

Manufacturing process influences properties of probiotic bacteria

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Abstract

Production and manufacturing methods and the food carrier may influence the properties of probiotic strains, and have an impact on the outcome of clinical intervention studies. The aim of the present study was to establish whether the properties of a specific probiotic strain, *Lactobacillus rhamnosus* GG, may differ depending on the product and source of the strain. In total, fifteen different *L. rhamnosus* isolates, among them fourteen labelled as *L. rhamnosus* GG, were isolated from specific probiotic products. The micro-organisms were phenotypically and genotypically characterised. Their adhesion properties were compared using the human intestinal mucus model, and the ability of the isolates to influence model pathogen adhesion to human colonic mucus was assessed. All *L. rhamnosus* isolates used were confirmed as members of the species *L. rhamnosus*. Except the reference strain OL, all *L. rhamnosus* isolates showed randomly amplified polymorphic DNA, enterobacterial repetitive intergenic consensus and pulsed-field gel electrophoresis profiles identical to that of *L. rhamnosus* GG (ATCC 53103). All *L. rhamnosus* isolates showed similar tolerance to acid and were able to bind to human colonic mucus. However, pathogen exclusion by inhibition and competition varied significantly among the different *L. rhamnosus* isolates and pathogens tested. The results suggest that different sources of the same probiotic may have significantly altered strain properties. This should be considered in *in vivo* studies on human subjects and also for quality control of probiotic products.

Key words: Adhesion: Food matrix: *Lactobacillus rhamnosus* GG

In 2002, a joint FAO/WHO Working Group defined a probiotic as a 'live microorganism which when administered in adequate amounts confers a health benefit on the host'⁽¹⁾. Until now, specific probiotics have been selected and characterised based on their *in vitro* properties, for example tolerance to gastrointestinal conditions, adhesion to the intestinal mucus or epithelial cells and competitive exclusion of target pathogens^(2–4). Unfortunately, published information frequently lacks accurate information on strain identity and characteristics, for example International Culture Collection deposits and deposit numbers, on the one hand, and the mode of administration and the preclinical documentation and the selection criteria on the other. This makes strain comparison difficult and may influence the results of clinical intervention studies. We have extended this concern to the food matrix used to deliver the probiotic to the study subject, providing experimental evidence that the impact of *Lactobacillus rhamnosus* GG (ATCC 53103) on antigen transport depends on the quality of protein, unhydrolysed *v.* hydrolysed⁽⁵⁾ in the diet.

One further cause of variation in the outcomes of clinical studies, beyond differences in populations, selection criteria and study design, may arise when different production conditions, growth media, drying conditions or cryoprotectants are used for the same strain or when a successful probiotic is combined with other bacteria or strains. For example, the adhesion properties of the *L. rhamnosus* GG strain (ATCC 53103) have been shown to depend on the composition of the growth media and the number of starter culture transfers⁽⁶⁾. Clinical and immunological effects were not achieved when *L. rhamnosus* GG was combined with *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* ssp. *shermanii*⁽⁷⁾. Similarly, as far back as in 1983, a distinction in the clinical outcome for *L. acidophilus* was shown to depend on the production lot⁽⁸⁾.

The use of probiotics is currently extending from research to recommendations^(9,10), but rigorous scientific effort is still required to validate specific strains with anti-allergic potential for preventive and therapeutic applications. The production of functional probiotic foods

Abbreviation: PFGE, pulsed-field gel electrophoresis.

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presupposes stability of strain features in the final product. This requirement is emphasised in treating young infants with impaired gut barrier function, aberrant gut microbiota and sensitisation to dietary substances. For this purpose, we evaluated in the present study the strain characteristics, tolerance to acid pH and the adhesion and competitive exclusion properties of putative *L. rhamnosus* GG isolates obtained from different commercial products and production lots used in clinical intervention studies. As a prerequisite step, each isolate was genotypically and phenotypically characterised to ensure identical profiles, also excluding the possibility of contamination or misidentification of the strain obtained from different probiotic products.

We hypothesised that there are differences among the properties of the same probiotic strain, which depend on the production processes using different methods and media, and these influence strain properties with a consequent impact on the clinical and nutritional properties of probiotics.

