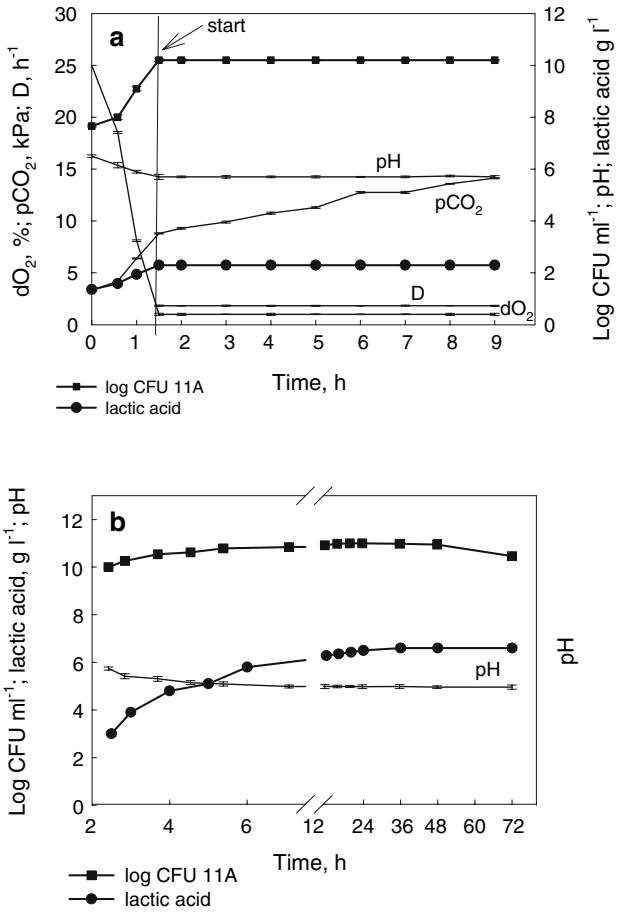

Fig. 1 Growth of *S. thermophilus* 11A in milk at 37°C under conditions of continuous (pH-stat 5.7) prefermentative process (**a**), subsequent batch fermentation until full coagulation of milk (pH 4.8), and 72-h storage of the fermented culture at 4°C (**b**)

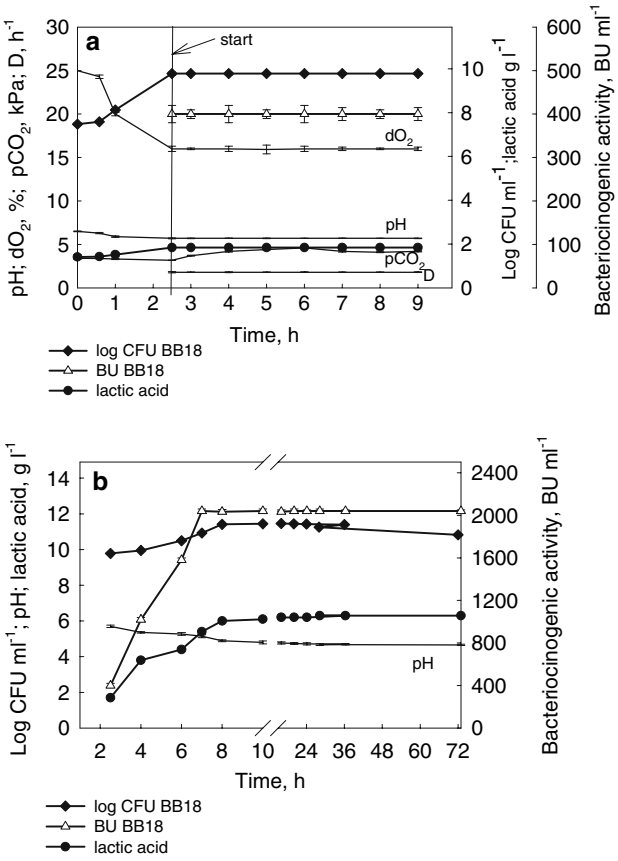

Fig. 2 Growth and bacteriocin production by *Lb. bulgaricus* BB18 in milk at 37°C under conditions of continuous (pH-stat 5.7) prefermentative process (**a**), subsequent batch fermentation until full coagulation of milk (pH 4.8), and 72-h storage of the fermented culture at 4°C (**b**)

