A combination of calcium phosphate and probiotics beneficially influences intestinal lactobacilli and cholesterol metabolism in humans

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Abstract

Background & aims: The study focuses on the influence of a probiotic supplement alone and in combination with a calcium supplement on faecal lactobacilli colonisation and beneficial health effects such as lowering of blood cholesterol.

Methods: Thirty-two men and women participated in the double-blind, placebo-controlled, cross-over study. All participants consumed a probiotic drink containing 10^10 CFU/d Lactobacillus paracasei (LPC37) for four weeks. In addition, one group consumed bread enriched with pentacalcium hydroxy-triphosphate (CaP; 1 g Ca/d) and the other group had bread without CaP. After a two-week washout and a two-week placebo period, the intervention was switched for further four weeks.

Results: After intervention with LPC37 + CaP, total cholesterol and LDL-cholesterol concentration in plasma decreased significantly compared to LPC37 and placebo. The faecal concentration of L. paracasei and that of all lactobacilli increased significantly after LPC37 + CaP and LPC37 compared to placebo. Moreover, secondary bile acids in faeces increased significantly after LPC37 + CaP intervention compared to placebo.

Conclusions: CaP modulates the colonisation of LPC37 in the human gut under combinatory supplementation of CaP and LPC37. The combined supplementation also decreases plasma LDL-cholesterol and the LDL/HDL ratio in healthy, moderately hypercholesterolemic men and women, which could be also due to the CaP supplementation.

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1. Introduction

Due to the intake of calcium from milk and fermented milk products an increasing resistance of rats to salmonella infections was demonstrated by Bovee-Oudenhoven et al. in 1996. Calcium phosphate not only increased resistance to salmonella infections but also stimulated intestinal lactobacilli colonisation. The underlying mechanism may be explained by an alteration of the ileal bile acid composition to a less cytotoxic one due to a precipitation of fatty and bile acids by dietary calcium phosphate. Based on the authors line of reasoning, this precipitation probably gives rise to a less aggressive environment for the endogenous lactobacilli, thereby leading to a stimulation of growth. Consequently, calcium phosphate may beneficially influence the colonisation of probiotic lactic acid bacteria.

Probiotic lactic acid bacteria are associated with several health promoting aspects in terms of nutritional benefits such as vitamin production, improving availability of minerals and trace elements, promoting barrier effects, stimulating of the immune system as well as supporting cholesterol-lowering effects. The health promoting effects of Lactobacillus paracasei, a gram-positive homofermentative rod, were described in human studies mainly in the context of immune system modulation.

To the best of our knowledge, there are no published studies examining the influence of a combinatory supplement consisting of probiotics and calcium phosphate on the faecal microbiota and on cholesterol metabolism in humans. The aim of the present study was to increase the intestinal colonisation of L. paracasei by means of a calcium phosphate supplement and to promote beneficial health effects, particularly the cholesterol-lowering actions, due to a combinatory supplementation with probiotics.
2. Materials and methods

2.1. Supplements

Two supplements were used in this study: pentacalcium hydroxy-triphosphate (Ca$_5$(PO$_4$)$_3$OH; cfb Germany; CaP) and *L. paracasei* LPC37 (Danisco Germany; LPC37). In order to achieve a calcium supplementation of 1 g/d, CaP was incorporated in wholewheat bread. Participants consumed approximately 135 g of this bread daily. The probiotic, *L. paracasei* LPC37, was added to a yoghurt drink at a concentration of $10^{10}$ CFU/g. The daily intake of the probiotic drink was 100 ml. Both placebo bread and placebo yoghurt drink were prepared in the same manner as the supplementation products, but without LPC37 or CaP. Taste and visual properties of supplement and placebo products were comparable.

