Dairy Sci. Technol. 89 (2009) 219–231
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DOI: 10.1051/dst/2009012

Available online at:
www.dairy-journal.org

Original article

The effect of *Lactobacillus rhamnosus* HN001 on mineral absorption and bone health in growing male and ovariectomised female rats

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Received 23 September 2008 – Accepted 27 February 2009

Abstract – Prebiotics affect mineral absorption, in part by increasing solubility due to short chain fatty acid generation. Prebiotics stimulate the growth of probiotics in the gut and probiotics may affect mineral absorption by similar mechanisms. The objectives of this study were to measure (1) the effect of *Lactobacillus rhamnosus* strain HN001 (HN001) on mineral absorption in growing male rats and (2) whether HN001 can reduce bone loss and affect bone properties in female ovariectomised (OVX) rats. *Study 1*: Twenty-two 3-week-old Sprague-Dawley rats were weaned onto a milk powder-based diet supplying 20% protein and 0.5% calcium. After a week, the rats were randomised into two groups: one continuing on the base diet and the other receiving 10^9 cfu HN001 per day in the diet. After 3 weeks, the animals were housed in metabolic cages for 8 days. Food intake was measured, and urine and faeces were collected for 3 days. Calcium and magnesium balances were calculated. *Study 2*: Forty 5.5-month-old female Sprague-Dawley rats were ovariectomised or sham-operated and randomised into three groups. The sham (n = 10) and OVX control groups (n = 15) remained on a caseinate-based control diet, while the experimental group (n = 15) received 10^9 cfu HN001 per day in the diet. After 12 weeks, bone density was measured by dual energy X-ray absorptiometry and bone and blood samples were collected. HN001 improved calcium and magnesium retention in the growing male rats, but due to differences in food intake, data was inconclusive. However, HN001-fed OVX rats had a reduced rate of bone loss and a higher final bone density in the spine and femur at week 12 compared to the OVX control group. HN001 may improve mineral bioavailability and have a positive effect on bone mineral density and mineral content. The underlying mechanisms need to be researched.

ovariectomised rat / bone density / *L. rhamnosus* / biomechanical strength / mineral bioavailability

摘要 – 鼠李糖乳杆菌 HN001 对雄性和卵巢切除雌性大鼠矿物元素吸收和骨健康的影响。益生元影响矿物元素吸收的部分原因是产生的短链脂肪酸增加了矿物元素的溶解性。益生元在肠道中刺激益生菌的生长，而益生菌以同样的机制影响着矿物元素的吸收。本研究测定内容包括: (1) 鼠李糖乳杆菌 *Lb* HN001（*Lactobacillus rhamnosus*）对生长期雄性大鼠矿物元素吸收的影响; (2) *Lb* HN001 是否会降低卵巢切除雌性大鼠 (OVX) 的骨丢失和影响骨质性质。研究内容包括: (1) 22 只 3 周龄的 Sprague-Dawley 大鼠喂饲的基础饲料为含有 20% 蛋白质和 0.5% 钙的奶粉基饲料，一周后试验鼠被任意地分成为两组，一组大鼠继续喂基础饲料，另一组大鼠在基础饲料的基础上每天摄入 10^9 cfu 的 *Lb* HN001。3 周后将试验鼠放入代

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Article published by EDP Sciences

谢笼中继续喂养 8 d。测定食物的摄入量，每隔 3 d 收集尿和粪便，测定和计算钙和镁平衡。(2) 将 40 只 5.5月龄的雌性 Sprague-Dawley 大鼠进行卵巢切除手术和假手术，然后将这些大鼠任意的分成 3 组。假手术组 (n = 10) 和 OVX 对照组 (n = 15) 喂饲的基础饲料为酪蛋白基的半合成饲料，而试验组 (n = 15) 是在基础饲料的基础上每天补充 10^9 cfu 的 *Lb* HN001。12 周后，采用双能量 X 光吸光测定法测定骨密度并采集骨和血液样品。 研究结果表明，*Lb* HN001 能够改善钙和镁在生长期雄性大鼠体内的保持力，但是由于食物摄入量的差异，对此结果还不能给出准确的结论。然而，与 OVX 对照组比较，12 周龄的 *Lb* HN001-OVX 试验组大鼠的骨丢失量较低，并且脊骨和大腿骨的骨密度较高。*Lb* HN001 能够改善矿物元素的生物利用率，对骨中矿物元素密度和矿物元素含量有正面影响，但是更深入的机制有待进一步研究。

