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Use of *Lactobacillus helveticus* BGRA43 for Manufacturing Fermented Milk Products

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Summary

Lactobacillus helveticus BGRA43 isolated from human intestines shows antimicrobial activity against foodborne pathogens and during fermentation in milk releases peptides with demonstrated anti-inflammatory properties. In this study, it was found that strain BGRA43 exhibits antimicrobial activity against human pathogens *Yersinia enterocolitica*, *Shigella sonnei*, *S. flexneri* and *Streptococcus pneumoniae.* Strain BGRA43 was able to survive in simulated gastric juice containing milk and retained cell number stability during the incubation in simulated intestinal conditions. In addition, LC/MS/MS analysis showed the ability of BGRA43 to hydrolyze β-lactoglobulin. Abundant growth of strain BGRA43 occurred in the presence of prebiotics inulin or concentrated oat bran β-glucan (Nutrim®), even when used as the sole carbon source. Similarly, strain BGRA43 grew satisfactorily in pure cow's or goat's milk as well as in the milk containing inulin or Nutrim®. Using the probiotic strain BGRA43 as a single starter strain, fermented milk products obtained from cow's or goat's milk with or without inulin or Nutrim® contained about 10^7 CFU/mL. The products were homogeneous and viscous and the best sensory scores were observed for fermented milk beverage made from reconstituted skimmed milk, whole cow's milk and whole goat's milk supplemented with 1 % inulin.

Key words: Lactobacillus helveticus, milk fermentation, probiotics, prebiotics

Introduction

Gut microbiota has an important role in human health and diseases. Among them, some lactobacilli and bifidobacteria have been reported to exert health benefits ranging from interaction with harmful bacteria in the gut to the restoration of gut epithelial and immune homeostasis (*1*). The knowledge that different lactobacilli could be a source of new probiotics has been expanded (*2*). Moreover, fermented dairy products containing probiotics have been used to establish microbial balance in the intestines, facilitate dietary digestion or prevent pathogen colonisation (*3,4*). Furthermore, it was shown that milk, whey proteins and mucin could protect bacterial cells during transit through the gastrointestinal (GI) tract (*5*). Milk contains two main protein classes, which are the caseins (αS1-, αS2-, κ- and β-casein) and whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin and lactoferrin). Large population studies of infants allergic to cow's milk have shown that the major allergens are β-lactoglobulin (BLG) and αS1-casein (*6*). In the last years, the use of probiotics such as lactic acid bacteria (LAB) has been proposed as an alternative for the management of allergenic diseases. Proteolysis of

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BLG by LAB could increase its digestibility by releasing smaller peptides, which are known to be less immunoreactive and could be better absorbed in the intestinal tract (*7*).

The basis for selection of probiotic microorganisms includes their human origin, biosafety aspects, acid and bile tolerance, colonisation of the intestine, antimicrobial activity, immunomodulation, and/or prevention of pathogen growth (*2*). In addition, probiotic bacteria should have desirable technological properties such as superior growth rate in milk or other food bases and stability during processing and storage, which should result in a final product with satisfactory sensory properties and a significant impact on nutritional quality (*8*). One of the main criteria for selection of a probiotic strain is its survival in the gastrointestinal environment. Therefore, simple *in vitro* simulation tests for screening have been designed (*9*). Other characteristics required for a probiotic strain include the ability to inhibit the growth of various pathogens by production of lactic and acetic acids, hydrogen peroxide, bacteriocins, bacteriocin-like substances and biosurfactants (*10*). These antagonistic effects in fermented products could be very useful against foodborne pathogens, and also against human pathogenic bacteria, particularly enteropathogens like *Salmonella*, *Listeria* and *Campylobacter* (*11*).

In order to stimulate selectively the viability of a particular probiotic strain in fermented products or the GI tract, appropriate prebiotics have been used (*12*). Prebiotic inulin is a low-calorie carbohydrate found in certain roots and tubers. It affects different functions of the organism and colonic microflora, immune function, availability of minerals, lipid metabolism and normal functioning of the digestive tract (*13*,*14*). Experiments also showed that the ingestion of inulin improved calcium absorption and enhanced bone mineralization (*15*). Nutrim® is a new-generation oat bran that contains carbohydrates (hydrocolloids) formulated to reduce plasma cholesterol levels by promoting the increased excretion of bile acids (*16*). In addition, soluble β-glucans provide maintenance of optimal blood sugar level (*17*). Prebiotic-containing diets have been shown to change populations of *Bifidobacterium* and *Lactobacillus* in the GI tract increasing their number by 10-fold. On the other hand, prebiotics also specifically decrease the population of other anaerobes, for instance, sulphite-reducing clostridia (*18*).

Research regarding probiotic properties of *Lactobacillus helveticus* strains included studies on immunomodulation in animal models (*19*), as well as on their antagonism to pathogens (*3*). It was also demonstrated that peptides released during fermentation of reconstituted skimmed milk by *L. helveticus* had strong ACE-inhibitory activity (*20*,*21*). It had been shown previously that human intestinal isolate *L. helveticus* BGRA43 (formerly *Lactobacillus acidophilus* BGRA43) exhibited an inhibitory activity against various strains of *Lactococcus*, *Lactobacillus*, *Staphylococcus*, *Bacillus* and *Pseudomonas* species, as well as the foodborne pathogen *Clostridium sporogenes* (*22*). Additionally, the whole BGRA43 cells were able to hydrolyze α_{S1}-, β- and κ-caseins completely (*23*). Moreover, protein fractions released during the fermentation of milk with BGRA43 strain showed inhibitory effect on human monocyte RB, as well as anti-inflammatory effect on THP-1 cells (*24*).

