RESEARCH ARTICLE

Safety issues of *Lactobacillus bulgaricus* with respect to human gelatinases *in vitro*

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Abstract

In oral medicine and dentistry probiotics have shown promising results in controlling dental diseases and yeast infections. This study was made to investigate the effect of eight strains of *Lactobacillus bulgaricus* and their effects on human matrix metalloproteinase-9 (MMP-9). The hypothesis was that these bacteria used in yoghurt production for centuries are not proteolytic and thus can be safely used in the development of probiotic preparations. Bacterial cell fractions and supernatant specimens were prepared and studied with gelatinase zymography and MMP-9 activation was assessed by immunoblotting. The effect of synthetic MMP inhibitors and a serine protease inhibitor (Pefabloc) on bacterial proteinases was studied with zymography. The results showed very low gelatinolytic activity. There was a slight difference between the supernatant and cell fractions so that the supernatant specimens produced weak gelatinolytic bands in zymography while hardly anything was seen in the cell fraction series. The tested synthetic MMP inhibitors and Pefabloc did not affect the proteolytic activity of the lactobacilli strains. The lactobacilli did not seem to induce the conversion of proMMP-9 to its active form. Consequently, our study hypothesis was confirmed and the studied *Lactobacillus* strains are not likely to degrade host tissue components.

Introduction

The importance of administration of live microorganisms with health benefits has been advocated through many *in vitro* and *in vivo* studies during the past few decades. The term 'probiotic' defines this entity as 'live microorganisms, which when administered in adequate amounts, confer a health benefit on the host', a definition adopted by the International Scientific Association for Probiotics and Prebiotics in 2002 (Guarner *et al.*, 2005).

A variety of microorganisms have been tried as probiotics. Common bacterial species used in probiotic preparations include strains of *Lactobacillus, Bifidobacterium, Streptococcus* and *Saccharomyces*. The role of probiotics has been studied in prophylaxis and management of different pathological conditions, with a main focus on diseases of the gastrointestinal tract (Elmer *et al.*, 1996; Anderson & Gilliland, 1999; Van de Water *et al.*, 1999; Meydani & Ha, 2000).

A marked interest in the investigation of different bacterial species with respect to oral health has been observed during the past few years. Within dentistry, studies with *Lactobacillus rhamnosous GG* (Meurman *et al.*, 1994; Näse *et al.*, 2001; Ahola *et al.*, 2002) and *L. reuteri* (Nikawa *et al.*, 2004) have defined their potential in interacting with *Streptococcus mutans* by reducing the number of this caries pathogen, thus suggesting a role for probiotics in caries prophylaxis. An evident decrease in gum bleeding and reduced gingivitis has been observed by Krasse *et al.* (2006) with the application of *L. reuteri*. A reduction in the prevalence of *Candida albicans* in the oral cavity of the elderly after consumption of probiotic cheese containing *L. rhamnosus* GG and *Propionibacterium freudenreichii* ssp *shermanii* JS has been observed by Hatakka *et al.* (2007).

The number of strains that are potential probiotic candidates gradually increases. Thus, issues of safety demand

substantial consideration, and *in vitro* tests are critical when assessing the mechanisms of probiotic effect with no hazards being imposed on the host by the use of living microorganisms in therapy. According to the Guidelines for the evaluation of probiotics in food (FAO/WHO Working Group, 2002), any probiotic species should be assessed for safety by ruling out its deleterious metabolic activities, the possibility to induce systemic infections, excessive immune stimulation in susceptible individuals and gene transfer. To be considered as safe, a probiotic microorganism should not interfere with the intricate systems that maintain oral homeostasis. Safety issues of lactobacilli have been studied by evaluating adhesion to main constituents of extracellular matrix: collagen type IV and fibrinogen; binding to intestinal mucus; induction of respiratory burst in peripheral blood monocytes and resistance to serum-mediated killing (Vesterlund *et al.*, 2007). There are no studies presently addressing the issues of safety related to screening putative probiotic species with a scope of application in the oral cavity. In the current study we applied a method evaluating the effect of probiotic candidates on the activation of matrix metalloproteinases (MMPs), the enzymes responsible for extracellular matrix degradation and remodeling. Elevated levels of salivary MMPs have been associated with metabolic activity of various oral pathogens (Ding *et al.*, 1997; Mäntyla *et al.*, 2003; Söder *et al.*, 2006). Thus, the capacity of some microbial species to convert extracellular matrix enzymes into their active forms might be considered an inherent virulence factor. Consequently, a precise evaluation is needed before such microorganisms can be recommended as probiotics.