卵巢切除大鼠 / 骨密度 / 鼠李糖乳杆菌 / 生物机制 / 矿物元素的生物利用率

Résumé – Effet de *Lactobacillus rhamnosus* HN001 sur l'absorption minérale et la santé osseuse chez le rat mâle en croissance et la ratte ovariectomisée. Les prébiotiques ont un effet sur l'absorption minérale, en partie en augmentant la solubilité due à la génération d'acides gras à chaîne courte. Les prébiotiques stimulent la croissance des probiotiques dans l'intestin et les probiotiques peuvent affecter l'absorption minérale par des mécanismes similaires. Les objectifs de l'étude ont été de mesurer : (1) l'effet de *Lactobacillus rhamnosus* HN001 (HN001) sur l'absorption minérale chez le rat mâle en croissance, (2) si HN001 peut réduire la perte osseuse et affecter les propriétés des os chez la ratte ovariectomisée (OVX). *Étude 1* : 22 rats Sprague-Dawley de 3 semaines ont été sevrés puis soumis à un régime à base de poudre de lait apportant 20 % de protéines et 0,5 % de calcium. Après une semaine, les rats ont été répartis au hasard en deux groupes : l'un continuant le régime de base, l'autre recevant en plus 10^9 ufc HN001 par jour. Après 3 semaines, les animaux ont été installés dans des cages métaboliques pour 8 jours. La prise alimentaire a été mesurée, et l'urine et les fèces récoltés pendant 3 jours. Les équilibres en calcium et magnésium ont été calculés. *Étude 2* : 40 rattes Sprague-Dawley de 5 mois et demi ont été ovariectomisées ou opérées de façon fictive puis réparties au hasard en 3 groupes. Les groupes « fictif » (n = 10) ou OVX de contrôle (n = 15) étaient maintenus au régime contrôle à base de caséinate tandis que le groupe expérimental (n = 15) recevait le même régime additionné de 10^9 ufc HN001 par jour. Après 12 semaines, la densité osseuse a été mesurée par absorptiométrie à rayons X biphotonique et des échantillons d'os et de sang ont été collectés. HN001 améliorait la rétention de calcium et magnésium chez les rats mâles en croissance, mais, en raison de différences dans la prise alimentaire, les données étaient peu concluantes. Cependant, les rattes OVX nourries avec HN001 montraient moins de perte osseuse et une densité osseuse finale plus élevée dans la colonne vertébrale et le fémur à 12 semaines que les rattes du groupe contrôle. HN001 pourrait améliorer la biodisponibilité minérale et avoir un effet positif sur la densité minérale osseuse et la teneur en minéraux. Les mécanismes qui sous-tendent cette activité restent à déterminer.

ratte ovariectomisée / densité osseuse / *Lactobacillus rhamnosus* / propriété biomécanique / biodisponibilité minérale

1. INTRODUCTION

Several studies in the past 10 years report the beneficial effects of prebiotics on mineral absorption. Prebiotics are non-digestible oligosaccharides (NDOs) that beneficially affect the host by selectively stimulating the growth or the activity of one or more specific bacterial species (probiotics) in the gastrointestinal tract thereby affecting the host's health, especially by enhancing mineral absorption [1–4, 7, 16–21].

NDOs pass into the large intestine where they are fermented by bacteria into short chain fatty acids (SCFAs), acetic, propionic and butyric acids [1, 7]. Thus, they provide probiotics with a source of food that in turn provides the host with energy, metabolic substrates and nutrients [16]. Several studies in animals and in human beings have shown

that prebiotics improve mineral absorption by producing an acidic environment in the gut with resultant increases in bone mineral density (BMD) or bone mineral content (BMC) [17–21].

Alternatively, the endogenous bacterial population can be manipulated by the use of probiotics, where exogenous bacteria are introduced into the colonic microflora. Probiotics may be defined as viable microorganisms that (when ingested) have a beneficial effect on the health and metabolism of their host. The most popular strains are represented by the following genera: *Lactobacillus*, *Streptococcus* and *Bifidobacterium* [3, 16, 17].

The mechanisms by which probiotics exert biological effects are still poorly understood. One possible mechanism is whereby the indigenous anaerobic flora limits the concentration of potentially pathogenic flora in the digestive tract. Although probiotic bacteria are thought to mediate their effects using some of the same mechanisms as the native intestinal flora, probiotics may also work through other modes of action such as supplying enzymes or influencing enzyme activity in the gastrointestinal tract [1, 3, 7, 16, 17].

There have been few studies published on the effect of probiotics on mineral absorption. Ghanem et al. showed that yoghurt with probiotics when fed to rats, increased SCFA production and improved calcium absorption [4]. Perez-Conesa et al. [14] fed functional follow-on infant formulae containing pre- and/or probiotics to rats, and found that calcium and magnesium absorption was enhanced and bone calcium improved in groups fed with synbiotics, a mixture of pre- and probiotics. The formula containing only probiotics improved tibial calcium content significantly compared to the control diet devoid of pre- and probiotics. These results suggested that probiotics even in the absence of prebiotics may affect mineral balance. Narva et al. showed that *Lactobacillus helveticus* increased calcium absorption and improved bone mass in growing rats fed with fermented milk for 14 weeks [9].