Experimental methods

Bacterial isolates and culture conditions

Altogether thirteen *L. rhamnosus* isolates claimed to be *L. rhamnosus* GG (ATCC 53103) were isolated from different probiotic products from different countries (identification codes: AL, BL, CL, DL, EL, FL, HL, IL, JL, KL, LL, ML and NL). Among them, four isolates (AL, EL, FL and NL) were isolated from capsule products from different countries, two isolates (LL and ML) were derived from commercial infant foods, three isolates (DL, HL and KL) were isolated from freeze-dried powders of different products and four isolates (BL, CL, IL and JL) were provided from soft agar. For comparison, the original *L. rhamnosus* GG (original *L. rhamnosus* GG strain isolate donated by Professor Sherwood Gorbach of Tufts University, Boston, MA, USA) was included. The original *L. rhamnosus* GG strain is the foundation strain from which other cultures used in probiotic products were derived. An additional commercial probiotic *L. rhamnosus* strain (identification code OL) of the same species but known to be phenotypically different from *L. rhamnosus* GG was included as an external reference strain.

The bacterial pathogens used were *Cronobacter sakazakii* (ATCC 29544), *Staphylococcus aureus* (DSM 20231), *Clostridium perfringens* (DSM 756) and *Salmonella enterica* serovar Typhimurium (ATCC 12028).

The *L. rhamnosus* isolates were grown in de Man, Rogosa and Sharpe broth (Oxoid Limited, Basingstoke, Hampshire, UK) and incubated at 37°C under anaerobic conditions (10% H₂, 10% CO₂ and 80% N₂; Concept 400 anaerobic chamber, Ruskin Technology, Leeds, UK). For adhesion, competitive exclusion, displacement and inhibition assays, the isolates were grown for 18 h,

harvested and then washed twice with PBS buffer. All micro-organisms were metabolically labelled by the addition of 10 µl/ml tritiated thymidine (5-³H-thymidine 1.0 mCi/ml; Amersham Biosciences, Little Chalfont, Bucks, UK) to the media.

Species identity of isolates by partial sequence analysis of the 16S rRNA gene

The *L. rhamnosus* isolates used in the present study were identified at the species level by partial sequence analysis of the 16S rRNA gene, followed by Blast analysis. In brief, micro-organisms were grown overnight, 1 ml of cells was harvested and the DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma, St Louis, MO, USA) following the manufacturer's instructions. Partial amplification of the 16S rRNA gene and species identification were carried out as described previously⁽¹¹⁾. Amplified PCR products were purified using the GenElute™ PCR Clean-Up Kit (Sigma), and automated sequencing of the amplicons was carried out at Secugen SL (Madrid, Spain) in an automated sequencer ABI Prism (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared with those held at the GenBank database by using the Blast application at the NCBI webpage.

Genetic typing of the isolates

Randomly amplified polymorphic DNA-PCR. DNA extracts from the different *L. rhamnosus* isolates were used for strain typification by randomly amplified polymorphic DNA analysis using previously described conditions and the primer for *L. rhamnosus*⁽¹²⁾ (Sigma Genosys, St Louis, MO, USA). PCR assays were run in a Unocycler (VWR International Eurolab S.L., Barcelona, Spain) thermocycler. Amplification products were subjected to electrophoresis on 1% agarose (Sigma), and the gels were stained and visualised by ethidium bromide staining.

Enterobacterial repetitive intergenic consensus-PCR. Enterobacterial repetitive intergenic consensus-PCR is based on the use of oligonucleotides targeting short repetitive sequences distributed throughout the bacterial genome. DNA was amplified using primers 5'-ATGTAAGC-TCCTGGGGATTCAC-3' and 5'-AAGTAAGTGACTGGGGT-GAGCG-3' (Sigma Genosys) as described previously⁽¹³⁾.

DNA restriction patterns by pulsed-field gel electrophoresis. The *L. rhamnosus* isolates were typified using analysis of DNA restriction patterns by pulsed-field gel electrophoresis (PFGE). Intact high-molecular weight genomic DNA was isolated and digested in agarose plugs. Cells were grown to an OD₆₀₀ of 1.5, harvested by centrifugation, washed three times in buffer Tris-HCl (10 mM)-EDTA (1 mM), pH 8.0, and resuspended in 500 µl of the same solution. To form agarose plugs, the cell suspension was heated to 50°C, mixed with an equal volume of 2% PFGE agarose (Bio-Rad Laboratories, Richmond, CA, USA)

in 0.5 × Tris–borate–EDTA buffer and added to moulds. The plugs were incubated for 24 h at 37°C in 1 ml lysis buffer (per plug) containing 50 mM-EDTA (pH 8.0), N-laurylsarcosine (0.5 mg/ml; Sigma), Brij58 (5 mg/ml; Sigma), deoxycholate (2 mg/ml; Sigma), lysozyme (2 mg/ml; Sigma), mutanolysin (15 U/ml; Sigma) and RNase (2 µg/ml; Sigma) and were then deproteinised by incubation at 50°C for 24 h in a solution containing 0.5 M-EDTA pH (8.0), 40 mM-Tris–HCl (pH 8.0), 1% (w/v) SDS (Sigma) and proteinase K (1.5 mg/ml; Sigma). They were finally washed for 1 h in Tris–HCl (10 mM)–EDTA (1 mM), pH 8.0, and incubated for 24 h at 37°C in Tris–HCl (10 mM)–EDTA (1 mM), pH 8.0, containing Pefabloc SC (0.29 mg/ml; Merck, Darmstadt, Germany).

