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ORIGINAL ARTICLE

Influence of a combination of two potential probiotic strains, *Lactobacillus rhamnosus* IMC 501® and *Lactobacillus paracasei* IMC 502® on bowel habits of healthy adults

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Abstract

Aims: This study aims to investigate the effect of different kinds of food products enriched with a combination of two potential probiotic strains, *Lactobacillus rhamnosus* IMC 501® and *Lactobacillus paracasei* IMC 502®, on bowel habits of healthy adults.

Methods and Results: Fifty healthy volunteers took part in a double-blind placebo probiotic feeding study (25 fed probiotics, 25 fed placebo) for 12 weeks. Each volunteer ingested daily one or more food products enriched with a combination of the two potential probiotic strains (probiotic group) or the same food products without the probiotics (control group). Faecal samples were collected before, at the end and 2 weeks later the intervention period, and some of the main groups of faecal bacteria were enumerated by plate count and real-time PCR. Questionnaires on bowel habits were submitted to volunteers. After the intervention, a significant increase in faecal lactobacilli and bifidobacteria were observed in the probiotic group, and stool frequency and stool volume were higher in the probiotic group than in the placebo group.

Conclusions: Daily consumption of food products enriched with the two potential probiotic strains, *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502®, contributes to improve intestinal microbiota with beneficial properties and enhances bowel habits of healthy adults.

Significance and Impact of the Study: The study revealed that *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502® exert a positive effect, in terms of improved bowel habits, on healthy adults.

Introduction

The condition and function of the gastrointestinal (GI) tract are essential to our well-being that's why the biological and clinical importance of bacteria resident in the gut is becoming increasingly recognized. The intestinal microbiota is significantly related to the health and diseases of the host. The beneficial effects of gut microbiota are guarantees when friendly and potentially pathogenic micro-organisms are balanced. Indeed, several studies have shown that shifts in the composition of commensal micro-organisms are implicated in the aetiology of some human diseases and in a reduced resistance towards infections (Ouwehand *et al.* 2001; Sullivan *et al.* 2001; Verdu and Collins 2004; Possiemers *et al.* 2009). Considering these facts, it is quite useful to improve the intestinal microbiota, that is, to increase the indigenous beneficial bacteria and, by this, decrease harmful bacteria, for both host defence and nutritional benefits. The scientific concept to modulate the human gut microbiota towards a beneficial composition has been developed over the last decade (Collins and Gibson 1999; Isolauri *et al.* 2008). This modulation can be achieved by the use of specific functional foods containing probiotics or prebiotics. In

particular, the enrichment of lactobacilli and bifidobacteria is considered a relevant parameter to evaluate the probiotic effect, because their biological functions in the intestinal ecosystem are well recognized (Servin 2004). Oral administration of probiotics is suggested to have a positive effect on, i.e. the composition of the intestinal microbiota and the colonization resistance against pathogens, as well as to promote beneficial immune responses (Collins and Gibson 1999). In the present work, we carried out an oral administration study of food products enriched with the two potential probiotic strains, *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502® (provided by Synbiotec S.r.l., Camerino, Italy) (Verdenelli *et al.* 2009), on healthy Italian adult volunteers to elucidate the strain-specific effects of the two *Lactobacillus*. In particular, the impact of *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502® on some important groups of bacteria present in the faecal samples was determined by both culturable and nonculturable methods; moreover, the effects of the ingestion of the two probiotic strains on bowel functions were assessed.

Materials and Methods

Study subjects

A total of 50 healthy adult human volunteers (27 females and 23 males) with ages ranging from 23 to 65 years were included in the study. The subjects followed their habitual diet during the study. The exclusion criteria were critical illness, inflammatory bowel diseases, lactose intolerance, frequent GI disorders or metabolic diseases. The use of antibiotic was not allowed for 1 month prior to and for the whole duration of the study. The study was approved by the local Ethic Committee, and a written informed consent was obtained from the volunteers.