In light of these findings we:

- investigated the effect of *Lactobacillus rhamnosus* HN001 on mineral absorption and retention in the growing male rat (Study 1);
- tested whether long-term intake (3 months) of *L. rhamnosus* HN001 affected bone loss and bone properties in the female ovariectomised (OVX) rat (Study 2).

2. MATERIALS AND METHODS

2.1. Materials

The high-calcium milk powder (HCMP; Anlene™) was supplied by Fonterra Brands Pty Ltd. (Auckland, New Zealand). This milk powder contained 2000 mg calcium, 140 mg magnesium and 1480 mg phosphorus per 100 g. The probiotic *L. rhamnosus* strain HN001 was obtained from Fonterra Cooperative Group Ltd. (Auckland, New Zealand).

2.2. Study 1

2.2.1. Animals

Twenty-two 3-week-old male Sprague-Dawley rats ($n = 11$ per group) were obtained from the Small Animal Production Unit, Massey University. The animals were housed separately in shoebox cages, and kept at 22 ± 2 °C, under a 12-h light: 12-h dark cycle in temperature and light-controlled room in the Small Animal Production Unit. Animals had ad libitum access to deionised water and food intake was recorded daily. Ethical consent was obtained from the Massey University Animal Ethics Committee (Approval Number 05/07).

Table I. Diet formulation for the growing rats (Study 1) and for the ovariectomised rats (Study 2).

Constituents (amount in g)	Study 1	Study 2
HCMP	250.0	
Caseinate		140.0
Sucrose	50.0	57.5
Amino acids[a]	40.2	
Mineral mix[b]	50.0	50.0
Vitamin mix	50.0	50.0
Cellulose	50.0	50.0
Corn oil	50.0	40.0
Calcium carbonate		12.5
Starch	459.8	600.0
Total	1000.0	1000.0

HCMP, high calcium milk powder.
[a] Amino acids included: alanine, cysteine, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, threonine and valine.
[b] Cysteine was added to the mineral mix for Study 2.

2.2.2. Diets

Animals were weaned onto the base diet 10 days prior to the start of the trial (week 1). At week 1, animals were randomised into one of the two dietary groups, i.e. HCMP supplying 20% protein, 0.5% calcium and 5% fat ± HN001 (group HCMP+ or HCMP−). The probiotic HN001 was mixed into a weekly ration, which was then subdivided into daily doses for each diet, to ensure that animals received a constant dose of probiotics. Probiotic diets were stored at 4 °C to prevent oxidation and death of bacteria. Both diets were formulated on the recommendations AIN93G according to the Subcommittee on Laboratory Animal Nutrition (Tab. I) [12].

2.2.3. Balance studies

At week 3, animals (aged 7 weeks) were placed in individual metabolism cages suitable for rats up to 600 g (Techniplast; Buguggiate, Italy) for 8 days (5 days acclimatisation and 3 days collection). Daily food intake was measured. Urine and faeces were collected in pots positioned at the bottom of the cage; 500 µL of 1 mol·L^{-1} HCl was added to the urine-collection pots to prevent bacterial growth and nitrogen breakdown. The collection pots were emptied daily and the samples were frozen at −20 °C until assayed. After 8 days, animals were returned to shoebox cages.

Urine and freeze-dried homogenised faecal samples, together with a sample of each diet, were analysed for calcium and magnesium. Samples were digested in a mixture of 2 mL nitric acid and 0.5 mL HCl for 1 h at 85 °C. The samples were then diluted in water (containing a small amount of Triton to aid passage through instrument tubing), and were analysed using a Thermo Elemental Intrepid II XDL (Intrepid) Inductively Coupled Plasma-Optical Emission Spectrophotometer (ICP-OES) (Franklin, TN, USA).

Absolute mineral absorption (mg·day^{-1}) was calculated by subtracting the amount lost in urine and faeces from the total intake from the diet. Fractional mineral absorption (%) was calculated by dividing the mineral balance by intake and multiplying by 100.

2.2.4. Statistical analysis

Results were analysed using SPSS version 15.0.1.1 (SPSS, Inc., Chicago, IL, USA). A $P < 0.05$ was considered significant. Levene's test was used to ensure that groups had equal variances. The two groups were compared using a one-tailed Student's t test. Values are expressed as mean ± standard error of the mean (SEM).

