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ORIGINAL CONTRIBUTION

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Abstract

The possibility of using microbes to maintain health, and to prevent or treat disease is a topic as old as microbiology. The research of novel probiotic strains is important in order to satisfy the increasing request of the market and to obtain functional products in which the probiotic cultures are more active and with better probiotic characteristics than those already present on the market.

In this study, the probiotic potential of *Lactobacillus* strains isolated from Italian elderly human faeces was investigated.

The *Lactobacillus* strains were identified and examined for resistance to gastric acidity and bile toxicity, adhesion to HT-29 cells, antimicrobial activities, antibiotic susceptibility and plasmid profile. Survival of the strains through human intestine was examined in a 3 months human feeding trial.

Two strains, *Lactobacillus rhamnosus* IMC 501 and *Lactobacillus paracasei* IMC 502, tolerated well low pH and bile acids. In antimicrobial activity assays, both strains showed inhibitory properties towards selected potential harmful microorganisms, particularly against *Candida albicans*. The two selected strains expressed high in vitro adherence to HT-29 cells increasing this characteristic when they are used in combination and they were resistant to vamcomycin, colistin sulphate, gentamicin, oxolinic acid and kanamycin. Moreover, the two strains could be recovered from stools of volunteers after the feeding trials.

Lactobacillus rhamnosus IMC 501 and *L. paracasei* IMC 502 present favourable strain-specific properties for their utilisation as probiotics in functional foods and the high adhesion ability of the *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 used in combination, confirmed by both in vitro and in vivo study, indicate that the two bacterial strains could be used as health-promoting bacteria.

Keywords

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Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit on the host [17]. The definition probiotic requires that the efficacy and safety of all probiotics be verified and thus, assessment of this constitutes an important part of their characterisation for human use [23]. The criteria for selecting a good probiotic strain have been listed comprehensively by several authors [28]. The strains should preferably be of human origin, possess a generally regarded as safe (GRAS) status and able to survive through the gastrointestinal tract [35]. One of the important criteria for a potential probiotic strain is believed to be its ability to adhere to mucosal surfaces of the human intestinal tract. Probiotic strains should also have desirable antibiotic resistance and sensitivity patterns, antagonistic toward

potentially pathogenic microorganisms and have metabolic activities beneficial to the well-being of the host [35]. Characteristics ascribed to a probiotic strain are, in general, strain specific and individual strains have to be tested for each property.

The most studied probiotics are the lactic acid bacteria, particularly *Lactobacillus* and *Bifidobacterium*. Most of *Lactobacillus* species are normal and non-pathogenic inhabitants in human and animal intestine and their presence is important for the maintenance of the intestinal microbial ecosystem [24]. Lactobacilli have been shown to possess inhibitory activity towards the multiplication of enteropathogens and they are highly competitive largely due to their production of several antimicrobial compounds [6]. The research of novel probiotic strains is important in order to satisfy the increasing request of the market and to obtain functional products in which the probiotic cultures are more active [10] and with better probiotic characteristics than those already present on the market.

The aim of this study was to screen isolates of human intestinal lactobacilli for their probiotic characteristics suitable for the preparation of functional food and food supplements that provide improvement or restoration of the intestinal microflora.

Materials and methods

Bacterial strains and culture conditions

The 11 intestinal lactobacilli strains used in this study were isolated from the faecal samples of Italian elderly subjects of both sexes and ranging in age from 60 to 90 years as previously described [30, 38]. The strains were analysed for morphological, cultural and physiological characteristics and the sugar fermentation profiles have been performed using API 50 CHL (bioMérieux, Marcy-l'Etoile, France) [38].

pH and bile tolerance

The isolated *Lactobacillus* strains were tested for their ability to resist to low pH and bile salt.

The pH value of gastric acid varies in the range of about 1.5–4.5 in a period of 2 h, depending on the entering time and the type of gastric contents. In the present study, pH 3 was used as a representative gastric pH value. A 24-h-old culture of each lactobacilli (10^8 CFU/ml) was suspended in a citrate buffer pH 3 for 5 h at 37 °C. The suspensions were then centrifuged at 3,000 rpm for 10 min at 4 °C twice and washed in sterile saline solution to eliminate the citrate buffer. Cells were suspended in physiological solution and a series of tenfold dilution (10^{-2} to 10^{-10}) was prepared. A given amount of each dilution (100 µl) was plated on to Man Rogosa Sharpe (MRS) agar (Oxoid, Basingstoke, Hampshire, UK) and incubated anaerobically at 37 °C for 24–48 h. The percentage of the viable bacteria was calculated.

