Proceedings of the 13th ISTRC Symposium, 2007
pp. 417 - 422

Probiotic and prebiotic properties of lactic acid bacteria isolated from cassava fermentations

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Abstract. This work was a preliminary investigation of the putative prebiotic and probiotic properties of lactic acid bacteria (*Lactobacillus* sp., *Pediococcus* sp.) isolated from cassava and sorghum fermentations. Exopolysaccharides produced by a small number of isolates were tested for their ability to stimulate *Bifidobacterium*, but not *Escherichia coli* or *Clostridium perfringens*. At the same time, a number of isolates were screened for basic probiotic properties (resistance to stomach acidity and bile) and for production of specific anti-microbial agents. Results showed that EPS produced by some isolates was fermented by *Bifidobacterium*, but not by *Escherichia coli* or *Clostridium perfringens*. At the same time the basic probiotic properties of resistance to stomach acidity and bile could be demonstrated. Some isolates also produced anti-microbial compounds. In the present study, however, isolates producing EPS with prebiotic properties were not those producing anti-microbial compounds. Although considerable substantiation is required, isolates of lactic acid bacteria (LAB) from cassava and sorghum fermentations appear to have considerable potential as both probiotic and prebiotic ingredients in foods. These properties could be exploited either to enhance the nutritional value of existing foods, such as cassava, or in development of new food products based on traditional foods.

Introduction

The relationship between the intestinal microflora and health has been extensively studied in recent years and the importance of bacteria of the genera *Bifidobacterium* and *Lactobacillus* in promoting good health is recognised. Dietary modulation of the microflora to increase the proportion of these beneficial (probiotic) bacteria is the subject of significant research effort. Two approaches are taken: production of dietary supplements or foods, such as yoghurts, containing live cultures of probiotic bacteria and incorporation of ingredients that favour the growth of *Bifidobacterium* already present in the intestine. Such ingredients are described as prebiotics (Gibson and Roberfroid, 1995) and are substances that are not digested by humans and that are metabolised by bifidobacteria (and possibly lactobacilli), but not by other members of the gut microflora. The probiotic approach is considered limited by loss of viability of bacteria during product storage and by the more fundamental problem of introducing new strains of bacteria to an established microflora. The prebiotic approach may, therefore, be seen as preferable, but there can be digestive problems if intake is excessive. Probiotics can be combined with prebiotics, the resulting symbiotics being considered highly effective (Collins and Gibson, 1999). This study was carried out to

investigate prebiotic and probiotic properties of lactic acid bacteria from cassava and sorghum fermentations.

Materials and Methods

Isolation of extracellular polysaccharide-producing lactic acid bacteria. Model fermentations were set up using cassava, sorghum, maize and dried beans in water. Materials were purchased at local markets in London, but all were of African origin. Fermentations were incubated at 37ºC and samples taken after 24, 48 and 72 hours. Samples (10g) were homogenised in a paddle-type blender and decimal dilutions, prepared in maximum recovery diluent, were plated onto MRS (Oxoid, UK) medium and, on some occasions, MRS medium with sucrose replacing glucose as fermentable carbon source. After incubation (37ºC for 48 hours under anaerobic conditions), colonies developing were examined for formation of slime as a marker of EPS production.

EPS-producing colonies were purified by streaking and identity as LAB was confirmed by a negative reaction in the catalase test and by microscopic examination of Gram-stained smears. Gram-positive, rod-shaped bacteria were presumptively identified as *Lactobacillus* and Gram-positive, coccal-shaped bacteria occurring in tetrads were presumptively identified as *Pediococcus*. In some cases, fermentation profile was determined using the API 50 CHL system (bioMérieux, UK), which also permitted tentative identification to species level. The identity of two isolates was confirmed genotypically by sequencing the 16S rRNA gene.

Conditions for production of extracellular polysaccharides. The influence of fermentable carbon source was determined by visually comparing the quantity of EPS produced during growth with glucose, fructose, sucrose or maltose as carbon source. Effect of temperature was determined comparing EPS production during incubation at 30ºC and 37ºC. Attempts to quantify yield in relation to cell numbers by growing cultures in MRS broth containing the four different carbon sources were unsuccessful due to isolates losing the ability to produce EPS in liquid culture.

