Technological and probiotic potential of BGRA43 a natural isolate of Lactobacillus helveticus

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Lactobacillus helveticus BGRA43 is a human intestinal isolate showing antimicrobial activity, amongst others, against Yersinia enterocolitica, Shigella sonnei, Shigella flexneri, and Streptococcus pneumoniae. BGRA43 produces PHH proteinase with proteolytic activity on both casein and β-lactoglobulin (BLG). BGRA43 is able to reduce the allergenicity of BLG. Bioactive peptides released in BGRA43 fermented milk are potent modulators of innate immunity by modulating the production of proinflammatory cytokines IL-6 and TNF-α. BGRA43 is able to survive in simulated gastric and intestinal conditions. The growth of BGRA43 in milk results in a fast acidification lowering the milk pH to 4.53 generating mild, homogeneous, and viscous yogurt-like product. The strain BGRA43 grows suitably in pure cow or goat’s milk as well as in milk containing inulin or nutrim even when they are used as the sole carbon source. It is suggested that strain BGRA43 could be used as a single-strain culture for the preparation of yogurt-like products from bovine or caprine milk. Overall, L. helveticus BGRA43 could be considered as a potential probiotic candidate with appropriate technological properties attractive for the dairy industry.

Keywords: Lactobacillus helveticus, milk fermentation, probiotics, prebiotics

INTRODUCTION

Lactobacillus helveticus belongs to a homofermentative L. acidophilus/L. delbrueckii subgroup of lactic acid bacteria (Felix and Del-laglio, 2007) associated to fermented milk, meat, and plant products as well as the gastrointestinal (GI) and urogenital (UG) tracts of humans and animals. L. helveticus has been traditionally used as a starter in the production of Italian and Swiss-type cheeses (Gurza et al., 1998; Borelo et al., 2001). In the last 10 years, the scientific knowledge about different L. helveticus strains with health-promoting properties has accumulated (de Moreno de LeBlanc et al., 2005; Guglielmetti et al., 2010; Ohland and MacNaughton, 2010; Slattery et al., 2010). Significant features that provide for the broad use of L. helveticus in food and feed biotechnology are often complemented with demonstrable probiotic characteristics such as antimicrobial, immunomodulatory, and antihypertensiv activies (LeBlanc et al., 2004; Zhao et al., 2011). The general characteristics of BGRA43 are as follows: (a) growth is optimal under anaerobic conditions in the presence of 10% CO2; (b) it grows in the presence of 2% NaCl but not in the presence of either 4 or 6.5% NaCl; (c) during its growth in milk, it forms a homogeneous curd showing extensive auto-aggregation giving the curd a slimy appearance, and (d) it efficiently hydrolyzes lactose. The BGRA43 strain grows well in MRS broth at 37 and 45°C after successive sub-cultivations but not at 15°C. The optimal growth conditions for the BGRA43 strain have been determined and results show that the shortest generation time can be achieved when the cells are grown in MRS broth at 37°C. The stationary growth phase is reached after 12 h. However, it was shown that during its growth at 42°C, the log period is longer and the stationary growth phase is attained after 14 h, although at this temperature the total cell number is slightly higher compared to growth at 37°C. A detailed characterization of the BGRA43 strain was also performed, including tests of its antimicrobial and proteolytic activity, as well as a study of its probiotic and technological properties. The results revealed that BGRA43 exhibits significant antimicrobial activity against a variety of microorganisms, including Staphylococcus aureus, Escherichia coli C600, Bacillus mycoides, Pseudomonas sp., Yersinia enterocolitica, Shigella sonnei, and Shigella flexneri, as well as Streptococcus pneumoniae. In addition, the experiment performed in sulfite agar showed
that BGRA43 exhibited an inhibitory effect on the growth of Clostridium spongens, widely distributed in nature as a food contaminant, as well as a human and animal pathogen. It was found that any growth of dextran could not be detected in co-culture with BGRA43 (Banina et al., 1998). Additionally, the nature of the BGRA43 antimicrobial activity was tested using fresh bacterial cultures (grown for 9, 12, and 16 h) from which cell-free and neutralized cell-free filtrates were prepared and examined in parallel. The possibility that the compound possessing an antimicrobial activity is of a proteinaceous nature was excluded, since in the well-diffusion assay for detection of bacteriocins the characteristic reduction of the inhibitory zone of indicator strain growth was absent after the addition of the proteolytic enzymes – pronase E, proteinase K, trypsin, and pepsin (Strahinic et al., 2012). While combination of lactic and acetic acids can reduce bacterial growth, lower degree of dissociation of these acids (under pH 4–4.6) might be associated with strong antimicrobial activity (Ouwehand, 1998). Inhibitory effect of BGRA43 is completely lost when supernatant was used instead of fresh bacterial culture. These results suggested that organic acid(s) are involved in antimicrobial activity of BGRA43. We can only speculate that synergistic effect of lactic and acetic acid in combination with some currently unknown compounds provided antimicrobial activity to a wide range of bacteria.

