Comparison of the Kinetics of Intestinal Colonization by Associating 5 Probiotic Bacteria Assumed Either in a Microencapsulated or in a Traditional, Uncoated Form

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Background: Beneficial findings concerning probiotics are increasing day by day. However, one of the most important parameters able to significantly affect the probiotic value of a microorganism is its survival during the transit through the stomach and the duodenum. Some techniques may be applied that aim to improve this parameter, but microencapsulation of bacterial cells remains one of the most important. A recent study assessed the kinetics of intestinal colonization by a mixture of 2 probiotic strains, given either in a microencapsulated or in a traditional, uncoated form.

Methods: A comparison between the intestinal colonization by associating 5 microencapsulated bacteria and the same uncoated strains was performed by a double-blind, randomized, cross-over study. The study (December 2007 to January 2009) involved 53 healthy volunteers. In particular, subjects were divided into 2 groups: group A (27 subjects) was given a mix of probiotic strains Probiotical S.p.A. (Novara, Italy), *Lactobacillus acidophilus* LA02 (DSM 21717), *Lactobacillus rhamnosus* LR04 (DSM 16605), *L. rhamnosus* GG, or LGG (ATCC 53103), *L. rhamnosus* LR06 (DSM 21981), and *Bifidobacterium lactis* BS01 (LMG P-21384) in an uncoated form, whereas group B (26 subjects) received the same strains microencapsulated with a gastroprotected material. The uncoated strains were administered at 5×10^9 cfu/strain/d (a total of 25×10^9 cfu/d) for 21 days, whereas the microencapsulated bacteria were given at 1×10^9 cfu/strain/d (a total of 5×10^9 cfu/d) for 21 days. At the end of the first period of supplementation with probiotics, a 3-week wash-out phase was included in the study setting. At the end of the wash-out period, the groups crossed over their treatment regimen; that is, group A was administered the microencapsulated bacteria and group B the uncoated bacteria. The administered quantities of each strain were the same as the first treatment. A quantitative evaluation of intestinal colonization by probiotics, either microencapsulated or uncoated, was undertaken by examining fecal samples at the beginning of the study (time 0), after 10 days and after 21 days of each treatment period. In particular, fecal total *Lactobacilli*, heterofermentative *Lactobacilli*, and total *Bifidobacteria* were quantified at each checkpoint. A genomic analysis of an appropriate number of colonies was performed to quantify individual *L. rhamnosus* strains among heterofermentative *Lactobacilli*.

Results: A statistically significant increase in the fecal amounts of total *Lactobacilli*, heterofermentative *Lactobacilli*, and total *Bifidobacteria* was registered in both groups at the end of each supplementation period compared with d_0 or d_{42} (group A: $P = 0.0002$, $P = 0.0001$, and $P < 0.0001$ at d_{21}, $P = 0.0060$, $P = 0.0069$, and $P < 0.0001$ at d_{63} for total *Lactobacilli*, heterofermentative *Lactobacilli*, and *Bifidobacteria*, respectively; group B: $P = 0.0002$, $P = 0.0006$, and $P < 0.0001$ at d_{21}, $P = 0.0015$, $P = 0.0016$, and $P < 0.0001$ at d_{63} for total *Lactobacilli*, heterofermentative *Lactobacilli*, and *Bifidobacteria*, respectively), confirming the ability of each strain in the administered composition to colonize the human gut, whether supplemented in a gastroprotected or in a traditional freeze-dried form. On the contrary, subjects receiving microencapsulated bacteria reported a kinetics of intestinal colonization that was entirely comparable with those who were given uncoated strains at a 5 times higher amount.

Conclusions: The microencapsulation technique used in this study is a valid approach aimed to significantly improve the survival of strains during gastroduodenal transit, thus enhancing their probiotic value and allowing the use of a 5 times lower amount.

Key Words: probiotic strain, microencapsulation, intestinal colonization, gastroduodenal transit

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The concept of "normal microflora" came about at the end of last century, when both physiologists and microbiologists discovered that, besides pathogenic bacteria, many other nonpathogenic microorganisms normally reside in the human body. The microbiota of the human intestine influences health and well-being.

In light of the apparent impact of intestinal microflora on the health and well-being of the host, different strategies aiming to modulate its composition were defined, namely the control of external factors such as diet or drugs intake, or the oral administration of specific microbial bacteria called "probiotics."