A commonly used vehicle for probiotic administration is yoghurt and other dairy products based on milk fermentation by lactic acid bacteria. There is some debate about whether or not yogurt starter bacteria should be considered probiotics. The yogurt starter cultures *L. bulgaricus* and *S. thermophilus* are used in milk fermentation. However, these cultures are not very resistant to the conditions in the stomach and small intestine and generally they do not reach the gastrointestinal tract in high numbers. Therefore, they cannot mediate any probiotic effects. But these starter culture bacteria have been shown to improve lactose digestion in people lacking lactase and have demonstrated some immune enhancing effects (Pereyra & Lemannier, 1993; Meydani & Ha, 2000). For these reasons these bacteria have been suggested to have probiotic characteristics. Regardless of its broad availability in dairy products, however, *L. bulgaricus* has not yet been studied as a probiotic with the scope of activity in the oral cavity.

The long-established history of *L. bulgaricus* consumption with dairy products allowed us to hypothesize that this species has little or no detrimental effect on basic components of extracellular matrix. We further anticipated that it has an unsubstantial capacity to induce or increase the activity of MMPs, the enzymes responsible for extracellular matrix degradation and often associated with oral diseases (Sorsa *et al.*, 2004, 2006). The specific aim of the present study was to assess the proteolytic activity of different *L. bulgaricus* strains utilized in yogurt production and to study their effect on human progelatinase B (proMMP-9). Subsequently, this study addressed the issue of safety as a prerequisite for further research on the role of this species in oral and general health.

Materials and methods

Bacterial strains

This study included seven *Lactobacillus delbrueckii* spp. *bulgaricus* dairy strains isolated from fermented milk and one laboratory strain *L. bulgaricus* ATCC 11842. The dairy strains were kindly provided from LB Lactis (Scientific-Applied Laboratory for Starter Cultures and Probiotic Products, Plovidv, Bulgaria) culture collection. Lactobacilli were grown in de Man, Rogosa and Sharpe broth at pH 6.4 (MRS broth, LAB M™, IDG Ltd., Lancashire, UK) at 37 °C in 5% CO_2 atmosphere for 24 h. The pH of the culture medium after 24 h' bacterial growth was measured for each test strain and OD was defined at 497 nm wave length. Cells were harvested by centrifugation at 5000 *g* for 20 min, and the supernatants were dialyzed against distilled water for 2 h at 4 °C using Spectra/por Membrane MWCO: 12–14 000 (Spectrum Laboratories Inc., CA, USA). The pH of the supernatants was measured with a pH meter, and they were lyophilized and stored at 4 °C. Harvested cells were washed twice with PBS, pH 7.4, suspended in 1 mL of PBS and stored at − 75 °C. Prior to use they were sonicated on ice to disrupt the cells; this was monitored by phase-contrast microscopy. Both the cell fractions and the supernatant fractions were used in this study.

Assessment of the gelatinolytic activity of bacterial strains

The method evaluating the proteolytic activity of *L. bulgaricus* strains in terms of matrixmetalloproteinase activity was applied corresponding to protocols used for assessing the gelatinolytic activity of defined oral pathogens (Grayson *et al.*, 2003). The presence of gelatinolytic proteases was assayed with the use of an enzymography in 0.75-mm-thick 11% SDS-PAGE gels impregnated with 1 mg mL^{-1} gelatin which had been labeled with fluorescent dye using 2-methoxy-2,4-diphenyl-3-(2H)furanone (MDPF, Fluka, Buchs SG, Switzerland). Lyophilized supernatants were concentrated 10 times by adding TNC buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM $CaCl_2$, pH 7.6). Samples were mixed with 5 μL Laemmli's sample buffer without reductant

