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IMPACT OF CONSUMPTION OF DIFFERENT LEVELS OF *BIFIDOBACTERIUM LACTIS* HN019 ON THE INTESTINAL MICROFLORA OF ELDERLY HUMAN SUBJECTS

M. AHMED[2], J. PRASAD[1], H. GILL[2], L. STEVENSON[2], P. GOPAL[1]

1. Fonterra Research Centre, Fonterra Co-operative Group Limited, Private Bag 11029, Palmerston North, New Zealand. 2. Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand. Correspondence: Jaya Prasad, Fonterra Research Centre, Fonterra Co-operative Group Ltd, Private Bag 11029, Palmerston North, New Zealand, Phone: +64 6 350 4600 ext 7053, Fax: +64 6 356 1476, E-mail: prasad.jaya@fonterra.com

Abstract: *Background:* Age-related changes in the physiology and intestinal function of the elderly render them more susceptible to gut-related illnesses. Probiotic dietary supplementation has been shown to enhance the health indices in the elderly. *Objective:* To determine the effect of three different doses [5 x 10^9 CFU/day (high), 1.0 x 10^9 CFU/day (medium) and 6.5 x 10^7 CFU/day (low)] of *Bifidobacterium lactis* HN019 (DR10™) on the intestinal flora of elderly human subjects and the dose response effect. *Design:* Randomised, double-blind and placebo-controlled human dietary intervention study consisting of four groups of 20 elderly (over 60 years old) volunteers. Each volunteer consumed 250 mL per day of reconstituted skim milk (RSM) which either did not contain any probiotic supplement (placebo group) or contained *B. lactis* HN019 at different levels (low, medium and high dose groups). The study comprised three stages: a 2-week pre-intervention (without any supplement), followed by 4 weeks of test feeding (dietary intervention) and then a 2-week washout period. *Results:* After dietary intervention, statistically significant increases in bifidobacteria, lactobacilli and enterococci were observed. At the end of the 4-week feeding period the mean number of bifidobacteria recorded in the placebo group were 9.31 ± 0.01 log CFU/g of faeces. In the high, medium and low dose groups the bifidobacteria levels were significantly ($p<0.006$) higher (9.88 ± 0.1, 9.75 ± 0.14 and 9.74 ± 0.11 log CFU/g of faeces, respectively), when compared to the respective pre-intervention levels. There were no significant differences ($p > 0.05$) between the responses of the different dose groups, indicating that even the lowest dose tested augmented the changes in bifidobacteria. Similar trends were observed for lactobacilli and enterococci. In contrast, the counts of enterobacteria were reduced in all the probiotic dose groups. *Conclusion:* The present study showed that dietary supplementation with *B. lactis* HN019 significantly increased the number of resident bifidobacteria and reduced the enterobacteria counts. In addition, enterococci and lactobacilli were also increased. Based on this study and already published clinical evidence (4, 5, 8, 9) we conclude that, *B. lactis* HN019 is a suitable probiotic for elderly human subjects and even the lowest dose (6.5 x 10^7 CFU/day) tested is able to confer desired changes in the intestinal microflora.

Key words: Probiotics, intestinal flora, elderly, aging, bifidobacteria, dose response.

Introduction

Functional foods have become an important and rapidly expanding segment of the food market as processed food manufacturers seek to improve market share by promoting the health benefits provided by functional ingredients in their products. Functional foods targeted towards improving the balance and activity of the intestinal milieu currently provide the largest segment of the functional food market in Europe, Japan and Australia (1). Certain species of *Lactobacillus* and *Bifidobacterium* are known probiotics that are extensively used in yoghurts, dietary adjuncts and other health-related products (2). By definition, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (3).

Dietary supplementation is seen as an attractive, non-invasive means of enhancing the health status among individuals (such as the elderly) with sub-optimally functioning immune systems. It has been shown that the general health of the aging population may be improved using a probiotic approach (4, 5, 6). In a controlled pilot study aimed at evaluating the effect of supplementing fermented milk with *Lb. casei* DN-114 001 on the incidence of winter infections in elderly human subjects, the duration of all pathologies tested was significantly lower in the treatment group than in the control group (7). The immune-enhancing properties of dietary supplementation with *B. lactis* HN019 (DR10™) in elderly humans has been established (4, 5, 8, 9).