Thin slices of the agarose plugs were cut and washed six times for 30 min at room temperature in Tris–HCl (10 mM)–EDTA (1 mM), pH 8.0, buffer. DNA within the plugs was digested with 20 U of the appropriate restriction enzyme in 200 µl of the buffer recommended by the supplier. Two different restriction enzymes, SfiI and NotI, were tested. Electrophoresis was carried out at 6 V/cm and 14°C using a CHEF DRII apparatus (Bio-Rad Laboratories) in 1% PFGE certified agarose (Bio-Rad Laboratories) gels with 0.5 × Tris–borate–EDTA buffer. Pulse times ranged from 2 to 30 s during the 24 h electrophoresis. A DNA pulse marker (LowRange PFG Marker N0350S; New England Biolabs, Hitchin, Herts, UK) was used as molecular size standard. Gels were stained and visualised by ethidium bromide staining.

Characterisation of the isolates by carbohydrate fermentation profiles

The fermentation ability of the *L. rhamnosus* isolates was obtained in API 50 CH strips (Bio-Mérieux, Marcy l'Etoile, France) following the manufacturer's instructions.

Tolerance to acid

Bacterial cultures (5 ml) were grown overnight, cells were harvested, washed twice with 0.85% NaCl and resuspended in 500 µl of the same solution; 100 µl of the bacterial suspensions (approximately 1×10^8 cells) were added to 900 µl of simulated gastric juice (125 mM-NaCl, 7 mM-KCl, 45 mM-NaHCO₃ and pepsin (3 g/l; Sigma)) adjusted to pH 2.0 or 2.5 with HCl. Suspensions were then incubated for 90 min. Plate counts were made at time point 0 and after 90 min of incubation.

In vitro assay of adhesion to human intestinal mucus

Human intestinal mucus was obtained as described elsewhere⁽³⁾ from the human colon. Colonic mucus was dissolved (0.5 mg protein/ml) in HEPES–Hanks buffer (10 mM-HEPES, pH 7.4). Radiolabelled bacterial absorbance ($A_{600\text{nm}}$) was adjusted to 0.25 (SD 0.05) to

standardise the bacterial concentration (10^8 cells/ml). The adhesion assessment of the *L. rhamnosus* isolates and bacterial pathogens was carried out as described previously⁽¹⁴⁾. Adhesion was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilised mucus. Adhesion was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

Exclusion by inhibition assay

To test the ability of the *L. rhamnosus* isolates to inhibit the adhesion of pathogens, the procedure described by Collado *et al.*^(14,15) was used. In brief, unlabelled isolates (10^8 cells/ml) were added to the wells and incubated for 1 h at 37°C; they were then removed by washing with HEPES–Hanks buffer. Radiolabelled pathogens (10^8 cells/ml) were then added to the wells and incubated at 37°C for 1 h. Thereafter, the wells were washed, and the bound bacteria were recovered after lysis. Radioactivity was measured by liquid scintillation. The percentage of adhesion inhibition was calculated as the difference between the adhesion of the pathogen in the absence and presence of the different isolates. Inhibition was determined in three independent experiments, and each assay was performed in triplicate.

Exclusion by displacement assay

The ability of the *L. rhamnosus* isolates studied to displace pathogens already adhered was assessed according to Collado *et al.*^(14,15). Radiolabelled pathogens were added to the wells containing mucus. After washing and removal of unbound pathogens, non-radiolabelled isolates were added. Wells were incubated and washed; thereafter, bound bacteria were recovered after lysis and radioactivity was measured. Displacement of pathogens was calculated as the difference between the adhesion of pathogens before and after the addition of the *L. rhamnosus* isolates. At least three independent experiments were carried out. Each assay was performed in triplicate to calculate intra-assay variation.

Exclusion by competition assay

Competitive inhibition of the model pathogens by the *L. rhamnosus* isolates studied was determined as described previously⁽¹⁵⁾. For the competition test, equal quantities of a given bacterial suspension of isolates and radiolabelled pathogens were mixed and then added to the intestinal mucus and incubated as indicated previously. The cells of the pathogen bound to the mucus were then removed, and adhesion was calculated as described earlier.