(Laemmli, 1970) in the ratio of three to one, and incubated at room temperature for 2 h. Low-range prestained SDS-PAGE standards (BioRad, CA, USA) served as molecular weight markers. Human saliva was used as a positive control. SDS-PAGE was run at 110 V for 60 min in an ice-bath in nonreducing conditions. The gels were washed for 30 min with 50 mM Tris-HCl buffer, pH 7.5, containing 25% Tween 80, 0.02% NaN_3, and then for 30 min with the same buffer supplemented with 0.5 mM $CaCl_2$ and 1 μM $ZnCl_2$. Finally, the gels were incubated in 50 mM Tris-HCl buffer, pH 7.5, containing 0.02% NaN_3, 0.5 mM $CaCl_2$ and 1 μM $ZnCl_2$ overnight and for 7 days at 37 °C, respectively. The pH values of the buffers were adjusted at 7.5, 6.5 and 5 to study whether the different pH values affect the gelatinolytic activity of bacterial proteases. The degradation of gelatin was visualized under long-wave UV light. White zones of lysis indicating gelatin degrading were revealed by staining with 1% Coomassie Brilliant Blue. Gels were scanned with Scanner, GS-700 Imaging densitometer (Bio-Rad) and analyzed by QUANTITY ONE – program (Bio-Rad).

ProMMP-9 activation assay by *L. bulgaricus* strains

The molecular forms of MMP-9 were detected by a modified (Sorsa *et al.*, 1997) ECL Western blotting kit according to protocol recommended by the manufacturer (GE Healthcare, Amersham, UK). Aliquots of 2.5 μL (2.5 ng μL^{-1}) of human recombinant proMMP-9 (Invitek GmbH, Berlin-Buch, Germany) were incubated with 12.5 μL bacterial samples for 6, 12 and 24 h at 37 °C. For the positive control pro-MMP-9 was coincubated with aminophenylmercuric acetate (APMA, Sigma, St Louis, MO, USA), an optimal organomercurial pro-MMP activator (Sorsa *et al.*, 1997), for 2 h at 22 °C. Pro-MMP-9 without APMA was used as a negative control. Samples with Laemmli's buffer (without reductant) were boiled for 5 min to intercept enzymatic activity. The proteins were separated by 11% SDS-PAGE and electrotransferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Nonspecific binding was blocked with 5% milk powder (Valio Ltd, Helsinki, Finland) in TBST buffer (10 mM Tris-HCl, pH 7.5, containing 22 mM NaCl and 0.05% Triton-X) for 1 h, followed by 4 × 15 min washes in TBST buffer. Membranes were incubated with 1 : 500 polyclonal primary antibody (rabbit anti-human MMP-9) (Calbiochem, Darmstadt, Germany) overnight, followed by anti-rabbit IgG, horseradish peroxidase-linked whole antibody from donkey (1 : 800 dilution) (GE Healthcare) for 1 h. The membranes were washed four times for 15 min in TBST between each step. The proteins were visualized using the enhanced chemiluminescence (ECL) system (GE Helathcare) and scanned for data analysis as described above for the zymograms.

Effect of synthetic MMP inhibitors and Pefabloc on bacterial proteinases

To determine the inhibitory effect of different synthetic MMP inhibitors on *L. bulgaricus* proteases, Ilomastat (Chemicon International Inc., CA, USA), EDTA (Merck KGaA, Darmstadt, Germany), CMT3, CMT308 (Collagenex Inc., Newtown, PA, USA), CTT1 (Koivunen *et al.*, 1997) and a serine protease inhibitor, Pefabloc (Boehringer Mannheim GmbH, Mannheim, Germany), were employed in this study. The inhibitors (final concentration of 0.2 mM) were incubated together with the supernatant and bacterial cell samples for 2 h at 37 °C in dark. The same sample volumes as in the zymography assay were used. The MDPF-zymography was assayed as previously to detect the residual gelatinolytic activity. The respective samples without inhibitors were used as controls.