2.2. Subjects, study design and sample collection

The study was conducted at the Friedrich Schiller University Jena, Department of Nutritional Physiology between March and August 2008. Based on the results of a previous study with CaP, a sample size of 30–40 volunteers would be sufficient to detect significant differences.7

Thirty-four omnivorous, moderately hypercholesterolemic subjects (men, $n = 19$; women $n = 13$) participated in this double-blind, placebo-controlled, cross-over study (Fig. 1). All volunteers were informed of the purpose, the course and the possible risks of the study and gave their written consent. The study protocol was approved by the Ethical Committee of the Friedrich Schiller University Jena (no.: 2222-02/08). Two participants dropped out because of personal reasons (Fig. 1). The remaining 32 volunteers aged 25 ± 5 y and had a BMI of 22 ± 3 kg/m$^2$.

At the start of the study, the participants were randomly divided into two groups. All subjects consumed 100 ml of the probiotic drink daily. In addition, one group consumed 135 g calcium-enriched bread (LPC37 + CaP) and the other consumed 135 g bread without calcium (LPC37) for a period of four weeks. At the end of the four weeks a two-week washout and a two-week placebo period followed. Thereafter, the intervention groups were switched for another four weeks (Fig. 2). In the last week of each study period, subjects consumed a defined diet for three days. The defined diet, which contained all the food required per subject over the three days, was prepared and pre-weighed in the study centre. The subjects were instructed to consume no other foods than provided. Food intake was calculated by weighing food residues. Samples from each food component were frozen and stored at −20°C until analysis. Furthermore, the subjects collected faeces quantitatively for three days, beginning at the second day of the defined diet. Faeces were collected for three days as a compromise between obtaining a mixed sample over several days and to ensure a good patient compliance. On the third day of the defined diet, a fasting venous blood sample was taken.

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**Fig. 1.** Study flowchart. LPC37, *Lactobacillus paracasei* LPC37; LPC37 + CaP, *Lactobacillus paracasei* LPC37 + pentacalcium hydroxy-triphosphate.
2.3. Sample preparation

Blood samples were drawn by venipuncture and collected in lithium heparin tubes. Tubes were centrifuged (2000 × g, 10 min, 20 °C) and the plasma supernatants were stored at −20 °C until analysis.

The faecal samples were transported to the study centre without delay. Each specimen was weighed, frozen and stored at −20 °C. At the end of the study, faeces samples were homogenised, portioned, and the pH-value was determined. One portion was used for the analysis of minerals and lactobacilli and the other fraction was freeze dried for the analyses of neutral sterols and bile acids. Both parts were stored at −20 °C.

2.4. Food analysis

The intake of energy, fat, proteins, and carbohydrates was verified using the Prodi®, 5.4 software (Nutri-Science GmbH, Freiburg, Germany). For intake of minerals, the respective contents in the provided foods were analysed instead of using the calculation software. Mineral contents of all food samples were determined by means of ICP-OES (iCAP 6000 ICP Spectrometer, Thermo Scientific, Waltham, USA). Before analysis, the samples were ashed at 525 °C. The ash was dissolved in HCl (25%) and diluted with distilled water.

2.5. Blood analysis

Triacylglycerols, total cholesterol, HDL- and LDL-cholesterol in plasma were ascertained by enzymatic methods using the autoanalyser ARCHITECT C16000 (Abbott, Illinois; USA) according to the methods of the Institute of Clinical Chemistry and Laboratory Medicine, Friedrich Schiller University Jena.

2.6. Faeces analysis

2.6.1. Minerals

Mineral concentrations in faeces were determined as described above.

