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Antimicrobial Potential of Probiotic or Potentially Probiotic Lactic Acid Bacteria, the First Results of the International European Research Project PROPATH of the PROEUHEALTH Cluster

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The EU-funded PROPATH project addresses the important health issue of prevention of gastrointestinal disorders through probiotics and prebiotics. Seven European laboratories are co-operating in this project, which aims to isolate and characterize the relevant antimicrobial agents to combat Gram-negative bacteria including *Helicobacter pylori* and *Salmonella enterica* serovar Typhimurium. In this paper, the first results on the screening for probiotic or potentially probiotic lactobacilli that exhibit antimicrobial activity towards these Gram-negative pathogenic bacteria are presented. Spot-on-lawn assays, well-diffusion assays and time-kill studies were performed among the lactic acid bacteria strains that were either collected from fermented foods and faeces (breast-fed babies, infants and animals) or isolated from commercial products to investigate whether any of the collected strains were inhibiting growth of or were killing certain indicator bacteria. Strains inhibiting the gastrointestinal pathogens mentioned above were found. Evidence has been obtained that compounds different from organic acids are produced. *Key words:* probiotics, antimicrobial agents, gastrointestinal pathogens.

INTRODUCTION

The gastrointestinal tract is a complex ecosystem that associates a resident microbiota and cells of various phenotypes lining the epithelial wall. One of the basic physiological functions of the resident microflora is to act as a barrier against microbial pathogens (1). The mechanisms by which species of the microflora exert this barrier effect remain largely unknown. The following hypotheses have been formulated: (i) competition for substrates between the pathogen and the resident microflora; (ii) prevention of adherence of the pathogen to the epithelium; (iii) production of antimicrobial substances by a resident or transient microflora to remove the pathogen from the gut; and (iv) enhancement of the host immune response in the presence of the pathogen.

Lactic acid bacteria (LAB) are present in large numbers in the normal human and animal gastrointestinal tract. The use of LAB strains as probiotics to enhance health has been proposed for many years (2). In general, probiotic strains are defined as live microorganisms which, when consumed in appropriate amounts in the food, confer a health benefit to the host. The LAB probiotics most often investigated at present are *Lactobacillus* spp. and *Bifidobacterium* spp. (3). In parallel, pharmaceutical preparations of probiotics are used as biotherapeutic agents (4). They contain selected living or heat-killed, lyophilized strains of LAB. In recent years, specific LAB strains have been shown to contribute to the health of humans, some of which develop antimicrobial activities that participate in the host's gastrointestinal system of defence (5).

Gastroenteritis can be caused by a variety of pathogens including rotavirus, *Escherichia coli*, *Campylobacter* and *Salmonella*. It is a worldwide health problem among children and infants. Certain probiotics have been found to be helpful in preventing and treating some types of bacteria-induced diarrhoea because of their ability to alter

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the activity of the intestinal microflora and compete with potential pathogens (6). In addition, several LAB have been shown to inhibit *Helicobacter pylori*. For instance, it has been demonstrated that cell-free culture supernatant (CFCS) of *Lactobacillus johnsonii* La1, a commercial probiotic strain, decreases *H. pylori* density in healthy volunteers (7–9). *H. pylori* can be eradicated from the human stomach by administration of a cocktail of antibiotics and drugs, affecting the stomach acidity, which leads to healing of peptic ulcers and consequent reduction in cancer risk development (10). Problems encountered with the current eradication of *H. pylori* include the increasing rates of eradication failure, mainly due to increased rates of resistance of *H. pylori* to the administered antibiotics.

The fundamental basis of the inhibition of Gram-negative, gastrointestinal pathogens by probiotic LAB has not been elucidated. The objective of the EU-funded PROPATH project is to examine the mechanism(s) by which LAB develop antimicrobial activities. For this purpose, the European research teams involved in the PROPATH project are carrying out a set of comprehensive and well-conducted experiments, including screening for LAB strains showing a clear inhibition of Gram-negative pathogenic bacteria, purification strategies for the antimicrobial substance(s), and *in vitro* human cellular models, animal models and clinical trials. This paper reports the first results on the screening for probiotic or potentially probiotic lactobacilli that exhibit antibacterial activity towards *H. pylori* and/or *Salmonella enterica* serovar Typhimurium.