Tolerance to bile salts was verified inoculating 100 µl of bacterial suspension of each strains (10^8 cells/ml) on to MRS agar containing Bile salt (Oxoid) at different concentrations (0.1; 0.3; 0.5%) and on to MRS agar containing Bile salt N.3 (Oxoid) at different concentrations (0.05; 0.1; 0.2%). Survival of the *Lactobacillus* strains was examined by counting the cells after 24 and 48 h of incubation at 37 °C.

Only those strains, which survived these two resistance tests, were unequivocally identified and further investigated for in vitro probiotic properties.

Molecular characterisation

The 16S rRNA gene sequencing was used to validate the phenotypic characterisation of the *Lactobacillus* strains. The 16S rDNA of the selected strains were amplified by PCR using P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′) universal primers corresponding to position 27f (forward) and 1495r (reverse) of *Escherichia coli* 16S rDNA [21]. The DNA extraction was conducted using the Qiagen Dneasy Tissue kit (Qiagen, Hilden, Germany). One µl of cell lysate was added to 49 µl of PCR mixture containing 45 µl of PCR supermix (Invitrogen srl, Milan, Italy) and 1 µl of each primers (18 pmol/ml). The reaction mixtures, after incubation at 94 °C for 1 min and 30 s, were cycled through the following temperature profile: five cycles of 30 s at 95 °C, 30 s at 60 °C and 4 min at 72 °C; five cycles of 30 s at 95 °C, 30 s at 55 °C and 4 min at 72 °C; 25 cycles of 30 s at 92 °C, 30 s at 50 °C and 4 min at 72 °C; one final cycle of 10 min at 72 °C and 10 min at 60 °C. The PCR was conducted in a Tpersonal Thermal Cycler (Biometra, Gottingen, Germany). The PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 µg ml^{-1} (w/v) of ethidium bromide (GIBCO BRL Gaithersburg, USA). The PCR products were purified using QIAquick PCR Purification Kit (Qiagen), sequenced by MWG The Genomic Company (M-Medical, Milan, Italy) and aligned on GeneBank (http://www.ncbi.nml.nih.gov/Web/Genebank/index.html) using BLAST algorithm [4].

The identities of the sequences, obtained after analysis of amplified PCR products, were verified by a BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov. Strains showing homology of at least 97% were considered to belong to the same species.

RAPD fingerprinting

Randomly amplified polymorphic DNA was used to differentiate the *Lactobacillus* isolates from each other [42]. RAPD was performed with the following random primers: M13 minisatellite core sequence (5′-GAG GGT GGC GGT TCT-3′), RP (5′-CAGCACCCAC-3′) and R5 (5′-AACGC GCAAC-3′). Reactions were carried out in 25 µl amplification mixtures with 12.5 µl of 2× Master Mix (Fermentas, Burlington, Canada), 0.5 µl of primer, 1 µl of total DNA and 11 µl of water. The reaction mixtures with M13 primer, after incubation at 94 °C for 2 min, were cycled through the following temperature profile: 30 cycles 94 °C for 60 s, 42 °C for 20 s and 72 °C for 2 min. Final extension was carried out at 72 °C for 10 min. The primer RP was used under the following amplification conditions: one cycle 94 °C for 3 min, 45 °C for 45 s, 72 °C for 1 min; 30 cycles 94 °C for 45 s, 45 °C for 45 s, 72 °C for 1 min; one cycle 94 °C for 45 s, 45 °C for 45 s, 72 °C for 5 min. The reaction mixtures with R5 primer, after incubation at 94 °C for 5 min, were cycled through the following temperature profile: 40 cycles 94 °C for 60 s, 29 °C for 90 s and 72 °C for 2 min. Final extension was carried out at 74 °C for 5 min. The PCR was conducted in a Tpersonal Thermal Cycler (Biometra). Amplification products were separated on a 2% agarose gel, containing 0.5 µg/ml (w/v) of ethidium bromide (GIBCO BRL).