Probiotics are defined as live microorganisms that resist gastric, bile, and pancreatic secretions, attach to epithelial cells, and colonize the human intestine.[1]

Specific probiotic microorganisms of the intestinal microflora, such as *Lactobacilli* and *Bifidobacteria*, have been associated with beneficial effects on the host, including promotion of gut maturation and integrity, antagonism against pathogens, and modulation of the immune system.[2]

Research over the past 20 years has defined implantation in the bowel as the critical feature a strain must possess to influence the intestinal milieu.[3]

The actual mechanism of action of probiotics is subject to constant, increasingly in-depth studies, both with regard to their phenotypic-genotypic characterization and their effectiveness in many different conditions.

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The authors declare that they have nothing to disclose.

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The most extensively studied probiotic applications involve the intestinal tract.[4,5] In lactose intolerant people, the ability of yoghurt and probiotics to improve lactose digestion has been proven, mainly by delaying gastric emptying and the intestinal transit, thus causing slower delivery of lactose to the intestine, optimizing the action of residual β-galactosidase in the small bowel, and decreasing the osmotic load of lactose.[6,7] The bacterial β-galactosidase activity of probiotics is considered to be another important factor responsible for improving lactose digestion.[8]

Several studies have assessed the effectiveness of *Lactobacilli* in the prevention and treatment of antibiotic-associated diarrhea and confirmed the true effectiveness of many strains, especially in the adult population.[9,10] Benefits have also been proven in the prevention of acute childhood diarrhea, the reduction of both the severity and duration of rotavirus-associated acute diarrhea and the reduction in the risk of contracting traveler's diarrhea.[11,12]

Further evidence suggests that the use of probiotics can benefit patients affected by type 2 diabetes mellitus, self-immune diseases, and chronic inflammatory diseases, such as rheumatoid arthritis and bowel inflammatory diseases, and also enhance immunization against flu through increased immunoglobulin (Ig)G and IgA serum levels.[13–15]

A positive impact has also been seen in the treatment of atopic diseases, with a reduction of the SCORAD (SCORing Atopic Dermatitis) in children and adults affected by atopic dermatitis or atopic eczema, asthma, food intolerances, and allergies.[16–22]

More recent efficacy trials have shown the potential beneficial effect of probiotics on the respiratory system, specifically with regard to the prevention and reduction of the duration and severity of acute respiratory infections with a concomitant increase in IgA-secreting cells in the bronchial mucosa.[23,24] It has been reported that probiotics can also reduce the incidence and severity of respiratory infections in children.[25]

To be effective, any probiotic strain has to pass through the gastroduodenal tract before arriving at the gut, and an unavoidable reduction in the number of viable cells occurs. To be effective and confer health benefits to the host, probiotics must be able to reach the gut in a sufficient quantity to impact the bowel microenvironment. This means that they must tolerate the acidic and protease-rich conditions of the stomach and survive and grow in the presence of bile acids, at least to a certain extent. This feature is strongly strain dependent, although it could be assumed that on average 10% to 25% of the intaken cells are able to reach the gut, thus exerting their probiotic activities.[26]

The gastric juice is generally the worst barrier for probiotics, whereas bile salts and pancreatic secretion together are responsible for no more than 35% to 40% mortality of the cells coming from the stomach.

Some in vitro studies could be performed with real human juice or with specific simulations of gastric juice, bile salts, and pancreatic secretion. These in vitro evaluations could represent a reliable prediction of the number of cells that could be delivered to the human gut after oral intake.[26]

In a recent study by Del Piano et al,[27] 7 *Lactobacillus plantarum* probiotic strains were tested for resistance to both simulated gastric juice and human gastric juice taken in an empty stomach from healthy individuals. It was found that <20% of the bacteria survived after an hour of exposure to simulated gastric juice, whereas human gastric juice allowed a survival rate of between 15% and 45%.

Another recent study by Del Piano et al[28] demonstrated that many probiotic strains are clearly less sensitive to human bile than to bovine bile, whereas sensitivity of probiotics to real human or simulated pancreatic secretion is very comparable.[29]

Even though for most strains the quantity of viable cells that are able to survive through the stomach and the duodenum is sufficient to ensure a probiotic effect, there are some specific strategies that can significantly improve the effectiveness of probiotic microorganisms.

The microencapsulation of bacteria with a gastroprotected material could be applied to anticipate and amplify the onset of the beneficial effects. Microencapsulation is the process by which small particles or droplets are surrounded by a coating to produce capsules in the micrometer to millimeter range known as microcapsules.[30] The concept of microencapsulation allows the functional core ingredient (in this case the probiotic cell) to be separated from its environment by a protective coating. Separation of the functional core ingredient from its environment continues until the release of the functional ingredient is desired (after duodenum for probiotics).[31]

A very recent study by Del Piano and colleagues[32,33] compared the kinetics of intestinal colonization by associating the 2 probiotic strains *L. plantarum* LP01 (LMG P-21021) and *Bifidobacterium breve* BR03 (DSM 16604), which demonstrated a 5 times higher efficacy of these bacteria when administered in a microencapsulated, gastroprotected form.