The human large intestine is the most heavily colonised region of the digestive tract (10, 11). At least 50 different genera of bacteria reside in the colon, comprising several hundred individual species. Although considerable variation in the intestinal flora can be found among individuals, studies have shown that it remains quite stable in an individual over time. The mechanisms that lead to a dynamic equilibrium of the gastrointestinal tract microflora and the host are not entirely known, but are thought to comprise both host and microbial factors that may differ at different levels of the intestine.

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Numerous factors influence the balance of the intestinal bacterial flora, e.g. congenital or acquired immuno-deficiencies, illnesses, intestinal motility disorders and digestive stasis. Recent studies suggest that age affects the intestinal microflora, with a decrease in total anaerobes and bifidobacteria and an increase in enterobacteria (12). These changes and the reduced intestinal immunity of the aged may favour gastrointestinal infections, which are frequent in the elderly and have a profound effect on morbidity, mortality and health costs (12). Further, bifidobacteria have been shown to decline in numbers as a result of the aging process (13,14,15), whereas clostridia and enterobacteria, which are viewed as being detrimental to health, increase (16). Modern polyphasic analysis of faecal bacteria showed that significant structural changes occur in the microbiota with aging, and this was especially evident with respect to bifidobacteria (17). In spite of the evidence supporting the health benefits associated with supplementation of probiotic bacteria in elderly human subjects, there is a paucity of information describing the effect of different levels of probiotic supplementation on the gut microflora. The present investigation describes a randomised, double-blind, placebo-controlled human dietary intervention study aimed at determining the effect of three (high, medium and low) different doses of *B. lactis* HN019 on the intestinal flora of elderly human subjects in order to ascertain the minimum probiotic dose that can significantly influence the intestinal microecology.

Methods

Subjects

Eighty healthy free-living elderly human volunteers (mean age of subjects 69.5 years with a range of 60 to 87 years) with no history of gastrointestinal disease and no recent treatment with antibiotics gave their written informed consent to participate in the study protocol. The Manawatu-Wanganui Ethics Committee, New Zealand, approved the study.

Experimental design

This was a randomised, double-blind and placebo-controlled study consisting of four groups of 20 volunteers (The mean ages of different intervention groups were as follows: placebo, 69 years; high dose, 67 years; medium dose, 70 years and low dose, 70 years). Each volunteer consumed 250 ml per day of reconstituted skim milk which either did not contain any probiotic supplement (placebo group) or contained *B. lactis* HN019 at three different levels [total daily dose of , 6.5 x 10^7 colony forming units (CFU) (low dose), 1 x 10^9 CFU (medium dose) and 5 x 10^9 CFU (high dose)]. The study comprised three stages: a 2-week pre-feed (without any supplement), followed by 4 weeks of test feeding (dietary intervention) and then a 2-week washout period. During the study period, the volunteers consumed their normal diet except that they were asked to refrain from consuming yoghurt or any other probiotic-containing foods. Faecal samples were collected at six time points: weeks 0, 2, 4, 6, 7 and 8. The faecal samples were collected in sterile specimen containers and brought to the laboratory immediately. The samples were analysed immediately and the residual samples were stored at -80°C.