All images that were scanned were analyzed using the GS-700 Model Imaging Densitometer and BIORAD QUANTITY ONE analysis program.

Results

All the strains included in the study demonstrated similar growth in MRS broth. Table 1 gives the values for the pH and $OD_{492\,nm}$ measurements after the 24 h incubation.

Gelatinolytic activity

Gelatin zymography with a labeled substrate enables the detection of type I and type IV collagenolytic activity. Zymography has the advantage that in addition to detecting enzyme activity it can be used to provide information about the molecular weight of an enzyme and thus help identify the enzyme. The gelatinolytic activity of all strains tested was very low compared with positive human saliva controls. Degradation of gelatin was not detected after an 18 h incubation period. However, the prolonged 7-day incubation time yielded molecular weight bands at the area of 106 kDa and around 150 kDa (Fig. 1). There was no significant difference in the gelatinolytic activity when the

Table 1. *Lactobacillus bulgaricus* strains tested in the study

Strain	pH of MRS broth	$OD_{492\,nm}$
L. bulgaricus LBL-12	4.5	0.8
L. bulgaricus LBL-22	4.6	1.4
L. bulgaricus LBL-6	4.4	1.4
L. bulgaricus LBL-83	4.8	0.9
L. bulgaricus LBL-9	4.6	1.4
L. bulgaricus LBL-11	4.7	0.9
L. bulgaricus LBL-23	4.7	0.9
L. bulgaricus ATCC 11842	4.5	0.8

pH values and OD were measured 24 h after incubation.

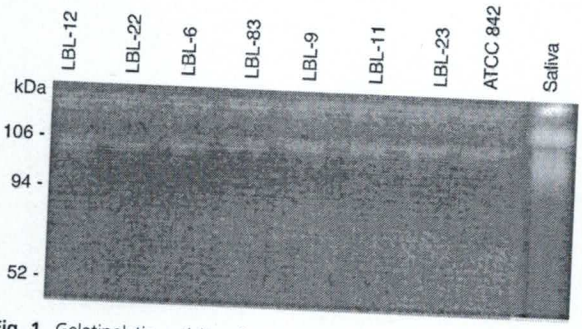

Fig. 1. Gelatinolytic activity of *L. bulgaricus* supernatants. All strains tested show similar bands of activity after a 7-day incubation period.

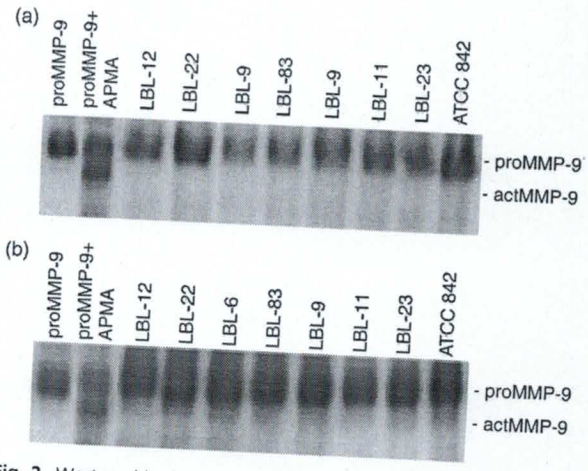

Fig. 2. Western blotting images demonstrate the ineffectiveness of *L. bulgaricus* strains to convert proMMP-9 to its active form by bacterial supernatant fraction (a) and cell fraction (b).

different pH values of the buffers were used. Supernatant samples, although showing only weak proteolytic activity, were more potent in degrading gelatin than the cell fraction samples which yielded no visible bands on the UV light picture. No difference was observed among the strains in the degradation of gelatin.

