Influence of a Probiotic Milk Drink, Containing *Lactobacillus Paracasei* Lpc-37, on Immune Function and Gut Microbiota in Elderly Subjects

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ABSTRACT

Immunosenescence and alterations in the intestinal microbiota are associated with aging. Immune functions, as well as the intestinal microbiota, can potentially be modified and improved by probiotics, thus being particularly beneficial to elderly consumers. The ability of *Lactobacillus paracasei* Lpc-37 to modulate immune markers including phagocytic activity, natural killer (NK) cell activity and cytokine profiles, as well as composition and activity of the intestinal microbiota, in healthy elderly subjects was investigated in a randomised, double-blind, placebo-controlled study.

Only very limited effects in the measured blood or faecal immune markers or intestinal microbiota could be detected between the fermented milk drinks with or without probiotics. Thus, no significant immunological or microbial effects of the probiotic fermented milk could be detected in this study population.

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1. INTRODUCTION

During the past century, life expectancy increased by almost 30 years, elderly being the most rapidly expanding sector of the population. In fact, in most developed countries life-expectancy for those born during the last decade is expected to exceed 100 years (Christensen et al., 2009). This represents a challenge for modern societies and new approaches to improve health and reduce disease-risk among elderly are needed.

Aging has been related with changes in immune function (Mazari and Lesourd 1998) and gut microbiota composition (Claesson et al., 2011). These age-related changes represent targets for the development of nutritional intervention strategies specifically directed to the elder population. Among these strategies, the development of functional foods including probiotics constitutes a promising approach. Whilst in healthy adults the immune system is in balance and will unlikely be affected by probiotics, elderly represent a group with a 'challenged' immune system.

Elderly are more prone to infections and gastrointestinal problems (Bhutto and Morley 2008). In fact, an altered immune response has been described in elderly, a phenomenon known as immune-senescence (Candore et al., 2008). In elderly, these changes often include a reduced natural killer (NK) cell activity (Mocchegiani et al., 2009). Cytotoxic activity of NK cells plays a role on the response against viruses and cancer cells (Kazue et al., 2000) and therefore a reduced NK activity may, at least partially, explain the higher incidence of infections in elderly subjects. The intestinal microbiota has an important role in the health and well being of humans of all ages, but it is also important during old age; among others in modulating immune function. The intestinal microbiota composition in elderly is different from that of younger adults, showing reduced levels of potentially beneficial microorganisms and higher numbers of harmful bacteria in their intestines (Hayashi et al., 2003; Woodmansey 2007; Hopkins et al., 2001, 2002). This change is likely due to factors that are related to ageing, like reduced mobility and suboptimal nutrition.

It has been shown that the immune system of different population groups (i.e. healthy vs atopic adults) may react differently to probiotics (Roessler et al., 2008) and, therefore, probiotic containing products targeting elderly should be tested in this specific population. Different probiotic strains have been studied in the elderly population and some strains have been found to show immunomodulatory properties (Gill et al., 2001; Ouwehand et al., 2008; Ibrahim et al., 2010; Takeda and Okumura 2007; Sheih et al., 2001) or to reduce the duration of infections (Guillemard et al., 2010).

The objective with this study was to determine the properties of a milk drink containing *Lactobacillus paracasei* Lpc-37 on immune function and intestinal microbiota in healthy elderly subjects. This strain has been shown to pose immunomodulatory properties in healthy adults (Paineau et al., 2008), and suggested to be able to alter the activity of intestinal microbiota (Valerio et al., 2010). Additionally, the Lpc-37 strain has been reported to modulate the immune system in adults (Roessler et al., 2008). Thus, we studied whether *L. paracasei* Lpc-37 is able to modulate immune markers including phagocytic activity, NK cell activity, cytokine profiles or faecal IgA, as well as the influence of the product on composition and activity of the intestinal microbiota, on elderly subjects.

2. SUBJECTS AND METHODS

2.1 Volunteers and Study Design

Faecal and blood samples were obtained from 40 elderly (67 to 95 years, mean 82.3 years) volunteers recruited from 3 nursing elderly homes in Gijon city area (Asturias, Spain). Exclusion criteria for the subjects were: consumption of antibiotics in the past month, lactose intolerance, regular consumption of probiotics/prebiotics, history of colon cancer, inflammatory bowel diseases or intestinal surgery.

