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Assessment of a new synbiotic preparation in healthy volunteers: survival, persistence of probiotic strains and its effect on the indigenous flora

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Abstract

Background: Use of synbiotic preparations as dietary supplement is believed to be a valid approach to restore and maintain colonic microflora. However, only few papers have been published on the assessment of these food supplements and none of them have used molecular biology techniques to evaluate the effects of the probiotic components.

Methods: Twelve healthy volunteers were recruited. Faecal samples were taken before and at various time points during the administration period and at day 3 in the post-treatment period. Stool culture were performed and amplified ribosomal DNA restriction analysis was used to detect *L. paracasei*, the major bacterial component of the synbiotic products.

Results: An increase of at least 1 log of *L. paracasei*-like bacteria was observed in all subjects. An increase of as much as 3 log was seen in subjects who had a low number of *L. paracasei*-like lactobacilli at the baseline. The counts of *L. paracasei*-like lactobacilli were found to persist for at least 3 days after discontinuation of intake in healthy volunteers in 7 subjects. Genetic analysis showed that the maiority of vancomicin insensitive lactobacilli were real *L. paracasei*, as the strains administered with the tested product.

Conclusion: This study has shown that the strains of *L paracasei* administered with a synbiotic dietary supplement are able to survive through the gastrointestinal tract and to persist for at least a few days. It was also shown the efficacy of a synbiotic preparation to positively affect the microflora of healthy volunteers.

Background

For a long time colonic microflora has been considered to play an important role in the maintenance of the health and well-being of the host [1]. In addition to promote normal gastrointestinal functions and protecting against pathogenic bacteria, the microflora exerts beneficial effects on systemic metabolism and immune system [2].

The ability to control the growth and the pathogenic potential of these bacteria depends on the proper function of the microflora [3].

Imbalance in the colonic microflora with relative predominance of aggressive bacteria and insufficient concentration of protective species has been associated with colonic inflammation [4,5] and pouchitis[6].

Intake of probiotics (living micro-organisms), prebiotics (non-digestible oligosaccharides) and synbiotics (mixture of probiotics and prebiotics) has been demonstrated to modify the composition of the microflora, restore the microbial balance and therefore have the potential to provide health benefits [7–9]. However it has only been during the last few years that well designed clinical studies have provided clear evidence of health promoting effects, such as prevention of antibiotic-associated diarrhoea [10], treatment of acute diarrhoea[11], inflammatory bowel disease[12], eradication of *C difficile* infection[13] and enhancement of intestinal immunity [14,15].

The current state of evidence suggests that probiotic effects are strain-specific and even strains belonging to the same species may have marked or no probiotic effect [16].

As a result, in recent years there has been an increasing demand to select, by means of *in vitro* and *in vivo* tests, new strains with potential superior probiotic effects [17]. There is a general consensus that probiotic strains should be of human origin, as these bacteria have a greater chance of competing with resident bacteria, and of becoming numerically predominant after short intake and to persist in the colonic environment for some time after discontinuation of use.

Prebiotic substances are non digestible food ingredients which could be fermented by selected groups of beneficial bacteria; their positive influence on intestinal flora has been assed by a number of studies (for a review see [18]) The use of probiotic strains together with prebiotic substances will provide a combined effect, named "synbiotic" [19].

A large number of new lactobacilli strains have been previously isolated from faecal samples of newborns [20]; they were identified, by means of genetic analysis, as naturally persisting in the same subjects for several following days [20]. Following phenotypic characterisation and *in vitro* evaluation, three new lactobacilli strains (*L. paracasei* strain B 21060, *L. paracasei* B21070 and *L. gasseri* strain B21090) have finally selected in view of their use as probiotics.

A synbiotic formulation, consisting of a mixture of the above selected strains, oligosaccharides as prebiotic ingredients, glutamin, vitamin B6 and zinc, has been developed. The rationale of this formulation is to exploit a complementary probiotic action resulting from the different intrinsic properties of each individual strain and the promotion of bifidobacterial growth due to oligosaccharides. This formulation has been here assessed in a nutritional trial aimed at evaluating the ability of the selected strains to survive, grow and persist along the gastrointestinal tract and its efficacy and safety in various gastrointestinal disorders when administered in the final pharmaceutical formulation, in order to follow the recently issued FAO/WHO guidelines [16].