2.6.2. Lactobacilli population

DNA extraction. The bacterial DNA was extracted from faeces as described previously.6

Quantitative real-time polymerase chain reaction. The PCR mastermix for the quantification of L. paracasei and all lactobacilli contained 1 × Power Sybr Green PCR Mastermix (Applied Biosystems, Foster City, USA), 0.2 μM of the primers Lp1 and Lc2 for LPC37 and 0.5 μM of the primers L159-1, and L677-1 for all lactobacilli.9 For the PCR of LPC37 16 μl PCR Mastermix and 4 μl DNA template, and for all lactobacilli 24 μl PCR Mastermix and 1 μl DNA template were added to the reaction tube. The amplification program for LPC37 consisted of 40 cycles of 30 s at 94 °C, 60 s at 61 °C, and 60 s at 72 °C. For all lactobacilli, it consisted of 45 cycles of 30 s at 95 °C, 60 s at 61 °C, and 60 s at 61 °C. The PCR was carried out on a MX3000P QPCR System (Stratagene, La Jolla, USA). Absolute quantification of L. paracasei and all lactobacilli was carried out using a standard curve obtained from amplification of known DNA quantities from LPC37 and L. reuteri, respectively.

2.6.3. Neutral sterols and bile acids

Faecal neutral sterols (cholesterol and its metabolites coprostanol, coprostanone, cholesterol, cholestanone and cholestenone) as well as bile acids (iso-lithocholic acid, lithocholic acid, isodeoxycholic acid, deoxycholic acid, cholic acid, chenodeoxycholic acid and 12 keto-deoxycholic acid) were analysed as described previously.10 Briefly, neutral sterols were extracted with cyclohexane following a mild alkaline hydrolysis. Analysis was performed on the GC-FID (GC17A-AF Vers.3, Shimadzu, Kyoto, Japan). Bile acids were extracted with diethyl ether after a strong alkaline hydrolysis. Thereafter, extracts were methylated, silylated and analysed by GC-MS (GC17-QP5000, Shimadzu, Kyoto, Japan).

2.7. Statistics

Samples of each participant were coded to protect volunteer identity and to mask treatment groups during the analysis. Data analysis was performed using the statistical software package PASW Statistics 18 (SPSS Inc., Chicago, USA). Values were reported as means ± standard deviation. Differences were considered significant at p ≤ 0.05. Variance homogeneity was tested using the Levene test. In case of variance heterogeneity, the data were log_{10} transformed (signed with superscript t in the text). The differences between the study periods were tested using the paired Student’s t-test. Pearson’s linear correlations were used to test for the degree of associations.

3. Results

3.1. Nutrient intake during the standardised diet

During the last three days of each study period, the subjects consumed a defined diet. There were differences regarding the nutrient intake between the interventions and the placebo because the defined diet was just restricted with regard to the supplied food components; the quantitative food intake was not limited (Table 1). Following supplementation with LPC37 + CaP, the energy intake was significantly lower in comparison to placebo (p ≤ 0.05). This was obviously due to a significant decrease in fat intake (p ≤ 0.05). However, carbohydrate and protein intake remained constant.

Due to the supplementation of calcium phosphate, the subjects consumed more calcium and phosphorus after LPC37 + CaP compared to LPC37 and placebo (p ≤ 0.001 between all periods; Table 1).
Due to the LPC37 CaP intervention, faecal excretion of calcium increased significantly compared to LPC37 alone, from 5 \( \pm \) 2.9 mg/d to 9.7 \( \pm \) 2.6 mg/d \( (p < 0.001) \) and placebo (Ca: 4.6 \( \pm \) 2.6 mg/d; P: 3.8 \( \pm \) 2.2 mg/d; \( p < 0.001 \)). After LPC37 intervention, the excretion of calcium and phosphorus remained unaffected compared to placebo.

### Faecal parameters

#### 3.3.1. General excretion parameters

The faecal mass did not change during the interventions. The pH-value of the faeces increased significantly after LPC37 + CaP (pH = 6.7 \( \pm \) 0.3) compared to LPC37 (pH = 6.4 \( \pm \) 0.3; \( p < 0.001 \)) and placebo (pH = 6.4 \( \pm \) 0.3; \( p < 0.001 \)). LPC37 alone had no influence on the pH-value of the faeces.