The double-blind, placebo controlled, parallel study design consisted in three phases. A 4 weeks run-in phase, a 4 weeks intervention phase in which the volunteers were randomly allocated to the placebo or test groups and a 3 weeks wash-out period. Randomization was achieved by using GraphPad program (www.graphpad.com/quickcalcs/index.cfm) by using random numbers to assign subjects to treatment groups within each elderly home. During the run-in period volunteers were asked not to consume probiotic/prebiotic products or yogurt. During the intervention phase the subjects consumed daily one bottle of placebo (100 ml of fermented milk drink) or Lpc-37 product (the same product containing 3×10^9 CFU *L. paracasei* Lpc-37, ATCC SD 5275, (Danisco, Niebüll, Germany). Both the fermented placebo milk drink and the fermented probiotic milk drink were manufactured by Senoble (Jouy, France) using proprietary starter strains. During the whole duration of the study volunteers continued consuming their habitual diet.

The number of subjects in the study is based on previous studies (Sheih et al., 2001) in which the same parameters were determined. To detect differences of 20% between placebo and test products, on NK or phagocytic activity, at a significance level of 0.05 with an 80% power, a total of 34 volunteers would be needed. Thus, we recruited 40 allowing for some drop-outs. The study was approved by the Regional Ethical Committee on Clinical Research from the Asturias Principality (Asturias, Spain). All volunteers, as well as a close relative, gave written informed consent to participate in the study.

Faecal and blood samples were collected from the volunteers at the end of each study phase. Faecal samples were immediately frozen at -80 °C and stored until further analysis. Heparinized peripheral blood was drawn for assessment of immune parameters and blood serum was immediately frozen at -80ºC and stored until further cytokine analyses. At the different sampling points the volunteers also answered a Diet and Quality of Life-questionnaire.

2.2 Diet and Quality of Life Questionnaire

Dietary intake was assessed by determination of the average frequency of each food offered by the institution, after they had provided us with the menus, so that estimation of consumption does not depend upon participant´s memory. The menus served on the different elderly homes were nutritionally assessed and on the basis of these menus a food frequency questionnaire was specifically designed for each nursing home by an experienced nutritionist. The dietitian asked each kitchen chef about cooking practices and number and amount of ingredients used in each recipe, as well as questions concerning menu preparation (such as type of oil used, type of milk, etc.). During interviews, subjects were asked item-by-item whether they usually ate each food and, if so, how much of it did they ate.

At the different sampling points the questionnaire including the food consumption, as well as questions related to habits and quality of life introduced in the questionnaire by trained nutritionists, was filled in by means of a personal interview. These questions related to sleeping habits (hour of dream, having siesta, awakens during night, perception of rest), bowel function (depositions per week, consistency of faeces, use of laxatives, pain during defecation), mood (happiness self-perception, depression) and diet (appetite, ration size, chewing problems). When appropriate the volunteers were asked to rank the responses on a scale from 0 to 4. In the same interview an anthropometry was carried out.

Nutrient intake was calculated by using the Nutrient Composition tables CSIC 1994 and Wander Sandoz Nutrition 1990.

2.3 Immune Measurements

Heparinized whole blood was used to determine phagocytic activity. To this end phagocytosis of fluorescein (FITC) labelled *E. coli* cells was determined by using the Phagotest® kit (Orpegen Pharma, Heildelberg, Germany), according to the manufacturer's instructions, by using a FACSCanto II flow cytometer (Becton Dickinson, BD Biosciences, San Diego, CA).

Human peripheral blood mononuclear cells (PBMCs) from each volunteer were obtained from heparinized blood by centrifugation over a Ficoll-Hypaque gradient (Lymphoprep, Nycomed, Oslo, Norway). Cells were counted by trypan blue exclusion test and adjusted to 5×10^6 cells/mL. Then, NK cell activity was determined by specific target lysis of labelled K562 cells by flow cytometry, using a commercially available kit (NKtest®, Orpegen Pharma) following the manufacturer's instructions.