The effect of *L. bulgaricus* strains on proMMP-9

Considered as a specific marker reflecting gingival health, MMP-9 was chosen to assess the effect of bacterial proteinases on its inactive pro-form (proMMP-9). ProMMP-9 was incubated for three different time periods with supernatants and cell fraction suspensions of the *L. bulgaricus* strains. The conversion of proMMP-9 into its active form was not detected after 24 h of incubation as shown by Western blotting with the anti-MMP-9 antibodies (Fig. 2a and b).

Proteolytic activity of bacteria in the presence of synthetic MMP inhibitors

To investigate if the synthetic inhibitors of MMPs affect the gelatinolytic activity of bacterial proteases, the *L. bulgaricus* strains tested were incubated with five different synthetic MMP inhibitors and Pefabloc. After 7 days of incubation at 37 °C, no significant changes in gelatinolytic activity were observed on Coomassie Brilliant Bule stained gels. Both the supernatant specimens and cell fraction suspensions showed low proteolytic activity which was similar to that in the control group and to the test series where the different inhibitors were added. Synthetic MMP inhibitors and Pefabloc did not affect the proteolytic activity of the supernatants or the cell fraction suspensions of the *L. bulgaricus* strains investigated (data not shown).

Discussion

This study was the first in addressing the effect of *L. bulgaricus* strains on human gelatinases. A forefront requirement for a probiotic microorganism is to be safe to the host when administered. Putative oral probiotic species should be able to colonize or at least temporarily harbor the oral cavity by adhering to both hard and soft tissue surfaces in the mouth. Considering the attachment to oral mucosa, it is essential that the microorganism is not harmful to mucosal cells and extracellular matrix and basement membrane (BM) components. A damaged or disintegrated oral epithelium facilitates a microbial invasion, providing a propitious environment for further bacterial growth. Nevertheless, little is yet known about the possible interaction of probiotic strains with components of the extracellular matrix (ECM).

The ECM is a stable macromolecular structure, underlying epithelial and endothelial cells and surrounding connective tissue cells (Štyriak *et al.*, 2003). Under various physiological and pathological conditions ECM and BM undergo different structural and functional changes that are strongly dependent on the coordinated functioning of a large family of zinc-dependent enzymes, the MMPs. These are genetically distinct but structurally related proteinases consisting of five subgroups, namely collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs. MMPs are expressed at low levels in the absence of inflammation, wound healing or other pathological processes (Woessner, 1991). Collagenases (MMP-1, − 8, and − 13) and gelatinases (MMP-2 and MMP-9) are MMPs capable of degrading almost all ECM and basement membrane components. Most bacterial proteinases, however, have weak degrading activity against collagen (Okamoto *et al.*, 2004). Once activated human collagenolytic MMPs might provide suitable substrate for further activity of human gelatinases or other bacterial proteinases. The test strains investigated in our study demonstrated very low

gelatinolytic activity even after the longest incubation period, which validates their relative safety as probiotic candidates.

MMP-9 and other endogenous proteinases hydrolyze and degrade the fragments of denatured collagens, for example gelatin, into smaller fragments. It has been shown in many studies that MMP-9 is a specific marker for periodontal destruction (Ejeil *et al.*, 2003) and elevated levels of this enzyme are related to the severity of periodontal breakdown. The integrity of basement membrane corresponds to the stability of periodontal health and it consists of molecules such as type IV collagen, laminin, fibronectin and heparan sulfate proteoglycans. Referred to as type IV collagenase MMP-9 is particularly implicated in the degradation of the basement membrane (Reynolds & Meikle, 1997). The proteolysis of the ECM seems to play an important role in initiating the progression of the inflammatory process, and thus conversion of proMMPs into their active forms is a crucial step here, facilitating bacterial adhesion and infection. Studies on the activation of human MMPs have shown that some bacterial species with clear pathogenic potential are capable of activation of MMPs. For example, *Vibrio* proteinase and *Pseudomonas* elastase have shown stronger activation of pro-MMP-9 than did APMA (Okamoto *et al.*, 1997). MMPs are secreted as proenzymes and their activity is low in intact normal tissues but could undergo activation by a broad range of stimuli (Sorsa *et al.*, 1997; Johnson *et al.*, 1998; Potempa *et al.*, 2000; Okamoto *et al.*, 2004). A key event in the activation of proMMPs is the removal of the propeptide domain in their structure that usually consists of *c.* 80 amino acid residues (Nagase *et al.*, 1990). *Lactobacillus bulgaricus* strains in our study were incapable in converting the proMMP-9 to the 60–80 kDa forms considered active.