In vitro adhesion assays

The adhesion of *Lactobacillus* strains was studied using HT-29 intestinal epithelial cell line [1, 11]. The HT29 cell-lines were grown routinely in Dulbecco's Modified Eagle's Medium (DMEM) 4,500 mg/ml glucose supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% foetal bovine serum. For adhesion assays, monolayer of HT-29 cells was prepared on tissue culture plates. After incubation at 37 °C under 5% CO_2 atmosphere for 24 h the HT-29 cell cultures were washed twice with PBS and 10 ml of bacterial suspension at a concentration of 10^8 cells/ml was applied to each plate. The plates were incubated at 37 °C for 2 h followed by washing three times with PBS to collect non-adhering bacteria. The adherent bacteria were released by applying a solution of PBS and EDTA (0.2%) and resuspended in 10 ml of saline solution. After a centrifugation for 5 min at 3,000 rpm, the cells were suspended in 5 ml of saline solution and a series of tenfold dilution (10^{-1} to 10^{-5}) was prepared. A given amount of each dilution (50 µl) was plated on to MRS agar (Oxoid) and incubated anaerobically at 37 °C for 24–48 h.

The adhesion percentage was calculated by comparing the number of adhered cells to the total cells of the bacterial suspension used. Each adherence assay was conducted in triplicate.

The adhesion assay was applied, using the same protocol, to a combination (1:1) of the best strains selected after the screening tests.

Antimicrobial activity assay

Antimicrobial activity of the selected strains was tested against *E. coli* ATCC 11775, *Staphylococcus aureus* ATCC 25923, *Clostridium perfringens* ATCC 13124, *Candida albicans* ATCC 10261 and *Streptococcus mutans* ATCC 20523 using a modification of the "deferred cross-streak" technique [16]. Briefly, MRS agar plates were streaked with the probiotic strain tested (10^6 CFU/ml) in the centre of the plate covering a 1 cm × 2 cm area and then incubated anaerobically at 37 °C until grown to confluence. After incubation, the probiotic growth was outlined and then removed. The plate was incubated again over chloroform for 1 h to inactivate any remaining cells and air dried for 45 min. The plate was then spread with 100 µl of potential pathogen tested at 10^7 CFU/ml and incubated at 37 °C for 24 h. The inhibition activity of the probiotic strains was evaluated measuring the zone of inhibition around probiotic growth.

Antibiotic susceptibility testing

Antibiotic resistance patterns of the selected probiotic strains were studied by disk diffusion method [8] on MRS agar plates. A total of 12 antibiotic substances were tested: ampicillin, amoxicillin, colistin sulphate, erythromycin, gentamicin, kanamycin, neomycin, oxolinic acid, penicillin G, tetracycline, vancomycin and rifampicin. All the antibiotic substances were from Oxoid. The agar plates were incubated anaerobically for 24 h at 37 °C. The diameters of inhibition zones were measured and the results (average of three readings) were expressed as sensitive (S) and resistant (R) according to NCCLS standard [31].

Plasmid profiles

The isolation of plasmid DNA from the selected bacterial strains and from *E. coli* ATCC 13706, as a positive control, was performed following the methods described by Anderson and McKay [5] and Frere [20] with minor modifications. One colony with typical morphology was picked from agar plates, inoculated in 10 ml of broth, which was incubated at 37 °C for 8–12 h. When the absorbance (A_{660}) of cultures reached 1–1.5, they were chilled on ice and then centrifuged at 5,000×*g* for 10 min at 4 °C. Cell pellets were washed with 5 ml ice-cold TES buffer (50 mM Tris–HCl, 5 mM EDTA, 25% sucrose),

resuspended in 300 µl of TES containing RNase (100 µg/ml), lysozyme (20 mg/ml) and mutanolysin (40 µg/ml) and incubated on ice for 1 h. Following addition of 250–300 mg of acid-washed glass beads (<106 µm, Sigma), the mixtures were vortexed briefly and the DNA extracted using chloroform:isoamyl alcohol (24:1) as described by Anderson and McKay [5]. Purified DNA preparations were analysed by electrophoresing (3.5 V/cm for 5 h) on 0.7% agarose gels stained with ethidium bromide.