In light of such recent findings, the aim of this study was to investigate the ability of another 5 probiotic strains, namely *L. acidophilus* LA02 (DSM 21717), *L. rhamnosus* LR04 (DSM 16605), *L. rhamnosus* GG, or LGG (ATCC 53103), *L. rhamnosus* LR06 (DSM 21981), and *B. lactis* BS01 (LMG P-21384) to colonize the human gut when given in a microencapsulated form in comparison with uncoated bacteria given at a 5 times higher daily concentration.

MATERIALS AND METHODS

Study Design

A total of 53 healthy volunteers (29 males, 24 females) between 32 and 61 years of age were enrolled between December 2007 and January 2009.

The criteria for study participation included: age between 18 and 65, no known health problems at the time of enrollment, no medical conditions requiring antibiotic treatments, and willingness to comply with the study protocol and provide informed consent.

The subjects were also selected according to a few basic exclusion criteria: age younger than 18 years, ongoing pregnancy or breast-feeding, severe chronic degenerative diseases, severe cognitive deficits, previous abdominal surgery, diverticulitis, immunodeficiency states, concomitant organic bowel disease, antibiotic treatment.

After informed consent was obtained, each volunteer was provided with 21 sachets containing the probiotics, which was a sufficient quantity for the first treatment period. This was a double-blind, randomized, cross-over study.

The consumption of fermented dairy products containing viable *Bifidobacteria* or *Lactobacilli* was prohibited during the entire study to avoid a rise in fecal bacterial counts because of external sources.[34]

In addition to the probiotic strains, each volunteer received sterile plastic containers and instructions for feces collection, storage, and delivery to the laboratory for analysis.

Volunteers were then randomly divided into 2 groups (A and B). Group A was given 21 sachets containing 50 mg each of the 5 probiotic strains *L. acidophilus* LA02 (DSM 21717), *L. rhamnosus* LR04 (DSM 16605), *L. rhamnosus* GG, or LGG (ATCC 53103), *L. rhamnosus* LR06 (DSM 21981), and *B. lactis* BS01 (LMG P-21384) in an uncoated form (corresponding to 5×10^9 cfu/strain/sachet, for a total of 25×10^9 cfu/sachet) and 2.25 g of potato maltodextrin. Group B received 21 sachets containing 10 mg each of the same 5 microorganisms microencapsulated with a gastroprotected coating material (corresponding to 1×10^9 cfu/strain/sachet, for a total of 5×10^9 cfu/sachet) and 2.45 g of potato maltodextrin.

Volunteers were directed to consume 1 sachet every day in the morning in an empty stomach after dissolution in a glass of cold water.

The sachets containing the 2 different active formulations were identical in appearance. They could only be distinguished from one another by a unique randomization code on each sachet.

At the end of the first treatment period of 21 days, a 3-week wash-out phase was included in the protocol. After the wash-out period, the groups crossed over their treatment regimen: each subject belonging to group A received 21 sachets containing 10 mg each of microencapsulated *L. acidophilus* LA02, *L. rhamnosus* LR04, *L. rhamnosus* GG (LGG), *L. rhamnosus* LR06, and *B. lactis* BS01 (corresponding to 1×10^9 cfu/strain/sachet, for a total of 5×10^9 cfu/sachet), whereas group B was given the same strains in an uncoated form (5×10^9 cfu/strain/sachet, for a total of 25×10^9 cfu/sachet).

Volunteers were directed to consume 1 sachet every day in the morning in an empty stomach after dissolution in a glass of cold water, the same recommendation as during the first probiotic supplementation period.

Probiotic strains used in this study, either microencapsulated or uncoated, were manufactured and provided by Probiotical. The microencapsulation of bacteria was performed in accordance with an exclusive, internationally patented technology.

Collection of Fecal Specimens

The feces were collected before treatment with probiotics (d_0), after 10 days of the first treatment (d_{10}), at the end of the first supplementation period (d_{21}), at the end of the 3-week wash-out period (d_{42}), after 10 days of the second treatment (d_{52}), and at the end of the second supplementation period (d_{63}).

Fecal samples for evaluating specific parameters of the microflora (about 10 g) were collected by volunteers in sterile plastic containers previously filled with 20 mL of Amies transport liquid (BD Italia; Milan, Italy), stored at 4°C in the volunteer's home and delivered to the laboratory within 24 hours after collection.