Statistical analysis

The adhesion (%) measured in different conditions was the primary endpoint. One-way ANOVA was used to test the overall difference in the adhesion properties between strains. When $P < 0.10$, Dunnett's (two-tailed) t test was used to compare each strain with the reference strain *L. rhamnosus* GG without any other pairwise comparisons. The cut-off point was not strictly determined at 0.05 to indicate statistical significance due to the small sample size and large type II error. Based on Dunnett's t test, the exact P values are given, and in which both 0.05 and 0.10 were used as cut-off points to indicate the degree of statistical significance in the difference between strains.

Statistical analysis was conducted using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Identification at the species level

All *L. rhamnosus* isolates used were confirmed as members of the species *L. rhamnosus* by partial sequencing of the 16S rDNA. Sequence analysis showed 100% sequence homology among the different *L. rhamnosus* isolates studied as well as with *L. rhamnosus* 16S rDNA sequences held in the databases (data not shown).

Genotypic and phenotypic characterisation of the isolates

All *L. rhamnosus* isolates, except the reference strain OL, showed randomly amplified polymorphic DNA, enterobacterial repetitive intergenic consensus and PFGE profiles identical to that of the original *L. rhamnosus* GG isolate obtained from Professors Gorbach and Goldin. These three techniques allowed differentiation of the isolate *L. rhamnosus* OL from the other *L. rhamnosus* isolates.

With regard to phenotypic characterisation, none of the *L. rhamnosus* isolates fermented glycerol, erythritol, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl- β -D-xylopyranoside, inositol, methyl- α -D-mannopyranoside, D-melibiose, D-saccharose, inulin, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. All isolates fermented D-arabinose, D-ribose, D-galactose, D-glucose, D-mannitol, D-fructose, D-mannose, dulcitol, D-sorbitol, N-acetyl glucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-trehalose, D-melezitose, gentiobiose, D-tagatose, L-fucose and gluconate. All putative *L. rhamnosus* isolates showed identical sugar fermentation profiles, being different from those of the reference strain OL, which in addition was able to ferment L-sorbose, L-rhamnose, methyl- α -D-glucopyranoside, D-maltose and D-lactose.

Low pH tolerance

Tolerance to acidic pH (pH 2 and 2.5) varied from 43.0 to 79.1% and showed no significant differences ($P > 0.05$) among the tested *L. rhamnosus* isolates.

In vitro adhesion assay to colonic mucus

All the tested *L. rhamnosus* isolates had a good ability to adhere to human colonic mucus, although this varied between different isolates. The adherence of the original *L. rhamnosus* GG was 19 (SD 7.5)%, and that of the other isolates varied from 12.1 to 24.3%. Only the adhesion of the reference strain OL differed significantly from that of the original *L. rhamnosus* GG ($P = 0.01$), and it also showed the lowest percentage of adhesion among all the isolates tested (data not shown).

Among the pathogenic strains tested, the most marked ability to adhere to colonic mucus was detected for *S. aureus* (10.2 (SD 3.0)%), followed by *C. perfringens* (4 (SD 3.1)%), *S. enterica* serovar Typhimurium (2.4 (SD 0.7)%) and *C. sakazakii* (2 (SD 0.7)%).

Exclusion by inhibition assay

The ability to inhibit the adhesion of pathogens to colonic mucus by the *L. rhamnosus* isolates differed significantly (see Fig. 1). The original *L. rhamnosus* GG was able to inhibit all pathogens tested, and this micro-organism reduced the adhesion of *C. sakazakii* (31%), *S. enterica* serovar Typhimurium (25%), *C. perfringens* (25%) and *S. aureus* (24%).

Differences were between *L. rhamnosus* isolates in the adhesion of *C. sakazakii*, *S. enterica* serovar Typhimurium and *C. perfringens* (ANOVA, $P < 0.001$ for each) but not in the adhesion of *S. aureus* ($P = 0.352$).

Four *L. rhamnosus* isolates (AL, LL, ML and NL) showed different inhibition ($P = 0.083$, $P = 0.015$, $P < 0.001$, $P = 0.025$, respectively) of *C. sakazakii* compared with the original *L. rhamnosus* GG. The isolate ML, again, differed ($P < 0.001$) from the original *L. rhamnosus* GG in the adhesion inhibition of *S. enterica* serovar Typhimurium. Two *L. rhamnosus* isolates (ML and OL) differed ($P < 0.001$, $P = 0.032$, respectively) in the adhesion inhibition of *C. perfringens* from the original *L. rhamnosus* GG. The adhesion inhibition of *S. aureus* did not differ between the tested *L. rhamnosus* isolates and the original *L. rhamnosus* GG.