In vivo feeding trial

Ten healthy volunteers participated in this study. Eligible participants were of both sexes and aged 24–65 years. Each subject signed an informed consent after he/she had been made full aware of the purpose of the study. Six different food products were used as carriers for delivering the same probiotic bacterial strains: yoghurt,"ricotta" cheese, "mozzarella" cheese, chocolate, chocolate mousse and ice-cream. The selected bacterial strains were mixed in the same concentration and inoculated in the food products to reach a final concentration of approximately 10^9 CFU/g of product. The viability and concentrations of bacterial strains in the products were checked by plate count and real-time PCR at day of inoculum of product and at expire date of the product (data not shown). The study had three periods: 1 month screening and baseline period (30 days till day 0), 3 months intake period (day 1–day 90) and 2 weeks post-treatment period (day 90–day 104). During the administration period, the subjects consumed one or more of the probiotic food products daily. Faecal samples were collected at day 0, day 90 and day 104. At each sampling, microbial analysis and reisolation of the strain were done as follows. Faecal samples were suspended (1:10 w/v) in physiological solution and tenfold serially diluted and 100 µl of appropriate dilutions was plated on Rogosa agar (Oxoid) with or without 12 µg/ml of vancomycin and gentamicin (Sigma-Aldrich, Missouri, USA). Vancomycin and gentamicin-resistant lactobacilli were enumerated on Rogosa-vancomycin and gentamicin agar. Plates were incubated anaerobically for 3 days at 37 °C. Ten to 20% of total colonies, randomly selected from countable Rogosa vancomycin and gentamicin agar plate, were isolated and checked for purity. DNA was extracted using the Qiagen Dneasy Tissue kit and analysed using the RAPD technique. The number of isolates selected from each sample was chosen on the basis of results obtained in previous studies where it was shown that ten randomly selected colonies gave good coverage of the numerically predominant strains cultured on a selective medium [25, 29]. The DNA extracted for RAPD was also used to verify 16S sequence identity following the methods reported at the Sect. "Molecular characterisation".

Results

pH and bile tolerance

Eleven isolated candidate probiotic strains of *Lactobacillus* from the faeces of elderly humans were screened for their survival to low pH and bile salts. Only two probiotic candidate strains survived, phenotypically identified as *Lactobacillus rhamnosus* and *Lactobacillus paracasei*, and were therefore selected for further study of their probiotic characteristics.

Molecular characterisation

The 16S rDNA of the two bacterial strains that survived from the tests above described were sequenced and they were identified by alignment as *L. rhamnosus* IMC501 (accession no. EU483094.1) and *L. paracasei* IMC502 (accession no. FJ542303.1).

In vitro adhesion assays

Lactobacillus rhamnosus IMC501 and *L. paracasei* IMC502 were examined for their ability to adhere to human intestinal cell line HT29. Results for adhesion tests and comparison among the adhesion percentage of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 and some commercial *Lactobacillus* strains were summarised in Fig. 1. Both tested lactobacilli strains expressed a higher in vitro adherence to human HT29 cell line than the commercial *Lactobacillus* strains belonging to the same species. *L. rhamnosus* exhibited an adhesion rate of 14.9 ± 3.2% and *L. paracasei* of 4.7 ± 1.5%. Moreover, the adhesion assay applied to a combination (1:1) of the *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502, showed an increased adhesion on HT29 cells (Fig. 2).

Antimicrobial activity assay

The inhibitory activity of the two probiotic bacterial strains was ranked according to the size of zones of inhibition against common intestinal pathogens (Table 1). The two *Lactobacillus* strains had an inhibitory effect on potentially pathogenic microorganisms such as *E. coli*, *S. aureus*, *C. albicans*, *C. perfringens* and *S. mutans*. Both *Lactobacillus* strains exhibited a particularly enhanced antipathogenic activity against *C. albicans* (inhibition zone >2.5 × 3 cm) (Fig. 3).

Antibiotic susceptibility testing

Both strains were found resistant to vancomycin, colistin sulphate, gentamicin, oxolinic acid and kanamycin and