TABLE 1. Quantification of Fecal Total *Lactobacilli*, Heterofermentative *Lactobacilli*, and Total *Bifidobacteria* (Mean ± SEM, log10 cfu/g) Before and After the 2 Treatment Periods, Including the Wash-Out Phase: Comparison Between Time 0 (d_0), or d_{42}, and the Following Analysis Within Each Group

	Group A		Group B	
Time	**log cfu/g**	***P*†**	**log cfu/g**	***P*†**
d_0				
Total *Lactobacilli*	6.80 ± 0.17	*	6.78 ± 0.17	*
Heterofermentative *Lactobacilli*	6.73 ± 0.22		6.61 ± 0.21	
Total *Bifidobacteria*	8.85 ± 0.20	*	8.94 ± 0.16	*
d_{10}				
Total *Lactobacilli*	7.57 ± 0.19	0.0090	7.62 ± 0.17	0.0021
Heterofermentative *Lactobacilli*	7.39 ± 0.19	0.0028	7.53 ± 0.19	0.0034
Total *Bifidobacteria*	9.62 ± 0.14	0.0018	9.61 ± 0.16	0.0058
d_{21}				
Total *Lactobacilli*	7.85 ± 0.12	0.0002	7.82 ± 0.14	0.0002
Heterofermentative *Lactobacilli*	7.70 ± 0.12	0.0001	7.71 ± 0.15	0.0006
Total *Bifidobacteria*	9.69 ± 0.12	< 0.0001	9.81 ± 0.11	< 0.0001
d_{42}				
Total *Lactobacilli*	6.75 ± 0.17	*	6.98 ± 0.16	*
Heterofermentative *Lactobacilli*	6.70 ± 0.22		6.89 ± 0.21	
Total *Bifidobacteria*	8.89 ± 0.14	*	9.02 ± 0.14	*
d_{52}				
Total *Lactobacilli*	7.62 ± 0.20	0.0103	7.67 ± 0.21	0.0047
Heterofermentative *Lactobacilli*	7.38 ± 0.19	0.0034	7.51 ± 0.22	0.0069
Total *Bifidobacteria*	9.70 ± 0.14	0.0028	9.63 ± 0.15	0.0034
d_{63}				
Total *Lactobacilli*	7.99 ± 0.13	0.0060	7.91 ± 0.17	0.0015
Heterofermentative *Lactobacilli*	7.83 ± 0.13	0.0069	7.78 ± 0.19	0.0016
Total *Bifidobacteria*	9.79 ± 0.11	< 0.0001	9.85 ± 0.10	< 0.0001

*Comparison reference time (d_0 for the first treatment period and d_{42} for the second one).
†Comparison between time 0 (d_0), or d_{42}, and the following analysis within each group.
cfu indicates colony forming units.

TABLE 2. Quantification of Fecal Total *Lactobacilli*, Heterofermentative *Lactobacilli*, and Total *Bifidobacteria* (Mean ± SEM, log10 cfu/g) Before and After the 2 Treatment Periods, Including the Wash-Out Phase: Comparison Between the 2 Groups at d_0 and Following Analysis

	log cfu/g		
Time	Group A	Group B	*P* (A vs. B)*
d_0			
Total *Lactobacilli*	6.80 ± 0.17	6.78 ± 0.17	0.9063
Heterofermentative *Lactobacilli*	6.73 ± 0.22	6.61 ± 0.21	0.6589
Total *Bifidobacteria*	8.85 ± 0.20	8.94 ± 0.16	0.5158
d_{10}			
Total *Lactobacilli*	7.57 ± 0.19	7.62 ± 0.17	0.8121
Heterofermentative *Lactobacilli*	7.39 ± 0.19	7.53 ± 0.19	0.4535
Total *Bifidobacteria*	9.62 ± 0.14	9.61 ± 0.16	0.9394
d_{21}			
Total *Lactobacilli*	7.85 ± 0.12	7.82 ± 0.14	0.8502
Heterofermentative *Lactobacilli*	7.70 ± 0.12	7.71 ± 0.15	0.9383
Total *Bifidobacteria*	9.69 ± 0.12	9.81 ± 0.11	0.2673
d_{42}			
Total *Lactobacilli*	6.75 ± 0.17	6.98 ± 0.16	0.2133
Heterofermentative *Lactobacilli*	6.70 ± 0.22	6.89 ± 0.21	0.3195
Total *Bifidobacteria*	8.89 ± 0.14	9.02 ± 0.14	0.3004
d_{52}			
Total *Lactobacilli*	7.62 ± 0.20	7.67 ± 0.21	0.7843
Heterofermentative *Lactobacilli*	7.38 ± 0.19	7.51 ± 0.22	0.4906
Total *Bifidobacteria*	9.70 ± 0.14	9.63 ± 0.15	0.6589
d_{63}			
Total *Lactobacilli*	7.99 ± 0.13	7.91 ± 0.17	0.6617
Heterofermentative *Lactobacilli*	7.83 ± 0.13	7.78 ± 0.19	0.8045
Total *Bifidobacteria*	9.79 ± 0.11	9.85 ± 0.10	0.5607