Microbiological analysis of faecal samples

Immediately upon receipt the fecal samples were weighed into 50 ml sterile plastic tubes (0.5 to1.0 g) and transferred to an anaerobic glove box (Coy Laboratory Products Inc., Grass Lake, MI, USA). Anaerobic conditions were achieved using a gas mixture containing 5% carbon dioxide and 10% hydrogen in nitrogen. Further, 1:10 faecal suspensions were made in pre-reduced brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) supplemented with vitamin K_1 (0.1 μg/mL), haemin (5 μg/mL) and L-cysteine hydrochloride (0.5 mg/mL). The faecal suspensions were homogenised using 10 glass beads (3 mm diameter) and mixing on a vortex. Further dilutions were made in the same broth. To enumerate total anaerobes, Brucella agar (Difco, Detroit, USA) supplemented with 5% (v/v) sheep blood as well as vitamin K_1 (0.1 μg/mL), haemin (5 mg/mL) and L-cysteine hydrochloride (0.5 μg/mL) were incorporated (18). After spreading the relevant dilutions, the plates were incubated anaerobically at 37°C for 48 to 72 h. Bacteroides were enumerated using BBE (bacteroides-bile-esculin) agar followed by incubation of the plates at 37°C for 48 to 72 h. Bifidobacteria were enumerated using Beerens medium (19). Lactobacilli were enumerated on Rogosa SL agar (20). The incubation conditions for both bifidobacteria and lactobacilli were similar to those for total anaerobes. Enterobacteria and enterococci were enumerated on MacConkey agar (Oxoid, Hampshire, England) and bile esculin azide agar (Difco, Detroit, USA). The plates were incubated aerobically for 24 h. Yeasts were enumerated on Sabaroud dextrose agar containing chloramphenicol (50 μg/ml) (Oxoid, England). The plates were counted after 48 h of aerobic incubation at 37°C.

Statistical analysis

Analysis of variance (ANOVA) was used (Proc GLM, SAS version 8, SAS Institute, 2000; MINITAB™ statistical software, MINITAB Inc., 2000) to test the statistical significance between treatments and time periods, as well as their interaction. The variation between individual subjects in the trial was used as the error term. To determine the difference between weeks (time interval), the student t-test or Tukey honest significant differences and Dunnett's test were used. A probability value of $p < 0.05$ was considered to be sufficient to reject the null hypothesis of no treatment effect. The results were expressed as mean log CFU/g of wet faeces with standard error (SE).

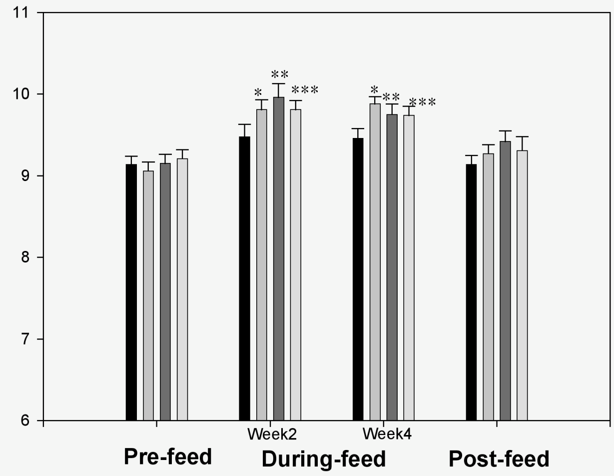
Results

The microbiological analyses of faecal samples were performed to determine the effect of different daily doses of *B. lactis* HN019 (6.5 x 10^7, 1 x 10^9 and 5 x 10^9 CFU/day) on the resident populations of bifidobacteria, lactobacilli, faecal streptococci, enterobacteria, total anaerobes, bacteroides, and yeast and mold. The mean (± SE) values of faecal counts of different bacteria present during three periods (pre-intervention period, during intervention period and post-intervention period) of this study are summarised in Figures 1 and 2 and Table 1.

The changes in the populations of resident bifidobacteria upon ingestion of three different doses (high, medium and low) of B. lactis HN019 are shown in Fig. 1. The ANOVA analysis of data showed significant differences over time ($p < 0.0001$) and between the groups ($p < 0.008$). In the placebo group, the resident bifidobacteria remained at relatively lower levels (mean 9.31 ± 0.01 log CFU/g of faeces) throughout the period of study. The bifidobacteria population increased as a result of consumption of three (high, medium and low) different doses of the probiotic. At the end of the 4-week feeding period, bifidobacteria were present at levels of 9.88 ± 0.1, 9.75 ± 0.14 and 9.74 ± 0.11 log CFU/g of faeces in the high, medium and low dose groups respectively. These levels were statistically significant (p<0.006 to p<0.0005), as compared to the respective pre-intervention levels. Relatively small increase in bifidobacteria count (appox. 0.3 log units) was observed during dietary intervention in placebo group. When the probiotic dietary intervention was stopped for 2 weeks (after wash out period), the bifidobacteria counts decreased in treatment groups, but remained higher than the respective pre-intervention levels. The bifidobacteria mean counts ranged from 9.14 ± 0.11 to 9.42 ± 0.13 log CFU/g of faeces.