The aim of our study is further examination of probiotic features of the BGRA43 strain including antimicrobial activity against several human pathogenic bacteria, resistance to simulated gastrointestinal conditions (pH=2, bile salts, presence of pepsin and pancreatin) and its ability to hydrolyze BLG. The final goal of this study is the implementation of potential probiotic strain as starter culture in dairy production. Consequently, the fermentation abilities of BGRA43 during growth in skimmed and whole cow's milk and whole goat's milk are examined. Moreover, the fermentation ability of BGRA43 strain in all three milk samples containing prebiotic inulin or Nutrim® is tested. The survival of bacteria in the resulting fermented milk product after 7 days of storage at 4 °C is also reported.

Materials and Methods

Bacterial strains, media and culture conditions

Strain *L. helveticus* BGRA43 isolated from faecal samples of healthy men was cultured in liquid MRS broth (Oxoid, Basingstoke, UK) or in a medium prepared from reconstituted skimmed milk (RSM) powder 10 % (by mass per volume), obtained from Subotica dairy plant, Serbia, and sterilized by autoclaving at 110 °C for 20 min. The isolates were stored at –80 °C in MRS broth (Oxoid) supplemented with 15 % (by volume) glycerol. When the active culture was needed, the frozen culture was inoculated in MRS broth at 37 °C during 16 h.

In order to determine the growth rate in the presence of different sugars, BGRA43 strain was grown in a chemically defined medium (CDM) for lactobacilli containing in g/L: sodium acetate 6, ammonium citrate 1, K_2HPO_4 3, KH_2PO_4 3, $MgSO_4{\cdot}7H_2O$ 0.5, $MnSO_4{\cdot}H_2O$ 0.032, $FeSO_4{\cdot}7H_2O$ 0.02, casitone 20 g/L; in mg/L: *p*-aminobenzoic acid 0.2, folic acid 0.1, nicotinic acid 1, pantothenic acid 1, pyridoxal 2, riboflavin 1, biotin 1; and in mL/L: polyethylene sorbitan monooleate 1. Either glucose, lactose (Sigma Chemie GmbH, Deisenhofen, Germany), inulin (FutureCeuticals, Santa Rosa, CA, USA) or the low-glycemic carbohydrate product, Nutrim® (FutureCeuticals) were added to the CDM at 1 % (by mass per volume) final fraction. All components were filtered or autoclaved (121 °C for 15 min) prior to use.

In this study, in addition to RSM, commercial ultra-heat treated (UHT) whole cow's milk (WCM) obtained from a local supermarket and whole goat's milk (WGM) obtained from a local farm were used. Inulin (1 %, by mass per volume) or Nutrim® (1 %, by mass per volume) were added to the RSM, WCM and WGM at the same time as BGRA43 inoculation was performed.

The human pathogenic strains used as indicators in the antimicrobial activity assay were cultivated in the following media: Baird-Parker medium (*Staphylococcus aureus*), blood agar with tryptone (15 g/L), bovine heart extract (3 g/L), and NaCl (5 g/L) with the addition of 7 % (by volume) sheep blood (*Streptococcus pneumoniae*), Columbia agar with the addition of 5 % (by volume) of horse blood (*Bacillus* sp.), Mueller-Hinton medium (*Shi-*

gella and *Candida* species, *Micrococcus flavus* and *Yersinia enterocolitica*), all obtained from Torlak (Institute of Virology, Vaccines and Sera, Belgrade, Serbia). When a solid medium was required, agar (2 %, by mass per volume; Torlak) was added to each medium. The plates were incubated overnight at 30 or 37 °C depending on the strain involved.

Assay of antimicrobial activity

For the detection of antimicrobial activity, BGRA43 strain was inoculated in MRS broth and incubated at 37 °C for 16 h. The antimicrobial activity was tested by the agar well diffusion assay (22) against the following indicator strains: *Streptococcus pneumoniae*, *Yersinia enterocolitica*, *Shigella* spp., *Bacillus* spp., *Staphylococcus aureus*, *Micrococcus* spp. and *Candida* spp. The results are expressed as the mean diameter of triplicate independent experiments for each sample.

The cumulative effect of simulated gastrointestinal juices on L. helveticus BGRA43 survival

The viability of BGRA43 strain during simulated gastrointestinal digestion (gastrointestinal passage) was tested as previously described (*25*) with minor modifications. Artificial gastric juice was formulated using (in g/L): NaCl 7.31, KCl 0.52, $NaHCO_3$ 3.78, adjusted to pH=2 using concentrated HCl. After sterilisation, filtered pepsin (3 g/L) was added. Two artificial intestinal juices (A and B) were composed to simulate conditions in the duodenum and ileum during intestinal transit. Intestinal juice A was prepared by suspending 0.6 % (by mass per volume) of bile salts in distilled water, adjusted to pH=8 using 10 M NaOH and autoclaved. Intestinal juice B consisted of 0.3 % (by mass per volume) of bile salts in distilled water, and, after autoclaving, pancreatin (1 g/L) was added without filtration. Pepsin (from porcine stomach mucosa) and pancreatin (from porcine pancreas) were purchased from Sigma. Bile salts were obtained from Torlak.