Study food products

The study products consisted of six different food products (yoghurt, 'ricotta' cheese, 'mozzarella' cheese, chocolate, chocolate mousse and ice cream). The products were enriched from the producers with a mixture of the two potential probiotic strains (1 : 1, about 10^9 CFU per serving), *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502®, during their normal production process, as previously reported (Verdenelli *et al.* 2009). The choice to use a combination of the two bacterial strains is justified from the results obtained by Verdenelli *et al.* (2009) where the mixture expressed higher *in vitro* adherence of the two *Lactobacillus* strains to intestinal cell line. During the intervention period, the subjects were instructed to consume during the day one or more food portions of *c.* approximately 80–120 g. The use of test products was recorded daily, and the records were checked by the investigator at each visit. Volunteer compliance was determined by verbal assessment by the investigator. The amount of product returned was recorded to confirm compliance. The subjects were free to consume their favourite foods among those provided, at least one probiotic-enriched product per day.

The stability of the products was monitored up to its expiry date, and no significant changes in viable numbers were detected (results not shown). The control products consisted of the same food products without the probiotic enrichment.

Study design

Volunteers were randomly distributed into two groups: control group (25 subjects) and probiotic group (25 subjects). Both study subjects and investigators were blinded to the nature of the products. The study was performed in a parallel manner, with a 4-week run-in period followed by a 12-week intervention period and finished with a 2-week wash-out period. During the intervention period, the subjects consumed one or more potential probiotic food products daily. Faecal samples were collected at the end of each study period. All faecal samples were anaerobically stored using AnaeroGen sachet (Oxoid Unipath Ltd., Basingstoke, Hampshire, England) at 4°C, and a faecal microbiota analysis was performed within 24 h, using the standard plate count technique. *Lactobacillus* and *Bifidobacterium* have been also quantified by real-time PCR.

Microbial analysis

A sample of faeces of *c.* approximately 1 g (wet weight) was immediately placed in an anaerobic cabinet (Concept 400; Ruskin Technology Limited, Leeds, West Yorkshire, UK), suspended in 9 ml reducing solution (Holdeman *et al.* 1977) and homogenized with Stomacher Lab Blender Model 80-BA 7020 (Seward Medical, London, UK). One millilitre of the faecal homogenate was suspended in 9 ml of reducing solution, and a series of 10-fold dilution (10^{-1}–10^{-10}) was prepared. A given amount of each dilution (100 μl) was spread onto a nonselective medium, Columbia +5% sheep blood Agar (bioMérieux, Marcy l'Etoile, France) for both aerobic and anaerobic total bacteria counts, and onto five selective media, Rogosa agar (Oxoid) for *Lactobacillus* counts, Raffinose Bifidobacterium medium (Hartemink *et al.* 1996) agar for *Bifidobacterium* counts, Sulphite Polimixin Sulphadiazine agar (Oxoid) for *Clostridium* counts, Brain Heart Infusion agar (Oxoid) supplemented with vancomycin (0·75 mg ml^{-1})

and kanamycin (10 mg ml^{-1}) (bioMérieux) for *Bacteroides* counts, MacConkey agar (Oxoid) for *Enterobacteriaceae* counts. The nonselective inoculated media were incubated aerobically and anaerobically at 37°C for 72 h, and the selective inoculated media were incubated anaerobically under the same conditions.

To verify the presence of the two potential probiotic strains in the faeces of human volunteers before and after oral intake of the probiotic-enriched foods, ten colonies randomly selected from countable MRS (de Man, Rogosa & Sharpe) agar plates were isolated and checked for purity. DNA was extracted using the Qiagen Dneasy Tissue kit (Qiagen, Hilden, Germany) and analysed using the randomly amplified polymorphic DNA (RAPD) technique as previously described (Verdenelli *et al.* 2009).