Differential fermentation of extracellular polysaccharides. EPS was harvested from the surface of agar media, washed in Ringer's solution and pasteurised by heat. Reinforced clostridial broth containing no added fermentable carbon source was used as basal medium. Fermentation of EPS by a "cocktail" of *Bifidobacterium* species was determined using basal medium alone (negative control), basal medium containing 2% EPS and basal medium containing lactulose (a known prebiotic) as positive control. An experiment was also made with a single type of EPS in which growth of the "cocktail" of *Bifidobacterium* was compared in basal medium containing glucose and basal medium containing glucose and EPS. Fermentation of EPS by *C. perfringens*, a non-pathogenic strain of *E. coli* (555) and a non-toxigenic strain of *E. coli* serovar O157: H7 (NCTC 12990) was determined using the same basal medium. Glucose was used as carbon source in the positive control in place of lactulose, which is not fermented by these bacteria.

Tolerance of gastric acidity and bile. Isolates of LAB were grown in MRS broth to a density of ca. 10^8 cells/ml. Tolerance of gastric acidity was determined by inoculating MRS broth at pH 2.5 with ca. 10^5 cells/ml and incubating at 37ºC for 4 hours. Microbial numbers were determined immediately after inoculation and at the end of incubation. A similar procedure was used for determination of bile tolerance, cells being exposed to 0.3% purified ox-gall bile (Sigma-Aldrich UK). A known probiotic strain of *Lb. plantarum*, 299v, was included in the study for comparison.

Production of inhibitors by isolates of lactic acid bacteria. Inhibition was tested against *C. perfringens* and *E. coli* O157: H7. LAB were grown in MRS broth, cells removed by centrifugation and the supernatants

pasteurised. Sterile filter paper discs were moistened with culture supernatant and placed on the surface of lawn plate cultures of test bacteria. After incubation for 24 hours at 37ºC (*C. perfringens* in anaerobic conditions, *E. coli* in air), plates were examined for zones of inhibition around the discs. *L. plantarum* 299v was included for comparison.

Results

The present work is concerned with investigations into the EPS-producing lactic acid bacteria (LAB) *Lactobacillus* sp. and *Pediococcus* sp. isolated from fermenting cassava and sorghum. The work has involved preliminary investigations of EPS production and determination of its potential prebiotic properties. Isolates were screened for their ability to survive simulated gastric passage at low pH value and in the presence of bile. In addition, a small number of isolates have been screened for production of inhibitors of *Escherichia coli* and *Clostridium perfringens*.

Results have shown that some types of EPS produced by LAB isolated from traditional fermentations have prebiotic properties and that basic probiotic characteristics are present in some isolates. Further work and substantiation is required, but if the preliminary results are confirmed, in situ production of EPS by LAB provides a means of nutritionally enhancing cassava and other fermented food products. At the same time, the putative probiotic properties offer the potential for control of enteropathogens. In addition to enhancement of traditional fermented foods, suitable cultures could be used as the base for development of novel probiotic and prebiotic foods.

Isolation of extracellular polysaccharide-producing lactic acid bacteria. EPS-producing LAB comprised as many as 50% of the total population in cassava and sorghum fermentations (ca. 5 x 10^7 cfu/g). Quantity of EPS produced varied, but diversity of bacteria isolated from cassava was low and usually only one type of EPS-producing colony was present. EPS production was more prevalent amongst isolates from sorghum than cassava and diversity was greater, with some colonies producing two distinct types of EPS. EPS-producing LAB included both *Lactobacillus* sp. and *Pediococcus* sp. Lactobacilli were tentatively identified as on the basis of fermentation profiles and Pediococci as atypical strains of *P. damnosus*. Identity of two *L. plantarum* isolates was confirmed by sequencing the 16S rRNA gene. Fermentation profiles showed EPS-producing isolates to be capable of utilising a wide range of carbohydrates. These included glucose, fructose, maltose and sucrose, but not starch.

Conditions for production of extracellular polysaccharides. EPS was produced on all substrates tested. There were two patterns of response to different carbohydrates. The first response involved similar levels of EPS production on all substrates. The second response showed slightly greater production on fructose than on glucose and maltose and markedly greater production on sucrose. EPS production appears to mainly occur towards the end of incubation when the cells are assumed to be in the stationary phase. There are indications with some, but not all isolates, that production is greater at 30ºC than at 37ºC, the optimal temperature for growth.