A fresh overnight culture of BGRA43 (16 h) prepared in MRS was split in two tubes each containing 10 mL. Cells were collected by centrifugation at 3200×*g* for 15 min and resuspended in 2 mL of saline (tube 1) or 2 mL of 10 % RSM (tube 2). Subsequently, gastric juice (8 mL) was added to each tube. The addition of milk resulted in the increase of pH of the suspension from pH=2 to 5.63. The obtained mixtures were incubated for 90 min at 37 °C with agitation (~58 rpm) to simulate peristalsis. Afterwards, the two bacterial suspensions were centrifuged at 3200×*g* for 15 min, the pellets resuspended in 10 mL of simulated intestinal juice A and incubated for 10 min at 37 °C under anaerobic conditions. After that, the cells were pelleted at 3200×*g* for 15 min and finally resuspended in intestinal juice B (10 mL) and incubated anaerobically for 120 min at 37 °C. Aliquots (100 μL) from each tube were taken for enumeration of viable cells at 0, 30, 60, 90, 100, 160 and 220 min.

Hydrolysis of β-lactoglobulin

Proteolytic activity of the BGRA43 strain was analyzed as described previously (*23*) with slight modifications. Briefly, after growth on milk citrate agar (MCA) plates, cells were collected, resuspended in 100 mM sodium phosphate buffer (pH=6.8) and the absorption (UV-VIS spectrophotometer, Cary®, Varian, NC, USA) was measured at 600 nm. As substrate, 3 mg/L of β-lactoglobulin (Lactalis, Rétiers, France) dissolved in the same buffer and heated at 80 °C for 30 min was used. The cell suspension was diluted in 100 mM sodium phosphate buffer (pH=6.8) with the substrate in 1:1 ratio to obtain a final $A_{600\ nm}$=20 and incubated for 0, 9 and 24 h at 37 °C. The samples were then centrifuged (at 10 000×*g*, 10 min, 4 °C) and the supernatants with the products of hydrolysis were analyzed by reversed phase high performance liquid chromatography (RP-HPLC). The samples were made in three independent experiments before further analysis by RP-HPLC.

Liquid chromatography-tandem mass spectrometry analyses

The peptides released from BLG hydrolysis by *L. helveticus* BGRA43 were analyzed by mass spectrometry using an LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) as described previously (*26*) with minor modifications. For peptide reduction, samples were dissolved in a reduction buffer (50 mM Tris, 10 mM dithiotreitol, and 0.6 M guanidinium chloride, pH=8.5), and incubated for 1 h at 45 °C. Alkylation of free sulphydryl group was performed by adding 1.0 M iodoacetamide to a final concentration of 5 μM. After incubation for 30 min at room temperature in the dark, the reaction was stopped by adding trifluoroacetic acid (TFA) to a final volume fraction of 0.1 %. Samples were desalted again by solid-phase extraction (SPE) on a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). After rinsing the cartridge with 0.1 % TFA (by volume) solution, 5 mL of the sample were loaded into the cartridge, and the peptides were eluted using a water/acetonitrile/TFA (20:80:0.09, by volume) solution.

Raw data collected during the LC-MS/MS run were processed by Bioworks v. 3.1 browser software (Thermo Fisher) and converted into MASCOT generic format (mgf) files to be further searched against databanks using MASCOT server v. 2.2 (Matrix Science, London, UK).

Growth of L. helveticus BGRA43 strain in different milk types

The ability of BGRA43 to coagulate milk was studied in nine prepared milk combinations (RSM, RSM+1 % inulin, RSM+1 % Nutrim®, WCM, WCM+1 % inulin, WCM+1 % Nutrim®, WGM, WGM+1 % inulin and WGM+1 % Nutrim®) under laboratory conditions. For this purpose, 2 L of RSM were inoculated with 60 mL of fresh overnight culture of BGRA43 strain previously grown in MRS broth and incubated for 16 h at 37 °C. After incubation, 30 mL (3 %) of this culture were used for inoculation of nine different milk combinations (1 L each). The fermentation was carried out at 42 °C. After 4 h, the fermentation was stopped, since milk was coagulated. The pH value of milk and fermented milk products was measured during 0, 2, 3, 3.5 and 4 h of fermentation with a pH meter (model HI 9311, HANNA Instruments, Lisbon, Portugal) at room temperature. At the same time, titratable acidity of milk and fermented milk products was

determined according to the method proposed elsewhere (*27*) and expressed in Soxhlet-Henkel degrees (°SH). In addition, CFU, pH and °SH values were also measured after 1 and 7 days of storage at 4 °C.