*Comparison between the 2 groups at d_0 and following analysis. cfu indicates colony forming units.

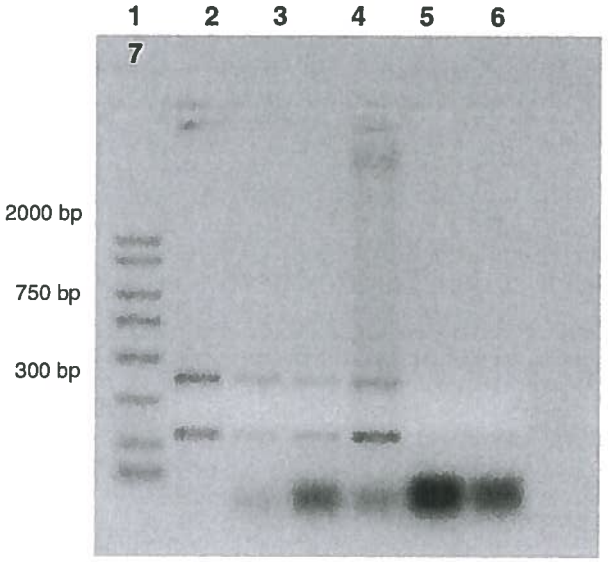

FIGURE 1. Species-specific polymerase chain reaction (PCR) of the 3 *Lactobacillus rhamnosus* strains. From the left: Lane 1, PCR Marker (Sigma 50 to 2000 bp); Lane 2, positive reference: *L. rhamnosus* DSM 20021; Lane 3, *L. rhamnosus* GG; Lane 4, *L. rhamnosus* LR04; Lane 5, *L. rhamnosus* LR06; Lane 6, negative reference: *L. casei* DSM 20011; Lane 7, experimental blank.

Evaluation of Fecal *Lactobacilli* and *Bifidobacteria*

Fecal samples were processed as soon as they were received, and in any event within 24 hours after collection. The weighed samples (about 30 grams) were transferred to a sterile container (Stobag), diluted with Amies liquid to achieve 1:10 wt/vol and homogenized in a Stomacher. Samples were then decimally diluted using a sterile saline, and 1 mL of the appropriate dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} for total *Lactobacilli* and heterofermentative *Lactobacilli*, and 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} for total *Bifidobacteria*) was plated on selective culture agarized media. In particular, for total *Lactobacillus* spp. Rogosa acetate agar medium (Oxoid; Milan, Italy) and for heterofermentative (vancomycin insensitive) Lactobacilli Rogosa acetate agar medium (Oxoid) added with 12 µg/mL of vancomycin (Sigma-Aldrich; Milan, Italy) were used,[35] whereas the selective count of total *Bifidobacteria* was performed using TOS propionate agar medium added with 50 µg/mL of mupirocin and 2 mg/mL of lithium chloride (Sigma-Aldrich).[36–38] Plates were incubated for 48 to 72 hours at 37°C under anaerobic conditions (Gas Pak system) with Anaerocult A (Merck; Darmstadt, Germany). The colonies were counted and the results expressed as $\log_{10}$ of colony forming units (cfu) per gram of fresh feces. The medium used for vancomycin-insensitive *Lactobacilli* was able to count the 3 *L. rhamnosus* strains of the association, whereas the medium used for total *Lactobacillus* spp. was able to enumerate all the 4 *Lactobacillus* strains.

Quantification of total *Lactobacilli*, heterofermentative *Lactobacilli*, and total *Bifidobacteria* in the fecal specimens was undertaken at Biolab Research Laboratory of the Mofin Alce Group.