The levels of IL-10, TNF-α, IL-8, IL-17, and IL-12p70 in blood sera were quantified by a multiplex immunoassay (Cytometric Bead Array, CBA, BD Biosciences) using a FACSCanto II flow cytometer. The concentrations of TGF-β were measured using an ELISA kit (BD OptEIA^{TM}, BD Biosciences). C-reactive protein (CRP) levels in plasma were determined by using the Human CRP Instant ELISA (Bender Medsystems, Vienna, Austria) kit according to the manufacturer's instructions and results were expressed as ng/mL. Faecal concentrations of IgA, calprotectin and prostaglandin E2 (PGE_2) were determined by using Human IgA ELISA Quantitation Kit E80-102 (Bethyl Laboratories Inc., Montgomery, TX, USA), Phical ELISA test (Calpro AS, Oslo, Norway), and PGE_2 EIA Kit (Cayman Chemical Company, MI, USA) according to the manufacturers' instructions. The results were expressed as µg/g of faeces (fresh weight) for IgA and calprotectin, and pg/g for PGE_2.

2.4 Microbiological Analyses

One g of faecal sample was used for DNA extraction by using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) as previously described (Gueimonde et al., 2004). Quantification of the different bacterial populations in faeces was performed by quantitative PCR in 25 µL PCR reactions by using primers and conditions shown in Table 1.

Table 1: Oligonucleotides and conditions used for the bacterial qPCR assays

Target	Primer	Conc. (nM)	AnnealingTm	Reference
Akkermansia spp.	F:CAGCACGTGAAGGTGGGGAC R:CCTTGCGGTTGGCTTCAGAT	200	60	Collado *et al.* 2007
Bacteroides–Prevotella – Porphyromonas	F:GAGAGGAAGGTCCCCCAC R:GCKACTTGGCTGGTTCAG	200	60	Layton *et al.,* 2006 Ramirez-Farias *et al.,* 2009
Bifidobacterium spp.	F:ATTCTGGCTCAGGATGAACGC R:CTGATAGGACGCGACCCCAT	200	60	Gueimonde *et al.* 2004
Clostridia XIVa *Blautia coccoides–* *Eubacterium rectale*	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	200	55	Rinttilä *et al.,* 2004
Clostridia IV *Clostridium leptum–* *Faecalibacterium prausnitzii*	F: TTAACACAATAAGTWATCCACCTGG R: ACCTTCCTCCGTTTTGTCAAC	200	60	Ramirez-Farias *et al.,* 2009
Clostridium difficile	F:TGAGCGATTTACTTCGGTAA R:CCATCCTGTACTGGCTCACCT	150	60	Lahtinen *et al.* 2011
C. difficile TcdA	F: TCTACCACTGAAGCATTACAGCTACAG R: TGTAGGTACTGTAGGTTTATTGATTTGAGAT P: GGATTTTGAATCTCTTCCTCTAGTAGCGCGTG	300 200	60	This study
Enterobacteriaceae	F:TGCCGTAACTTCGGGAGAAGGCA R:TCAAGGACCAGTGTTCAGTGTC	200	58	Matsuda *et al.,* 2007
Lactobacillus group	F: AGCAGTAGGGAATCTTCCA R: CATGGAGTTCCACTGTCCTC	200	60	Peso Echarri *et al.* 2011
Lactobacillus paracasei	F:ACATCAGTGTATTGCTTGTCAGTGAATAC R:CCTGCGGGTACTGAGATGTTTC P:TGCCGCCGGCCAG	300 200	60	Haarman & Knol 2006

Standard curves were made with pure cultures of appropriate strains (*Akkermansia muciniphila* CIP107961, *Bacteriodes thetaiotaomicron* DSMZ2079, *Bifidobacterium longum* NCIMB8809, *Blautia coccoides* DSMZ935, *Clostridium leptum* DSMZ753, *Clostridium difficile* ATCC 1382, *Escherichia coli* ATCC 11775, *Lactobacillus gasseri* IPLAIF7/5 and *L. paracasei* Lpc-37) which were grown overnight in GAM medium (Nissui Pharmaceutical Co, Tokio, Japan) under anaerobic conditions. The *Akkermansia, Bacteroides, Bifidobacterium, Bl. coccoides-E. rectale* group, *C. leptum* group, *C. difficile*, and *Lactobacillus* qPCR assays were performed with a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems). For *C. difficile TcdA* and *L. paracasei* a TaqMan qPCR assay was used (TaqMan Master mix, Applied Biosystems). Faecal DNA extracts were analyzed and the mean quantity per g faecal wet weight was calculated.