cooperation of *S. thermophilus* and *Lb. bulgaricus*. The change in the oxygen concentration in the systems was noteworthy. If the studied process were to be provisionally divided into three phases, in the first phase (batch) *S. thermophilus* 11A assimilated the milk-dissolved oxygen faster and to a fuller extent than *Lb. bulgaricus* BB18. In the streptococcus culture, after a 1.2-h growth, the oxygen concentration dropped below 1% and remained about 0.5% to the end of the process (Fig. 1). In the *Lb. bulgaricus* BB18 culture the oxygen concentration decreased over a period of 2.5 h to 16% and retained that level until the end of prefermentation (Fig. 2). These results indicate that the thermophilic streptococcus is more microaerophilic than *Lb. bulgaricus* BB18.

The obtained results indicate the exceptional role of O_2 in the mutual metabolism of the two species. They suggest that the milk-dissolved oxygen is a major factor not only in the mutual stimulation of growth of the streptococcus and lactobacillus, but also a factor in the stimulation of products from the combined metabolism of the starter culture (lactic acid, bacteriocins). It was proved that *S. thermophilus* 11A, which is more aerophilic, assimilated milk-dissolved oxygen faster and to a fuller extent, creating the anaerobic conditions necessary for growth of the less aerophilic *Lb. bulgaricus* BB18. The stimulated *Lb. bulgaricus* BB18 developed actively and became a crucial factor in the metabolism of the starter culture. The growth of *S. thermophilus* 11A and *Lb. bulgaricus* BB18 in the starter culture commenced immediately after milk inoculation (Figs. 3, 4). *Lb. bulgaricus* BB18 growth in the individual culture started after a 35-min lag phase (Fig. 2). The final concentrations of lactobacilli and streptococci in the starter cultures were about 1 log unit higher than in the individual cultures (Figs. 1b, 2b, 3b). The concentration of the lactic acid produced in milk prefermented by starter cultures was about 1.4 times higher compared to the individual culture *Lb. bulgaricus* BB18 and *S. thermophilus* 11A. During the initial batch phase and the continuous prefermentation, there was high-bacteriocinogenic activity and about 16% of the total amount of bacteriocins was produced. In the starter-prefermented culture *Lb. bulgaricus* BB18 produced about 1.4 times more bacteriocins than the single-strain prefermented