2.3. Study 2

2.3.1. Animals

Forty 5.5-month-old female Sprague-Dawley rats were obtained from the Small

Animal Production Unit, Massey University. The animals were sham-operated or ovariectomised aged 6 months (week 0 of the study). Sham-operated animals (n = 10) were anaesthetised, an incision made, but the ovaries were left intact. In the OVX animals, the ovaries were removed, and these OVX animals were randomised into two groups (n = 15 per group).

The animals were separately housed in shoebox cages, and kept in a temperature- (22 ± 2 °C) and light-controlled (12 h day/night cycle) room in the Small Animal Production Unit, Massey University. These animals had ad libitum access to deionised water. The test animals were fed a casein-based semi-synthetic diet to which HN001 was added. The sham-control group and the OVX control group received the base diet with no probiotics added. The daily food intake of the animals was measured, and the intake was adjusted weekly according to the sham group's intake to prevent excessive body weight gain in the OVX groups. The trial ran for 3 months with monthly measurements. The Massey University Animal Ethics Committee approved the study (02/76).

2.3.2. Diets

The animals were fed a balanced semi-synthetic diet consisting of 14% caseinate, 5% cellulose, 4% corn oil, 0.5% calcium, 60% starch and 5% added vitamins and minerals as needed from week −2 (Tab. I). Once ovariectomised, the animals were fed with either the control diet (sham and OVX control) or a diet to which 10^9 cfu HN001 per rat per day was added. The control diet was prepared in bulk and the probiotics were added weekly. The diet was prepared a week in advance and stored in the absence of oxygen in a sealed bag at −20 °C. Monthly plate counts were conducted to check bacterial viability.

2.3.3. Dual energy X-ray spectrometry scans

Animals were scanned for baseline measurements at week −2, and then ovariectomised at week 0. During the trial, the animals were scanned at 4 and 11 weeks under anaesthesia, and they were weighed and anaesthetised with an appropriate dose of anaesthetic, i.e. 0.05 mL·100 g^{-1} body weight. The anaesthetic was a mixture of 0.2 mL acepromazine, 0.5 mL ketamine, 0.1 mL xylazine and 0.2 mL sterile H_2O in 1 mL, and was administered via an *intraperitoneal* injection using a 25 g × 5/8″ needle and 1 mL syringe. The animals attained a suitable level of anaesthesia ~ 5–10 min after injection and remained under anaesthesia for 2 h.

Bone mineral measurements were taken using a Hologic QDR4000 bone densitometer pencil beam unit (Bedford, MA, USA). On each day the scans were performed, a quality control (QC) scan was taken to ensure that its precision met the required coefficient of variation (CV). The CV for the QC data was 0.98−1.01. Regional high-resolution scans were performed using a 1.5-mm diameter collimator with 0.31-mm point resolution and 0.5-mm line spacing. Rats were placed on an acrylic platform of uniform 37.5-mm thickness. Each rat underwent three regional high-resolution scans of the spine and left and right femurs. They were positioned supine with right angles between the spine and the femur, and between femur and tibia.

The CV for the femurs ranged between 0.92% and 0.85% with and without repositioning between scans. These values ranged between 1% and 0.98% for the spine.

2.3.4. Terminal heart puncture

After 12 weeks, the animals were weighed and anaesthetised with an appropriate dose of anaesthetic, i.e. 0.1 mL·100 g^{-1}

body weight. A 19 g × 1½″ needle and 5 mL syringe were used to withdraw blood directly from the heart. The animals were then euthanised by exsanguination under anaesthesia (19 g × 1½″ needle and 5 mL syringe), and dissected. Both the right and the left femur and spine were excised with some flesh remaining and frozen (−20 °C) in phosphate-buffered saline (PBS) for further analysis. The uteri were removed and weighed to confirm that OVX had been successful.

2.3.5. Mechanical properties of bone

The fracture load was defined as the maximum force (N) required to break the bone using the three-point bending method. Breaking energy (J) is an integration value (area under the force/displacement curve) of force that is required to fracture the bone, or can be defined as the total amount of energy a bone must absorb in order to cause a break. Breaking strength reflects the mineral content as well as the protein component of the bone, while breaking energy is thought to reflect the collagen content of the bone. Measured stiffness ($N \cdot mm^{-2}$) reflects the distance in mm by which bone can bend under the applied load of 400 N, without permanent deformation (plasticity). Break stress is the force per unit area which causes the bone to break [8].

The right femurs were scraped clean of adhering flesh and stored in PBS solution at −20 °C. Before biomechanical testing, the bones were thawed. The length of the femurs was measured using an electronic calliper. The midpoint was marked with a waterproof pen and the width and thickness of the femurs at midpoint were also recorded. The femurs were then held at 23 °C to be at room temperature before and during the test. The femurs were placed in a testing jig constructed for a three-point bending test. The distance between the supporting rods had a fixed length of 12 mm. Load was applied at a constant deformation rate of 50 $mm \cdot min^{-1}$. Maximum load (N), stiffness ($N \cdot mm^{-2}$) and energy (J) were measured using a Shimadzu Ezi-test texture analyser (Kyoto, Japan).