MATERIALS AND METHODS

Strains and cultivation conditions

Strains were collected from fermented foods (dairy products, fermented vegetables) and from faeces (breast-fed babies, infants and animals); known strains were also included. Known strains were either commercial strains, strains with assumed or reported inhibitory effects towards the relevant Gram-negative pathogens, or strains for which validated literature data were available. The strains that were finally selected for further examination are listed in Table I. The indicator bacteria used are also listed in Table I.

The *S. enterica* serovar Typhimurium strain SL 1344 (11) was cultured in Luria broth (Difco, Detroit, MI, USA) at 37°C.

Helicobacter pylori strains (SS1, CCUG 38770, CCUG 38771, CCUG 38772) were grown at 37°C under micro-aerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) on Chalgren's-Wilkins agar enriched with 7% (v/v) horse blood and 1% (v/v) vitox (all purchased from Oxoid, Basingstoke, UK) and antibiotics (vancomycin, 10 μg/ml; trimethoprim, 10 μg/ml; polymyxin B, 104 IU/l; amphotericin B, 2 μg/ml; nalidixic acid, 10 μg/ml; bacitracin, 30 μg/ml and fluorocytosine, 5 μg/ml; all purchased from Sigma, St Louis, MI, USA). *H. pylori* liquid cultures were prepared in Brain Heart Infusion (BHI; Oxoid) or Brucella Broth (BB; Oxoid), supplemented with 10% (v/v) horse serum, under the same conditions, in a shaking incubator. *H. pylori* ATCC 43504 was grown on Columbia blood agar plates, consisting of Columbia medium (Oxoid) supplemented with 7% (v/v) lysed horse blood (Oxoid) and Dent *Helicobacter pylori* selective supplement (Oxoid) under micro-aerophilic conditions at 37°C.

Identification and confirmation of the authenticity of LAB strains at the genus and species level

Identification and confirmation of the authenticity of LAB strains at the genus and species level was done by SDS-PAGE of whole cell proteins (12), species-specific PCR (13) and/or 16S rRNA DNA sequencing (14).

Screening of probiotic or potentially probiotic LAB strains for inhibition of Gram-negative, gastrointestinal pathogenic bacteria

The study investigated whether any of the collected LAB inhibited the growth of or killed certain indicator bacteria, including *H. pylori* and *S.* Typhimurium. The following screening methods were used.

(i) Spot-on-lawn assay (15). Antibacterial activity was examined by spotting 10 μl of CFCS on an overlaid agar plate inoculated with the indicator bacterium. Overlaid agar plates were prepared by pouring of a soft agar layer (0.7% m/v) inoculated with a liquid culture (2.9% v/v) of an exponentially growing indicator bacterium with an optical density at 600 nm of 0.45, on top of the agar medium (1.5% m/v). Plates were investigated for inhibition zones after incubation at 37°C overnight.

(ii) Well-diffusion assay (16). In the well-diffusion assay 10^5 CFU/ml of the pathogenic strains were incorporated into soft agar (1% v/v) plates; BHI for the enteric pathogens and tryptone soya (TS) agar (Oxoid) for the *Helicobacter* strains. Then, 50 μl of the CFCS were transferred into holes drilled into the agar. The plates were incubated at 37°C, under aerobic conditions for the enteric pathogens or using a GasPak kit (Oxoid) for the *Helicobacter* strains. Antimicrobial activity of the CFCS was evident as inhibition zones around the well or the spotted area.