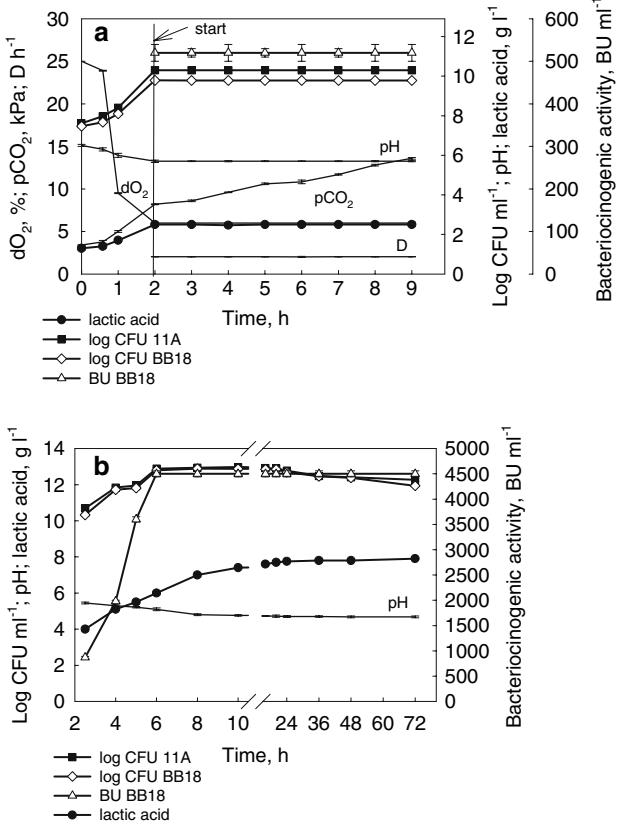

Fig. 3 Growth and bacteriocin production by *Lb. bulgaricus* BB18 co-cultivated in starter culture with *S. thermophilus* 11A in milk at 37°C under conditions of continuous (pH-stat 5.7) prefermentative process (**a**), subsequent batch fermentation until full coagulation of milk (pH 4.8), and 72-h storage of the fermented culture at 4°C (**b**); initial ratio *S. thermophilus* 11A:*Lb. bulgaricus* BB18 = 1:1

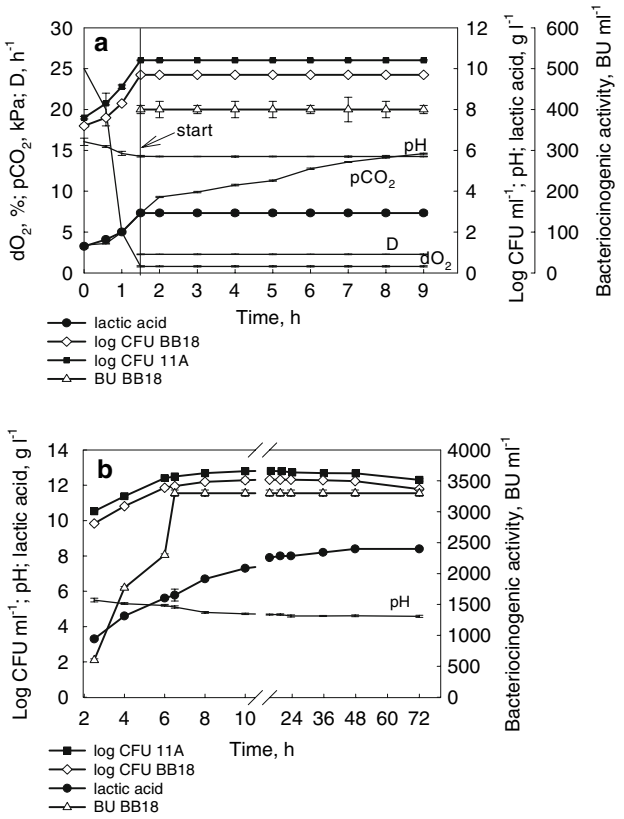

Fig. 4 Growth and bacteriocin production by *Lb. bulgaricus* BB18 co-cultivated in starter culture with *S. thermophilus* 11A in milk at 37°C under conditions of continuous (pH-stat 5.7) prefermentative process (**a**), subsequent batch fermentation until full coagulation of milk (pH 4.8), and 72-h storage of the fermented culture at 4°C (**b**); initial ratio *S. thermophilus* 11A:*Lb. bulgaricus* BB18 = 3:1

culture (Figs. 2, 3, 4), and about 2.5 times more bacteriocins than the single-strain batch culture (for the same period of time from the beginning of the batch phase to the onset of batch fermentation after a pH-stat process).