The primary aim of this study was to evaluate the ability of the probiotic strains delivered by the synbiotic preparation to survive following passage through the gastrointestinal tract and to persist in the stools after discontinuation of the intake in healthy volunteers.

The secondary aim was to evaluate the effects of the synbiotic formulation on some members of the indigenous flora.

Methods

Subjects

Twelve healthy volunteers participated in this study. Eligible participants were of both sexes and aged 24–48 years. Subjects were considered healthy on entry into the study if they did not have a history of chronic gastrointestinal diseases including chronic constipation and any episode of diarrhoea (> 3 bowel movements/day for 3 consecutive days) during the last month and did not present any current sign or symptom of gastrointestinal disorder or infection. Individuals were not included in the study if they were pregnant or breast-feeding, had a history of diabetes or had received antibiotics over the last 3 months before admission.

Subjects taking probiotic preparations including fermented milk had to discontinue the intake at least 2 weeks before entry into the study. Standard yoghurt, containing *Lactobacillus bulgaricus* and/or *Streptococcus thermophilus* only, was not prohibited. Before entry, all participants were screened medically for their suitability for the study.

Each subject signed an informed consent after he/she had been made fully aware of the purpose of the study.

Synbiotic administration

For the present study we used a new synbiotic preparation containing a combination of viable freeze-dried new lactobacilli strains of human origin with prebiotics

(inuline, oligosaccharides), glutamine, zinc and vitamin B6.

The product was available as a powder and dispensed in 6-g bag. Each bag contained 5×10^9 of both *L. paracasei* strain B21060 and strain B21070 and 0.5×10^9 of *L. gasseri* strain B 21090 [Flortec, Bracco SpA, Milan]. Each subject was instructed to take one bag three times a day (before breakfast, lunch and dinner) for 15 days. The content of the powder had to be dissolved in 50 ml of water before oral intake.

Strains are deposited at the Collection Nationale de Cultures de Microorganismes, Institute Pasteur (Paris).

Study procedures

The study had three periods: 7-day screening and baseline period (day-7 till day 0), 15-day intake period (day 1-day 15) and 3-day post-treatment period (day +1-day+3) Subjects' medical history, physical examination, and routine laboratory tests were taken at day-7. Faecal samples were collected at day-7 and day 0.

During the administration period subjects returned to deliver stool samples collected at day 5, 10 and 15, a general daily questionnaire on daily well-being, stool consistency and frequency and verification of compliance to the study procedures. Information on tolerability and possible adverse events was recorded at each visit.

In the post-treatment period subjects returned to deliver stool samples collected at day +3. The faecal samples were collected in sterile disposables with 9 ml of AMIES liquid (Difco, Detroit, Michigan), stored at 4–8 °C and delivered to the Department of Microbiology within 12 hours after collection.

Microbiological analysis of faeces

Processing of samples occurred within 12 hours after collection.

Weighted samples (about 1 g) were homogenised for 30 s in a stomacher (Stomacher 400, Seward, London, England) before dilution in a pre-reduced brain hearth infusion broth and cultivation on the appropriate selective media.

Appropriate dilutions were plated using Rogosa Acetate agar (Difco) and Rogosa Acetate agar (Difco) added with 12 μg/ml of vancomycin (Sigma) to enumerate total *Lactobacillus* spp. and vancomicyn insensitive lactobacilli (i.e. *L. paracasei* group, including *L. paracasei, L. casei and L. rhamnosus)*, respectively.

Bifidobacterium strains were enumerated using TPY agar added with 12 μg/ml of nalidixic acid (Sigma, St. Louis, USA), while enterobacteria were counted on VRBA, enterocci on SB agar and *Cl. perfringens* was counted on *Clostridium perfrigens* agar base.

All plates for lactobacilli were incubated for 48 hrs at 37 °C in anaerobic jars (GasPak, BBL, Coskeysville, MD, USA), while the incubation for clostridia was extended to 3 days. Enterocci were incubated in aerobic conditions for 24 hours and enterobacteriaceae for 12 hours.