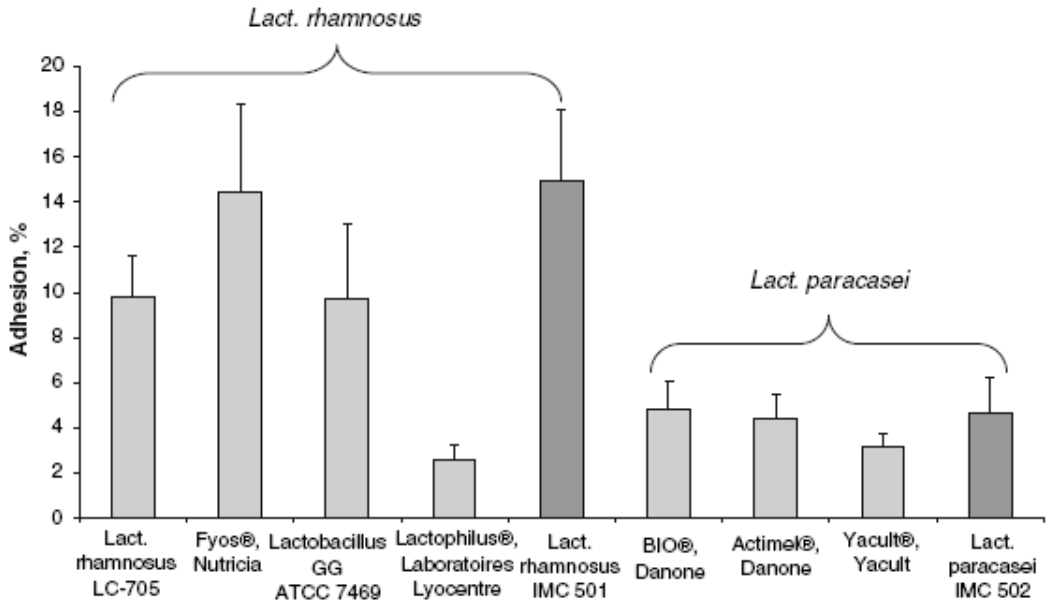

Fig. 1 Comparison among the adhesion percentages of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 and some commercial *Lactobacillus* strains to human intestinal cell lines. The adhesion values of commercial strains are from Tuomola and Salminen [40]

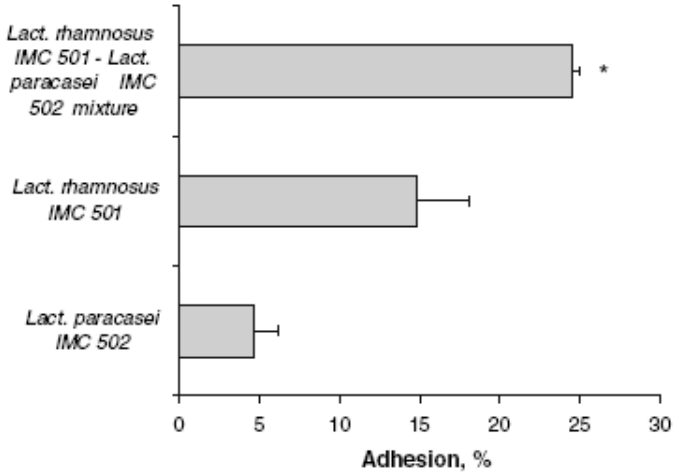

Fig. 2 Adhesion percentages of *Lactobacillus paracasei* IMC 502, *Lactobacillus rhamnosus* IMC 501 and two-strains mixture (1:1). Each value represents the mean ± SD of three measurements. *Significantly different from the adhesion of the single strain, $P < 0.05$ (*t* test)

susceptible to the other antibiotics tested. Moreover, the erythromycin and neomycin inhibited the growth of *L. paracasei* IMC 502 but not of *L. rhamnosus* IMC 501 (Table 2).

Plasmid profiles

Analysis of plasmid profiles revealed that the two *Lactobacillus* strains did not contain plasmids (results not shown) whereas a plasmid was isolated from *E. coli* (positive control).

In vivo feeding trial

This test was carried out in order to confirm intestinal transit survival in humans. The recovery of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 from ten out of ten faecal samples (collected at the end of the consumption period) demonstrated the presence of the strains during the consumption of the probiotic-enriched foods (Table 3). The strains were not detected in any of the faeces samples obtained before the consumption of probiotic food products. The strains were also still detected in nine volunteers at the end of follow-up (day 104) (Table 3). An example of the RAPD profiles obtained from colonies isolated from the faecal samples of volunteer "C" at day 104 using primer M13 is shown in Fig. 4: the profiles reported in lanes 5, 6, 8, 9, 12, 13 were identical to the pattern obtained from

Table 1 Degree of inhibition of tested potential human pathogens by *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502

Bacterial strain	Inhibition of growth[a] of				
	E. coli (ATCC 11775)	*Staph. aureus* (ATCC 25923)	*C. albicans* (ATCC 10261)	*Cl. perfringens* (ATCC 13124)	*Str. mutans* (ATCC 20523)
L. rhamnosus IMC 501	+++	++	++++	+++	+
L. paracasei IMC 502	+	+++	++++	+++	+