S. thermophilus 11A and *Lb. bulgaricus* BB18 grew independently in a continuous mode at similar and sufficiently high-dilution rates (D−1.83 h^{-1}—*S. thermophilus* 11A; D = 1.80 h^{-1}—*Lb. bulgaricus* BB18) without addition of stimulating substances. The kinetics of the continuous process of cultivation of the starter culture and the subsequent batch fermentation for milk coagulation was studied for different initial ratios between *S. thermophilus* 11A and *Lb. bulgaricus* BB18 in the inoculum as given in Figs. 3 and 4. The preliminary studies with various *S. thermophilus*: *Lb. bulgaricus* ratios in the starter (1:1–3:1) showed that the kinetics of the parameters of the state of the microbial cultures and the parameters of the state of the process in its batch phase until initiation of the prefermentation was entirely dependent on that ratio. At 3:1 ratio in favour of the streptococci the alteration of pH, dO_2 and O_2 was analogous to that of the monoculture *S. thermophilus* 11A (Fig. 1). It should be noted that the increase in the relative share of the lactobacillus in the starter yogurt culture (*S. thermophilus* 11A: *Lb. bulgaricus* BB18 = 1:1) led to modifications in parameters because of the lesser starting position of *Lb. bulgaricus* BB18 (poorer aerophily than *S. thermophilus* 11A, active growth at pH below 6.0). The time to reach pH 5.7 was extended by 30 min. The active assimilation of the oxygen dissolved in milk began 35 min after inoculation, while at ratio of 3:1 (*S. thermophilus* 11A: *Lb. bulgaricus* BB18) for the same period of the time there was a dramatic decline in the concentration of dissolved O_2 in milk (Figs. 3, 4). The production of CO_2 at a ratio 1:1 was delayed by nearly 20 min, then the curve followed the course of the starter culture with a ratio *S. thermophilus* 11A: *Lb. bulgaricus* BB18 = 3:1. In the continuous pH-stat process a new ratio was formed in which the streptococcus dominated about three times over the lactobacillus at a initial ratio 1:1, and about six times at a ratio 3:1. In the prefermentation continuous process there was recorded a positive interaction between the two cultures whose effect was strongly expressed by: (1) the high level of the populations of both cultures in the

prefermented milk (0.6×10^{10} CFU ml^{-1}—lactobacilli and 2.0×10^{10} CFU ml^{-1}—streptococci at a ratio of 1:1 (*S. thermophilus* 11A: *Lb. bulgaricus* BB18 = 1:1), and 0.4×10^{10} CFU ml^{-1}—lactobacilli and 2.6×10^{10} CFU ml^{-1}—streptococci at a ratio 3:1 (*S. thermophilus* 11A: *Lb. bulgaricus* BB18 = 1:1); (2) the rate of dilution—the associated cultures developed in a stream at high-dilution rates (D = 2.03–2.28 h^{-1}); (3) high level of bacteriocins in the prefermented milk; (4) high concentration of lactic acid in the prefermented milk (about 1.4 times higher than that in the individual culture *Lb. bulgaricus* BB18). In each experiment the starting ratio between the two types of lactobacteria determined specific rates of dilution.

After continuous prefermentation of the yogurt starter culture, milk fermentation commenced with active yogurt bacteria with high-bacterial concentrations, which ensured active growth, acidification and bacteriocinogenesis.

Maximum cell growth was recorded 5.5–6.0 h after milk inoculation at initial ratio of cocci and lactobacilli 1:1; maximum concentration of bacteriocins 2.5 h earlier (4,200 BU ml^{-1}); maximum concentration of lactic acid was 7.9 g l^{-1} (Fig. 3). *Lb. bulgaricus* BB18 produced bacteriocins actively in the exponential growth phase of the batch fermentation. *Lb. bulgaricus* BB18 growth in the starter followed a notable course of development until end product (probiotic yogurt): growth onset 35 min after inoculation and exponential growth for 2 h (batch phase); stationary growth during the continuous prefermentation of milk; restart of the exponential phase when the prefermented milk was set for fermentation proper to total coagulation of milk at 37°C to pH 4.8. The accumulation of cell mass was accompanied by active bacteriocin production; upon entering the late log phase bacteriocin production ceased.