DNA extraction and quantitative PCR analysis

For the quantitative real-time PCR analyses, DNA extraction was performed on faecal samples using the Ultra-Clean Faecal DNA kit (Mo Bio Laboratories, Inc., Carlsbad, CA 92010, USA) according to the manufacturer's instructions. The purified DNA samples were subjected to quantitative PCR analysis to determine the number of bacteria using a Mx3000P (Stratagene, La Jolla, CA 92037, USA). Amplifications of the 16S rRNA gene were performed in a total volume of 25 μl consisting of Brilliant SYBER Green QPCR Master Mix (Stratagene), 0·2 μmol l^{-1} of each primer and 5 μl of DNA extract. Amplifications were carried out with the following temperature profiles: one cycle at 95°C (3 min), 35 cycles of denaturation at 95°C (30 s), primer annealing (30 s) and primer extension at 72°C for 45 s, with a final extension step at 72°C for 5 min. For bifidobacteria quantification, Bif164F and Bif601R (Langendijk *et al.* 1995) were used as primers and *Bifidobacterium longum* ATCC 15707 was used as standard strain. For lactobacilli quantification, Lac1 (Walter *et al.* 2001) and S-G-Lab-0677-a-A-17 (Heilig *et al.* 2002) were used as primers and *Lactobacillus acidophilus* DSM 20079 was used as standard strain. Primers and reaction conditions are indicated in Table 1. The standard strains were cultivated on selective broth media to the stationary phase, and a 10-fold dilution series of each strain in the same media was prepared. DNA was extracted from 1 ml of these suspensions and used to quantify the number of bacteria in faecal samples. The viable counts of bacterial suspensions were determined by plate counting on selective agar plates. The standard curves representing the correlation between C_t values and initial bacterial concentration (CFU g^{-1}) in standard samples were generated by Stratagene software of Stratagene Mx3000P system. Each faecal sample was run in triplicates. In each run of real-time PCR, at least five serial dilutions of standard DNA samples were included. The concentration of the bacteria genera in faecal samples was calculated by comparing the C_t of the sample with that of the respective standard curve. To determine the specificity of amplification, analysis of samples melting curve was performed after the last cycle of each amplification. Results of real-time PCR were compared with those obtained by plate counts.

Bowel habits

At the end of probiotic administration, volunteers were asked to note their overall bowel habits during the administration period compared to their normal status before intake. This was performed by using a scale ranging from 0 to 10, being five the individual status before the study. The following matters were used to monitor the conditions of the subjects: the intestinal regularity, the volume of stools, the constipation and the flatulence. The volunteers were personally instructed as to how to complete the questionnaire form and were also given written instructions. They had to submit the questionnaire at the time of their scheduled visit 3 months later. Questionnaires were completed at home in the subjects' own time and took 10–15 min on average. The questions were in the multiple-choice format, and individuals were simply required to circle the answer most closely corresponding to their health status. The questionnaire was reviewed with the subject by the investigator, and any unanswered questions or unclear answers were completed and clarified.

Table 1 Primer sets used for real-time PCR amplification

Target bacteria	Primer set	Sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference or source
Bifidobacterium genus	Bif164F Bif601R	GGGTGGTAATGCCGGATG TAAGCGATGGACTTTCACACC	278	59	Langendijk *et al.* 1995
Lactobacillus genus	Lac1 G-Lab-0677-a-A-17	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	341	58	(Walter *et al.* 2001; Heilig *et al.* 2002)

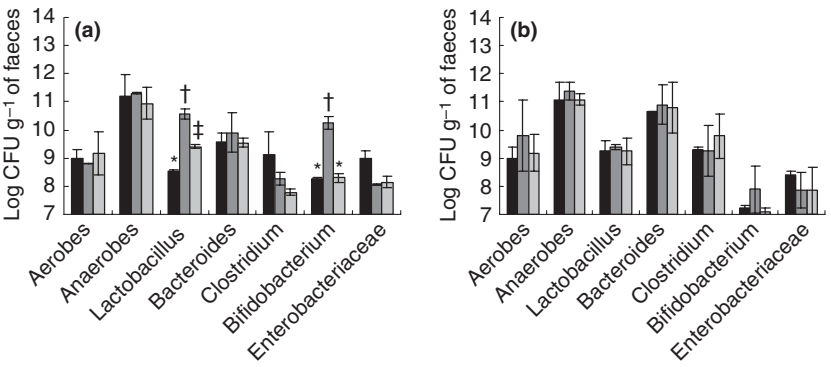

Figure 1 Bacterial counts of the main intestinal bacteria genera during the study period. (■) before the consumption; (▩) after the consumption; (▨) after the washout. (a) Probiotic group. (b) Control group. *†‡Different symbols correspond to significantly different values within bacterial groups, according to the Student's *t*-test ($P < 0.05$).