Due to the LPC37 + CaP intervention, faecal excretion of calcium (1591 \( \pm \) 525 mg/d) and phosphorus (909 \( \pm \) 301 mg/d) increased significantly compared to LPC37 (Ca: 722 \( \pm \) 336 mg/d; P: 504 \( \pm \) 214 mg/d; \( p < 0.001 \)) and placebo (Ca: 744 \( \pm \) 306 mg/d; P: 514 \( \pm \) 172 mg/d; \( p < 0.001 \)). After LPC37 intervention, the excretion of calcium and phosphorus remained unaffected compared to placebo.

#### 3.3.2. Lactobacilli population

After supplementation of LPC37 alone or in combination with CaP, faecal concentration of L. paracasei increased significantly compared to placebo (all \( p < 0.001 \); Fig. 3). After LPC37 + CaP, the concentration of L. paracasei was higher compared to LPC37 alone, but the effect was not significant. The concentration of all lactobacilli in faeces increased after LPC37 + CaP and LPC37 intervention compared to placebo (all \( p < 0.05 \); Fig. 3).

### 3.3.3. Neutral sterols

Eight of the 32 subjects showed an inverse faecal neutral sterols conversion profile in at least one period. These subjects metabolised less than 25% of cholesterol to coprostanol (low converters). Thus, the faeces content of low converters was high in cholesterol and low in coprostanol. These eight volunteers were excluded from the statistical analyses, referring to Ditscheid et al. Data are given for \( n = 24 \) high converters.

Total neutral sterols as well as cholesterol and neutral sterol metabolites in faeces did not change due to the supplementation compared to placebo (Fig. 4).

### 3.3.4. Bile acids

The concentration of total bile acids and that of primary bile acids remained unaffected following supplementation compared to placebo (Fig. 4). The faecal concentration of secondary bile acids increased significantly after LPC37 + CaP intervention compared to placebo (\( p < 0.05 \)).

### 4. Discussion

Lactic acid bacteria constitute the major representatives of probiotics. The main properties of probiotic bacteria, such as resistance to pancreatic enzymes and acid as well as adhesion to intestinal mucosa according to Ouwehand et al., have also been shown for L. paracasei. Moreover, beneficial health effects due to L. paracasei, as for example, modulation of the immune system have also been demonstrated in several human studies. The supplemented strain L. paracasei LPC37 is available commercially by Danisco (Madison, WI, USA) and has according to Danisco proven health benefits. In a human study by Rössler et al. the strain was used in the context of immune system modulation. Further human studies are planned. As a consequence the results of the current human study are mainly compared with studies using L. paracasei strains.

In the present study, faecal analysis of the supplemented L. paracasei LPC37 was performed using quantitative real-time PCR with specific primers for the species L. paracasei and a L. paracasei LPC37 standard was used for the quantification. Consequently, the increase in faecal concentration of L. paracasei after the probiotic interventions in comparison to placebo was due to the supplemented LPC37. The significant increase of the concentration of L. paracasei from 5 \( \times \) \( 10^3 \) CFU/g faeces after placebo to 3 \( \times \) \( 10^5 \) and 4 \( \times \) \( 10^5 \) CFU/g faeces after LPC37 and LPC37 + CaP intervention, respectively, indicates its successful passage through the human gut. This forms the basis of the beneficial health effects of the probiotic. Healthy volunteers and patients with atopic dermatitis received yoghurt supplemented with a combination of LPC37, Lactobacillus acidophilus 74-2 and Bifidobacterium animalis subsp. lactis DGCC 420 at a concentration of 3.9 \( \times \) \( 10^9 \) CFU LPC37/g in a study by Rössler et al. After the intervention, faecal concentration of L. paracasei was approximately 3 \( \times \) \( 10^5 \) CFU/g faeces (original data published log \( _{10} \) 8.4 CFU/g faeces). Moreover, Jahreis et al. supplemented the probiotic \( 10^9 \) CFU L. paracasei LTH 2579/ d in a sausage. Following the supplementation, faecal concentration of L. paracasei LTH exceeded \( 10^8 \) CFU/g faeces in more than 50% of the volunteers. The differences in absolute concentrations are probably a result of using either diverse analytical methods, modes of administration or varying quantities. Moreover, the concentration of all lactobacilli was very low, too. However, real-
time PCR as used in the current study is suitable for the quantification of *L. paracasei*. Furthermore, by employing the same analytical treatment for all samples, it was possible to examine the influence of CaP on the colonisation of LPC37. Bovee-Oudenhoven et al. reported beneficial effects of calcium supplementation, especially calcium phosphate on the intestinal growth of lactobacilli. In the present study, the faecal concentration of the supplemented LPC37 and of all lactobacilli was 10^5 CFU/g faeces and 10^7 CFU/g faeces higher after LPC37 + CaP intervention compared to LPC37 alone respectively (Fig. 3). However, the increase was not statistically significant. Here, since both interindividual and intra-individual differences regarding the microbiota were immense, it was difficult to show a statistically significant effect for such a supplementation. Nevertheless, an attempt was made to minimise these differences with the concept of the defined diet during the time of faeces collection. Besides, there was a significantly positive correlation between the faecal concentration of calcium and the colonisation of *L. paracasei* (r = 0.277; p ≤ 0.05). This supports the hypothesis that CaP positively influences the colonisation of *L. paracasei* in the human gut.