The total bacterial load was determined by using a flow cytometric FACSCalibur-system (BD Biosciences) as previously described (Apajalahti et al., 2002). For determination, the samples were fixed with formaldehyde (final concentration 4%) and stained with a fluorescent, nucleic acid binding dye, SYTO 24 (Molecular Probes, Leiden, the Netherlands).

2.5 Chemical Analyses

Analysis of short chain fatty acids (SCFAs) was performed using gas chromatography essentially as described before (Salazar et al., 2011), analyzing the concentration of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. Concentrations of biogenic amines (methylamine, putrescine, piperidine, cadaverine, histamine, tyramine, spermidine, and spermine) were determined as dansyl derivatives by reversed phase HPLC as previously reported by Saarinen (Saarinen 2002).

2.6 Statistical Analysis

The data from the study was analyzed with linear mixed effects models in a repeated measures fashion, using a random effect for the subject accounting for repeated measures. The models had fixed effects for time point, treatment, gender, nursing home, and an interaction term for combination of time point and treatment. Continuous valued variables body mass index and age were also included in the models as covariates. In addition, there was a baseline regression coefficient accounting for individual baseline differences between the subjects. Based on the residual analysis, some of the original variables were log-transformed. The linear model analyses were conducted with statistical programming language R (version 2.11.1) using nlme package (version 3.1-97). Wilcoxon test was used for before-after comparisons within each treatment group. The obtained P-values were considered as statistically significant at $P < 0.05$.

3. RESULTS

3.1 Diet and Quality of Life

Differences in background data (gender ratio, age, weight, use of anticoagulant and antihypertensive drugs, etc.) were not observed between the placebo and probiotic groups (data not shown). With regard to drop-outs only 1 person (placebo group) dropped out for claiming self-reported lack of tolerance (but when asked for specific symptoms was not able to describe them) and one was moved from the elderly home. In this group there were also two volunteers that dropped-out before the end of the study because they were hospitalized

because a respiratory infection and breaking a hip (these last two subjects were excluded and not considered for the study). In the probiotic group, one volunteer was moved to another elderly home and another one went on holidays with her family before the end of the study, and thus, some samples were not available.

Intake of nutrients did not differ between the groups neither at baseline nor at the end of intervention or during washout. At baseline energy intakes were 1810 ± 380 and 1740 ± 390 (kcal/day, mean ± SD) for placebo and intervention groups, respectively, 1899 ± 321 and 1824 ± 287 at the end of intervention, and 1869 ± 242 and 1678 ± 341 at the end of the washout period. With regard to macronutrients, intake of proteins ranged between 72 and 81 g/d, those of lipids between 73 and 85 g/d and those of carbohydrates ranged between 170 and 218 g/d, without differences between placebo and probiotic groups. No differences were found for intake of fiber (14-19 g/d), cholesterol (222-286 mg/d) or fatty acids (28-42, 25-30 and 13-16 g/d for saturated, monounsaturated and polyunsaturated fatty acids respectively) or for micronutrients. Statistical analysis of the QoL questionnaires did not show any significant differences in the parameters included (sleeping habit, bowel function, mood or appetite) (data not shown).

3.2 Immune Variables

Both the placebo and probiotic milk drinks increased significantly (P = 0.020 and P = 0.048, respectively) the NK activity at the end of the intervention (34.1 ± 12.8% and 27.4 ± 13.0% for placebo and probiotic group, respectively) when compared with those at baseline (25.2 ± 8.2% and 22.1 ± 5.9%). However, between-group analyses did not show any statistically significant difference on NK activity. The percentages of phagocytic cells ranged between 89 and 93% in the different groups and time points without showing any statistically significant difference. However, when the phagocytic activity per cell was determined, by measuring the mean fluorescent intensity per cell, whilst statistically significant differences were not observed at baseline (placebo: 12686 ± 8050 vs probiotic: 13210 ± 8654), a significantly (P = 0.0137) lower activity was observed in the probiotic group at the end of the intervention (placebo 16946 ± 7820 vs. probiotic: 14162 ± 7751).