[a] + zone of inhibition <2 × 1.5 cm, ++ zone of inhibition <2 × 2.5 cm, +++ zone of inhibition <2.5 × 3 cm, ++++ zone of inhibition >2.5 × 3 cm

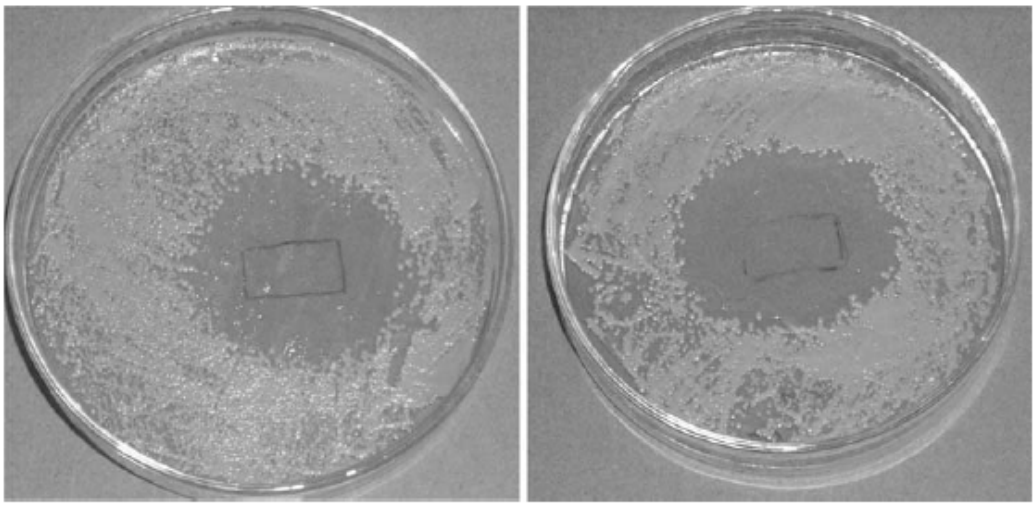

Fig. 3 In vitro inhibition of *Candida albicans* with *L. paracasei* IMC 502 (*left*, zone of inhibition >2.5 × 3 cm) and *L. rhamnosus* IMC 501 (*right*, zone of inhibition >2.5 × 3 cm)

Table 2 Antibiotic susceptibility test of *Lactobacillus rhamnosus* IMC 501 and *Lactobacillus paracasei* IMC 502

Antibiotic	Diameter (mm) of inhibition zone	
	L. rhamnosus IMC 501	*L. paracasei* IMC 502
Ampicillin	20 ± 1.2[a] (S)	26 ± 0.9 (S)
Amoxicillin	35 ± 0.6 (S)	31 ± 0.8 (S)
Colistin sulphate	0 (R)	0 (R)
Erythromycin	0 (R)	39 ± 0.7 (S)
Gentamicin	0 (R)	0 (R)
Kanamycin	7 ± 1.1 (R)	0 (R)
Neomycin	9 ± 0.4 (R)	13 ± 0.5 (S)
Oxolinic acid	0 (R)	0 (R)
Penicillin G	36 ± 1.2 (S)	36 ± 0.9 (S)
Rifampicin	31 ± 0.8 (S)	29 ± 1.2 (S)
Tetracycline	28 ± 1.1 (S)	31 ± 0.8 (S)
Vancomycin	0 (R)	0 (R)

S sensitive (NCCLS standard [31]), *R* resistant (NCCLS standard [31])

[a] Values are means ± SD

L. paracasei IMC 502, used as positive control (lane 2) and confirmed by the 16S DNA sequencing.

Discussion

Despite the difficulties encountered in reliably characterising probiotic strains using in vitro methods, the initial screening of strains in this manner remains a useful preliminary step in the detection of probiotic candidates.

The selected bacterial strains isolated from human faeces, *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 showed good probiotic characteristics. They survived under low pH conditions for 5 h and they tolerated well the bile acids under in vitro conditions even at concentrations higher than those previously used by other authors [18, 24]. Acid tolerance of bacteria is an important factor as well as to assure their resistance of gastric stresses also for their use as dietary adjuncts in acid foods such as yoghurt.