Genetic Identification and Quantification of Individual *L. rhamnosus* Strains

A certain number of colonies from appropriate dilutions plated on Rogosa acetate agar medium added with vancomycin (vancomycin-insensitive *Lactobacilli*) were selected and inoculated into 10 mL of Rogosa Acetate broth added with 0.05% cysteine before overnight incubation at 37°C. The broth cultures were then centrifuged at 13,000 rpm for 10 minutes after which the supernatant was removed and the remaining pellet was subjected to total genomic DNA extraction using the Wizard Genomic DNA Purification Kit (Promega; Madison), according to the manufacturer's instructions.[39] Species-specific polymerase chain reaction (PCR) assay using primers RHA/PR1[40,41] was then performed on genomic DNA to identify *L. rhamnosus* species among the vancomycin-insensitive *Lactobacillus* colonies. Pulsed field gel electrophoresis was performed according to the protocol described by Tynkkynen et al[42] using the Not I restriction enzyme with the purpose of enumerating colonies of individual *L. rhamnosus* strains (LR04, LGG, and LR06) within the total number of colonies of the species *L. rhamnosus*, as assessed by species-specific PCR assay.

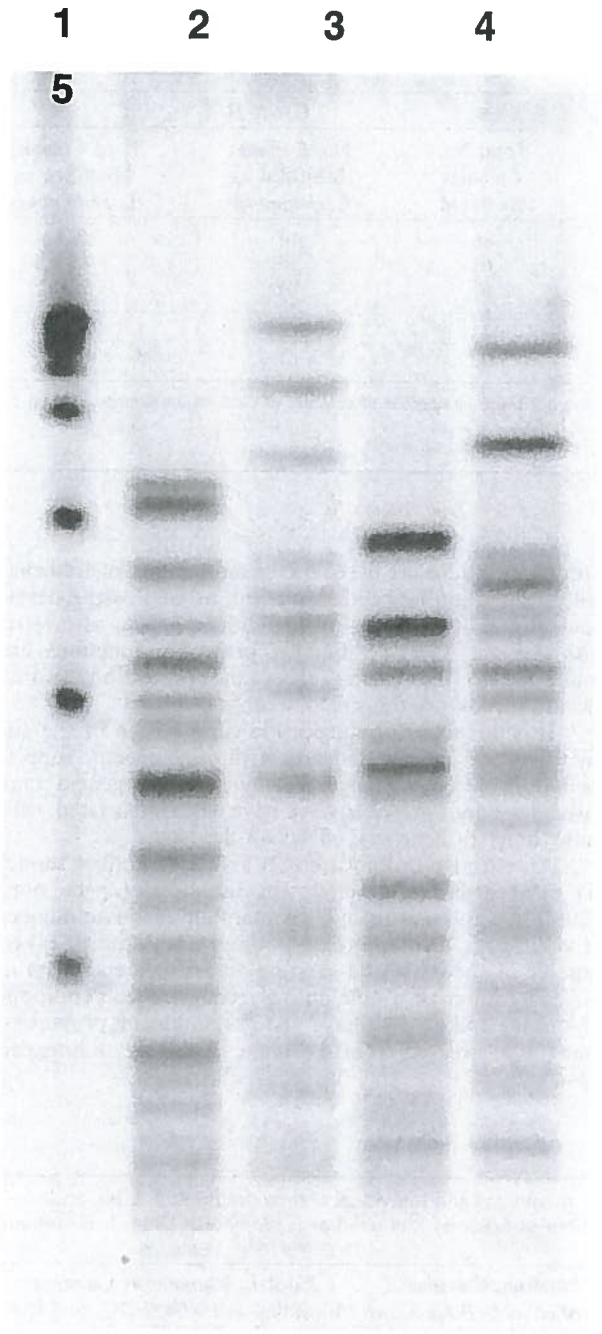

FIGURE 2. Pulsed field gel electrophoresis of the 3 *Lactobacillus rhamnosus* strains using Not I restriction enzyme. From the left: Lane 1, electrophoretic marker (Sigma 50 to 1000 bp); Lane 2, *L. rhamnosus* GG; Lane 3, *L. rhamnosus* LR06; Lane 4, *L. rhamnosus* LR04; Lane 5, positive reference: *L. rhamnosus* commercial strain.

Statistical Analysis

All values relative to the concentrations of fecal *Lactobacilli* and *Bifidobacteria* are expressed as mean ± SEM. Parallel quantification of fecal total *Lactobacilli*, heterofermentative *Lactobacilli*, and *Bifidobacteria* was performed at time 0 and after 10, 21, 42, 52, and 63 days, in accordance with the study protocol. Paired and unpaired 2-tailed *t* test statistical analysis was used to compare the results. *P* values were calculated for the comparison between the number of total *Lactobacillus* spp., heterofermentative *Lactobacilli* and *Bifidobacteria* concentrations in the 2 groups at d_0, d_{10}, d_{21}, d_{42}, d_{52}, and d_{63} (unpaired *t* test). *P* values were also calculated for each parameter at d_{10} and d_{21} compared with d_0, as well as at d_{52} and d_{63} compared with d_{42} within each active group to quantify intestinal colonization by the ingested strains (paired *t* test). Differences were considered significant at $P \leq 0.05$.