Figure 1

Impact of different *Bifidobacterium lactis* HN019 doses on the bifidobacteria population of elderly subjects. Data are expressed as mean log CFU/g of wet faeces ± standard error. Placebo (n = 14), High dose (n = 19), medium dose (n = 15), low dose (n = 18) were administered between week 0 and week 4. Bifidobacteria counts were enumerated before (pre-feed), during (during-feed) and after (post-feed) the probiotic administration.



When compared to the respective pre-feed/intervention levels the differences were significant *(P<0.0005); **(P<0.006); ***(P<0.005)

Table 1

Impact of different doses of B. lactis HN019 on resident total anaerobes, faecal streptococci and coliforms

Group/Microflora	Pre-intervention	During-intervention Week 2	During-intervention Week 4	Post-intervention
Placebo group				
Total anaerobes	10.37 ± 0.07	10.49 ± 0.10	10.51 ± 0.14	10.11 ± 0.21
Faecal streptococci	5.35 ± 0.23	5.38 ± 0.17	5.36 ± 0.10	5.20 ± 0.17
Faecal coliforms	8.32 ± 0.32	8.36 ± 0.20	8.02 ± 0.17	8.05 ± 0.26
High dose group				
Total anaerobes	10.31 ± 0.08	10.46 ± 0.11	10.46 ± 0.10	10.10 ± 0.15
Faecal streptococci	5.28 ± 0.27	6.64 ± 0.33[a]	6.54 ± 0.27[a]	6.03 ± 0.17
Faecal coliforms	8.36 ± 0.09	8.24 ± 0.21[a]	8.04 ± 0.17[a]	7.95 ± 0.26
Medium dose group				
Total anaerobes	10.38 ± 0.06	10.51 ± 0.07	10.52 ± 0.10	10.05 ± 0.15
Faecal streptococci	5.35 ± 0.14	6.22 ± 0.21[b]	5.87 ± 0.19[b]	5.62 ± 0.29
Faecal coliforms	7.90 ± 0.28	7.98 ± 0.18	7.68 ± 0.15[b]	7.57 ± 0.16
Low dose group				
Total anaerobes	10.18 ± 0.10	10.17 ± 0.11	10.08 ± 0.14	10.23 ± 0.17
Faecal streptococci	6.31 ± 0.30	6.63 ± 0.30	6.23 ± 0.30	6.11 ± 0.27
Faecal coliforms	8.21 ± 0.16	8.25 ± 0.14	7.98 ± 0.16	7.64 ± 0.26

The data are expressed as mean log CFU per gram of wet faeces (± SE). When compared with the respective pre-intervention levels the differences were significantly different a(P<0.005); b(P<0.05)

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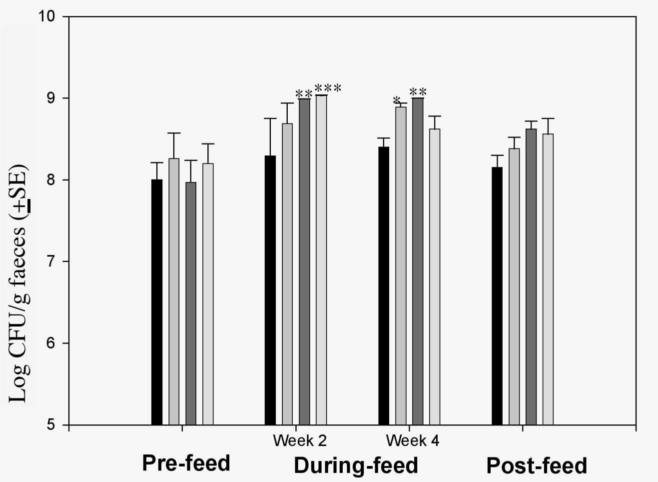
Figure 2