Genetic identification of L. paracasei

PCR-ARDRA (amplified ribosomal DNA restriction analysis) was performed to identify species among the vancomycin-insensitive *Lactobacillus* colonies (*L. paracasei* group). We used a set of four enzymes and five primers to amplify the 16S-rDNA sequences of the tested lactobacilli. This is a reliable and rapid method to recognise *L. paracasei* strains from *L. casei* and *L. rhamnosus* [21].

L. gasseri was not sought due to the lack of a selective medium with antibiotics able to reduce the number of CFUs to be checked by means of genetic analysis.

Study endpoints

In the planning of the study the ability to survive passage through the gastrointestinal tract was defined as successful if an increase of least one log in the counts of *L. paracasei* group was observed in the stool sample at the end of treatment compared to baseline. Persistence was considered adequate if the concentration in the faecal sample after a 3-day discontinuation of intake was equal or only slightly decreased (max 1 log) compared to end of treatment.

In addition, an increase of total lactobacilli and *Bifidobacterium* and a decrease of *Enterobacteriacee*, enterococci and clostridium in the stool sample were considered as potential beneficial effects.

Results

Microbiological assessment

All the 12 subjects (5 M, 7 F, range 24 to 48 years old) completed the study. The preparation was tolerated and accepted very well by each participant. No adverse effect was registered during the study.

The microbiology examination of faecal samples showed an increase of at least 1 log of *L. paracasei* group in all subjects over the intake period. The increase in the counts was rapid and mostly evident after 5 day-administration. Six subjects who had a low number of counts of *L. casei* group at the baseline had in increase of even 3 log during the intake (Table 1).

Table 1: Changes in the counts of lactobacilli and bifidobacteria following intake of synbiotic preparation. Individual values. Values are in log of CFU/g of faeces

Time-Bacteria/Vol. no.	1	2	3	4	5	6	7	8	9	10	11	12
Day 0												
Total Lactobacilli	1×10^6	7.5×10^5	1.6×10^5	1×10^5	1×10^6	9.3×10^4	7.6×10^7	5×10^5	4×10^4	2.6×10^6	6.8×10^6	1×10^7
L. paracasei-like	1.3×10^4	2.2×10^6	0×10^3	8.6×10^3	3×10^3	9×10^4	6.8×10^7	8×10^3	4×10^3	2.4×10^6	5.5×10^5	0×10^3
Bifidobacteria	8×10^8	3.7×10^8	0×10^6	3.5×10^9	9×10^7	1.8×10^8	1.5×10^9	4.6×10^6	0×10^4	2.2×10^9	3.4×10^7	1.6×10^9
Day 5												
Total Lactobacilli	1.2×10^6	2.4×10^7	2.6×10^6	5×10^7	9.8×10^6	8.5×10^5	2.7×10^8	9.5×10^6	3.3×10^7	1.4×10^8	3.8×10^6	2.8×10^7
L. paracasei-like	1.5×10^6	2.3×10^7	2.1×10^6	3.9×10^7	9.5×10^6	8.3×10^5	2×10^8	9.5×10^6	2.7×10^7	1.4×10^8	3.4×10^6	1.7×10^7
Bifidobacteria	7.7×10^8	4.2×10^8	1.5×10^9	4.5×10^7	3.4×10^6	3×10^7	2.4×10^8	4.3×10^8	2.6×10^7	2.4×10^8	3.7×10^6	4.2×10^9
Day 10												
Total Lactobacilli	4×10^5	2×10^8	8.3×10^6	2.8×10^7	3.2×10^6	2.2×10^6	4.1×10^8	1.6×10^7	8.6×10^7	9.7×10^5	7.5×10^6	7.6×10^6
L. paracasei-like	1.7×10^5	1.1×10^8	6.5×10^6	2.8×10^7	2.8×10^6	2.1×10^6	3.5×10^8	1.5×10^7	1.7×10^7	9.4×10^7	1×10^6	1×10^6
Bifidobacteria	9.2×10^8	1.4×10^{10}	2.3×10^7	2.6×10^9	2.4×10^6	2.3×10^8	7.5×10^8	3.4×10^9	4.3×10^7	2.3×10^7	3.9×10^7	2.5×10^9
Day 15												
Total Lactobacilli	6×10^6	3.7×10^8	9×10^6	2.5×10^7	1.5×10^7	2.8×10^6	4.4×10^8	5.9×10^8	3×10^7	1.5×10^8	5.7×10^5	4.1×10^6
L. paracasei-like	5.7×10^6	4.2×10^8	9×10^6	2.2×10^7	1.3×10^7	2.8×10^6	3.7×10^8	2×10^7	2×10^7	1.1×10^8	1.5×10^5	4.1×10^6
Bifidobacteria	4.6×10^9	1.4×10^{10}	7.2×10^8	3.1×10^9	2.3×10^7	5.5×10^9	1.3×10^{10}	2.9×10^8	3×10^7	3.5×10^9	2.6×10^8	2.6×10^7