Apparent viscosity measurement

Viscometer VISCO BASIC Plus (Fungilab S.A., Barcelona, Spain) was used to measure the viscosity of fermented milk products after 1 and 7 days of storage at 4 °C. Spindle no. 3 was used. All measurements were performed at (18±1) °C. Viscosity readings were done at a constant speed. The spindle speed was initially 100 rpm for 30 s, then it was adjusted to 20 rpm and the readings were carried out every 30 s (a total of six readings). The change of viscosity of fermented milk products at 20 rpm was investigated by the method described by Labropoulos *et al*. (*28*). According to this method, viscosity values were recorded during an estimated time following the moment of the first value shown on the display, which was taken as time zero. Viscosity values were recorded every 30 s, during 3 min. This research was conducted in two repetitions, and for each repetition a new sample was used.

Sensory evaluation

The sensory evaluation of nine manufactured fermented milk products was done by a panel of 5 sensory analysts. The products were equilibrated at room temperature when the sensory session started. Acceptable sensory characteristics (colour, taste, odour and consistency) were evaluated on a 5-point scale (1 – the worst; 5 – the best). The data were collected from each analyst independently. The reference sensory properties are as follows: for colour – typical, characteristic, intensive white; for taste – sour, characteristic, yoghurt-like; for odour – characteristic and intensive; and for consistency – uniform and compact, creamy, not lumpy and without syneresis.

Statistical data analyses

Presented results are the mean values ($\bar{x}$) calculated based on three independent measurement results ($\bar{x}\pm s$), where s is standard deviation under the conditions of repeatability.

Results

Antimicrobial activity

The inhibitory activity of fresh overnight culture of BGRA43 strain, cell-free filtrate and neutralized cell-free filtrate were determined by an agar well diffusion assay. The results showed that the antimicrobial activity could be detected only in case when wells were loaded with fresh overnight culture of BGRA43. The strongest effect was expressed against enteropathogenic strains of *Yersinia enterocolitica*, *Shigella sonnei* and *Shigella flexneri* (London 9950), as well as *Streptococcus pneumoniae*. Fresh overnight culture of BGRA43 did not inhibit the growth of *Micrococcus*, *Salmonella* and *Candida* species tested in this study (Table 1). Moreover, cell-free filtrate and neutralized cell-free filtrate were fully ineffective in all cases.

Table 1. Antimicrobial activity of *Lactobacillus helveticus* BGRA43 against various pathogenic strains

Indicator strain	BGRA 43
Streptococcus pneumoniae ATCC 496	+++
Yersinia enterocolitica O3 (clinical isolate) Torlak collection	+++
Shigella sonnei (Hamburg IO5) Torlak collection	+++
Shigella flexneri (London 9950) Torlak collection	+++
Shigella flexneri (clinical isolate) Torlak collection	+
Shigella dysenteriae (serotype 5 and 7) Torlak collection	+
Bacillus subtilis ATCC 8	+
Bacillus cereus ATCC 11778	+
Staphylococcus sp. Met^r (clinical isolate) Torlak collection	+
Micrococcus flavus ATCC 10240	–
Salmonella sp. (clinical isolate) Torlak collection	–
Candida sp. (clinical isolate) Torlak collection	–

diameter of inhibition zone: + up to 2 mm, +++ over 4 mm, – no inhibition zone

Effects of simulated gastrointestinal tract conditions on the viability of L. helveticus BGRA43 cells

The ability of BGRA43 cells to survive in simulated GI tract conditions was monitored. The starting overnight culture at zero time had $(3.12\pm0.32)\cdot10^8$ CFU/mL. After incubation for 90 min in pure gastric juice, the CFU decreased by approx. 5 log units. Successive 10-minute incubation periods in intestinal juice A containing a high bile salt fraction (0.6 %) led to an additional decrease in the number of living cells and CFU were undetectable (<1 log CFU/mL). The small number of CFU remained after further incubation of cells in intestinal juice B (Fig. 1).

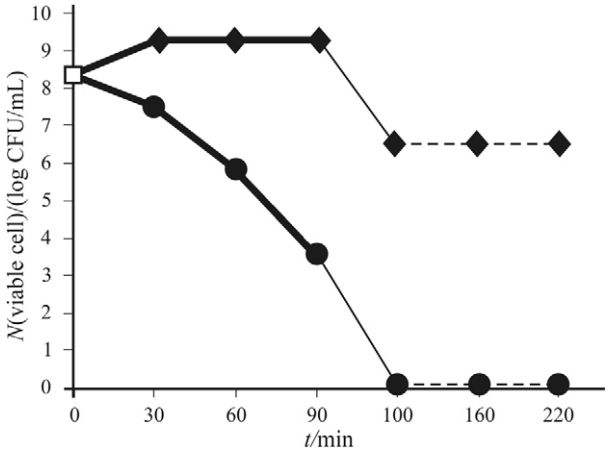

Fig. 1. Survival of *Lactobacillus helveticus* BGRA43 in simulated GI tract conditions in the presence or absence of milk. Pure gastric juice (pH=2.0) contained pepsin 3 g/L. Intestinal juice A (pH=8.0) contained 0.6 % bile salts, while intestinal juice B (pH=8.0) contained 0.3 % bile salts and pancreatin 1 g/L
□ – number of cells at the beginning of the experiment, –●– – BGRA43 in pure gastric juice, –●– – BGRA43 in intestinal juice A after incubation in pure gastric juice, --●-- – BGRA43 in intestinal juice B after incubation in intestinal juice A, –◆– – BGRA43 in gastric juice containing milk, –◆– – BGRA43 in intestinal juice A after incubation in gastric juice containing milk, --◆-- – BGRA43 in intestinal juice B after incubation in intestinal juice A