Exclusion by displacement assay

All *L. rhamnosus* isolates were able to displace the preadhered model pathogens tested in colonic mucus. Differences were between *L. rhamnosus* isolates in the adhesion of *C. sakazakii*, *S. enterica* serovar Typhimurium (ANOVA, $P = 0.063$, 0.001, respectively) but not between

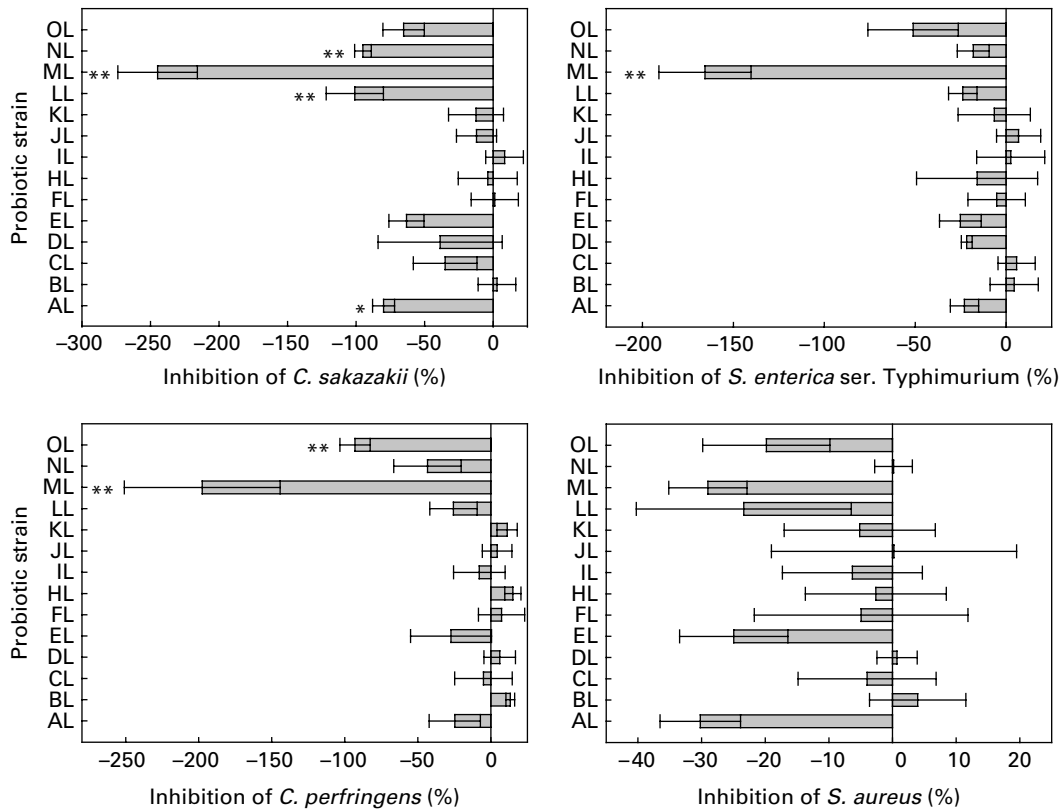


Fig. 1. Inhibition of the adhesion to colonic mucus of pathogenic bacteria by the tested *Lactobacillus rhamnosus* isolates. Results are expressed as percentages with regard to the inhibition obtained with the original *L. rhamnosus* GG isolate donated by Professor S. Gorbach. Values on the X-axis higher than 0 indicate adhesion inhibition higher than the original *L. rhamnosus* GG, and those lower than 0 indicate adhesion inhibition lower than the original *L. rhamnosus* GG. Adhesion inhibition significantly different from the original *L. rhamnosus* GG (*if $0.05 \leq P < 0.10$ and **if $P < 0.05$). Values are means, with standard errors represented by vertical bars. The OL indicates the external reference *L. rhamnosus* strain. *C. sakazakii*, *Cronobacter sakazakii*; *S. enterica* ser., *Salmonella enterica* serovar; *C. perfringens*, *Clostridium perfringens*; *S. aureus*, *Staphylococcus aureus*. Identification codes: AL, BL, CL, DL, EL, FL, HL, IL, JL, KL, LL, ML and NL.