Statistical analysis

The results of microbiological analysis are expressed as mean values ± standard deviation of at least three independent experiments. A Student *t*-test on log-transformed data was used to determine the significance of differences in bacterial counts between different time points within probiotic and control group and between probiotic and control group at the same time points. The same test was used for the statistical evaluation of differences between the mean values of plate counts and those obtained by real-time PCR. Statistical difference on the bowel habits between probiotic and control group after the intervention study was examined with chi-square test. The difference between means was considered significant at $P < 0{\cdot}05$.

Results

Study subjects

Forty-seven subjects of the fifty recruited completed the study. Two subjects were prescribed antibiotics during the intervention study and were excluded for this reason. Furthermore, one subject was excluded because of the difficult to follow the regular food products administration during the intervention period.

Microbial analysis

The effects of probiotic intervention on the faecal microbiota of probiotic and control group of volunteers are shown in Fig. 1. There were not statistically significant results except for *Lactobacillus* and *Bifidobacterium* genera. There was a significant higher concentration of *Lactobacillus* and *Bifidobacterium* genera during the treatment than before and after the washout. The *Bifidobacterium* levels declined after the washout, whereas the *Lactobacillus* levels, even if lower than those of the intervention period, remained significantly higher then the run-in. A reduction, even if not significant, in the number of clostridia and enterobacteria in probiotic group was also observed. Figure 2 shows the comparison on the faecal microbiota composition between probiotic and control group at the end of both the run-in and the intervention period. Lactobacilli and bifidobacteria were significantly higher in

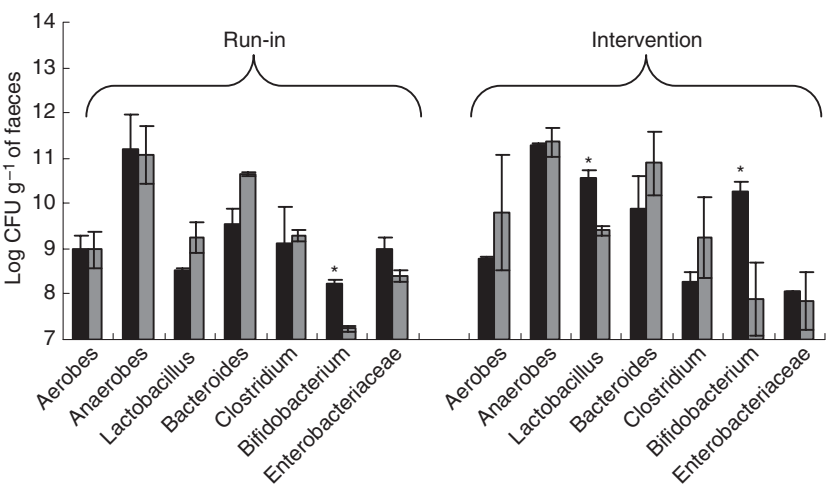

Figure 2 Comparison on faecal microbiota composition between probiotic (■) and control group (▩) after the run-in and the consumption period. *Significantly different from control group, according to the Student's *t*-test ($P < 0.05$).

the probiotic group after intervention as compared to control group. Bifidobacteria were already significantly higher in probiotic group with respect to control group at the end of run-in period. However, this difference was more pronounced after the intervention period with respect to the run-in. The results obtained by the real-time PCR for *Lactobacillus* and *Bifidobacterium* quantification were almost the same as those obtained by the culture method (Table 2).

To confirm whether the administration of the potential probiotic lactobacilli was playing a role in the increase in the Rogosa counts observed in the probiotic group, the RAPD technique was applied to colonies randomly selected from Rogosa plates. Both *Lactobacillus* strains have been detected from faecal samples of the probiotic group (Fig. 3) but not from those of the control group.