The influence of milk as a protector on the survival of BGRA43 under the simulated GI conditions was also determined. Incubation of BGRA43 in gastric juice containing milk enabled better survival. Interestingly, CFU/mL increased from $(3.12\pm0.32)\cdot10^8$ to $(1.2\pm0.12)\cdot10^9$ during the first 30 min of incubation in gastric juice with milk but then remained more or less the same over the next 60 min, *i.e.* up to 90 min of incubation in total. Transfer of cells into intestinal juice A, and a further 10-minute incubation resulted in a decline of CFU by 3 log units. After 2 h of incubation in intestinal juice B, there was a negligible decrease of living cell numbers that remained over 10^6 CFU/mL until the end of the experiment (Fig. 1).

LC/MS/MS analyses of peptides released from BLG hydrolysis by L. heleveticus BGRA43

SDS-PAGE and RP-HPLC analyses showed that the hydrolysis of BLG by BGRA43 observed after 9 and 24 h of incubation was without any significant differences (data not shown). Samples obtained after 24 h of incubation were used to obtain the highest quantity of peptides. The obtained peptides were identified by mass spectrometry analysis, which was performed on a sample hydrolyzed by BGRA43 without any modifications to avoid losing small-size peptides. The same hydrolysate was additionally desalted after the reduction and carboxymethylation to identified peptides linked by disulphide bridges. Recovery of the sequences after mass spectrometry analysis was not complete. Since our results are not quantitative, it is possible that peptides smaller than 400 Da present in very small amounts were not detected. The size of the peptides identified after hydrolysis of BLG with BGRA43 varied from 5 to 17 amino acids (559.95 to 1942.64 Da; Table 2 and Fig. 2). The 50 % of peptides have hydrophobic amino acid on their C-terminal end and another 30 % have basic amino acid lysine.

Technological properties of L. helveticus BGRA43

To compare the ability of BGRA43 to grow in the presence of lactose, glucose and the prebiotics inulin or Nutrim®, CDM containing one of the carbohydrate sources was inoculated with BGRA43 and incubated for 16 h. The results showed that total count of BGRA43 cells was fairly similar, ranging between $2.5\cdot10^7$ and $7.5\cdot10^7$ CFU/mL depending on the carbon source.

Furthermore, the ability of BGRA43 to grow and coagulate milk as a monoculture was followed in RSM, WCM and WGM with or without added inulin or Nutrim®. The total number of viable BGRA43 cells used as a starter was $8.4\cdot10^9$ CFU/mL. The initial number of viable cells counted in each nine milk combinations was between $1.9\cdot10^6$ and $5.6\cdot10^6$ CFU/mL. The number of viable cells after 4 h of incubation and cooling was about 10^8 CFU/mL, except in the case when cells were grown in RSM supplemented with inulin or Nutrim®. Despite the fact that total cell number was one log unit lower, it did not prevent the coagulation. Cell counts of BGRA43 were not lower than 10^7 CFU/mL in any case, even after 7 days of storage at 4 °C (Table 3).

Table 2. Peptides identified in the BLG hydrolysate produced by *Lactobacillus helveticus* BGRA43

m(peptide)/Da	Peptide identity*	Sequence
559.94	BLG (2–6)	IVTQT
572.18	BLG (71–75)	IIAEK
915.33	BLG (84–91)	IDALNENK
1436.43	BLG (127–138)	EVDDEALEKFDK
1567.57	BLG (43–56)	**VEELKPTPEGDLEI**
1680.67	BLG (43–57)	**VEELKPTPEGDLEIL**
1730.68	BLG (42–56)	**YVEELKPTPEGDLEI**
1942.64	BLG (41–57)	**VYVEELKPTPEGDLEIL**

*numbers refer to amino acid positions in the BLG protein sequence; sequences given in bold are part of allergenic epitopes in the BLG

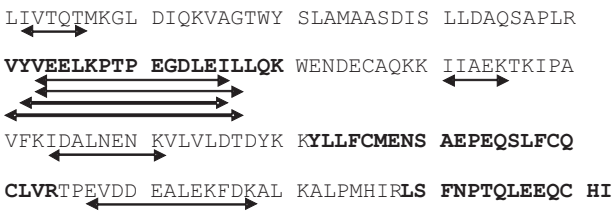

Fig. 2. Location of the main peptides (arrows) identified in the primary sequence of BLG released after hydrolysis with *Lactobacillus helveticus* BGRA43. Sequences given in bold are the allergenic epitopes in the BLG

Data for pH value measurements obtained after 4 h of fermentation were similar for all milk combinations and ranged between 4.35 (BGRA43+WCM+1 % Nutrim®) and 4.69 (BGRA43+RSM+1 % Nutrim®). After 7 days of storage at 4 °C, the pH value was maintained above 4 in all combinations when WGM was used. The most expressed changes of titratable acidity were detected between the first and seventh day of storage in all three fermented milk products prepared from RSM (Table 3).