C. perfringens and *S. aureus* (ANOVA, $P=0.128$, 0.717 , respectively). The original *L. rhamnosus* GG displaced 54% of adhered *S. enterica* serovar Typhimurium, and it differed ($P=0.084$) from the *L. rhamnosus* LL isolate. *C. perfringens* was displaced by 33% and *S. aureus* by 20% by the original *L. rhamnosus* GG, and all the other isolates showed no difference in the displacement from the original *L. rhamnosus* GG. The original *L. rhamnosus* GG displaced *C. sakazakii* by 12%, differing ($P=0.082$, 0.009 , 0.017 , 0.013 , 0.068 , 0.019 , 0.01 , 0.092 , 0.051 , respectively) from the other study isolates AL, BL, CL, DL, EL, HL, IL, KL and NL. The results are shown in Fig. 2.

Exclusion by competition assay

The inhibition by competition varied among the *L. rhamnosus* isolates and pathogens tested. The relevant data are shown in Fig. 3. The presence of some isolates increased the adhesion of *C. sakazakii*, *S. enterica* serovar Typhimurium and *C. perfringens* to colonic mucus. All but one isolate (BL) was able to reduce the adhesion of *S. aureus*. Differences were between *L. rhamnosus* isolates in the adhesion of *C. sakazakii*, *S. enterica* serovar

Typhimurium, *C. perfringens* and *S. aureus* (ANOVA, $P < 0.001$, $P < 0.001$, $P=0.005$, $P=0.023$, respectively). The original *L. rhamnosus* GG showed inhibition abilities for all pathogens tested. This micro-organism inhibited the adhesion of *C. perfringens* in 47%, being different ($P=0.057$, 0.059 , 0.07 , 0.091 , respectively) from the BL, LL, ML and NL isolates; *S. aureus* in 39%, being different ($P=0.019$) from the BL isolate; *S. enterica* serovar Typhimurium in 5%, being different ($P=0.064$, 0.014 , 0.026 , respectively) from the IL, ML and NL isolates; *C. sakazakii* in 9%, showing a difference ($P=0.001$, $P=0.003$, $P=0.048$, $P=0.001$, $P < 0.001$, $P=0.079$, respectively) from the AL, DL, LL, ML, NL isolates and the reference strain OL.

Discussion

The term 'probiotic' *per se* implies a health benefit. Based on the definition of probiotics, consumption of these bacteria present in food products should exert a range of health-promoting effects in the host⁽¹⁾. In several meta-analyses and reviews, however, strains with no such documentation are reported along with true probiotics^(16,17). Moreover, confusion is caused by reporting on