Table 2: Viable bacterial counts from faecal samples at day 3 of post-treatment period. Individual values. Values are in log of CFU/g of faeces.

Volunteer N.	1	2	3	4	5	6	7	8	9	10	11	12
Total Lactobacilli	8.1×10^6	7.8×10^8	9.5×10^7	8.8×10^5	6.5×10^6	1.5×10^4	1.2×10^7	1.4×10^7	3×10^5	2.1×10^9	2.3×10^8	3.8×10^5
L. paracasei-like	1.5×10^6	4.8×10^8	1.9×10^6	8.5×10^4	2.8×10^5	1.5×10^4	1.1×10^7	1.2×10^7	1.5×10^5	6.4×10^6	2.3×10^6	3.1×10^4
Bifidobacteria	2×10^9	3.6×10^9	5.2×10^8	3.1×10^9	6.5×10^8	0×10^5	2.3×10^7	1×10^7	4×10^5	6.4×10^9	3.8×10^8	4.4×10^9

In the post administration period, the counts of *L. paracasei* group in 7 subjects were similar to those achieved at the end of treatment whereas in 5 subjects a decrease of more than one log was found. At the same timepoint, only 3 subjects had low counts (< 10^5) of *L. paracasei* group compared to 7 subjects in the baseline period (Table 2).

In 8 out of 12 subjects, an increase of at least one log was observed in the counts of bifidobacteria at end of treatment compared to the baseline sample. In half of the subjects an increase of 3 log was observed. Similarly, an increase of total lactobacilli was found in 9 subjects at end of treatment (Table 1).

No consistent changes were found in the counts of *Enterobacteriacee*, enterococci and *Clostridium* during the study.

Genetic identification of L. paracasei

Genetic analysis was carried out to identify and quantify isolates *L. paracasei* really belonging to this species among all the vancomicin insensitive CFU of lactobacilli. Results actually showed that most of them were *L. paracasei* (Fig. 1). In fact 60 out of 65 (92%) CFU at 10-day, 49 out of 58 (84%) CFU at 15-day and 105 out of 132 (80%) vancomicin CFU at 3-day post-administration were *L. paracasei* (Table 3).

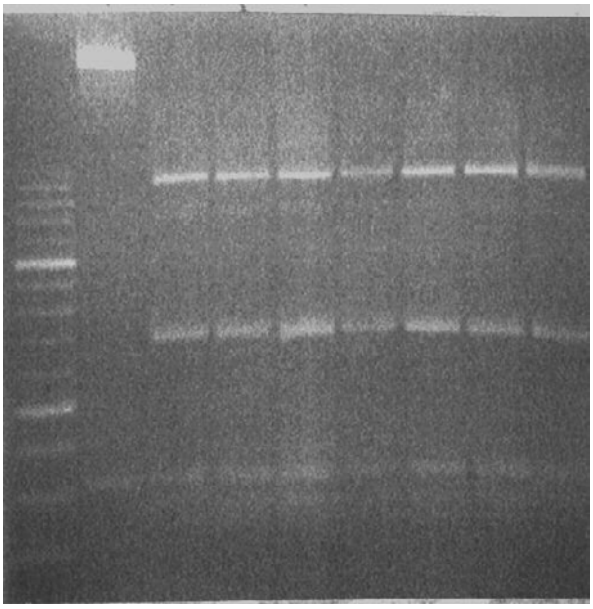
Discussion

A novel synbiotic preparation has been assessed by means of an *in vivo* nutritional trial. Quite surprisingly, only two papers are available on the assessment of the efficacy of synbiotic products [22,23] and none of them have used genetic tools to monitor the fate of the probiotic bacteria.