At the end of intervention, despite the slightly higher values of total faecal IgA (164 ± 174 vs. 106 ± 86 μg/g wet weight faeces), PGE_2 (1297 ± 498 vs. 834 ± 295 pg/g wet weight faeces) and calprotectin (124 ± 95 vs. 112 ± 82 pg/g wet weight faeces) observed in the probiotic group than in the placebo group, the differences did not reach statistical significance at any analyzed time point (Figure 1). Cytokines levels showed a large inter-individual variability and, as well as CRP, did not show any statistically significant differences between groups or time points (data not shown).
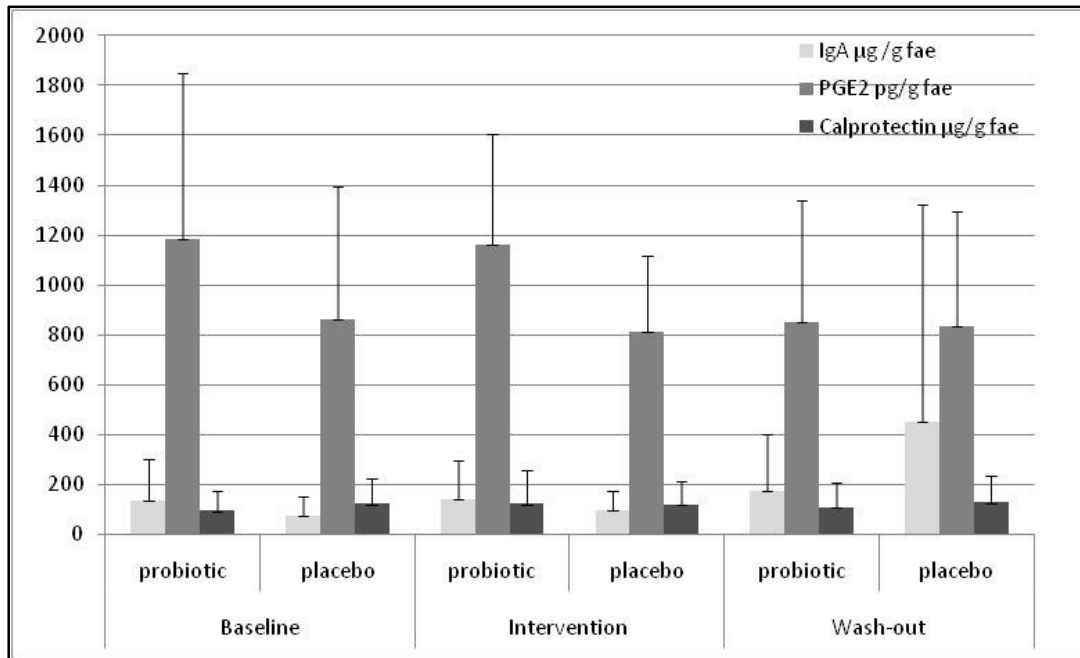
Figure 1: Faecal immune markers IgA, PGE₂ and calprotectin in placebo and probiotic groups.

Values are presented as means + SD.

3.3 Faecal Microbiota

Consumption of placebo milk drink or milk drink supplemented with *L. paracasei* Lpc-37 for 4 weeks resulted in only minor changes in the gut microbiota composition (Figure 2). Despite the high inter-individual variability, a trend (nonsignificant, $P = 0.0626$) towards higher levels of lactobacilli and a significantly higher number ($P = 0.0012$) of *L. paracasei* was detected in the probiotic group, than in placebo, at the end of the intervention. No other statistically significant effects on the gut microbiota composition were observed.