2.3.6. Blood assays

Heart puncture blood samples were taken while the rat was anaesthetised and the blood for plasma was mixed in tubes with the appropriate anticoagulant (heparin) and stored on ice until the plasma was separated by centrifugation at 1600× *g* for 10 min at 4 °C. Blood for serum was allowed to clot at ambient temperature for 30 min, then separated by centrifugation at 1600× *g* for 10 min at 4 °C.

Heparin plasma was stored on ice until aliquoted into tubes and frozen at −80 °C, within 2 h of collection, for bone marker analysis. Osteocalcin and CTx (C-terminal telopeptides of Type 1 collagen) concentrations were measured by enzyme-linked immunosorbent assay (Rat-MID® Osteocalcin ELISA or RatLaps® ELISA; Nordic Bioscience Diagnostics, Denmark).

2.3.7. Statistical analyses

Comparisons between the treatment groups were performed in two ways. Where no correction factor was applied, differences were tested using one-way analysis of variance with "least squares difference" post hoc comparison testing. Where data required correction for body weight or bone weight, they were analysed using the univariate analysis of variance with either of those variables as covariate, and using the same post hoc analysis. For the femur BMC and BMD analyses, the values for the two femurs of each rat were averaged. Differences were assumed significant at $P < 0.05$. Results were analysed using SPSS version 15.0.1.1. Values and graphs are expressed and shown as mean ± SEM.

3. RESULTS

3.1. Study 1

There were no significant differences in body weights or gain during the balance period of 3 days (Tab. II). The mineral content of the diets was analysed and was 5530 mg·kg^{-1} for calcium and 1310 mg·kg^{-1} for magnesium. The same base diet was used for both groups with probiotics added weekly.

Table II shows the effect of 3 weeks of feeding HCMP ± probiotics on calcium and magnesium balance. During the balance period of 3 days, the food intake, and calcium and magnesium intake were significantly higher in the HCMP+ group, which then resulted in a significantly higher absolute retention of both minerals. Fractional calcium as well as magnesium retention for groups fed with HCMP− and HCMP+ was not statistically different.

3.2. Study 2

There were no significant differences in body weight gain, the final body weights or food intake at week 12 (aged 9 months). Body weight varied between 404 and 427 g (P = 0.401) and daily food intake between 21 and 23 g per day (data not shown). Uterus weights were significantly lower in the two OVX groups as compared to the sham group (between 0.15 and 0.18 g in OVX vs. 0.75 g in the sham group).

Table III summarises in vivo BMD measured at weeks −2, 4 and 12, as well as biochemical markers for bone turnover and resorption measured at week 12. Plasma CTx as well as osteocalcin were increased in both OVX groups, and significantly higher than for the sham group. All OVX animals lost bone (BMC and BMD) compared to the sham group in the first 4 weeks after OVX. Thereafter, it appears that bone loss in the HN001-fed group was slower than that in the OVX group, resulting in the final BMC and BMD that were significantly lower than that of the sham group but significantly higher than the OVX group. Femoral BMC and BMD showed a similar pattern with bone loss due to ovariectomy in the OVX and HN001 groups, and final BMC and BMD for the *Lb* HN001 group was significantly higher than that of the OVX group but still lower than that of sham group. Figure 1 shows the percentage change in lumbar spine BMC and BMD, as well as femur BMC and BMD over time during the study.

Loss of lumbar spine BMC for the group fed with HN001 was not significantly different from sham or OVX during the time period, weeks 4–11 (Fig. 1A), while the loss for the OVX group was significantly more than that for the sham group. Lumbar spine BMD also indicated that bone loss of the HN001 group between weeks −2 and 4 was significantly more than that in the sham group, but between weeks 4 and 11 BMD increased in this group and final values were higher than OVX and not different from sham (Fig. 1B).

A similar pattern in the loss of BMC was observed for the pooled femur results (Fig. 1C and D). The BMC changes from week 4 to 11 in the HN001 group were significantly different from sham and also significantly different from OVX. Femur BMD indicated similar loss from weeks −2 to 4 and weeks 4 to 11 for the OVX as well as for the HN001 group, both being significantly lower than sham at week 12. The spine data and the femur BMC data suggest that once established in the gut, therefore after 4 weeks of ingestion, HN001 slows down bone loss significantly in both lumbar spine and femur.

Table IV summarises the biomechanical data for the right femurs collected from sham and OVX rats fed with HN001 for 12 weeks. There were no significant differences between the various parameters due to large variation in the data. However, HN001 seemed to consistently bring the measurements closer towards sham values.