The growth and bacteriocin production in the starter culture at initial ratio between *S. thermophilus* 11 and *Lb. bulgaricus* BB18 in the inoculum 3:1 during milk coagulation were different at 1:1 initial ratio. The progress of both processes (growth and bacteriocin production) was dependent on the initial ratio between the two microorganisms. The level of the 3:1 ratio in the inoculum increased during the continuous prefermentation process to the advantage of *S. thermophilus* 11A to a stationary level of 6:1, and decreased during milk coagulation to 3:1 in the end product. That ratio was retained in the cooled culture up to 92 h. The 1:1 ratio in the inoculum increased to stationary level of about 3:1 (*S. thermophilus* 11A:*Lb. bulgaricus* BB18) in the continuous prefermentation process, and during milk coagulation at 37 °C the level of lactobacilli increased, the ratio reaching levels of about 2:1–1:1 in the end product. In the prefermented milk the concentration level of lactobacilli was higher at 1:1 ratio than at 3:1 ratio; during batch fermentation and milk coagulation the level of the *Lb. bulgaricus* BB18 population at initial ratio 3:1 remained about two times lower than that at 1:1 ratio. The lower cell concentration of *Lb. bulgaricus* BB18 during milk coagulation at initial ratio 3:1 is most probably due to the higher level of the ratio in the prefermented milk (5:1), and streptococci predominating throughout the whole process of coagulation. That resulted in a lower level of the relative share of *Lb. bulgaricus* BB18 in the mixed culture, and in lower bacteriocinogenic activity (3,300 BU ml^{-1}) in the phase of milk coagulation (at initial ratio 3:1) (Figs. 3b,4b). The milk coagulated for 7.5 h from onset of inoculation; maximum bacteriocin concentration was reached after 7.0 h at initial ratio 3:1. At initial ratio 1:1 (*S. thermophilus* 11A:*Lb. bulgaricus* BB18) the milk coagulated for 6.5 h and maximum bacteriocinogenic activity was reached 0.5 h earlier (for 6.0 h) at a higher level (4,500 BU ml^{-1}). The activity remained in the milk cooled at 4°C up to day 14 for both variants. The starter cultures retained a high content of viable lactobacilli and streptococci (10^{12} CFU ml^{-1}) during a 14-day storage period at 4°C (Figs. 3b, 4b). Many of the probiotic strains do not survive to be present in a sufficient amount in the final yogurt product [34], or can even alter the traditional yogurt flavour. Although there are no fixed standards for the population of the probiotic microorganisms in the final yogurt or at the end of storage time, a final population of between 5 and 8 log CFU g^{-1} is generally considered acceptable [34, 38, 42]. According to other authors, a concentration of probiotic live cells of 10^9 CFU g^{-1} is sufficient for temporary colonization of the intestinal tract [18].

The effectiveness of the batch fermentative process for milk coagulation after continuous prefermentation (for both variants), and the effectiveness of bacteriocinogenesis were in correlation with the mutual growth stimulation and production of metabolic substances. This is evidenced by the high levels of the population dynamics of the lactobacteria and the high in situ produced bacteriocinogenic activity of *Lb. bulgaricus* BB18 co-cultivated with *S. thermophilus* 11A. The continuous prefermentation process increased 1.7 times the bacteriocinogenic activity of the batch starter culture, and 2.4 times the activity of the batch single culture *Lb. bulgaricus* BB18.

There are reports about high-bacteriocin titres of continuous processes for production of bacteriocins from selected LAB at low dilution rates—for enterocin 1146 from *Ec. feacium* DPC1146 (D = 0.14–0.56 h^{-1}) [30], plantaricin C from *Lb. plantarum* LL 441 (D = 0.055–0.25 h^{-1}) [2], nisin from *Lc. lactis* ATCC 11454 (D = 0.1–0.4 h^{-1}) [28] and nisin from *Lc. lactis* IO-1 (D = 0.1 h^{-1}) [11]. The bacteriocin titre of continuous prefermentation (pH-stat 5.7) process + batch fermentation of *Lb. bulgaricus* BB18 in milk was about ten times higher than that of continuous

processes for plantaricin C and nisin from *Lc. lactis* ATCC 11454. The yogurt culture *Lb. bulgaricus* BB18+*S. thermophilus* 11A in continuous prefermentation (pH-stat 5.7) process + batch fermentation increased the bacteriocin titre of *Lb. bulgaricus* BB18 in continuous process 2.0 times at a high-dilution rate (D = 2.03–2.28 h^{-1}).