Adhesion and colonisation of probiotic bacteria in the gastrointestinal tract of the host is believed to be one of the essential features required for the delivery of their health benefits [9]. It is known that good adhesion of probiotic microorganism to the intestinal cells is related to many beneficial effects. In fact, the adhesion is a prerequisite for colonisation [3], stimulation of the immune system [37] and for antagonistic activity against enteropathogens [13]. *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 expressed higher values of in vitro adhesion to HT29 cell line than other commercial strains belonging to the same species [40]. If this result is directly comparable with the in vivo situation, a smaller quantity of the two *Lactobacillus* strains need to be consumed to have the same number of organisms adhere to the intestinal epithelium as obtained with *Lactobacillus* strains isolated from commercial products. Combination of the two probiotic strains may have synergistic adhesion effects. The property of co-adhesion, having an improved adhesion effect, is a peculiar characteristic of the strain, *L. paracasei* IMC 502 and *L. rhamnosus* IMC 501 in our case. In fact, as reported in literature [7, 14, 32] not all probiotic bacteria, with good individual property of adhesion, are able to improve this characteristic in combination with other probiotic microorganisms.

An important aspect of the function of probiotic bacteria is the protection of the host gastrointestinal microenvironment from invading pathogens. It is generally believed that the resident gastrointestinal microflora in vivo provides protection for the host against possible colonisation by pathogenic bacteria [33]. Several reports have been documented on the ability of probiotic lactobacilli and bifidobacteria to inhibit the cell association and invasion by

Table 3 Total vancomycin and gentamicin-resistant *Lactobacillus* count in the faeces of ten healthy volunteers fed *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502-containing food products and recovery of the strains identified by RAPD

Day of sampling[a]	Subject	Lactobacillus spp. (CFU/g)	No. of *L. rhamnosus* IMC501 colonies/no. analysed	No. of *L. paracasei* IMC502 colonies/no. analysed
0	A	3.2×10^5	0/10	0/10
	B	1.4×10^3	0/10	0/10
	C	2.5×10^4	0/20	0/20
	D	1.7×10^5	0/20	0/20
	E	3.9×10^4	0/15	0/15
	F	1.2×10^3	0/10	0/10
	G	6.7×10^3	0/18	0/18
	H	1.5×10^4	0/10	0/10
	I	2.1×10^5	0/20	0/20
	L	1.9×10^5	0/10	0/10
90	A	1.7×10^7	10/10	0/10
	B	1.2×10^5	0/20	16/20
	C	3.1×10^6	0/15	11/15
	D	5.7×10^5	2/10	0/10
	E	2.0×10^5	7/18	7/18
	F	7.0×10^4	3/10	5/10
	G	1.1×10^5	6/10	0/10
	H	2.3×10^6	14/20	2/20
	I	4.6×10^5	0/10	8/10
	L	1.3×10^5	2/10	5/10
104	A	1.04×10^6	14/15	0/15
	B	1.30×10^3	0/10	6/10
	C	2.81×10^5	0/10	6/10
	D	1.30×10^4	0/10	0/10
	E	1.29×10^4	6/20	12/20
	F	2.20×10^3	1/10	3/10
	G	6.90×10^4	10/20	0/20
	H	1.73×10^5	14/20	0/20
	I	5.45×10^5	0/10	6/10
	L	3.31×10^4	3/10	5/10

Day 0 before consumption, *day 90* end of consumption, *day 104* 2 weeks after end of consumption

pathogenic bacteria [12, 22]. Lactic acid bacteria have been shown to inhibit the in vitro growth of many enteric pathogens and have been used in both human and animals to treat gastrointestinal disorders [34]. *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 have been shown in vitro to strongly inhibit some of the usual potentially harmful microorganisms and also particularly evident was the effect against *C. albicans*. For this reason, the two bacterial strains could be considered promising also in improving the response to *Candida* infections. Further research will investigate the nature of the antimicrobial agents produced by the bacteria.