(iii) Time-kill studies (17, 18). These involved liquid cultures supplemented with the sample to be tested, and viable counts of the indicator bacterium were measured. In the case of *H. pylori* SS1, the pathogen (10^8 CFU/ml) suspended in BHI, in the absence of antibiotics, was incubated under micro-aerophilic conditions at 37°C, in the presence of 10% (v/v) of CFCS of cultures of the lactobacilli (adjusted to pH 4.5 or 6.5 with 1 N NaOH) or the appropriate MRS medium controls. Viability of

H. pylori at 24 h and 48 h was evaluated by determination of viable CFU in Chalgren's-Wilkins agar plates following incubation at 37°C under micro-aerophilic conditions. In the case of *H. pylori* ATCC 43504, the pathogen (2.5×10^6 CFU/ml) was suspended in 100 µl of phosphate-buffered saline (PBS, pH 6.5) and incubated under micro-aerophilic conditions at 37°C, in the presence of an equal volume of concentrated CFCS (200-fold by extraction, lacking lactic acid) or control (PBS, pH 6.5). In the case of *S.* Typhimurium, the pathogen (100 µl in PBS buffer, pH 7.2, 10^8 CFU/ml) was incubated with 100 µl of concentrated CFCS (10-fold, by lyophilization) at 37°C for 24 h. Concentrated MRS medium was used as a control. At various time intervals, the surviving *S.* Typhimurium SL 1344 cells were determined by plating on BHI agar plates. Also, the pathogen (250 µl at 4×10^5 CFU/ml in DMEM–Dulbecco's Modified Eagle's Minimal Essential Medium; Invitrogen, Paris, France) was incubated with 250 µl of DMEM and 500 µl of CFCS or control. The pH of the incubation medium was 5.5. The controls were MRS medium adjusted to pH 4.5 with HCl, MRS medium adjusted to pH 6.5 with NaOH, MRS medium containing lactic acid (60 mM and adjusted to pH 4.5 with NaOH) and Luria broth (Difco).

RESULTS

Strain collection and identification

More than 850 strains were collected from fermented foods (dairy products, fermented vegetables) and from faeces (breast-fed babies, infants and animals). Most of food isolates belonged to the taxa *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus plantarum*. Also, strains from *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus gasseri* and *Lactobacillus rhamnosus* were isolated. More than 500 isolates were picked up from the faecal samples of breast-fed infants (between 1 and 9 months old). To date, a small fraction of them (30 isolates) have been determined to species level by 16S rRNA DNA sequencing. A surprisingly high fraction of the isolates belonged to the genus *Enterococcus*, but also some isolates of the genera *Lactobacillus* and *Bifidobacterium* were found.

The identity and the authenticity of the selected strains were confirmed by SDS-PAGE, species-specific PCR and 16S rRNA DNA sequencing (Table I).

Screening for inhibitory activity

Certain lactobacilli of both the collected strains and known ones inhibited the pathogenic bacteria tested (Table I). Direct plating of diluted faeces samples revealed that single

Table I

Project culture collection of probiotic or potentially probiotic lactic acid bacterium strains, their identification or confirmation of authenticity, and their inhibitory effect towards several Gram-negative, gastrointestinal pathogens

LAB strain (source)	Identification		Indicator bacteria					
			Helicobacter pylori					*Salmonella* Typhimurium
	SDS-PAGE	16S rRNA DNA sequencing and/or species-specific PCR	SS1	CCUG 38770	CCUG 38771	CCUG 38772	ATCC 43504	SL 1344
L. acidophilus IBB 801 (dairy product)	+	+	–	–	–	–	–	+
L. amylovorus DCE 471 (corn steep liquor)	+	+	–	–	–	–	+	–
L. brevis THT 030202 (fish)	ND	ND	–	–	–	–	NP	+
L. casei Shirota (Yakult)	+	+	–	–	–	–	+	+
L. fermentum ACA-DC 179 (kasseri cheese)	+	+	NP	NP	NP	NP	NP	+
L. johnsonii La1 (LC1)	+	+	+	+	+	+	+	+
L. plantarum ACA-DC 287 (xynotyri cheese)	+	+	NP	NP	NP	NP	NP	+
L. plantarum ACA-DC 2350 (feta cheese)	+	+	NP	NP	NP	NP	NP	+
L. rhamnosus GG (Gefilus)	+	+	NP	NP	NP	NP	–	+
L. rhamnosus THT 030902 (human faeces)	ND	+	–	–	–	–	NP	+
S. macedonicus ACA-DC 198 (kasseri cheese)	+	+	–	–	+	–	NP	–