Impact of different *Bifidobacterium lactis* HN019 doses on the lactobacilli population of elderly subjects. Data are expressed as mean log CFU/g of wet faeces ± standard error. High dose (n = 17), medium dose (n = 16), low dose (n = 14) and placebo (n = 13) were administered between week 0 and week 4. Lactobacilli counts were enumerated before (pre-feed), during (during-feed) and after (post-feed) the probiotic administration. The legend is as described in Fig. 1.



When compared to the respective pre-feed/intervention levels the differences were significant *(P<0.05); **(P<0.005); ***(P<0.006)

The effect of feeding different doses of *B. lactis* HN019 on the resident lactobacilli population is shown in Fig. 2. The mean lactobacilli population differed significantly in the different dietary groups ($p < 0.05$) and over the period of feeding ($p < 0.0001$). After feeding the three different doses of *B. lactis* HN019 for 4 weeks, the lactobacilli mean counts were 8.90 ± 0.13, 9.00 ± 0.01 and 8.62 ± 0.16 log CFU/g of faeces for high, medium and low dose levels, respectively. The levels for the high and medium doses were statistically significant ($p<0.05$ and $p<0.005$) from their respective pre-intervention levels. The placebo group also showed an increase in the lactobacilli count from 8.00 ± 0.21 log CFU/g of faeces in the pre-feed period to 8.4 ± 0.11 log CFU/g of faeces at the end of 4-week intervention period. The difference was not statistically significant ($p>0.05$). Further, the lactobacilli counts in treatment group remained higher than the respective placebo group. At the end of 2 weeks after dietary intervention (post-intervention period), the lactobacilli counts were 8.38 ± 0.14, 8.62 ± 0.1 and 8.56 ± 0.19 log CFU/g of faeces in the high, medium and low dose groups respectively. In all cases, the lactobacilli counts were higher than the respective pre-intervention levels (8.26 ± 0.31, 7.97 ± 0.27 and 8.2 ± 0.24 log CFU/g of faeces).

The response of the faecal streptococci and enterococci population to the different doses of *B. lactis* HN019 is summarised in Table 1. The mean enterococci counts differed significantly ($p < 0.0001$) in the different feeding groups (placebo, low, medium and high dose). Further, there were significant ($p < 0.0001$) differences in the mean enterococci counts at the various feeding intervals. At the end of 4 weeks of feeding different doses of the probiotic preparation, the mean counts of enterococci increased in all dose groups (Table 1). When compared to the respective pre-feed levels, statistically significant differences ($p<0.05$ to $p<0.005$) were observed in case of high and medium doses (Table 1). The mean placebo count, however, remained relatively unchanged over the study period. At the end of post-dietary intervention period, the enterococci remained comparatively higher in the treatment groups and the mean bacterial count ranged from 5.62 ± 0.29 to 6.11 ± 0.26 log CFU/g of faeces (Table 1).

Changes in the faecal coliform or enterobacteria population during this dietary intervention study are shown in Table 1. The differences in mean enterobacteria counts across the groups (placebo, and low, medium and high dose) showed moderate significance ($p < 0.02$). The changes in the mean coliform population over time showed a higher level of significance ($p < 0.009$). In general, coliforms showed a decreasing trend over the feeding time. The high dose group showed a decrease from 8.36 ± 0.09 log CFU/g during pre-intervention to 8.04 ± 0.17 at the end of 4 week intervention (Table 1). This difference was found to be statistically significant ($p < 0.005$). Similar trends were observed with medium and low dose groups.