The value of viscosity was the lowest in the fermented milk product obtained from WCM and it was 0.670 Pa·s after 0.5 min. The viscosity decreased during shearing and after 3 min it was 0.468 Pa·s. Viscosity value was the highest in the fermented milk product obtained from WGM supplemented with inulin or Nutrim®. In addition, viscosity values were decreased after 7 days of storage at 4 °C except in the case when Nutrim® was supplemented (Table 4).

Finally, the obtained products were evaluated sensorially by a group of five analysts one day after the production. The best sensory scores were observed for fermented milk products made from RSM, WCM and WGM prepared with 1 % inulin (Table 5). These fermented drinks were tasty, viscous, typically sour and refreshing, without any sense of bitterness. The worst sensory evaluation was given for fermented milk products prepared from RSM, WCM and WGM with 1 % Nutrim®, respectively. The reason for such evaluation mainly originates from the colour of the final products, because they became greyish.

Table 3. Monitoring of pH, titratable acidity (°SH) and cell survival (CFU/mL) of *Lactobacillus helveticus* BGRA43 in reconstituted skimmed milk (RSM), whole cow's milk (WCM) and whole goat's milk (WGM) with or without 1 % inulin and 1 % Nutrim®. Each milk was inoculated with 3 % BGRA43 culture previously incubated in RSM for 16 h at 37 °C

Fermentation time at 42 °C/h	BGRA43+RSM			BGRA43+RSM+1 % inulin			BGRA43+RSM+1 % Nutrim®		
	pH	°SH	*N*(viable cell)/CFU/mL	pH	°SH	*N*(viable cell)/CFU/mL	pH	°SH	*N*(viable cell)/CFU/mL
0	6.7±0.1	10.0±0.0	$(3.2\pm0.8)\cdot10^6$	6.6±0.1	13.6±1.0	$(2.8\pm0.14)\cdot10^6$	6.6±0.1	11.6±1.5	$(2.4\pm0.9)\cdot10^6$
2	5.3±0.2	19.2±2.4		5.3±0.2	18.8±1.7		5.4±0.8	18.4±1.9	
3	5.0±0.2	22.0±1.7		5.0±0.2	21.6±1.7		5.1±0.1	20.0±0.0	
3.5	4.8±0.2	24.8±1.8		4.7±0.2	24.4±2.4		4.9±0.2	21.2±2.2	
4	4.6±0.2	27.6±2.1		4.5±0.2	27.6±1.9		4.7±0.2	26.8±2.8	
Storage time at 4 °C									
1 day	4.4±0.3	32.8±1.4	$(4.1\pm0.9)\cdot10^8$	4.3±0.3	31.2±2.3	$(2.5\pm0.2)\cdot10^7$	4.5±0.2	30.4±3.3	$(4.8\pm0.7)\cdot10^7$
7 days	3.9±0.3	54.4±3.1	$(2.3\pm0.5)\cdot10^8$	3.7±0.4	49.6±3.7	$(1.2\pm0.3)\cdot10^7$	3.9±0.3	45.6±3.8	$(1.1\pm0.3)\cdot10^7$
Fermentation time at 42 °C/h	BGRA43+WCM			BGRA43+WCM+1 % inulin			BGRA43+WCM+1 % Nutrim®		
	pH	°SH	*N*(viable cell)/CFU/mL	pH	°SH	*N*(viable cell)/CFU/mL	pH	°SH	*N*(viable cell)/CFU/mL
0	6.7±0.1	10.8±0.07	$(2.9\pm0.3)\cdot10^6$	6.64±0.09	13.6±1.48	$(3.6\pm0.8)\cdot10^6$	6.6±0.1	12.8±2.2	$(4.3\pm0.6)\cdot10^6$
2	5.7±0.2	16.0±0.00		5.79±0.18	14.0±0.00		5.6±0.2	16.4±1.8	
3	5.1±0.1	23.2±0.92		5.17±0.17	20.4±0.92		5.0±0.2	22.8±1.5	
3.5	4. 8±0.2	28.8±1.11		4.81±0.17	26.8±2.64		4.7±0.2	30.4±2.1	
4	4.6±0.2	39.2±2.05		4.45±0.19	31.6±1.77		4.4±0.2	36.0±0.0	
Storage time at 4 °C									
1 day	4.3±0.2	41.6±2.8	$(1.9\pm1.0)\cdot10^8$	4.3±0.3	33.6±3.2	$(2.4\pm0.5)\cdot10^8$	4.2±0.3	39.2±2.1	$(3.7\pm0.5)\cdot10^8$
7 days	3.9±0.4	46.0±0.0	$(7.0\pm0.8)\cdot10^7$	3.8±0.4	46.8±3.1	$(7.0\pm0.4)\cdot10^7$	3.8±0.3	53.2±3.1	$(1.2\pm0.0)\cdot10^8$
Fermentation time at 42 °C/h	BGRA43+WGM			BGRA43+WGM+1 % inulin			BGRA43+WGM+1 % Nutrim®		
	pH	°SH	*N*(viable cell)/CFU/mL	pH	°SH	*N*(viable cell)/CFU/mL	pH	°SH	*N*(viable cell)/CFU/mL
0	6.2±0.2	11.6±1.2	$(5.6\pm0.7)\cdot10^6$	6.2±0.1	13.2±1.1	$(1.9\pm0.7)\cdot10^6$	6.2±0.1	12.4±2.6	$(3.1\pm0.6)\cdot10^6$
2	5.5±0.3	18.4±1.9		5.4±0.2	18.0±0.0		5.4±0.2	17.2±2.2	
3	5.1±0.3	22.0±1.3		5.1±0.1	22.0±0.0		5.0±0.2	22.8±3.1	
3.5	4.8±0.2	25.2±1.2		4.8±0.1	25.6±2.6		4.8±0.2	26.8±2.0	
4	4.6±0.2	29.6±2.0		4.5±0.2	30.8±2.1		4.5±0.2	32.4±3.5	
Storage time at 4 °C									
1 day	4.4±0.2	35.2±3.1	$(2.8\pm1.0)\cdot10^8$	4.4±0.2	36.8±4.1	$(2.2\pm0.2)\cdot10^8$	4.3±0.3	39.2±2.9	$(1.9\pm0.2)\cdot10^8$
7 days	4.3±0.3	41.6±2.6	$(6.0\pm2.3)\cdot10^7$	4.2±0.3	46.0±0.0	$(9.0\pm0.4)\cdot10^7$	4.07±0.3	50.8±3.7	$(5.0\pm0.2)\cdot10^7$