ND, not determined; NP, not performed.

colonies produced inhibitory substances that prevented growth of other bacteria in the sample. This was only seen with two different faeces samples and only at low dilution, when the plates contained a dense lawn of faecal bacteria. Also, it was found that approximately 85% of the faecal isolates showed inhibition of the Gram-negative indicators tested. However, the inhibition was most probably due to the production of organic acids (data not shown).

CFCS (adjusted to pH 6.5) of *Lactobacillus johnsonii* La1 inhibited the *H. pylori* strains tested (Table I), for instance *H. pylori* SS1 (Fig. 1). CFCS (adjusted to pH 6.5) of other LAB strains did not inhibit *H. pylori*, nor did the MRS control of pH 6.5. The specific activity of CFCS (pH 6.5) of *L. johnsonii* La1 was also observed in time-kill assays (Fig. 2). Also, concentrated CFCS, containing no organic acids, of *L. johnsonii* La1, *L. casei* Shirota and *Lactobacillus amylovorus* DCE 471 inhibited *H. pylori* ATCC 43504 in a killing assay (Table I). These data indicate that antibacterial substance(s) other than lactic acid or acetic acid is (are) responsible for the inhibitory activity.

Using the well-diffusion assay, CFCS (adjusted to pH 6.5) of cultures of *L. plantarum* (ACA-DC 124, ACA-DC 125, ACA-DC 127, ACA-DC 280, ACA-DC 281, ACA-DC 287, ACA-DC 288, ACA-DC 2350, ACA-DC 2411, ACA-DC 2414 and ACA-DC 2640), *L. paracasei* subsp. *paracasei* (ACA-DC 123 and ACA-DC 3301), *L. casei* ACA-DC 1520, *L. rhamnosus* ACA-DC 226/116, *L. fermentum* ACA-DC 179 and *L. gasseri* (ACA-DC 82 and ACA-DC85a) showed 'borderline' inhibition against *S.* Typhimurium SL 1344.

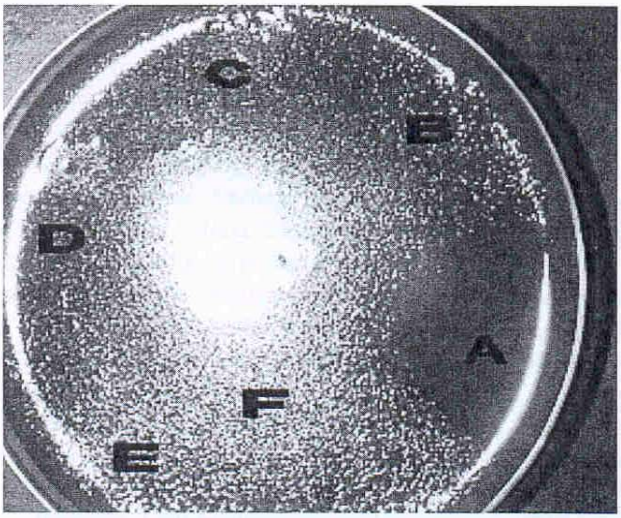

Fig. 1. Inhibitory effect of cell-free culture supernatant (CFCS, adjusted to pH 6.5) from a culture of *L. johnsonii* La1 spotted on an indicator lawn of *H. pylori* HpSS1 colonies (A). Also, CFCS (adjusted to pH 6.5) of *L. casei* Shirota (B), *L. rhamnosus* GG (C), *L. paracasei* subsp. *paracasei* ACA-DC 146 (D), *L. paracasei* subsp. *tolerans* ACA-DC 4037 (E), and MRS medium adjusted to pH 6.5 (F) were spotted.