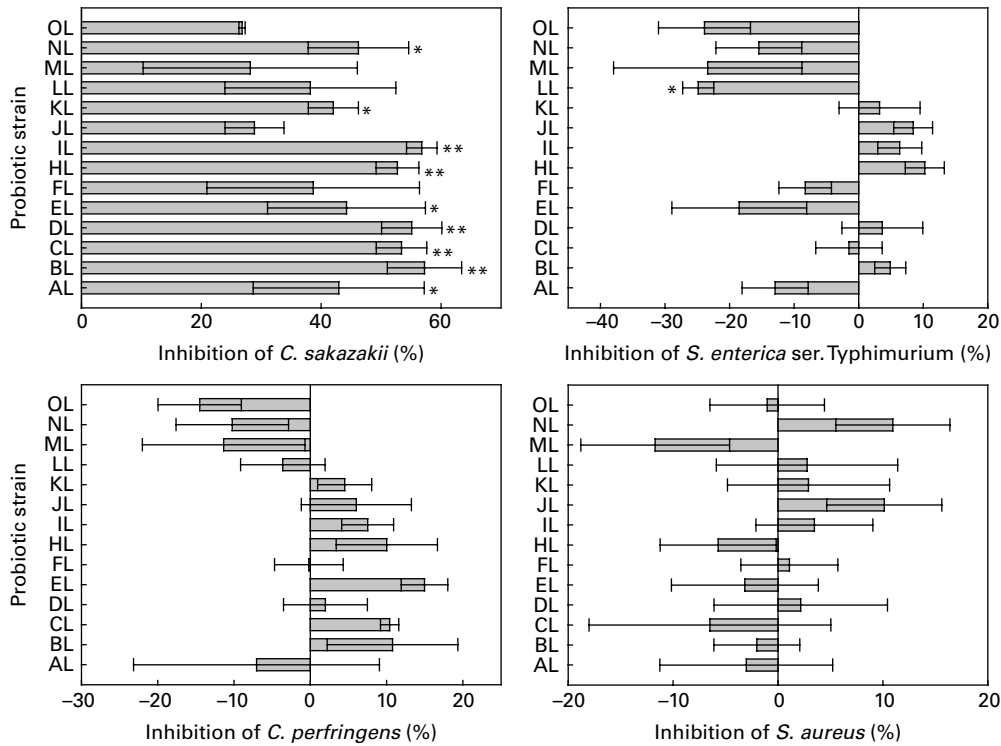


Fig. 2. Displacement of the adhesion to colonic mucus of pathogenic bacteria by the tested *Lactobacillus rhamnosus* isolates. Results are expressed as percentages with regard to the displacement obtained with the original *L. rhamnosus* GG isolate donated by Professor S. Gorbach. Values on the X-axis higher than 0 indicate adhesion displacement higher than the original *L. rhamnosus* GG, and those lower than 0 indicate adhesion displacement lower than the original *L. rhamnosus* GG. Adhesion displacement significantly different from the original *L. rhamnosus* GG (* if $0.05 \leq P < 0.10$ and ** if $P < 0.05$). Values are means, with standard errors represented by vertical bars. The OL indicates the external reference *L. rhamnosus* strain. *C. sakazakii*, *Cronobacter sakazakii*; *S. enterica* ser., *Salmonella enterica* serovar; *C. perfringens*, *Clostridium perfringens*; *S. aureus*, *Staphylococcus aureus*. Identification codes: AL, BL, CL, DL, EL, FL, HL, IL, JL, KL, LL, ML and NL.

the effect of probiotic strains and their combinations together. Indeed, scientifically it is not surprising that different micro-organisms have different effects.

We demonstrate in the present study, for the first time, the differences in the *in vitro* properties of several *L. rhamnosus* GG isolates from different probiotic products. As a preceding step, all micro-organisms were genotypically and phenotypically characterised to ensure that all putative *L. rhamnosus* GG isolates had identical profiles, obviating any contamination or misidentification of the isolates used in the different products. All putative *L. rhamnosus* GG isolates were members of the *L. rhamnosus* group proved by the use of profiling techniques such as randomly amplified polymorphic DNA-PCR, enterobacterial repetitive intergenic consensus-PCR and PFGE, and they showed profiles identical to that of the original *L. rhamnosus* GG isolate. The genotypic and phenotypic characterisation allowed distinction of the external reference strain OL from the *L. rhamnosus* isolates.

Adherence of bacteria to intestinal mucus or epithelium is known to be a prerequisite for colonisation and infection of the gastrointestinal tract by many pathogens⁽¹⁸⁾. The adherent probiotic strains may at least temporarily colonise the gastrointestinal tract and inhibit or compete with pathogens⁽¹⁹⁾. Several reports have described the adhesion abilities of probiotic strains and also their

inhibitory effects on model pathogen adhesion to human mucus^(14,20,21). Variations in these properties may affect the clinical outcome of intervention studies. In the present study, all tested *L. rhamnosus* isolates showed the ability to adhere to human colonic mucus, and the adhesion ability of different isolates varied but did not differ significantly. Potentially, the preculturing may have decreased the differences, and thus other adhesion assays without the preculturing step should be considered. However, all isolates had significantly higher adhesion ability than that of the reference strain.

The model pathogen exclusion results varied significantly and depended on the isolate in question. The results indicate differences in these important properties of probiotics among the different *L. rhamnosus* isolates obtained from different sources. Moreover, conducting our analyses required growing the different isolates under the same standardised laboratory conditions. Despite this culture step, which may have decreased the differences in physiological properties of the strain or the effect of different excipients used during manufacturing of the products, our observations suggest that the *in vitro* properties of probiotics may vary depending on the production conditions used for the same probiotic strain.

The present findings indicate that different isolates of the same strain may possess different properties which may