3.4 SCFA and Biogenic Amines Concentrations

The probiotic group had a slightly higher, although nonsignificant, total faecal SCFA concentration than the placebo group at the end of intervention. Acetic acid was the major SCFA in both groups, and a small increase of acetate could be detected for both groups during the intervention, contributing from 42% at baseline to 50% of total SCFA after the intervention (Figure 3). In general, the levels remained mainly unchanged and no statistically significant differences were observed. A high inter-individual variability was observed on both the faecal SCFA concentration as well as for the biogenic amines. No significant differences for the biogenic amines occurred between the probiotic and the placebo group (Figure 4), cadaverine being the predominant polyamine in both groups.
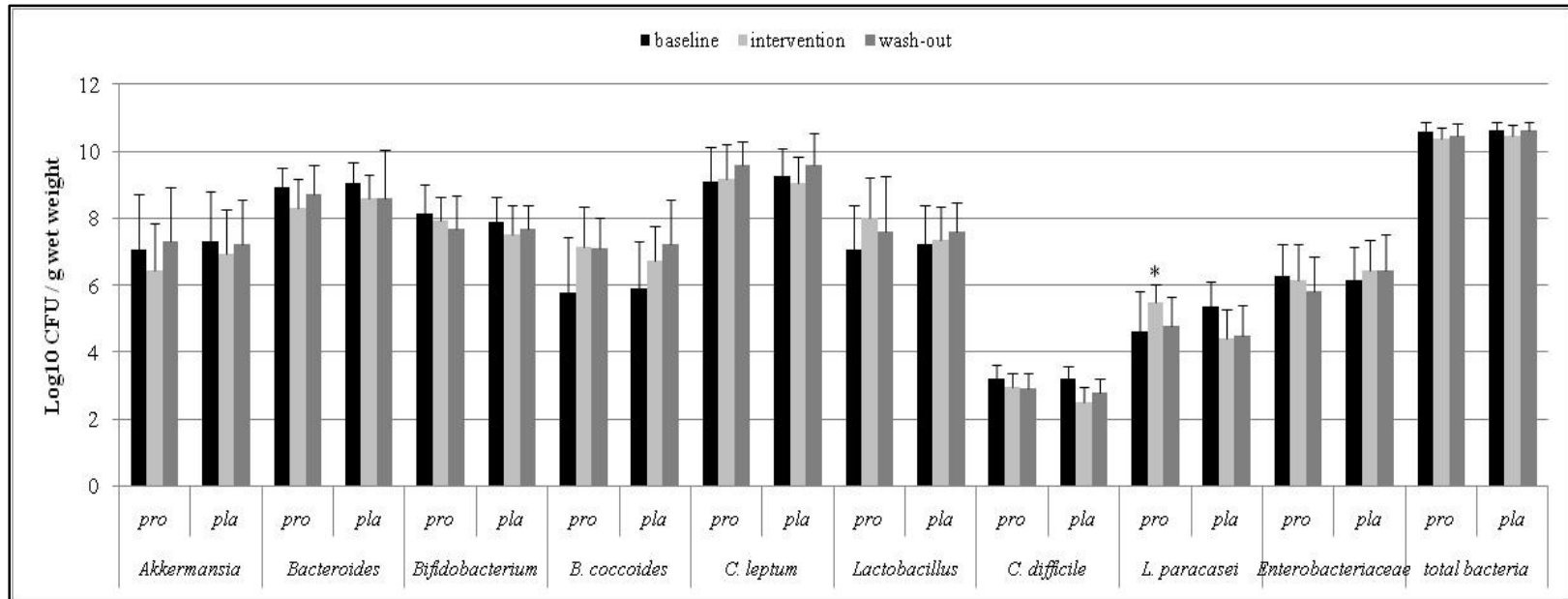
Figure 2: Levels (Log$_{10}$ microorganisms/g of faecal wet weight) of the different microbial groups analyzed (mean + SD).
Asterisk (*) indicates statistically significant differences between placebo (pla) and probiotic (pro) groups.
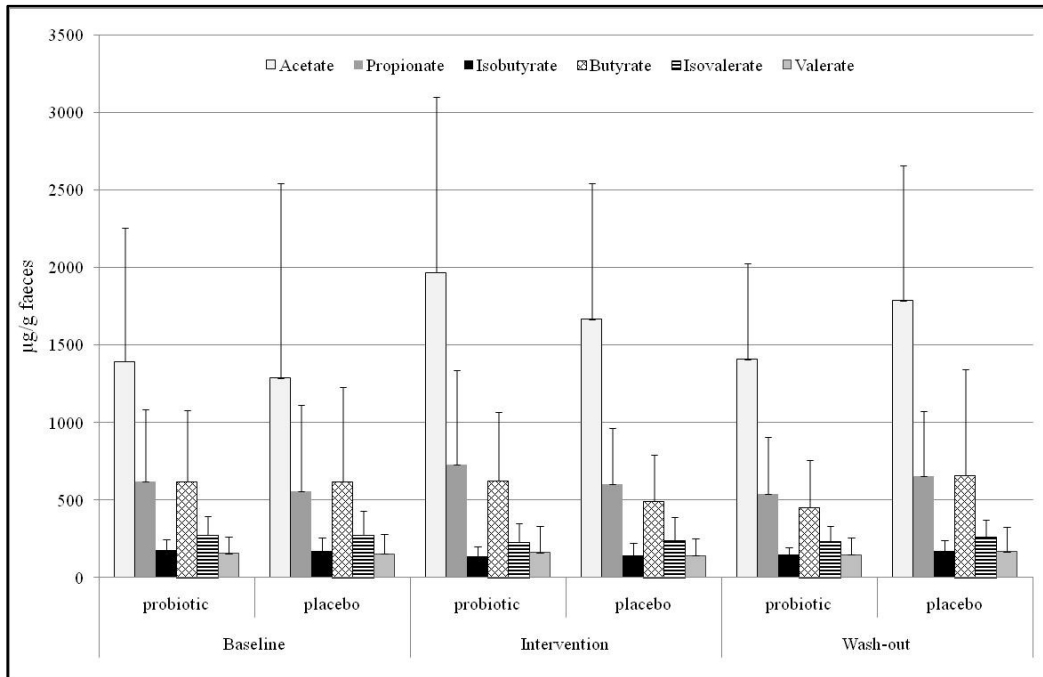
Figure 3: SCFA profiles (µg/g of faeces) determined by GC investigating the effects of the probiotic milk drink in comparison to the placebo (mean + SD).
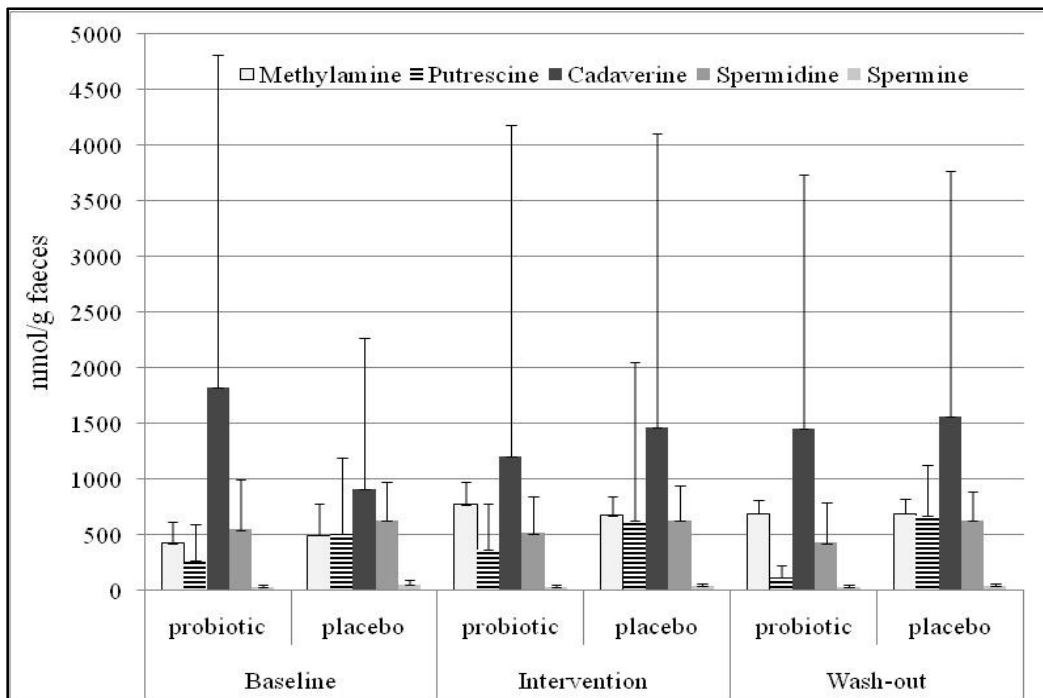

Figure 4: Biogenic amine profiles (nmol/g faeces) of the healthy elderly (mean + SD).