The results of the study give grounds for the following characteristic of the process bacteriocinogenesis of strain *Lb. bulgaricus* BB18 grown in milk with strain *S. thermophilus* 11A in a continuous (pH-stat) prefermentation process and subsequent milk coagulation for the purpose of forming a protective probiotic culture with health benefits: (1) Batch phase (after milk inoculation until entering a stationary regimen of continuous prefermentation (pH-stat = 5.7))—active growth of *S. thermophilus* 11A in the exponential phase, starting immediately after inoculation of the starter culture, simultaneously with growth onset, start of bacteriocin production by *Lb. bulgaricus* BB18; (2) Stationary phase of the continuous prefermentation process—stable microbial association developing in a stream at a high-dilution rate, increased level of the streptococcus in the *S. thermophilus* 11A: *L. bulgaricus* BB18 ratio depending on the level of the initial ratio in the inoculum, high concentration of viable cell mass and bacteriocins; (3) Batch fermentation process (fermentation proper for milk coagulation)—active production of bacteriocins during the exponential growth phase of the starter culture in the pH range of 5.7–5.1 with maximum concentration in the late log phase and the beginning of the stationary phase, decrease in the level of the streptococcus in the ratio *S. thermophilus* 11A:*Lb. bulgaricus* BB18 approaching the values of the initial ratio in the inoculum.

Under the chosen conditions of continuous prefermentation the obtained state of the culture *S. thermophilus* 11A+*Lb. bulgaricus* BB18 predetermined the activity of the subsequent batch prefermentation, i.e. biologically mature milk with high cell activity (6.2×10^{10} CFU ml^{-1}) was produced. It was established that the continuous prefermentation (pH-stat 5.7) process of cultivation of a starter culture *S. thermophilus* 11A+*Lb. bulgaricus* BB18 possessed important characteristics, some of which correlated with the main advantages of the process; (1) Microbial stability; (2) The cells of both bacterial cultures are exponentially growing without adaptation in the subsequent batch process; (3) A stable cocci:lactobacilli ratio in the prefermented milk during a 168-h working culture of 3:1 ratio, which is optimal for bacteriocin production and lactic acid fermentation. The starting ratio between the two types of bacteria in the inoculation can be manipulated and altered to a desired level (during the batch fermentation) for a given stationary state of the continuous prefermentation process to obtain a desirable ratio in the final product. The ratio is determinative for the biological activity of the culture resulting in the growth activity and production of bacteriocins.

The continuous pH-stat prefermentation process creates conditions for efficient fermentation and bacteriocinogenesis of the starter culture *S. thermophilus* 11A+*Lb. bulgaricus* BB18 during the batch process and induced the following characteristics of the subsequent batch fermentation process and bacteriocinogenesis: (1) Strong reduction of the time for bacteriocin production and the fermentation time compared to the batch single strain culture (by 4.5–5.0 h) and to the batch starter culture (by 1.5 and 2.0 h); (2) High cell productivity (lactobacilli—4×10^{12} CFU ml^{-1} and streptococci—6.2×10^{12} CFU ml^{-1}) compared to 1.1×10^{12} CFU ml^{-1} lactobacilli and 2.4×10^{12} CFU ml^{-1} streptococci in the batch starter culture, and 4.5×10^{11} CFU ml^{-1}—in the batch single strain culture; (3) High productivity of lactic acid; (4) High productivity of bacteriocins during the batch process—4,500 BU ml^{-1} compared to 2,600 BU ml^{-1} in the batch starter culture, and 1,880 BU ml^{-1} in the batch single culture.

The results provide grounds for the following conclusion: An exceptionally high level of physiological activity (concentration of bacteriocins and probiotic viable cells) of a probiotic starter yogurt culture was obtained by means of a continuous prefermentation process (pH-stat 5.7) of cultivation. The results will be expressed in the high characteristics of biological activity of the probiotic yogurt, and will be of significance for its use, purpose and health effect.

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