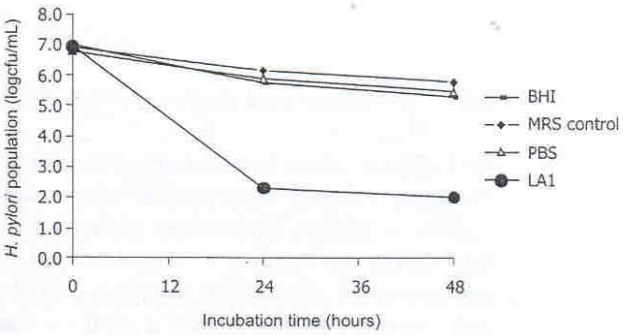

Fig. 2. Time-kill assay of *H. pylori* CCUG 38770 with cell-free culture supernatant (adjusted to pH 6.5) from a culture of *L. johnsonii* La1, at a 10% (v/v) ratio in the final incubation volume. The controls are MRS medium, BHI medium and PBS buffer.

Using time-kill studies, the reduction in cell counts of *S.* Typhimurium observed in the presence of concentrated CFCS of *L. plantarum* ACA-DC 287, *L. plantarum* 2350 and *L. fermentum* ACA-DC 179 ranged between 2 and 3 logs, while < 1 log reduction was observed in the controls over a 24-h period of incubation. The killing effect was more evident in the case of *L. plantarum* ACA-DC 287 (Fig. 3). In the presence of DMEM, the inhibitory effect of lactic acid on the viability of *S.* Typhimurium was eliminated. In contrast, a significant decrease in the viability of *S.* Typhimurium (3 log of decrease) was seen when CFCS of cultures of *L. rhamnosus* GG, *L. casei* Shirota and *L. johnsonii* La1 were examined. Moreover, the CFCS of cultures of *L. plantarum* ACA-DC 287, *L. plantarum* ACA-DC 2350 and *L. fermentum* ACA-DC 179 were effective in killing *S.* Typhimurium. These results suggest that an antibacterial substance(s) different from lactic acid is (are) present in the CFCS of these lactobacilli.

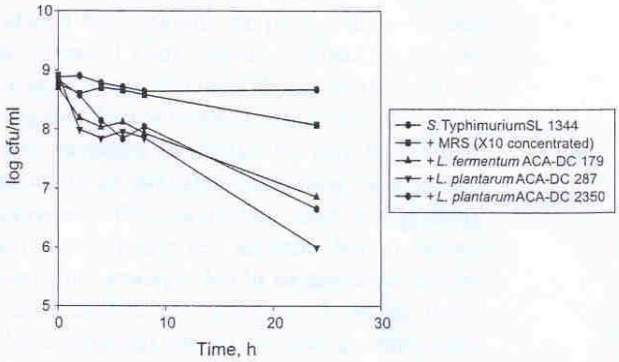

Fig. 3. Time-kill assay of *S.* Typhimurium SL 1344 with cell-free culture supernatant (10-fold concentrated by lyophilization and adjusted to pH 6.5) from cultures of *L. plantarum* ACA-DC 287, *L. plantarum* ACA-DC 2350 and *L. fermentum* ACA-DC 179. The controls are MRS medium (10-fold concentrated by lyophilization and adjusted to pH 6.5), BHI medium and PBS buffer.

DISCUSSION

It has often been hypothesized that probiotics interfere with gastrointestinal pathogens and this property may be a criterion in selecting new probiotic strains. However, it has been difficult to experimentally prove if such a property is of importance in preventing the establishment of pathogens in the gastrointestinal tract *in vivo*. In our work we have studied the antimicrobial potential of known probiotic LAB, and of new LAB isolates from various environments. We have shown that several strains inhibit gastrointestinal pathogens such as *H. pylori* and *S.* Typhimurium. Furthermore, we have obtained experimental proof that the faecal microflora in infants may contain species (including enterococci) that produce antimicrobial activity against other intestinal bacteria. Several mechanisms may be responsible for this interference, of which the production of antimicrobial compounds is of interest because of its possible direct interaction. Antimicrobial substances produced by LAB include organic acids (lactic acid, acetic acid, formic acid), other sugar catabolites (ethanol, diacetyl, carbon dioxide), oxygen catabolites (hydrogen peroxide), bacteriocins, antibiotic-like substances (reuterin and reutericyclin), and others (5).