Differential fermentation of extracellular polysaccharides. EPS was fermented by the cocktail of *Bifidobacterium*, growth being greater than with lactulose. There was also evidence of growth stimulation by EPS in the presence of glucose. *Clostridium perfringens* was unable to grow with lactulose as sole fermentable carbon source, but growth was stimulated by this substrate in the presence of glucose. Neither the non-pathogenic strain of *E. coli* nor the strain of *E. coli* serovar O157: H7 were able to ferment EPS and there was inhibition of serovar O157: H7.

Tolerance of gastric acidity and bile. Tolerance of gastric acidity and bile varied amongst both EPS- and non-EPS-producing lactic acid from cassava and sorghum fermentations. Two EPS-producing strains of *Lactobacillus* sp. showed higher levels of tolerance to both simulated gastric acidity and bile than the recognised probiotic strain *L. plantarum* 299v.

Production of inhibitors by isolates of lactic acid bacteria. Culture supernatant of some strains of *Lactobacillus* and *Pediococcus* from cassava and sorghum fermentations had an inhibitory effect on growth of *C. perfringens* and, to a lesser extent, both *E. coli* strains tested. The most effective strains, however, were not those producing EPS.

Discussion

EPS-producing strains of *Lactobacillus* sp. and *Pediococcus* sp. are of relatively high prevalence in cassava and sorghum fermentations. No chemical analyses have been made at present to determine structure of the EPS, but produced in equal quantities on different carbohydrates, is probably a heteropolysaccharide, containing more than one type of monosaccharide. The increased quantity of EPS produced by some isolates with sucrose as substrate is probably a homopolysaccharide of the fructan or glucan type, produced in addition to the heteropolysaccharide (personal communication, Dr G. Cote, US Department of Agriculture). Loss of ability to produce EPS when isolates are cultured in liquid medium is consistent with the environmental role of EPS in biofilm formation and would be unlikely to occur in fermentations containing a significant quantity of solid material.

The present studies provide evidence that EPS produced by *Lactobacillus* and *Pediococcus* sp. isolated from cassava and sorghum fermentations can be considered as a candidate prebiotic. This supports previous work demonstrating the prebiotic properties of EPS (Dai Bello *et al*., 2001; Yamomoto *et al*., 1999). Fermentation of EPS by *Bifidobacterium* was more rapid than that of lactulose and final cell numbers were higher. There was also some stimulation of the *Bifidobacterium* "cocktail" when glucose was also present as a fermentable carbon source.

It is probable that prebiotic efficiency varies between chemically distinct types of EPS. There is considerable diversity within each type and no systematic studies relating structure to prebiotic function has been undertaken. Selection of the most effective type is, therefore, likely to require extensive screening. In the current study, potential prebiotic activity has been defined by differential fermentability in batch cultures. This represents the first stage of identifying candidate prebiotic compounds. Although differential fermentability is a prerequisite for prebiotics, *in vitro* tests with pure strains of bacteria in batch cultures cannot necessarily predict efficacy in the human diet. Demonstration of prebiotic activity to an acceptable level of certainty requires that the number of bifidobacteria be increased in the gut without a corresponding increase in undesirable bacteria. This requires use of a suitable model, such as a two- or three-stage anaerobic fermenter, followed by volunteer feeding trials. The planned next stage of the work involves use of an anaerobic fermenter to extend knowledge of the prebiotic properties of EPS produced by different *Lactobacillus* sp. isolates.

Although prebiotics are incorporated into a range of foods, the compounds involved are added as ingredients to the formulation. Production of prebiotic EPS by bacteria involved in manufacture of fermented foods is a highly attractive concept, since it avoids additional ingredient formulation, procurement and purchase. *In situ* production of EPS in sourdough (rye) bread has been investigated (Korackli *et al*., 2002; Ticking *et al*., 2003). In most cases, production of EPS in-situ has been of technological interest in sourdough bread manufacture, but Korackli *et al*. (2002) emphasised the nutritional benefit through prebiotic activity. The evidence of the present work is that nutritional enhancement of cassava and other traditional

fermented food is feasible and probably occurs naturally under some conditions.