The present study was also conducted to investigate a potential cholesterol-lowering effect of LPC37 and a possible enhancing action of CaP. Therefore, moderately hypercholesterolemic subjects were recruited. The discussion in the literature regarding the cholesterol-lowering properties of probiotics is inconsistent, because of different modes of administration varying quantities and strains used in these studies. This leads to studies reporting an effect on blood lipids, and others demonstrate no effect. For example, in the study by Anderson and Gilliland, 14 subjects were supplemented with *L. acidophilus* L1 for four weeks. The authors observed a significant 3.2% reduction of blood cholesterol concentration. In contrast, Lewis and Burmeister supplemented *L. acidophilus* LA-1 to 80 study participants for six weeks and found no change in the concentration of blood lipids. In addition, Jahreis et al. supplemented 20 healthy men and women with *L. paracasei* LTH 2579 incorporated into sausages. After five weeks of intervention, the blood lipid concentrations did not change. Moreover, other research groups could not demonstrate a beneficial modulation of blood lipids after a supplementation with *L. paracasei*. Finally, in the present study supplementation with *L. paracasei* LPC37 alone did not influence the total blood cholesterol concentration. Thus, the above data show that LPC37 alone has apparently no modulatory effect on blood lipid concentrations after four weeks of supplementation.
However, results in the present study firstly show that the combination of CaP and LPC37 (LPC37 + CaP) decreases concentrations of total and LDL-cholesterol in plasma significantly (Table 2). There are two possible explanations for this observation: either LPC37 and CaP act synergistically, or the cholesterol-lowering effect of LPC37 + CaP is due to the action of CaP in the human gut. In fact, several studies in humans have examined the effect of calcium supplementations on blood lipids. For example, Ditscheid et al. used the same calcium phosphate compound as supplement and found a significant decrease of serum total cholesterol from 4.60 mmol/l in the placebo period to 4.36 mmol/l in the CaP-period. This modulation in serum total cholesterol is close to results achieved in the current study, suggesting that the cholesterol-lowering effect is solely due to the CaP supplement.