The two bacterial strains were tested for antibiotic susceptibilities and for the presence of plasmids to exclude the possibility that they may carry potentially transmissible plasmid-encoded antibiotic resistance genes, as shown for example in some *Lactobacillus* strains [2, 19, 39]. It is important to underline that the antibiotic resistance of some probiotic strains could be beneficial for people with an unbalanced intestinal microflora due to the administration of various antimicrobial agents [36]. At the same time, the presence of antibiotic resistance plasmids is considered a factor excluding the use of the strain as probiotics [36]. Among antibiotic resistances, vancomycin resistance is of major concern because vancomycin is sometimes the only available antibiotic left, that is effective against strains of microorganisms that have multiple resistance to antibiotics [43]. The antibiotic susceptibility tests indicated that both strains were resistant to vancomycin. The results were as expected as lactobacilli are known to be naturally resistant toward vancomycin [27] and such resistance is usually intrinsic, chromosomally encoded and no transmissible [26].

Moreover, our results indicate the absence of plasmids in both tested strains.

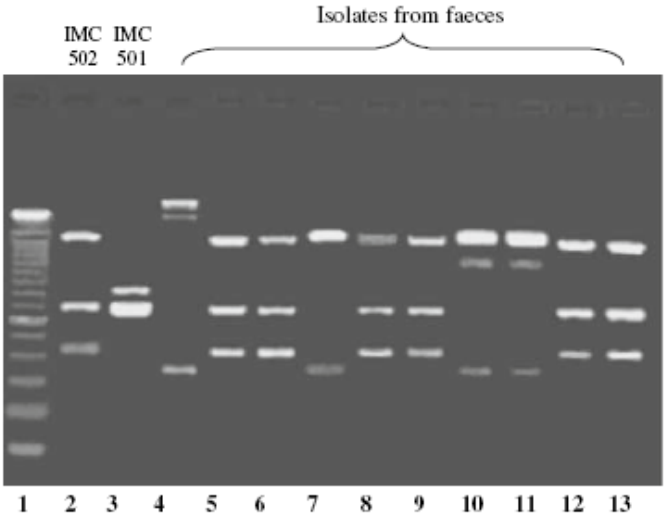

Fig. 4 Detection of *Lactobacillus paracasei* IMC 502 by RAPD with primer M13. *Lane 1* 100-bp DNA ladder; *lane 2* reference strain *L. paracasei* IMC 502; *lane 3* reference strain *L. rhamnosus* IMC 501; *lanes 4–13* strains from faecal samples of subject "C" at day 104; *lanes 5, 6, 8, 9, 12, 13* strain IMC 502; and *lanes 4, 7, 10, 11* other strains

In order to confirm intestinal transit survival in humans, analysis of faecal samples was conducted prior and following probiotic ingestion. The random selection of ten isolates from a given sample could, in theory, miss some of the strains present; however, several studies have shown that ten randomly selected colonies gives good coverage of the dominant strains [25, 41]. The results on recovery of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 from human faeces after different food products intake showed that both strains were recovered from the faecal samples of the volunteers even if in different proportion for each volunteer. This could indicate that the probiotic strains colonisation is host specific and confirm the high adhesion ability of the two bacterial strains and their persistence for 2 weeks after the treatment. However, little is known about the factors governing the colonization ability of probiotic strains in different individuals and the probability of strain interactions. Species specific attachment to the colonic mucosa may play some role in determining the varying persistence of a probiotic in different human hosts [3]. Included in human trial was observation monitoring of potential side effects of probiotic consumption. These included intestinal discomfort, increased flatulence, and changes in stool consistency and frequency. No adverse effects of probiotic administration were observed in the pilot study. The probiotics were well tolerated by all individuals and the fact that no side effects were detected in subjects ranging in age from 24 to 65 suggests that *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 are safe microbial food supplements. Adding weight to this argument is the strain's origin from the intestinal tract of a healthy human. In spite of the human, microflora is strictly related to geography, dietary habits, lifestyle, age, genetic factors, immune system [30]; the strains were from the intestinal tract of healthy humans chosen following experimental protocols corresponding to those quoted by Mueller et al. [30]. Moreover, it is accepted that a healthy, or balanced, flora is one that is predominantly saccharolytic and comprises significant numbers of bifidobacteria and lactobacilli. The exact numbers are difficult to give at present because a proportion of the gut flora has yet to be identified [15].

The present study indicates that *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 possess a number of interesting properties that constitute the basis for their use as health-promoting bacteria.

The two strains have been deposited in the culture collection Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ, Germany), the numbers DSM 16104 and DSM 16105 and they are Italian patent no. RM2004A000166.

We are now carrying on with further investigations on the probiotic activities of the two *Lactobacillus* strains, in particular, the technological characteristics of these microorganisms seem to confirm the actual use of them in the production of novel functional foods.

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