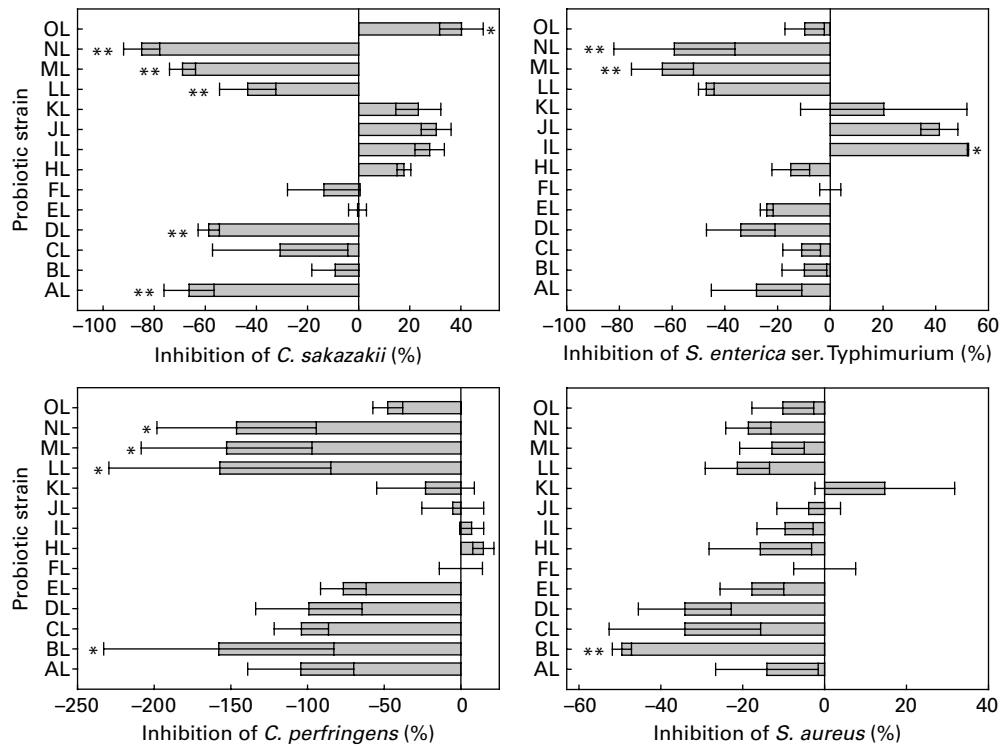


Fig. 3. Competition of the adhesion to colonic mucus of pathogenic bacteria by the tested *Lactobacillus rhamnosus* isolates. Results are expressed as percentages with regard to the competition obtained with the original *L. rhamnosus* GG isolate donated by Professor S. Gorbach. Values on the X-axis higher than 0 indicate adhesion competition higher than the original *L. rhamnosus* GG, and those lower than 0 indicate adhesion competition lower than the original *L. rhamnosus* GG. Adhesion competition significantly different from the original *L. rhamnosus* GG (* if $0.05 \leq P < 0.10$ and ** if $P < 0.05$). Values are means, with standard errors represented by vertical bars. The OL indicates the external reference *L. rhamnosus* strain. *C. sakazakii*, *Cronobacter sakazakii*; *S. enterica* ser., *Salmonella enterica* serovar; *C. perfringens*, *Clostridium perfringens*; *S. aureus*, *Staphylococcus aureus*. Identification codes: AL, BL, CL, DL, EL, FL, HL, IL, JL, KL, LL, ML and NL.

influence their *in vivo* effects, underlining the importance of control of the manufacturing process and the food matrix. The *L. rhamnosus* isolates tested in the present study were chosen to be from different products and origins. The manufacturing process may thus have had a significant impact on the strain properties. Often probiotics are used in fermented dairy products or as freeze-dried preparations. Both provide a matrix for the micro-organisms, and previous studies have indicated that the matrix may affect the strain properties^(22,23). This, along with the production conditions, may in part explain the different results obtained between strain isolates when characterising strain impact on pathogen adhesion to mucus, although the existence of point mutations in some of the different isolates cannot be overruled.

In addition to the differences between strains, Deepika *et al.*⁽²⁴⁾ have reported that the adhesion of the *L. rhamnosus* strain GG to Caco-2 cells was the highest when bacteria collected during the early stationary phase of growth were used. This finding suggests that the early stationary phase may be the optimum harvest point for *Lactobacillus* GG to obtain the most adhesive strain.

Though *L. rhamnosus* GG is one of the probiotics subjected to the largest number of human intervention studies, in many cases no attention has been paid to the specific properties of the strains used. The lack of such reports is surprising, especially considering the early

findings on changes in the *in vitro* properties of early *L. rhamnosus* GG strains following different production processes and a long-term series of reinoculations, suggesting deterioration of adhesion properties⁽⁶⁾ and the report on production lot differences in an *L. acidophilus* strain influencing the outcome of human intervention studies⁽⁸⁾.

Taken together, we conclude that the original properties used in the selection of specific probiotic strains may indeed be influenced by industrial production processes and conditions as well as by the food matrix used. This finding sets important prerequisites for quality control in probiotics. Ensuring the original properties is especially important when the strain or product is used in human intervention studies, as small changes may significantly influence the outcome. The finding also presupposes new quality-control measures for the manufacture of probiotics for food use to preserve the original properties, which may have an impact on efficacy in human studies.

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the study; Ł. G. and M. G. were responsible for the laboratory experiments of the study. All authors participated in the analysis of results and in writing the manuscript. We thank Tuija Poussa for statistical consultation during the data analysis and help in writing the manuscript.

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