Since the blood lipid modulating effect of CaP as well as of the probiotics can be due to a modulation of sterol excretion, it is worth examining the faecal sterol concentration. The influence of probiotics and calcium phosphate on sterol metabolism in the gut is well reported in the literature. Only 5% of the intestinal bile acids undergo bacterial metabolism in the large intestine. One of the most important bile acid transformations in the human gut is the hydrolysis mediated by a wide range of bacteria. The bile salt hydrolase splits the amino acids glycine or taurine from the bile acid resulting in a decreased solubility and emulsification capacity of the bile acid. During bile acid metabolism, 7α-dehydroxylation takes place and secondary bile acids are generated from primary bile acids. The 7α-dehydroxylase activity has been detected in the genera Clostridium and Eubacterium, but not in lactobacilli and bifidobacteria. L. paracasei is described to produce no deoxycholic acid or 7-keto deoxycholic acid. But secondary bile acids are formed from unconjugated primary bile acids, so it is plausible that a rise in bile salt hydrolase increases the formation of secondary bile acids. Cholesterol passing through the human gut can be metabolised by the microbiota. According to older literature the conversion was performed mainly via Eubacterium but Gérard et al. isolated Bacteroides sp. Strain D8 as the cholesterol-reducing bacteria in human faeces. There are two pathways: a direct conversion to coprostanol and an indirect conversion via the intermediate cholestenone. The indirect pathway is considered to be the major pathway. Cholesterol and its major metabolite in faeces, coprostanol, are beneficially influenced via the increased deconjugation of bile acids by probiotics. Different studies suggest that unconjugated bile acids are less water-soluble and are able to precipitate cholesterol in the human gut, ideally at a lower pH-value. On the one hand, this means an increased excretion of bile acids via faeces and on the other hand, a greater drain on the bile acid pool. This results in a reduction of the blood cholesterol concentration due to the loss of feedback inhibition on bile salt synthesis and in an increased conversion of cholesterol into bile salts. In the present study, the steroid concentration in faeces did not change after LPC37 supplementation. In contrast, Ahn et al. supplemented three volunteers for three weeks with L. acidophilus SNUL01 and analysed the faecal steroids. They observed that the slight increase in faecal cholesterol was due to a decrease in cholesterol solubility because of bile acid deconjugation. The faecal concentration of deoxycholic acid was found to increase because of the deconjugation of taurine or glycine by L. acidophilus and a 7α-dehydroxylation by other intestinal bacteria. However, since most of the studies examining a modulatory effect of steroids in the gut by probiotics are in vitro-studies, they do not reflect the
situation in the human gut. 28, 40, 41 De Boever et al. suggested that bile salt transformation by deconjugation has an impact on physicochemical properties, such as ionisation, solubility and micelle formation. 42 In addition to the influence on faecal cholesterol, the unconjugated bile acids are less resistant to precipitation by divalent cations such as Ca 2+ .

After LPC37 + CaP supplementation, the secondary bile acids increased compared to placebo. This can be due to a co-precipitation of bile acids by amorphous calcium phosphate, particularly when the bile acids are deconjugated by probiotics. The formation of amorphous calcium phosphate has been previously described in vitro 43, 44 and in vivo. 11, 44 For example, Ditscheid et al. showed an increase in total bile acids of 2.5 μmol/g faecal dry matter after supplementation with CaP. 13 This is in accordance with the findings of the present study (increase of 2.0 μmol/g faecal dry matter) and very likely due to the CaP supplementation independent of LPC37. Moreover, the modulation of faecal bile acids is in accordance with the changes in blood lipids.

In conclusion, the results of the present human study imply that CaP positively affects the colonisation of LPC37 in the human gut under conditions of combinatory supplementation of CaP and LPC37. This combined supplementation is also capable of beneficially modulating blood lipids in healthy, hypercholesterolemic men and women.

Conflict of interest statement

None of the authors had any financial or personal conflict of interests.

Statement of authorship

All authors made substantial contributions to one or more of the following: study conception and design, acquisition of data, analysis and interpretation of data, drafting and/or critically revising the manuscript. We thank VCI (Verband der Chemischen Industrie e.V.) and Zott GmbH & Co. KG for their financial support and for supplying the supplements. Study sponsors were not involved in data interpretation or in authoring the manuscript. Technical and scientific assistance is greatly appreciated from A. Roessler, S. Keller, U. Helms, K. Sachse and M. Leiterer. We thank Nasim Kroegel for language editing.

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