It is well known that the proteolytic activity of lactic acid bacteria, in terms of the degradation of casein fractions, plays an important role in the development of texture and flavor during the fermentation process and is very often essential for optimal growth in milk. Well-defined proteinases of lactic acid bacteria are the cell-envelope proteinases (CEP) that possess different domain organization. The characterization of the proteolytic activity of L. helveticus BGRA43 included the analysis of αs1-, β-, κ- and total-casein degradation. Additionally, the gene(s) encoding for CEP present in the BGRA43 genome were determined. The results revealed that the whole cells of BGRA43 possessed a strong casinolytic activity. It was shown that BGRA43 was able of completely hydrolyzing αs1- and β-casein fractions after 1 h of incubation in 100 mmol Na-phosphate buffer, pH 6.5 at 45°C. The whole cells also hydrolyzed κ-casein under the same conditions but after 2 h of incubation. The obtained results suggested that the release of the proteinase from the cell envelope of BGRA43 is not Ca²⁺ dependent. The activity of the crude proteinase extract was partially inhibited in the presence of Ca²⁺ and Zn²⁺ ions, but also by EDTA, EGTA as well as PMSF (Fiza et al., 2001).

From a genetic point of view the organization of genes encoding CEP in the BGRA43 strain appears to be different from all L. helveticus strains described so far. Until now, two CEPs have been described and completely analyzed in L. helveticus, PrtH2 and PrtH. Both belong to the subtilisin-like serine proteinase family with whom they share 22% amino acid sequence identity. According to the work of Genay et al. (2009), the prtH2 gene is a ubiquitous gene in L. helveticus, and it is present in all tested strains, whereas the presence of the prtH gene is strain-dependent. In our study, PCR analysis with primers corresponding to the specific regions of the prtH and prtH2 genes both present in L. helveticus CNRZ32, showed that BGRA43 possesses only the prtH2 gene, sharing 98.9% of identity with the same gene of CNRZ32. The hypothesis that PrtH is most probably the only active proteinase in the strain BGRA43 was further confirmed after obtaining the proteolytically inactive derivative of BGRA43 designated BGRA433. Results showed that the derivative BGRA433 carries a deletion within the prtH gene encoding the catalytic region of the proteinase (Lozo et al., 2011). Recently, a study on distribution of CEP paralogs among different strains of L. helveticus revealed that the most common CEP gene isprtH3, compared to theprtH, prtH2, and prtH4 (Broadbent et al., 2011). Only one strain tested in this study has onlyprtH gene, but its proteolytic activity, biochemical characterization and other features were not analysed.

The fact that the strain BGRA43 possesses only the PrtH proteinase, in contrast to other tested L. helveticus strains, could be especially interesting in relation to the production of the strain-specific bioactive peptides as a result of casein hydrolysis. Mass spectrometry analyses of the peptides released after β-casein hydrolysis by BGRA43 identified 22 peptides, varying from 5 to 46 amino acids. The uniqueness of the BGRA43 strain is reflected in the observation that the released peptides originated from the more hydrophobic N-terminus portion of β-casein, as well as that the numerous degradations of β-casein occurred after phenylalanine. The peptides identified after 24 h of β-lactoglobulin (BLG) hydrolysis varied from 5 to 17 amino acids; most of them had a hydrophobic amino acid on their C-terminus. Interestingly, the obtained results show that one of the three major allergenic epitopes of BLG at the Val¹¹, Tyr²², and Val³³ positions is hydrolyzed by the BGRA43 crude extract (Lozo et al., 2011; Strahinic et al., 2012). Moreover, four protein fractions released during BGRA43 milk fermentation exhibited an inhibitory effect on human monocyte RB, as well as anti-inflammatory effects on the Nd-THP-1 monocyte cell line due to the down-regulation of TNF-α and IL-6 production (Tompá et al., 2010).