RESULTS

No statistically significant differences were recorded at the beginning of treatment in the 2 active groups with regard to fecal total *Lactobacilli*, heterofermentative *Lactobacilli*, and *Bifidobacteria* (Table 2). This confirmed that the enrolled subjects were homogeneously distributed into the groups, which is essential to assess the intestinal colonization by the administered probiotic strains.

No significant adverse events were reported and only 3 drop outs were registered (1 in group A, 2 in group B) during the second treatment period.

Statistical significance increases in the fecal concentrations of total and heterofermentative *Lactobacilli* and total *Bifidobacteria* were recorded in both groups and with both types of probiotics administered (group A: $P = 0.0002$, $P = 0.0001$, and $P < 0.0001$ at d_{21}, $P = 0.0060$, $P = 0.0069$, and $P < 0.0001$ at d_{63} for total *Lactobacilli*, heterofermentative *Lactobacilli*, and *Bifidobacteria*, respectively; group B: $P = 0.0002$, $P = 0.0006$, and $P < 0.0001$ at d_{21}, $P = 0.0015$, $P = 0.0016$, and $P < 0.0001$ at d_{63} for total *Lactobacilli*, heterofermentative *Lactobacilli*, and *Bifidobacteria*, respectively), confirming the ability of the 5 bacteria to colonize the human gut, either in a microencapsulated form or an uncoated form (Table 1). After 21 days of wash-out, the fecal concentrations of the 2 bacterial genera were quite similar to those recorded at time 0, even if slightly higher especially for *Bifidobacterium* spp., but not to a significant extent.

A comparison between the 2 kinetics of colonization suggests that the fifth part of microencapsulated bacteria has an ability to colonize the gut that is entirely comparable with uncoated strains, as the statistical significance is similar (Table 2). This supports the evidence that the gastroprotection mediated by the microencapsulation material used is really effective in increasing the probiotic efficacy of strains. In this case, a 5 times lower number of viable protected cells has an effect which is comparable with 5 billion/strain/d of uncoated bacteria.

The results from genotypic analysis, namely species-specific PCR and strain identification by pulsed field gel electrophoresis, are reported in Figures 1, 2 and also in Tables 3 and 4.

The genotypic analysis performed highlighted, on the one hand, a significant increase in *L. rhamnosus* species inside the heterofermentative *Lactobacillus* spp. during and at the end of both treatments and, on the other hand, a substantially equivalent presence of the 3 *L. rhamnosus* strains administered. Not all colonies previously identified as *L. rhamnosus* proved to be one of the 3 LR04, LGG, or LR06 strains, but the percentage was >90% during both treatments, whereas after the wash-out phase the 3 strains substantially decreased but did not disappear completely,

TABLE 3. Species-specific PCR Assay and PFGE Identification of Total *L. rhamnosus* and Individual *L. rhamnosus* LR04, LGG, and *L. rhamnosus* LR06 Among the Vancomycin-insensitive *Lactobacilli*: Number and Percentage of Colonies Identified as Belonging to *L. rhamnosus* species Among the Vancomycin-insensitive *Lactobacilli*

	Group A			Group B		
Time	Total No. Colonies Analyzed	No. Colonies Identified as *L. rhamnosus*	% of Colonies Identified as *L. rhamnosus*	Total No. Colonies Analyzed	No. Colonies Identified as *L. rhamnosus*	% of Colonies Identified as *L. rhamnosus*
d_0	66	24	36.4	66	19	28.8
d_{10}	62	51	82.3	63	56	88.9
d_{21}	65	61	93.8	66	61	92.4
d_{42}	67	22	32.8	68	20	29.4
d_{52}	63	55	87.3	66	60	90.9
d_{63}	67	63	94.0	72	67	93.1

Values are expressed as positive identification for *L. rhamnosus*/no. total cfu analyzed and as number of colonies of each strain/number of total *L. rhamnosus* cfu.

cfu indicates colony forming units; PCR, polymerase chain reaction; PFGE, pulsed field gel electrophoresis.

especially in group B, whose subjects received micro-encapsulated strains during the first period.

The difference between total *Lactobacilli* and hetero-fermentative *Lactobacilli* provided a reliable quantification of *L. acidophilus* LA02 in human feces. This amount was between 20% and 30% of total *Lactobacilli*, thus confirming the theoretical relative ratio among all 4 *Lactobacillus* bacteria.