Proteolytic enzymes found in different species of lactobacilli have been characterized, revealing different protease activities and complex system of endo- and exopeptidases, which may differ in nature, specificity and cell location (Rul *et al.*, 1994; Kunji *et al.*, 1996). Proteolytic activity of some lactobacilli is an important feature in dairy production, for example it is essential for cheese ripening. *Lactobacillus bulgaricus* possesses high hydrolyzing activities towards substrates containing proline, alanyl-prolyl-*p*-nitroanilide and prolyl-*p*-nitroanilide (Sasaki *et al.*, 1995), to which trait the bands observed at 106 and 150 kDa in our zymographs may be attributed. Samples in our study did not show any activity at the region of molecular mass consistent with protease IV.

In the present experiments, human saliva was used as a positive control. Saliva is an essential for the health of teeth and oral mucosal surfaces. Human whole saliva contains a large number of enzymes derived from the salivary glands, oral commensal microorganisms, gingival crevicular fluid (GCF), and from epithelial and other cells. The activity of these enzymes is affected by ingestion of food, and certain foods themselves contain enzymes which temporarily contribute to the overall enzymatic activity of saliva. The main gelatinase in oral rinses, whole saliva and GCF of healthy individuals has been defined as MMP-9, originating mainly from GCF (Mäkelä *et al.*, 1994). The amount of this enzyme might be 10-fold higher ($50\,\mu g\,mL^{-1}$) in periodontitis patients compared to healthy individuals ($5\,\mu g\,mL^{-1}$). Compared with saliva samples, the proteolytic activity of lactobacilli tested was substantially low in the present series. This result also highlighted the safety of the strains we investigated.

It is generally assumed that proteases exert their proteolytic effect at neutral pH values. In the oral cavity, three buffering systems, namely bicarbonate, phosphate and protein buffers, are responsible for the maintenance of pH within the range of 6.0–7.5 (Lazarchik & Filler, 1997; Bardow *et al.*, 2004). Aframain *et al.* (2006) have recently registered that oral pH varies according to the test site, with the highest values found on the palate (pH 7.32), and lowest on the buccal mucosa (pH 6.28). A drop in salivary pH below 5.5 is potentially detrimental to the dental hard tissues and may also affect the soft tissues of mouth (Robb *et al.*, 1995; Aframian & Markitziu 1999). On the other hand, the main end product of *L. bulgaricus* metabolism is lactic acid that leads to lowering of the pH of the milieu. To assess whether different pH values might affect the proteolytic activity of *L. bulgaricus* strains we preadjusted the buffers used at pH 7.5, 6.5 and 5 during the 7 days of incubation. The results obtained gave no evidence that the different pH values investigated would affect the bacterial gelatinolytic activity.

The administration of synthetic inhibitors of MMPs is considered a therapeutic approach in the treatment of different pathological conditions in which elevated levels of MMPs are regarded as key factors in inflammation and tissue breakdown. The preserved and unaffected proteolytic activity of the test strains after addition of different synthetic MMP inhibitors and Pefabloc in the test system might additionally benefit the anticipated probiotic effect of those microorganisms. Consequently, a simultaneous administration of potential probiotics and inhibitors of MMPs should not be regarded contradictory when potential new treatment modes of infectious diseases are being considered.

In conclusion, the results of this study provided evidence that *L. bulgaricus* strains are harmless to main components of ECM, being unable to convert MMP-9 to its active form. Hence, intake of these bacteria is not anticipated to exert any deleterious effects on the regulatory enzymes and structures of the host extracellular matrix. However, further studies are called for in the assessment of other basic probiotic

requirements before these species can be considered for the development of functional products or for use in bacteriotherapy.

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