Table II. Fractional and absolute retention of calcium (Ca) and magnesium (Mg) in growing male rats (n = 11) following 3 weeks of feeding HCMP plus 10^9 cfu HN001 per day (HCMP+) or minus HN001 (HCMP−) in the formulated diets. Values are given as mean (SEM).

	HCMP−	HCMP+
Body weight (g; day 5)	213.98 (6.43)[a]	223.48 (3.14)[a]
Body weight (g; day 8)	227.62 (7.67)[a]	242.95 (3.06)[a]
Weight gain (g)	13.64 (2.79)[a]	19.47 (0.86)[a]
Total food intake (g; 3 days)	44.03 (3.48)[a]	58.05 (0.90)[b]
Ca intake (mg; 3 days)	239.13 (19.55)[a]	317.23 (5.00)[b]
Mg intake (mg; 3 days)	56.65 (4.63)[a]	75.15 (1.19)[b]
Absolute Ca retention (mg; 3 days)	112.75 (15.93)[a]	165.34 (4.17)[b]
Fractional Ca retention (%)	46.59 (5.34)[a]	52.14 (1.08)[a]
Absolute Mg retention (mg; 3 days)	9.41 (4.42)[a]	19.55 (0.91)[b]
Fractional Mg retention (%)	15.00 (8.70)[a]	26.15 (1.40)[a]

Values with different superscripts (a and b) denote significant differences between groups on the same row.

Table III. Baseline and final in vivo bone density and biochemical markers of bone turnover in rat plasma after being fed with *L. rhamnosus* HN001 for 12 weeks. Values are given as mean (SEM). Sham, sham-operated group (n = 10); OVX, ovariectomised group (n = 15); HN001, ovariectomised group fed with HN001 (n = 15).

Parameter	Week	Sham	OVX	HN001
CTx ($ng \cdot mL^{-1}$)	12	2.12 (0.39)[a]	3.63 (0.58)[b]	4.15 (0.61)[b]
Osteocalcin ($ng \cdot mL^{-1}$)	12	72.9 (8.8)[a]	184.6 (10.6)[b]	152.3 (20.8)[b]
Lumbar spine BMC (g)	−2	0.549 (0.021)[a]	0.509 (0.017)[a]	0.533 (0.031)[a]
	4	0.549 (0.024)[a]	0.499 (0.017)[b]	0.508 (0.026)[b]
	12	0.557 (0.021)[a]	0.456 (0.016)[b]	0.482 (0.021)[c]
Lumbar spine BMD ($g \cdot cm^{-2}$)	−2	0.253 (0.005)[a]	0.245 (0.017)[a]	0.253 (0.005)[a]
	4	0.248 (0.015)[a]	0.234 (0.015)[a]	0.234 (0.018)[a]
	12	0.246 (0.005)[a]	0.214 (0.006)[b]	0.225 (0.005)[c]
Femur BMC (g)	−2	0.572 (0.013)[a]	0.546 (0.012)[a]	0.563 (0.019)[a]
	4	0.571 (0.045)[a]	0.530 (0.035)[b]	0.552 (0.065)[a]
	12	0.586 (0.014)[a]	0.509 (0.010)[b]	0.545 (0.018)[c]
Femur BMD ($g \cdot cm^{-2}$)	−2	0.352 (0.006)[a]	0.341 (0.005)[a]	0.346 (0.006)[a]
	4	0.348 (0.006)[a]	0.322 (0.005)[b]	0.328 (0.006)[b]
	12	0.350 (0.006)[a]	0.307 (0.005)[b]	0.318 (0.004)[c]

Values with different superscripts (a, b and c) in a row are significantly different at $P < 0.05$. CTx, C-terminal telopeptides of Type 1 collagen; BMC, bone mineral content; BMD, bone mineral density.

For example, the breaking energy for the HN001 group is close to that of the sham group ($P < 0.08$). Energy is the ultimate measure of overall bone strength. The more energy a bone can absorb prior to breaking, the stronger the bone is. Similar values were apparent in maximum load and maximum stroke, plus break load, stroke and strain values; *Lb* HN001 group had values closer to sham group than OVX.

