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ABSTRACT

Purpose: Cheese is a dairy product with high calcium content. It has been suggested that calcium intake may increase fecal excretion of bile acids that would cause a regeneration of bile acids from hepatic cholesterol and thereby result in a lowering of plasma cholesterol concentrations. We aimed to test this hypothesis by assessing bile acid and calcium concentrations in fecal samples from humans after intake of cheese and butter.
Methods: The study was a randomized, 2×6 weeks crossover, dietary intervention study including 23 men and women who replaced part of their habitual dietary fat intake with 13% energy from cheese or butter.
Results: After 6 weeks of intervention cheese resulted in higher amounts of calcium excreted in feces compared to butter. However, no difference was observed in fecal bile acid output despite lower serum total, LDL and HDL cholesterol concentrations observed with cheese intake.
Conclusion: We were not able to confirm the hypothesis that calcium from cheese increases the excretion of fecal bile acids. Therefore, the mechanisms responsible for the lowering of cholesterol concentrations with cheese compared to butter intake remains unresolved.

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1. Introduction

Lately, there has been an increasing focus on cardiovascular disease (CVD) risk with dairy intake [1–4]. Cheese is a dairy product with high content of saturated fat. Therefore, it has been a general perception that cheese is associated with risk of CVD as saturated fat is known to increase cholesterol concentrations in plasma [5,6]. However, correlations studies did not find cheese to be related to CVD risk [7,8] which has been supported by several prospective studies also not finding cheese intake to be related to CVD risk [9–11]. In addition, human intervention studies suggest that cheese does not increase cholesterol concentrations despite the high saturated fat content when compared to butter intake [12–14]. The biological mechanism behind these findings has not yet been explained.

In the liver, cholesterol can undergo a series of steps to form the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA). This pathway includes the conversion of cholesterol to 7α-hydroxycholesterol by the enzyme, cholesterol 7α-hydroxylase as the rate limiting step [15]. CA and CDCA are excreted mainly as taurine and glycine conjugates via the bile duct in the small intestine where they are converted by bacterial 7 α-dehydroxylation to the corresponding secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA).

Calcium supplementation has been hypothesized to increase the excretion of bile acids in feces [16–18]. The suggested reason behind this is that calcium or calcium phosphate complexes bind to bile acids in the intestine. The latter will cause an efflux of bile acids from the enterohepatic cycle and thereby a need for regeneration of bile acids from hepatic cholesterol. Consequently, this will result in a lowering of cholesterol concentrations in plasma. As cheese has a high content of calcium with a high bioavailability [19] the objective of the present study was to investigate the hypothesis that the high calcium content may increase calcium and bile acid concentrations in feces and thereby decrease cholesterol concentrations in plasma. This hypothesis was tested by assessing bile acid and calcium concentrations in fecal samples from humans after intake of

cheese and butter for 6 weeks.

2. Materials and methods

The present study is part of a larger study, described in details elsewhere [12]. In short, 53 subjects (22–69 years of age) were recruited for a randomized, crossover intervention, with a two-week run-in period of habitual diet. Exclusion criteria included current or previous CVD, diabetes mellitus or other severe chronic disease, use of lipid-lowering agents, BMI above 32 kg/m^2, and known or suspected abuse of drugs, alcohol, or medication. Subjects consumed cheese or butter for six weeks each replacing approximately 13 energy percent (E%) of their daily fat intake. The participants were grouped into three groups according to their energy level and the amounts of cheese and butter to be consumed per day were adjusted accordingly in order to substitute the 13E% of fat: low ($\leq$9.8 MJ) corresponding to 111 g cheese/37 g butter, medium (9.8–12.5 MJ) corresponding to 143 g cheese/47 g butter and high ($\geq$12.5 MJ) corresponding to 176 g cheese/58 g butter. The two intervention periods were separated by a washout period of at least two weeks on habitual diet. The fatty acid composition of the cheese and butter were similar [12]. Thus, the fat quality and quantity from the cheese and butter was the same. No other dairy products were allowed during the cheese and butter period except from small amounts of low fat milk (0.5% fat, a maximum of 6 cl/d) that had to be consumed throughout the intervention. There were no dietary restrictions during the washout period. Subjects were instructed to refrain from blood donations and dietary supplements that might interfere with study measurements.

Twenty-three of the 53 subjects included in the intervention agreed to hand in 2 × 24 h feces collections at the end of both the cheese and butter period. A total of 23 participants agreed to deliver the faeces samples out of the 53 subjects participating in the main study. Baseline characteristics of these 23 subjects, included in this study, are listed in Table 1.