According to the FAO/WHO guidelines (FAO/WHO, 2006), the main criteria for probiotic selection and application is the survival of the bacterial cells during their passage through the GI tract. The strain BGRA43 was examined in a chemically simulated GIT transit, which included an assessment of its survival in gastric and intestinal juices. The obtained results show that L. helveticus BGRA43 survived GIT passage much better when it was included in a food matrix (skimmed milk), probably due to the buffering and protective effect of the skimmed milk. While the strain could not survive the simulated GIT conditions in a saline buffer, the survival of BGRA43 in simulated gastric juice supplemented with skimmed milk during 90 min of incubation was 100%. The first short exposure to bile salts (10 min, 0.6%) reduced the bacterial viability by three log units, but after lowering the concentration of bile salts to 0.3%, the number of surviving bacteria was higher (Strahinic et al., 2012). To evaluate the hydrophobic/hydrophilic cell surface properties of L. helveticus BGRA43, the affinity of the strain to the organic solvents hexadecane and chloroform was tested. Preliminary results pointed out a high affinity for both 70% hexadecane and 75% chloroform which is in agreement with values obtained for other human isolates (Ocana et al., 1999). Another criterion provided by the FAO/WHO guidelines for probiotic evaluation is the ability of a strain to colonize transiently the intestinal mucosa. Therefore, the adhesion ability
of L. helveticus BGRA43 to the Caco-2 epithelial intestinal cell line was determined. As controls, the exopolysaccharide (EPS)-producing strain L. paraplantarum BGCG11 and its non-ropy derivative BGCG11-NB1 were used (Nikolic et al., 2012). The BGCG11 strain, as shown previously, has an adherence property similar to the well-known probiotic strain L. rhamnosus GG. The derivative BGCG11-NB1 showed significantly higher adherence to epithelial intestinal cell lines compared to GG and BGCG11 strains. It seems that the EPS synthesized by BGCG11 strain covers the producing strain and hinder bacterial adhesion to enterocytes whereas in the absence of EPS other surface molecules could have been exposed and therefore act as adhesins. In general, the adherence similar to the well-known probiotic strain L. rhamnosus GG. The derivative BGCG11-NB1 showed significantly higher adherence to epithelial intestinal cell lines compared to GG and BGCG11 strains. It seems that the EPS synthesized by BGCG11 strain covers the producing strain and hinder bacterial adhesion to enterocytes whereas in the absence of EPS other surface molecules could have been exposed and therefore act as adhesins. In general, the adherence similar to the well-known probiotic strain L. rhamnosus GG. The derivative BGCG11-NB1 showed significantly higher adherence to epithelial intestinal cell lines compared to GG and BGCG11 strains. It seems that the EPS synthesized by BGCG11 strain covers the producing strain and hinder bacterial adhesion to enterocytes whereas in the absence of EPS other surface molecules could have been exposed and therefore act as adhesins. In general, the adhesion of BGRA43 to the Caco-2 cell line was similar to the adherence of the derivative BGCG11-NB1, and significantly higher (p < 0.05) than the adherence of BGCG11 (Figure 1A). These findings indicated that the BGRA43 strain has a significantly better adherence trait than the probiotic and reference strain L. rhamnosus GG.

The capability of the L. helveticus BGRA43 strain inactivated by UV-radiation to elicit the immune response was tested by examining the induction of proliferation of gut-associated lymphoid tissue (GALT) isolated from rats. The proliferation indices of GALT measured in the presence of BGRA43 and the L. paraplantarum BGCG11 strain, which served as a positive control, show that in the absence of stimulus the presence of both strains significantly (p < 0.05) decreased the proliferation of GALT in comparison with the control (Figure 1B). The results show that the strain BGRA43 has a strong immunomodulatory effect, revealed as a reduction in the proliferation of lymphocytes, and that it could play a role in the suppression of the immune response in vivo. Taking into account that the immunomodulatory ability of probiotics is not effective in all human populations it could be assumed that the strain BGRA43, which possesses a potential immunomodulatory effect, could be specifically used in a diet of autoimmune disorder patients that present an increased inflammatory status. However, according FAO/WHO and EFSA recommendations, before strains such as BGRA43 can be used for human consumption the strain needs to be tested in human clinical trials.