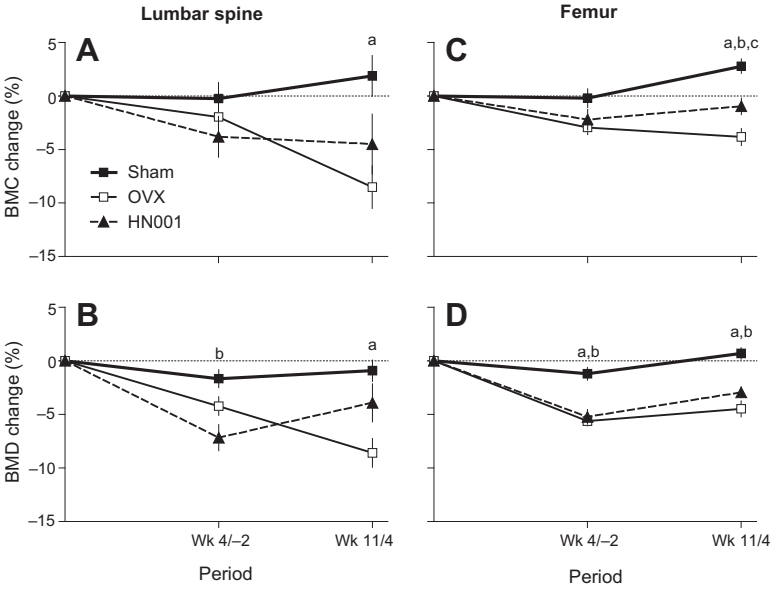

Figure 1. The percentage change of lumbar spine and femur BMC (A and C) and BMD (B and D) of female Sprague-Dawley rats between weeks 4 and −2, and weeks 11 and 4. Sham, sham-operated group (n = 10); OVX, ovariectomised group (n = 15); HN001, ovariectomised group fed with HN001 (n = 15). An 'a' represents $P < 0.05$ for sham vs. OVX, a 'b' for $P < 0.05$ for sham vs. HN001 and a 'c' for $P < 0.05$ for OVX vs. HN001.

4. DISCUSSION

In the two studies presented, we show that *L. rhamnosus* strain HN001 may improve mineral absorption in the growing rat and does reduce bone loss in the OVX female rat.

4.1. Study 1

In Study 1, *L. rhamnosus* strain HN001 significantly improved absolute calcium and magnesium retention. Fractional retention of these two minerals was also increased by feeding probiotics but not significantly. Although body weight gain and growth were not different between groups, it is possible that the higher intake for the HN001 group resulted in the increased retention.

However, several studies have suggested that prebiotics may promote calcium absorption from the large intestine but there is little data on the effect of probiotics on mineral absorption. Scholz-Ahrens et al. [17] investigated the effects of *Lactobacillus acidophilus* NCC90 on caecal pH and mineral absorption. In comparison to a group of rats fed with prebiotics or synbiotics, probiotics had no significant effect. However, this study used animals aged 6 months and older, while the present study used rats aged 4–8 weeks. Another study, using infant rhesus monkeys, fed with *Lactobacillus reuteri*, showed no effect on calcium, iron or zinc absorption [5].

It has recently been suggested that age may influence the effect which prebiotics may have on mineral absorption. Raschka and Daniel found that inulin-type fructans

Table IV. Parameters for bone biomechanics of the excised right femur from sham and OVX control animals as well as OVX rats fed with *L. rhamnosus* HN001 for 12 weeks. Values are given as mean (SEM).

Experimental group	Max		Break				Energy (J)	Width of bone (mm)	Thickness of bone (mm)
	Load (N)	Stroke (mm)	Load (N)	Stress ($N \cdot mm^{-2}$)	Stroke (mm)	Elastic ($N \cdot mm^{-2}$)			
Sham (n = 10)	235.22 (14.76)	1.68 (0.06)	223.7 (10.7)	131.04 (7.52)	1.65 (0.05)	1038.93 (69.04)	0.208 (0.018)	3.78 (0.06)	3.25 (0.07)
OVX (n = 15)	210.85 (8.74)	1.56 (0.06)	210.8 (8.7)	114.58 (5.49)	1.56 (0.06)	987.23 (65.19)	0.177 (0.012)	3.87 (0.07)	3.28 (0.06)
OVX + HN001 (n = 15)	226.74 (8.91)	1.68 (0.05)	224.1 (9.0)	111.47 (7.86)	1.69 (0.05)	861.98 (72.27)	0.201 (0.012)	3.95 (0.06)	3.42 (0.06)

only increased calcium, magnesium and zinc absorbed from the diet in rats in which mineral demand was particularly high such as at age 5–8 weeks and acquiring calcium [15]. This may also be true for probiotics. Ghanem et al. [4] reported that probiotic yoghurt containing strains of *Lactobacillus casei*, *L. reuteri* and *Lactobacillus gasseri* increased apparent absorption of calcium in growing rats. In this study, however, calcium intake between groups differed and food intake was not reported. Body weights of the rats were also not reported, so these findings have to be interpreted with caution. Perez-Conesa et al. [13] also showed that *Bifidobacterium bifidum* and *Bifidobacterium longum* improve apparent absorption and apparent retention of calcium in weanling rats. Although these authors did report findings supporting increased retention or absorption of minerals due to probiotics, our study had limitations in that the intakes were different, though body weights were not, and the group sizes were small. Our observations need to be confirmed in a larger trial.