Mean values±standard deviation for N=3

Table 4. The influence of the investigated parameters on the viscosity changes of *Lactobacillus helveticus* BGRA43 in RSM, WCM and WGM with or without 1 % inulin or 1 % Nutrim®

Product composition	*t*(storage at 4 °C) / day	*t*/min					
		0.5	1	1.5	2	2.5	3
		η/(Pa·s)					
BGRA43+RSM	1	0.879±0.009	0.843±0.009	0.812±0.009	0.785±0.009	0.772±0.008	0.740±0.008
	7	0.823±0.010	0.804±0.009	0.775±0.008	0.768±0.008	0.755±0.008	0.711±0.008
BGRA43+RSM+1 % inulin	1	1.186±0.010	1.152±0.011	1.116±0.011	1.071±0.010	1.020±0.010	0.999±0.009
	7	1.025±0.010	0.995±0.009	0.963±0.011	0.927±0.010	0.881±0.010	0.838±0.009
BGRA43+RSM+1 % Nutrim®	1	1.098±0.012	1.012±0.011	0.937±0.010	0.877±0.010	0.823±0.009	0.780±0.008
	7	1.186±0.011	1.117±0.011	0.956±0.010	0.914±0.010	0.896±0.010	0.831 ±0.010
BGRA43+WCM	1	0.670±0.007	0.622±0.008	0.601±0.007	0.560±0.007	0.519±0.006	0.468±0.006
	7	0.606±0.007	0.553±0.007	0.519±0.006	0.470±0.006	0.434±0.005	0.413±0.006
BGRA43+WCM+1 % inulin	1	0.810±0.010	0.799±0.008	0.761±0.009	0.724±0.008	0.700±0.008	0.660±0.007
	7	0.776±0.009	0.736±0.009	0.708±0.009	0.661±0.008	0.626±0.007	0.598±0.006
BGRA43+WCM+1 % Nutrim®	1	0.763±0.008	0.725±0.008	0.689±0.008	0.632±0.008	0.579±0.007	0.529±0.007
	7	0.789±0.008	0.758±0.008	0.727±0.008	0.681±0.007	0.646±0.008	0.606±0.007
BGRA43+WGM	1	1.165±0.013	1.033±0.012	0.976±0.011	0.961±0.011	0.948±0.011	0.924±0.010
	7	1.101±0.012	0.987±0.010	0.942±0.010	0.905±0.010	0.877±0.009	0.820±0.010
BGRA43+WGM+1 % inulin	1	1.322±0.012	1.257±0.013	1.171±0.012	1.054±0.012	1.002±0.011	0.955±0.011
	7	1.268±0.012	1.210±0.012	1.165±0.013	1.079±0.012	0.972±0.011	0.913±0.011
BGRA43+WGM+1 % Nutrim®	1	1.289±0.012	1.231±0.012	1.181±0.013	1.162±0.013	1.123±0.012	1.046±0.011
	7	1.326±0.013	1.289±0.012	1.271±0.012	1.224±0.013	1.201±0.012	1.159±0.012

Mean values±standard deviation for *N*=3

Table 5. Sensory evaluations (grades 1- 5) of fermented milk products after 1 day of storage at 4 °C

Products composition	Sensory properties				
	Colour	Taste	Odour	Consistency	General appearance
BGRA43+RSM	3.5	3.0	2.5	3.5	4.0
BGRA43+RSM+1 % inulin	3.5	4.0	3.5	4.5	4.5
BGRA43+RSM+1 % Nutrim®	1.5	3.5	3.5	3.5	3.0
BGRA43+WCM	4.0	4.0	4.0	4.0	3.5
BGRA43+WCM+1 % inulin	4.5	4.0	4.5	4.5	4.0
BGRA43+WCM+1 % Nutrim®	2.0	3.5	3.5	3.0	3.0
BGRA43+WGM	5.0	4.5	4.5	4.5	5.0
BGRA43+WGM+1 % inulin	4.5	5.0	4.5	4.5	5.0
BGRA43+WGM+1 % Nutrim®	2.0	4.0	4.0	3.5	3.5