TECHNOLOGICAL PROPERTIES OF L. helveticus BGRA43

Lactobacillus helveticus strains are traditionally used for the production of different types of cheeses primarily for long-ripening cheeses such as Parmesan, Emmental, Giana, Prowolone, and Gruyère (Torriani et al., 1994; Giraffa et al., 1998; Grayere and Neviani, 1999; Rossetti et al., 2008). During our work, we tried to apply BGRA43 as a starter in dairy fermentation to obtain a high-quality yogurt-like product.

The ability of BGRA43 to grow and curdle milk was initially tested in reconstituted skim milk (RSM). L. helveticus BGRA43 exhibited rapid growth at 37°C and 42°C in RSM, after 6 h of growth the bacterial culture reached approximately 3 x 10^8 CFU/ml (Banina et al., 1998). The growth of BGRA43 in a chemically defined medium (CDM) containing either inulin or nutrim as carbohydrate source, was tested in parallel. These two fructans are dietary fibers exploited as potential prebiotic stimulators of intestinal bacterial growth. BGRA43 displayed very satisfactory growth in the presence of either inulin or nutrim, even when they were the sole carbon source. The total cell number was approximately 10^7 CFU/ml and was comparable to the cell number obtained when the monosaccharides, glucose and lactose, were used in CDM as a carbon source.

The fermentation ability of BGRA43 in various milk sources on a laboratory scale was examined. For this purpose, aside from cultivation by RSM, commercial UHT sterilized whole calf’s milk (WCM), obtained from a local supermarket and whole goat’s milk (WGM), obtained from a local farm, were used. Furthermore, the fermentation ability of BGRA43 in RSM, WCM, and WGM containing inulin (1%) or nutrim (1%) was also tested. At the end of a 4 h fermentation at 42°C, the number of viable cells in each of the nine milk combinations increased from 10^6 to 10^7 CFU/ml, while the pH values ranged between 4.35 and 4.69, depending on the milk. At the same time, the titratable acidity of the yoghurt was maintained in the presence of BGRA43 and its non-ropy derivative BGCG11-NB1 to the human epithelial intestinal Caco-2 cell line. The statistical differences (p < 0.05) between the strains L. helveticus BGRA43 and control strain L. paraplantarum BGCG11 at a bacteria : cell line ratio of 5:1. The statistical differences (p < 0.05) between each stimulus and the control (L-cells) were determined by Student’s t-test.
the obtained yogurt-like products was measured. Acidity varied among the products. Thus, at the end of fermentation, acidity did not exceed 27.6°SH, 29.2°SH, and 29.8°SH for products obtained in RSM, WCM, and WGM, respectively. Similar results were obtained for the remaining fermented products containing inulin or nutrient. It is worthwhile mentioning that the highest viscosity of the yogurt-like products was recorded with either inulin or nutrient. This trend of viscosity was maintained even after 7 days of storage at 4°C. During the same storage conditions, the viable count of BGRA43 was 10⁶ CFU/ml in all of the tested product samples. These properties lend further support for the use of BGRA43 as a starter culture strain. Finally, after 7 days of storage, the yogurt-like products made from RSM, WCM, and WGM, all of which contained 1% inulin, retained sufficient acidity and received high scores by sensory analysts, which was not the case when nutrient was present (Strahinic et al., 2012). A test on yogurt-like production using B. amyloliquefaciens as a starter under industrial conditions was performed in one private SME. Obtained product was of good quality, that can find application in dairy industry. The optimal pH and temperature for BGRA43 growth was 7.4 and 4°C, respectively. This strain that can ferment lactose to its end products, and survived simulated digestion. More than 70% of BGRA43 bacteria adhere to non-polar solvents, hexadecane and chloroform, classifying it as a high hydrophobic strain. Strong adhesion ability of BGRA43 to intestinal epithelial cells was subsequently confirmed in vitro with Caco-2 cell line. Four oligopeptides showing anti-inflammatory effect were detected after BGRA43 milk fermentation. Moreover, reduction of lymphocytes proliferation confirmed that BGRA43 cells possess strong immunosuppressive potential.

Finally, technological properties of this strain were also satisfactory when tested on a laboratory and small industrial scale. However, for application as starter culture in production of fermented milk products, further experiments on a larger industrial scale are necessary.

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