The concept of EPS-producing LAB that also has probiotic attributes does not appear to have been considered elsewhere. Probiotic properties are associated with certain strains of a restricted number of species of *Lactobacillus*, although there appears to be wider distribution amongst *Bifidobacterium*. *Lactobacillus plantarum* is amongst species of lactobacilli known to contain probiotic strains, although the organism is not widely used in commercial products. Probiotic properties have also been associated with strains of *Pediococcus* sp.

Commercially used probiotic cultures are all derived from humans or other animals and there is a presumption that only strains from these sources can survive gastric passage and colonise the gastric epithelium. There is no body of evidence, however, for this presumption and some isolates from traditional fermentations were of higher resistance to stomach acidity and bile than the commercially used strain *L. plantarum* 299v. Resistance to stomach acidity and bile are prerequisites for successful use as a probiotic culture, but do not necessarily imply, however, that the organism will have any beneficial effect if ingested. A further recognised attribute of probiotic bacteria is the production of compounds with antimicrobial activity to enteropathogens. Antimicrobial activity to *E. coli* O157:H7 (*vt*$^-$) by *Lactobacillus* sp. and *Pediococcus* sp. isolated from cassava and sorghum has been demonstrated (unpublished work), although the isolates were not significant EPS producers. The number of strains tested was small, however, and there is no known reason why EPS synthesis and production of antimicrobial agents should not occur in the same isolate. It has also been suggested that EPS produced by a strain of *L. delbrueckii* has a beneficial function in stimulating macrophages (Nishimura-Uemura *et al*., 2003) independent of any effect of the bacterium.

Although antimicrobial activity is usually considered to involve production of specific inhibitors, such as bacteriocins, fermentation acids, such as lactic and acetic, are also important determinants of safety of fermented foods. A study of cassava fermentation for production of the Ghanaian food, agbelima, for example, concluded that fermentation acids rather than bacteriocins were responsible for the antimicrobial effect of LAB (Mante *et al*., 2003). Lactic acid is usually the major fermentation acid, but there is increasing recognition of the role of acetic acid in the safety of fermented foods, as well as in setting desirable conditions in the intestine. Examination of fermentation end products of LAB from cassava by gas-liquid chromatography showed high ratios of acetic to lactic acid (unpublished work). This may be due to a metabolic switch following depletion of fermentable carbohydrate levels and the need for an alternative energy source (Calderon *et al*., 2002). Production of acetic acid is not considered to be a probiotic attribute but high levels are desirable since its activity against enteropathogens is greater than lactic acid.

Although the research described is at a preliminary stage, prebiotic and probiotic properties associated with *Lactobacillus* sp. and other LAB appear to have considerable potential for enhancing the nutritional value of foods based on cassava and other traditional fermented plant products. Recent research has tended to be directed towards the preventative role of probiotic bacteria towards diseases that are prevalent in post-industrial societies, including bowel cancer and immune dysfunction. While these are of considerably less concern in the developing economies of Africa and Asia, modulation of the gut microflora provides a potentially important means of reducing morbidity and mortality due to diarrhoeal diseases as well as providing general health benefits.

A problem with both prebiotics and probiotics is devising a suitable vehicle of delivery (Reid, 2003). In the current situation, there are two options. The first involves setting conditions during the cassava fermentation to ensure maximum EPS yield.

The use of starter cultures is a possibility, but even undefined mixed cultures can be difficult to manage in village-scale operations. EPS-producing lactobacilli are common in natural cassava fermentations and a more effective approach might be to manage the fermentation to increase their prevalence. This may be possible by addition of additional carbohydrate, although better understanding of the dynamics of cassava fermentations may be required to ensure that the most effective lactobacilli are stimulated.

The second option would be the creation of a new functional (probiotic and prebiotic) product using a traditional fermented food as base. The European probiotic product ProvivaÔ is an oat-based, fruit-flavoured drink containing *L. plantarum* 299v. The concept was based on a traditional African fermented product, although strain 299v is a north European isolate from a human source. The use of microorganisms with both probiotic and prebiotic properties would offer considerable advantages with respect both to efficiency as a functional food and simplify production. In addition to health benefits, the development of functional fermented foods would offer new opportunities for local food industries and expand natural product utilisation.

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