DISCUSSION

Intestinal microflora is a well-recognized crucial factor for the health of the host as it has metabolic, trophic, and protective functions, and can be modulated by the exogenous administration of probiotics.[4]

Metabolic functions are primarily characterized by fermentation of nondigestible dietary fibers and molecules as well as endogenous mucus, production of short-chain fatty acids, different group B vitamins such as folates, riboflavin, and cobalamin, vitamin K, conversion of linoleic acid into conjugated linoleic acids, and absorption of ions. Trophic functions are based on the modulation of epithelial cell proliferation and differentiation, as well as the development and homeostasis of the immune system associated with the gut (GALT). Finally, protective functions are connected to the barrier effect and protection against harmful or even pathogenic microbes.

It is possible to influence the composition of the gut microbiota in infants and adults through dietary supplementation. Microorganisms belonging to the genera *Lactobacillus* and *Bifidobacterium* have been associated with many different beneficial effects on the host.

An effective probiotic strain and end product should (1) exert a beneficial effect on the host; (2) be non-pathogenic and nontoxic; (3) contain an adequate number of viable cells, also according to the efficacy studies; (4) be capable of surviving and exerting an active metabolism in the gut; (5) remain viable during recommended conditions of storage and use; (6) have good organoleptic properties; and (7) be isolated from the same species as its intended host.[4,26]

TABLE 4. Species-specific PCR Assay and PFGE Identification of Total *L. rhamnosus* and Individual *L. rhamnosus* LR04, LGG, and *L. rhamnosus* LR06 Among the Vancomycin-insensitive *Lactobacilli*: Number of Colonies Identified as *L. rhamnosus* LR04, *L. rhamnosus* GG, and *L. rhamnosus* LR06 Among the Total *L. rhamnosus* Colonies

Time	No. Colonies Identified as LR04	No. Colonies Identified as LGG	No. Colonies Identified as LR06	Total no. Colonies Identified as *L. Rhamnosus*	% of *L. Rhamnosus* Colonies Identified as LR04, LGG, or LR06
Group A					
d_{10}	14	18	15	51	92.2
d_{21}	20	19	19	61	95.1
d_{42}	1	2	1	22	18.2
d_{52}	17	17	17	55	92.7
d_{63}	20	19	22	63	96.8
Group B					
d_{10}	16	17	19	56	92.9
d_{21}	20	18	21	61	96.7
d_{42}	1	4	2	20	35.0
d_{52}	19	18	19	60	93.3
d_{63}	20	21	23	67	95.5

Values are expressed as positive identification for *L. rhamnosus*/no. total cfu analyzed and as number of colonies of each strain/number of total *L. rhamnosus* cfu.

cfu indicates colony forming units; PCR, polymerase chain reaction; PFGE, pulsed field gel electrophoresis.

However, one of the most important parameters that impacts the probiotic activity of a microrganism is its survival during gastroduodenal transit. To be effective and confer health benefits to the host, in fact, probiotics must be able to survive the passage through the stomach and upper intestine and be present in a sufficient quantity to influence the gut microenvironment.

It is generally accepted that between 10% and 25% of the ingested cells is delivered to the jejunum and the ileum, thus representing the real probiotic fraction. In any case, there are some relevant differences among strains even belonging to the same species, and some microrganisms suffer the gastroduodenal transit to a very high extent.

Many strategies have been developed to improve the number of viable bacteria delivered to the gut. A direct microencapsulation of probiotic cells with a gastroprotected material, for example, some special lipids or polisaccharides, proved to be very effective in this regard, as also confirmed by our group in a previous study involving the 2 probiotic strains *L. plantarum* LP01 (LMG P-21021) and *B. breve* BR03 (DSM 16604).[32,33]

This study, involving 5 different bacteria, further strenghtened the opportunity of using a lower number of certain strains if delivered in a special microencapsulated form able to confer a strong gastroprotection to the cells. For each single strain of the association tested, a 5:1 ratio was confirmed through microbiological and genomic analysis on fecal samples.

Additional evaluations may be needed in the future to assess the behavior of probiotic bacteria other than those used, even if the current results could be regarded as general evidence of strains with a 10% to 20% overall survival rate of the gastroduodenal transit when administered in an uncoated form.

In conclusion, this study confirms again that the microencapsulation of bacteria with the special gastroprotected coating utilized in this work is really effective in enhancing their probiotic efficacy, thus allowing the use of 5 times lower quantities of viable cells compared with the quantities positively tested during previous human clinical studies.[32,33]

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