Total anaerobes (shown in Table 1), remained relatively unaltered (a range of 10.05 ± 0.15 to 10.52 ± 0.10 log CFU/g) in the different groups (placebo, and high, medium and low probiotic dose groups). Following a similar trend, the bacteroides counts (mean 9.0 ± 0.3 log units) remained relatively unchanged between the feeding groups (data not shown). The yeast and mould counts showed large variations between individuals and no sensible interpretation was possible (data not shown).

Discussion

We have previously demonstrated the immune-enhancing (4, 5, 8, 9, 21), anti-infection (22, 23), safety profile (24, 25) and molecular characterisation (26) of the probiotic strain *B. lactis* HN019. The microflora changes as a result of dietary intervention with *B. lactis* HN019 at a relatively high dose of 3 x 10^{10} CFU/day and transient colonisation of *B. lactis* HN019 in healthy adult human subjects has also been demonstrated (27). The present investigation was designed to study the effect of *B. lactis* HN019 at three different doses (low, 6.5 x 10^7 CFU/day; medium, 1 x 10^9 CFU/day; high 5 x 10^9 CFU/day) on the intestinal flora of the elderly subjects. During the study period, no dietary-intervention related adverse health effects were reported by any of the elderly participants indicating its suitability to this age group. In order to keep the faecal flora variation to a minimum, the time lapse between the sample collection and analysis was kept below 3 hrs. Sometimes samples did not fit into this criterion, those samples were, therefore, excluded from the analysis (this is reflected in the data analysis as n value).

In this elderly population we found that the base level bifidobacteria count ranged between 9.14 ± 0.11 to 9.48 ± 0.14 log CFU/g of faeces (as observed from the data from placebo group and pre-feeding period of treatment groups).These values are slightly higher than the values reported for elderly population (aged 73 to 101 years) by Woodmansey *et al.* (13) who reported a mean bifidobacteria count of 8.1 ± 1.6 CFU/g in faeces. This variation may be a reflection of the fact that subjects were considerably older than the subjects in the present study. By contrast, in a dietary intervention study with B. lactis Bb12 and involving children (9 to 36 months), Fukushima et al. (28) reported a base level count of 10.14 ± 0.29 log CFU/g of faeces. In another investigation involving younger adults (ages between 21 to 34 years), Hopkins and Macfarlane (29) reported counts as high as 9.8 log CFU/g of wet faeces. These studies support generally held belief that the bifidobacteria counts decrease with age and our findings are consistent with this belief.

Bifidobacteria constitute a large proportion of the gut flora, they make a significant contribution to carbohydrate fermentation in the colon. Age associated reduction in the population of bifidobacteria could shift the homeostasis or microbial balance in favour of potentially harmful bacterial groups such as enterobacteria, clostridia and proteolytic bacteria. He et al (30), compared the *in vitro* adhesive properties of 51 Bifidobacterium strains from healthy younger adults (30 to 40 years) and seniors (over 70 years) and showed that mucosal adhesive properties of the human bifidobacteria were reduced with aging of the host. This could be one of the reasons for the reduced bifidobacteria levels in the elderly.

In the present study we found that the dietary intervention with all three dose levels of *B. lactis* HN019 resulted in statistically significant increase in the resident population of bifidobacteria in the elderly subjects (Fig. 1). We hypothesise that because of the natural low levels of bifidobacteria in elderly population, it is possible to effect the significant increases in the population through dietary intervention strategies. In some individual subjects in the current study we were able to achieve increments up to 0.8 log units in bifidobacteria at the end of 4 weeks of dietary intervention. As there were no statistically significant differences between the means of the three dose groups, we conclude that even the lowest dose (6.5 x 10^7 CFU/day) tested could achieve significant increases in the resident bifidobacteria population in elderly human subjects.