Data are mean values of evaluation scores obtained for each sample by five sensory analysts

Discussion

In this study, the antimicrobial potential of *Lactobacillus helveticus* BGRA43 against different human pathogens, its ability to survive in simulated gastrointestinal conditions in the presence of milk and its ability to hydrolyze BLG was evaluated before using it as a monoculture for manufacturing of fermented products from different milk samples at laboratory scale. Our study showed that BGRA43 exhibited broad antimicrobial spectrum. Additionally, taking into account the fact that living cells of BGRA43 are necessary for growth inhibition of pathogens, we can speculate that antimicrobial activity arises from the synergistic action of organic acids synthesized by BGRA43 cells. This assumption was supported by the observation that narrow zone of inhibition was obtained when acid-resistant *S. flexneri* clinical isolate was used as indicator strain. However, additional experiments are needed in order to make a final statement.

In vitro studies of bacteria have been used to evaluate various characteristics of potential probiotics. Among these, tolerances of the low pH in the stomach and bile components in the proximal parts of the intestine seem

to be very important (*29*). Strain BGRA43 is sensitive to gastric conditions, *i.e.* low pH, judging by the decrease of living cell numbers to 10^4 CFU/mL upon incubation for 90 min in simulated gastric conditions. However, BGRA43 showed remarkable survival in the simulated gastric juice with milk. Moreover, it retained stability in cell numbers during incubation in simulated intestinal conditions. Kos *et al.* (*5*) obtained similar results for *L. acidophilus* M92, showing that skimmed milk, whey protein concentrate and mucin function as buffering agents and inhibitors of digestive proteases *in vitro* and so increase their survival.

Published results show that three themophilic LAB strains were able to degrade BLG during growth in CDM and under starving conditions, giving different peptide profiles (*30*). Results obtained for *L. acidophilus* CRL 636 showed that this strain preferentially cleaved BLG after hydrophobic residues (*31*), as it was shown for BGRA43. As shown in Fig. 2, BGRA43 was able to hydrolyze one of the three major allergenic epitopes of BLG at Val^{41}, Tyr^{42}, and Val^{43} positions. In general, the degradation of BLG, α-lactalbumin and whey proteins was studied for *L. delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *L. acidophilus* (*30*), *L. paracasei* NCC2461 (*32*) and *Bifidobacterium lactis* NCC362 (*33*). To the best of our knowledge, this is the first report on the ability of *L. helveticus* strain to hydrolyse BLG.

Strain BGRA43 grew very well in the presence of either inulin or Nutrim® even when they were the only carbon source. Moreover, it also grew in cow's or goat's milk with or without inulin or Nutrim®. The initial number of viable BGRA43 cells was 10^6 CFU/mL, which correlates with the data reported by Dave and Shah (*34*) and Deutsch *et al.* (*35*). When BGRA43 was used as a single starter, the number of living cells in all prepared final products corresponded to the required minimum level of viable probiotic bacteria (*36*,*37*). In addition, our results showed that the viable count of BGRA43 during 7 days of storage slightly decreased, which makes it suitable for use as a potential probiotic starter strain.

According to the results obtained after viscosity measurements, it was found that the viscosity values of all products prepared with inulin or Nutrim® were higher than the viscosity values of those prepared without prebiotics. Havrlentová *et al.* (*38*) reported similar data. Viscosity values obtained for yoghurts prepared from commercial UHT whole cow's milk were lower than the viscosity values of yoghurts prepared from skimmed or whole goat's milk. Our results are in agreement with those obtained previously in study of Labropoulos *et al.* (*28*), who reported that high temperature treatments of milk cause a decrease in the viscosity of yoghurt. Additionally, it was noticed that viscosity of all tested fermented milk products made with Nutrim® became higher after 7 days of storage at 4 °C, which correlates with the results obtained by Inglett *et al.* (*39*). Sensory characteristics mainly depend on milk type and its composition, as well as on starter culture used for fermentation. In our study, all fermented milk products prepared with inulin showed very good to excellent sensory features. *L. helveticus* is used as starter culture in a variety of fermented dairy products as flavour enhancer and it is capable of reducing bitterness (*40*). This is probably the reason why the obtained fermented milk products containing inulin showed no bitter taste. The lowest scores given to fermented milk products supplemented with Nutrim® are related to the colour change of final products. According to the results presented by Park *et al.* (*41*), dark colour of Nutrim® is associated with polyphenol oxidase activity.

Conclusions

It was determined that *Lactobacillus helveticus* BGRA43 could be considered as a potential probiotic candidate with suitable technological properties. The strain is able to reduce the allergenicity of BLG, which can contribute to obtaining a product not only with probiotic characteristics but also with better digestibility, especially in people who are allergic to cow's milk. Moreover, it may be used as a single starter for the preparation of fermented milk products from bovine or caprine milk.

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