Table 2 Comparison of *Lactobacillus* and *Bifidobacterium* numbers determined by culture method and real-time quantitative PCR, during the study periods in probiotic group. Bacterial numbers are expressed as log colony-forming units (CFU) per g of faeces ± standard deviation

	Mean Log CFU g^{-1} ± SD			
	Lactobacillus		*Bifidobacterium*	
	Culture method	Real-time PCR quantification	Culture method	Real-time PCR quantification
Before the consumption	8·5 ± 0·1	9·4 ± 0·3	8·2 ± 0·1	8·5 ± 0·7
After the consumption	10·5 ± 0·2	11·1 ± 0·5	10·3 ± 0·2	10·1 ± 0·9
After the wash-out	9·4 ± 0·1	9·8 ± 0·6	8·4 ± 0·2	8·4 ± 1·0

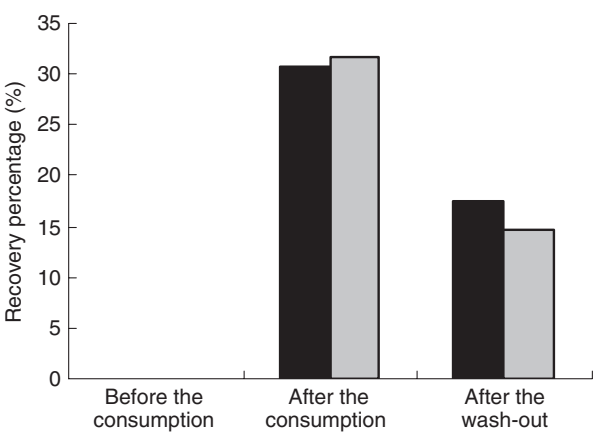

Figure 3 Recovery percentage of *Lact. rhamnosus* IMC 501® (■) and *Lact. paracasei* IMC 502® (■) with respect to the total lactobacilli, in faecal samples of volunteers belonging to probiotic group assessed by randomly amplified polymorphic DNA method.

The two *Lactobacillus* strains were recovered in 25 out of 25 faecal samples, collected at the end of the consumption period. Furthermore, the strains were also still identified in 22 volunteers at the end of washout. The average value of recovery percentages of the two potential probiotic strains (Fig. 3) reached the 31% of the total *Lactobacillus* spp. at the end of the consumption period and remained high (18% for *Lact. rhamnosus* IMC 501® and 15% for *Lact. paracasei* IMC 502®) after the washout with respect to the total *Lactobacillus* spp. recovery.

Bowel habits

Parameters related to the bowel habits of the volunteers were also measured to verify the potential impact of the potential probiotic strains on their bowel functions. The volunteers consuming the probiotic-enriched foods reported a significant increase in intestinal regularity and in faecal volume at the end of the consumption period with respect to the control group (Fig. 4). No significant differences were detected with respect to constipation and flatulence by both groups. The probiotic-enriched foods consumption was very well tolerated, and no side effects were experienced.

Discussion

The present study was inspired by the increasing interest in the potential of probiotic to improve human health even if it has been difficult to establish the existence of associations between specific probiotic bacteria and health benefits. This is even more difficult when the effects of probiotic are studied on healthy people who are the main consumers of functional foods. The consumption of the food products tested in this study, containing the two potential probiotic strains *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502®, determined a significant increase in lactobacilli and bifidobacteria in the probiotic group at the end of the consumption period. A mechanism of action for this effect was not investigated as part of this study, but we would speculate that the increase in bifidobacteria observed may be attributed to the intestinal pH decrease induced by lactic acid or other fermented products produced by *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502®. The probiotics may metabolize luminal components to generate a substrate that may be a preferred fuel source or create a preferred physical environment, such as localized pH, advantageous for the growth of *Bifidobacterium*. Moreover, the concentration of lactobacilli remained significantly higher also at the end of the washout with respect to the run-in. This finding could be correlated to the persistence of the two *Lactobacillus* strains in the faeces of volunteers after the