The lactobacilli are another group of lactic acid bacteria known to have probiotic properties. In our study, the lactobacilli population showed an increasing trend during dietary intervention with *B. lactis* HN019. This effect may have been due to the low pH environment prevailing in the lower gut (31) as a result of increased proliferation of bifidobacteria (major metabolic end products are acetate and lactate) and such environments would be favourable to the proliferation of lactobacilli. These findings are consistent with previous studies (28, 32). Increases in the lactobacilli population were observed for all the dose levels tested. This observation suggests that the lowest dose (6.5 x 10^7 CFU/day) of *B. lactis* HN019 may also augment the levels of lactobacilli in the intestinal tract of elderly subjects. We also found a placebo effect, by way of an increase in both bifidobacteria and lactobacilli during dietary intervention. This effect may be due to lactose present in the base milk powder consumed by the placebo group subjects and lactose is preferentially utilised by lactic acid bacteria.

Facultative bacteria, namely enterobacteria and faecal streptococci (or enterococci), were also monitored in this study (Table 1). Interestingly, upon administration of *B. lactis* HN019, irrespective of the dose, significant increases in the faecal streptococci population were observed. For example, with the highest dose administered for 4 weeks, the enterococci increased by one log unit to 6.5 ± 0.3 log CFU/g. Similar increases in enterococci have been reported (33) in healthy adult faecal flora after dietary intervention with *Lb. rhamnosus* HN001. It could be speculated that there may be a synergistic interaction between bifidobacteria, lactobacilli and enterococci in human gut.

The enterobacteria numbers are believed to increase in elderly human subjects as a consequence of aging. Hopkins *et al.* (17) reported a range of 6.0 to 7.5 log CFU in elderly as compared to 5.5 to 6.5 log CFU in young adults. The enterobacteria levels have been shown to reach over 8 log CFU units/g of faeces (34). Supporting this observation, our results (Table 1) showed enterobacteria counts of 8.3 ± 0.32 log CFU/g of faeces in the placebo group. In this study, as compared to the respective pre-intervention levels, the enterobacteria were significantly ($p < 0.005$) reduced at the end of 4 weeks after the administration of *B. lactis* HN019 (high dose). This may be due to antagonistic activity of *B. lactis* HN019 previously reported against enterobacteria such as *Escherichia coli* (22) *and Salmonella typhimurium* (23). Thus the consumption of *B. lactis* HN019 may confer additional benefit by decreasing the enterobacteria population in human gut, the natural levels of which increase during the aging process. This phenomenon may be translated into colonisation resistance against entero-pathogenic bacteria in the elderly. Mechanisms of such effect are likely to include the secretion of acids (lactate, acetate), competition for nutrients and gut receptor sites, immuno-modulation and the formation of specific antimicrobial agents (35).

The total anaerobes constitute several genera including Bacteroides, Eubacterium, Peptostreptococcus, Veillonella and Megasphaera. In this investigation, the population of total anaerobes did not alter significantly upon dietary intervention with any of the doses of B. lactis HN019 tested (Table 1). Similar observations have been reported for dietary intervention in adult human subjects with *B. longum* (36), in healthy children with *B. bifidum* Bb12 (28) and in healthy infants with *Lb. casei* (37).

In the present study, by using a dietary intervention strategy with *B. lactis* HN019, we observed that the bifidobacteria population, along with other potentially beneficial bacteria groups such as lactobacilli and faecal streptococci, were significantly increased in elderly human subjects. In addition, potentially harmful enterobacteria were reduced, shifting the microbial balance in favour of a healthy gut. Furthermore, an important finding from this study is that an effective daily dose of *B. lactis* HN019 for achieving a favourable change in microbial balance in the gastrointestinal tract of elderly is as low as 6.5 x 10^7 CFU/day. These changes may contribute to improvement in the intestinal microflora balance to the advantage of the host and hence well-being. Now it is established that the probiotic supplemented consumer products provide a daily dose of 10^7 to 10^8 CFU and this study indicates that regular consumption of *B. lactis* HN019 supplemented consumer products provide health benefit to the elderly by way of ameliorating the bifidobacteria population in the gut. The beneficial changes in the intestinal microflora may contribute to healthier gut and general well-being of the host. Systematic studies involving host microbe interaction combined with biochemical parameters associated with intestinal milieu would present an opportunity to further understand the basis of homeostasis and the probiotic effects associated with dietary intervention.

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