The major bacterial growth inhibitory principles produced by lactobacilli are organic acids that are most potent at low pH, but other antimicrobial substances, different from organic acids, may contribute to this killing. Among all these compounds bacteriocins have attracted most attention in recent years and it has been shown that many *Lactobacillus* strains produce different kinds of bacteriocins. Bacteriocins are antibacterial polypeptides that target their activity towards often closely related bacteria (19). Some of the LAB bacteriocins also inhibit food spoilers and/or food-borne pathogenic bacteria such as bacilli, clostridia, staphylococci and listeria, underlying their importance as potential, natural food preservatives. However, bacteriocins produced by LAB are almost exclusively active against Gram-positive bacteria (20). Gram-negative bacteria only become sensitive to bacteriocins when the cell surface structure is injured (21). It has also been shown that bacteriocins from LAB can synergistically enhance the antimicrobial activity of eukaryotic peptides against Gram-negative bacteria (22) and this may be another mechanism of action for LAB bacteriocins in the gastrointestinal tract. Nevertheless, it is highly likely that bacteriocins play a role in the gastrointestinal ecosystem, because many bacteriocins have been isolated from members of the *L. acidophilus* group, the predominant lactobacilli in the human colon. Moreover, it has been shown that bacterial isolates from human faeces produce bacteriocins (23, 24). Interestingly, in the faeces of gnotobiotic rats monoassociated with a human *Ruminococcus gnavus* strain, several *Clostridium* species (including *Clostridium perfringens*) were inhibited and a lantibiotic bacteriocin has been found to be produced by this bacterial strain (25–27). This so-called ruminococcin A was most probably produced *in vivo* and displayed its antibacterial activity *in vivo* as well, because the bacteriocin is resistant to trypsin and probably needs it to induce its biosynthesis. Moreover, this strain was isolated from a healthy human volunteer, free from *C. perfringens*, even after ingestion of a pure culture of *C. perfringens*. Yet, several *Lactobacillus* strains produce antimicrobial, low molecular mass, heat-stable, proteinaceous compounds, so-called bacteriocin-like peptides, with a broad inhibitory spectrum including both Gram-positive and Gram-negative bacteria (19). However, the well-known probiotic *L. johnsonii* La1 strain inhibits cell adhesion and cell invasion by enterovirulent bacteria that may be due to an antibacterial compound that is insensitive towards proteases and active *in vitro* towards several Gram-negative and Gram-positive pathogenic bacteria (17, 28). Similarly, the *L. acidophilus* LB strain secretes a heat-stable antimicrobial compound that is different to lactic acid and moderately sensitive towards several enzymes. Several characteristics of the component(s) supporting the antimicrobial activity suggest that it may contain an unusual acidic amino acid that is part of a novel peptide agent (29, 30). The well-studied *L. rhamnosus* GG (formerly *L. casei* GG) strain most probably produces an agent with antimicrobial activity (the nature of which is not known), that is active *in vitro* towards *S.* Typhimurium, and that blocks cell adhesion to Caco-2 cells and protects against infection in mice (31, 32).

CONCLUSION

During this study, evidence has been shown that compounds, inhibiting growth or killing gastrointestinal pathogens, are produced by probiotic or potentially probiotic LAB, but the chemical composition still has to be unravelled. The results of the PROPATH project are exciting, because over the past decade levels of bacterial resistance to antibiotics have increased dramatically, and 'superbugs' resistant to most or all available antibiotics have shown up in clinical practice. This means that there is a growing need to develop and introduce new antimicrobial drugs. An innovative therapeutic strategy should be envisaged on the basis of the antimicrobial molecules produced by LAB, which could be a potential source of new, antibiotic-like molecules.

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necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

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