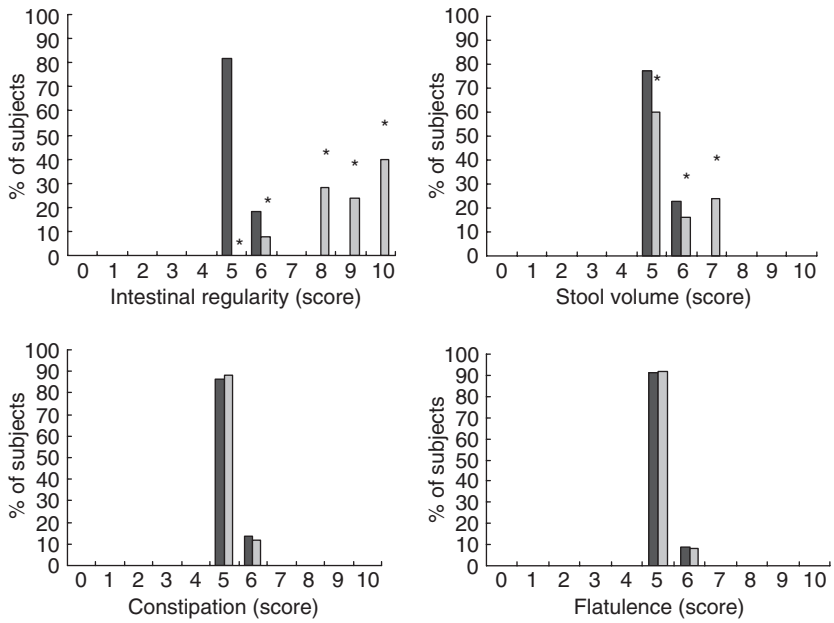

Figure 4 Bowel habit-related parameters. The evolution of the parameters is represented as a percentage of subjects recording a specific score for each parameter. 0 = worse, 5 = no change, 10 = best. (■) Control group. (▤) Probiotic group. *Significantly different from control group, according to chi-square test ($P < 0.05$).

washout, as shown by RAPD analysis. This implies that *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502® may multiply in the human GI tract for a certain period and confirms the previous study on the ability of the two probiotic strains to survive passage through the human GI tract (Verdenelli *et al.* 2009). This also highlights the peculiar characteristic of the improved adhesion effect of these two *Lactobacillus* strains when used in combination. There was a tendency not significant for the clostridia and enterobacteria to decrease in probiotic group after the consumption period. Similar results have been obtained also by other studies (Saito *et al.* 2002; Zanini *et al.* 2007) where the increase in lactobacilli during the dietetic treatment enhanced also the growth of bifidobacteria and reduced the number of potential harmful bacteria such as clostridia and enterobacteria. However, the main effect of probiotic consumption was to reinforce the intestinal *Lactobacillus* population without altering the balanced indigenous microecology of the healthy subjects. For the quantification of bacteria in the faecal samples, traditional plate counting was used. This method presents obvious disadvantages (Breeuwer and Abee 2000), but it is still a reliable, largely used and suitable method to assess the viable bacteria present in the faecal samples (Dommels *et al.* 2009; Pacheco *et al.* 2010). The consumption parameters assessed were bowel habits. Intestinal regularity and faecal volume were found to be significantly improved in probiotic group as compared to the control group at the end of the consumption. It is important to underlying that an increase in the faecal bulk dilutes carcinogens, mutagens and tumour promoters and results in a lower risk of colon cancer (Weisburger *et al.* 1993; Young *et al.* 2005), while a faster transit can determine a low prevalence of colonic disorders. In fact, it has been observed that slow transit time is associated epidemiologically with a high prevalence of large-bowel disorders, particularly diverticular disease and colon cancer, and it is implied that slow transit itself is important in determining metabolic events in the colon which are important in the aetiology of these disorders (Cummings *et al.* 2004).

This is a very interesting result because the recruited subjects had a normal bowel function before the intervention study; thus, the probiotic diet improves bowel habits without any side effects. Taken together, consumption of *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502® is likely to improve bowel movements through stimulating the growth and activity of bifidobacteria and lactobacilli as was reported in the studies with other strains of lactic acid bacteria (Yamano *et al.* 2006; Tuohy *et al.* 2007).

The possibility to have several kinds of food products enriched with the two potential probiotic *Lactobacillus* strains could result in the opportunity for people to consume daily oral doses of probiotic choosing their favourite foods. It is known that the colonization of new species in a well-established microbiota is quite rare and it happens often with a transitory manner (Roderick *et al.* 1999). That's way this is important to have a wide range of probiotic foods to assure a regular consumption of probiotics. Moreover, the participants belonging to the probiotic group preferred the bowel habits acquired

during the study. It was interesting that a number of subjects from the probiotic group enquired whether and where the products could be purchased as it made them 'feel good'.

In this work, we demonstrated that the consumption of the two potential probiotic strains, *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502®, was well tolerated and exerted a beneficial effect on the bowel habits of healthy adults increasing intestinal regularity and faecal volume.

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