4. DISCUSSION

In this study, *L. paracasei* Lpc-37 was used since previous reports have suggested that *L. paracasei* strains are able to alter the activity of intestinal microbiota (Valerio et al., 2010). Additionally, the Lpc-37 strain has been reported to modulate the immune system in adults (Roessler et al., 2008). However, in this study, with the sole exception of a lower phagocytic activity per cell, no significant effects of the probiotic fermented milk drink were detected on the measured immune functions. These results indicate a very limited effect on the immune system of the probiotic product, when compared to the placebo fermented milk, this maybe, at least partly, due to the use of a fermented milk drink as placebo, as pointed out by the increase in NK activity after the intervention phase observed not only for the probiotic product but also for the placebo. This fact, underlines the difficulties on obtaining a "neutral" placebo product when conducting studies with fermented products containing life bacteria. In addition, the dose used here (10^9 CFU/day) was lower than that used by Paineau and coworkers (Paineau et al., 2008) in healthy adults (2×10^{10}) and, whilst we used the strain together with the yogurt starter, Roessler and co-workers (Roessler et al., 2008) used it in combination with two other probiotic strains. Therefore the comparison of their results with those obtained in the present study is limited.

Quite large inter-individual variations were detected for the measured faecal immune markers IgA, calprotectin and PGE_2, within both groups. The faecal IgA and calprotectin levels were comparable to previous studies of healthy elderly and adults (Tiihonen et al., 2008; Ouwehand et al., 2008; Maiden et al., 2005). The faecal PGE_2 levels found were higher, already from the beginning, than those reported for elderly in another study (Ouwehand et al., 2008). In addition to immune modulation, PGE_2 has a central role in the normal physiological gastrointestinal functions including cytoprotection. In the present study, the faecal concentrations of PGE_2 were similar to what has been reported to healthy young adults (Tiihonen et al., 2008). Thus, this suggests that in the present study the mucosal protection in the stomach and duodenum is not decreased in the target population, in contrast to other studies (Tiihonen et al., 2008; Newton 2005).

No large differences between the placebo and the probiotic groups could be detected for the bacterial species or groups analysed, the levels being similar to those previously reported by other authors (Candela et al., 2010; Collado et al., 2007). As expected, the probiotic milk drink was found to significantly increase the faecal numbers of the administered microorganism, *L. paracasei. C. difficile* is a natural resident of the intestinal microbiota in infants (Jangi and Lamont 2010) and adults (Ozaki et al., 2004), but it may become harmful when overrepresented in the intestinal microbiota. Most individuals with *C. difficile* symptoms are elderly (>60 years) (Simor et al., 2002), however the detected levels of *C. difficile* in the present study were very low and none of the samples tested positive for *C. difficile* toxin.

Regarding the metabolic activity of the intestinal microbiota, and as expected from the lack of major effects on gut microbiota composition, only minor changes in the levels of SCFA were observed and these did not differ between the treatment groups. A slight increase in the total amount of SCFA could be detected in both the probiotic and the placebo group. However, since the majority of generated SCFA in the colon are absorbed during transit of digesta through the gut (Topping and Clifton 2001), large differences can be hard to detect. Biogenic amines play an important role in metabolic and physiological functions and in this

study no significant difference between the groups could be detected for the biogenic amines.

5. CONCLUSIONS

In this study, only very limited effects on the measured immune markers or intestinal microbiota of healthy elderly were detected for the Lpc-37-containing fermented milk when compared with the placebo fermented milk. The recruited elderly were among the healthiest in their age class, which may explain the limited response observed in this study. However, the results indicate that this bacterial strain will not have a significant impact on immune function or gut microbiota in this population.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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