Strains used in this work have been carefully selected by the most commonly used *in vitro* tests for the study of probiotic strains [24]. Three strains, *L. paracasei* B21060, B 21070 and *L. gasseri* B21190 have finally been selected. These strains have shown a resistance to gastric acidity equal or superior to the reference strains. Similar results were obtained in bile acid resistance tests.

Adhesion to human epithelial cells (buccal cells and intestinal cells) was previously assessed [24] and shows that adhesion is more pronounced for B21060 and 21070 than for B21190 and reference strains (ATCC 53103 and ATCC 23850).

Following the screening period, a combination of the selected probiotics were included in a synbiotic preparation with oligosaccharides, glutamin, vitamin B6 and zinc.



1 2 3 4 5 6 7 8 9

Figure 1
ARDRA analysis from the left: Lane 1. Molecular Weight Marker (Roche)Lanes 2: CFU not identified as *L. paracasei* Lanes 3 to 8: CFUs identified as *L. paracasei* Lane 9: ARDRA of the reference strain DSM 5622^T

Table 3: ARDRA identification of *L. paracasei* among the vancomycin insensitive lactobacilli. Values are expresses as positive identification/CFU analysed.

	Synbiotic Intake		Post-treatment
Vol. no.	Day 10	Day 15	Day + 3
1	3 / 4	5 / 5	9 / 9
2	6 / 8	10 / 18	4 / 6
3	3 / 3	0 / 0	3 / 3
4	3 / 3	5 / 5	9 / 9
5	5 / 5	9 / 9	16 / 18
6	7 / 7	3 / 3	2 / 2
7	5 / 7	1 / 2	7 / 10
8	20 / 20	2 / 2	6 / 6
9	4 / 4	2 / 2	11/ 11
10	2 / 2	9 / 9	33 / 33
11	2 / 2	2 / 2	3 / 23
12	0 / 0	1 / 1	2 / 2

In the present study, the ability of the probiotics strains to survive through intestinal transit and persist after discontinuation of intake was investigated in healthy volunteers. An increase of faecal counts of vancomycin insensitive group was consistently observed; in fact an increase of at least one log in the counts of *L. paracasei*-like was found in all subjects. A marked increase (>2–3 logs) was found in subjects who had low counts of this group of lactobacilli at baseline suggesting that the rate of growth of the administered strains is even greater in subjects who have reduced counts of this species and may be more exposed to the adverse consequences of ecological imbalance.

The above change was already apparent after 5 days of intake which suggests that potential probiotic benefits can be obtained after only few days of intake.

Analysis of faecal samples after 3 days in the post-treatment period shows that strains tend to persist as the counts of *L. paracasei* group (which includes the administered strains) are similar or only slightly decreased compared to those achieved at the end of treatment and higher than those observed at baseline.

Genotypic analysis confirmed that increase of strains phenotypically resembling *L. paracasei* group is related to actual increase of *L. paracasei* species.

With regard to the effects on other intestinal bacteria, the intake of the synbiotic preparation was accompanied by an increase of bifidobacteria. This beneficial effect could be related to the presence of oligosaccharide in the formulation. No consistent effects were seen in other indigenous bacteria, at least in those plate counted.

The tolerability of the preparation was excellent in all individuals. No gastrointestinal or systemic adverse effects were observed during the study

Conclusions

In this study we have shown that new human indigenous probiotic strains of *L. paracasei* administered in a synbiotic preparation can be recovered from the faeces of healthy human volunteers and rapidly become the numerically dominant *Lactobacillus* isolated in faecal samples. These strains seems to persist in the colon for at least 3 days after discontinuation of the oral intake. During the study a favourable increase in total lactobacilli and bifidobacteria was also found.

These results are very promising since the study on faecal samples may underestimate colonization of colonic mucosa by probiotic strains even if in the present study microbiological analysis were made on fresh faecal samples. However the results of this study should be con-

firmed by further studies designed to determine the minimum and optimal dose to achieve effective counts on the colonic mucosa and including a more prolonged period of observation to evaluate the actual duration of persistence of these strains in the large intestine.

Competing interests

Costs of the trial were funded by Bracco SpA.

Authors' contributions

Lorenzo Morelli, Daniela Zonenschain, and Maria Luisa Callegari have been in charge of the microbiological and genetic analysis.

Enzo Grossi, Federico Maisano and Michele Fusillo were responsible of the trial management.

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