4.2. Study 2

There were no significant differences in body weight between the animals at week 12. However, body weight affects BMD, so all statistical analyses on in vivo dual energy X-ray spectrometry were corrected for body weight [22]. The final weights of the uteri indicated that all animals were successfully ovariectomised.

Table III summarises the in vivo lumbar spine BMC and BMD at weeks −2, 4 and 12 of the study. The results indicate that all OVX animals had bone loss (BMC and BMD) compared to the sham group in the first 4 weeks after OVX. Thereafter, it appears that bone loss in the HN001-fed group was slower than that in the OVX group, resulting in the final BMC and BMD that were significantly lower than that of the sham group but significantly higher than the OVX group. Femoral BMC and BMD showed a similar pattern with bone loss due to ovariectomy in the OVX and HN001 groups, and final BMC and BMD for the HN001 group was significantly higher than that of the OVX group but still lower than that of the sham group. The observed effect by HN001 seems to have taken place between weeks 4 and 11 reducing the rate of bone loss during this time (Fig. 1).

Narva et al. studied the effect of a bioactive peptide, valyl-prolyl-proline (VPP) in water or *L. helveticus*-fermented milk in the OVX female rat. In this model, *L. helveticus* fermented the milk containing VPP attenuated bone loss due to OVX by 16% compared to water plus VPP [11]. The fermented milk also significantly increased tibial moment of inertia. One should interpret these results with caution as the calcium intake between the OVX control group and the OVX group receiving the fermented milk was significantly different. The authors did consider differences in nutritional intakes and concluded that the difference in calcium intake was probably too small to affect bone density significantly. They do point out that other nutrients in the fermented milk also could have had an effect. Perez-Conesa et al. found that *B. bifidum* and *B. longum* significantly increased femoral and tibial calcium content in weanling rats when they were fed for 30 days [13]. These authors showed that probiotics increase crypt depth in the colon, and lowered colon pH compared to a control diet with no probiotics and calcium absorption was correlated with the pH of the colonic contents.

Perez-Conesa et al. reported the effects of follow-up infant formulas on intestinal morphology and bone mineralisation [14]. They concluded that increased calcium absorption took place in the distal colon in the presence of pre-and/or probiotics, possible leading to increased calcium contents of the femur and tibia. Their study, however, did not report final weights of the animals

or calcium intake, making a comparison with our study difficult.

HN001 had no impact on the biochemical markers measured (CTx and osteocalcin). Therefore, it seems as if HN001 does not affect bone turnover and is not antiresorptive. None of the measured parameters for bone biomechanics were significantly different from sham or OVX (Tab. IV). When considering the change in maximum load the bones can withstand, there is a trend for HN001 to be closer to the sham value than that of the OVX group (not significant). The same is true for maximum stroke as well as break load; the HN001 value is the same as that for the sham group. However, due to large variation the results were not significantly different between groups.

Measures of both collagen and mineral aspects of bone strength indicated a trend for bones from HN001-fed animals to be similar to sham as compared to OVX. HN001 affected bone strength on the level of calcification as well as the collagen component, but due to variation in the results none of these were significant. Feeding the probiotic diet for a longer study period may be needed for these observed trends to become significant. The results presented above indicate that *L. rhamnosus* HN001 may affect both calcium and magnesium bioavailability and bone loss due to OVX.

Possible mechanisms of action may include increased solubility of minerals due to increased bacterial production of SCFAs, and enlargement of the absorption surface due to promotion of proliferation of enterocytes mediated by bacterial fermentation products [13]. It is also possible that SCFAs can directly stimulate calcium and possibly magnesium absorption in the rat colon [4, 13–15], and that calcium at least could pass through the cell membrane more readily in the form of a less-charged complex (calcium acetate), via a passive pathway [6]. Further work is needed to investigate these suggested mechanisms of action by *L. rhamnosus* and other probiotics on mineral absorption and bone properties.

Narva et al. reported the results of a small study where they compared the effect of milk fermented with *L. helveticus* compared to a control on acute changes in calcium metabolism in postmenopausal women. They reported a reduction in serum parathyroid hormone and a rise in serum calcium but no effect on a marker of bone resorption [10]. The reported results of the present animal studies do, however, indicate that probiotics may affect calcium absorption and possibly reduce bone loss over a period of time. The study, however, was small, and the results only suggest some effects on bone density. A larger animal trial with controlled feeding is required to confirm the preliminary observations. The results by Narva et al. also suggest that further research is warranted in older women, who are at risk of high bone turnover and bone loss at menopause.

Acknowledgements: Financial support for this study was provided by Fonterra Brands Ltd., Auckland, New Zealand.

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