In order to provide information about dietary intake during the intervention and ensure stable weight subjects completed a 3-day dietary record during the last two weeks of each intervention period. Two of these days were in connection with the fecal collection, whereas one day was the nearest weekend day to the fecal collection which was included to take any differences in nutrient intake during weekdays and weekends into account. Dankost 3000 dietary assessment software (Dankost, Copenhagen, Denmark) was used to estimate the dietary intake. The study was approved by the Danish National Committee on Biomedical Research Ethics.

Subjects were instructed to collect all feces excreted in pre-weighed containers during the last two days of each intervention period. All fecal samples were weighed when handed in. The samples from the same day were pooled and blended with milliQ-water (1:1) and pH was measured in the homogenate. The mean pH and fecal weight was calculated for each period. Bile acids in the fecal samples were analyzed quantitatively. Fecal samples were frozen at −80 °C until analyses by LC-MS/MS of the homogenate (a) and the fecal water (b), as described below. A pool of all samples

Table 1
Baseline characteristics of the 23 subjects collecting fecal samples.

	Mean ± SD
Men/women (no (%))	15 (65%)/8 (35%)
Age (years)	56.7 ± 11.1
Height (cm)	173.2 ± 8.5
Body mass index (kg/m^2)	25.4 ± 3.3
Smoking (no (%))	4 (17%)

was used as a calibration standard and analyzed 3 times in triplicate together with the individual samples. The bile acids DHCA (dehydrocholic acid, internal standard), CDCA, UDCA, LCA and HDCA were purchased from Sigma−Aldrich (Brøndby, Denmark); CA and DCA were purchased from Merck (Hellerup, Denmark); Bile acid standards: 13C GCA was purchased from Sigma−Aldrich, (Brøndby, Denmark); 13C DCA and 13C CA (internal standards) were purchased from Cambridge Isotope Laboratories (Andover, USA); 13C UDCA and LCA-d4 (internal standards) were purchased from CDN isotopes (Quebec, Canada);

a. Internal standards consisting of a mix of 13C- labeled and deuterated bile acids were added to 0.3 g homogenized samples and extracted once with 60% ethanol at 60 °C, then two times with 96% ethanol. The combined ethanolic supernatant was diluted by a factor of 9 with 0.1% formic acid in water and retained on a pre-activated Oasis HLB LP 96-well plate (Waters, Milford, Massachusetts, USA). After washing with 1 mL 0.1% formic acid, the bile acids were eluted first with 0.8 mL 50% acetonitrile with 24% methanol, then with methanol containing 0.1% formic acid. The eluate was evaporated to dryness and re-dissolved in a mixture of 15% acetonitrile, 30% methanol, 0.1% formic acid and water. Analyses were performed on Waters Acquity UPLC using a 5 cm BEH phenyl column and a pre-column of the same type using a gradient from phase A to B over 5 min. The mobile phases were 30% methanol and 0.1% formic acid (mobile phase A) and 100% ACN and 0.1% formic acid (mobile phase B) at a total flow rate of 0.9 mL/min. Detection was performed with a triple quadrupole detector (TQD) operated in MRM mode (cone voltage, collision energy, and transitions in brackets): CA (80, 18, 407.3 → 407.3); CDCA (90, 20, 391.3 → 391.3); DCA (70, 15, 391.3 → 391.3); hyodeoxycholic acid (HDCA) (80, 20, 391.3 → 391.3); LCA (90, 20, 375.2 → 375.2); ursodeoxycholic acid (UDCA) (80, 18, 391.2 → 391.2); all transitions were 4Da higher for the tetradeuterated internal standards. CV% of pooled samples was: 5.7 for CA, 4.0 for CDCA, 3.5 for DCA, 10.3 for HDCA, 6.2 for LCA, and 6.4 for UDCA. The taurine and glycine conjugates were also analyzed using labeled standards, however concentrations in the fecal samples were below detection limits. Standard curves were prepared for all bile acids for quantitation and internal standards were used to assess relative losses and ion suppression of each analyte. The fecal bile acid concentrations from the two consecutive collection days at the end of each intervention period were averaged before the statistical analysis.

b. Bile acids in fecal water were measured to be able to calculate percent bound bile acids in the fecal mass. Homogenized fecal samples were centrifuged at 20,000 g for 2 h at 4 °C. The aqueous supernatant was removed and filtered through Q-Max Syringe filters (Membrane Cellulose Acetate, Pore size 0.20 μm, Filter dia: 25 mm, Cat. No. CA250250S, Frisenette). The samples were then diluted with a mixture of 15% acetonitrile, 30% methanol, 0.1% formic acid in water and analyzed on a UPLC-TQD as described for the homogenate (a). Fecal samples from four of the 23 subjects contained insufficient amounts of fecal water for determination of the bile acids therefore only 19 subjects are included in the results. CV% of pooled samples for fecal water analyses were: 14.1 for CA, 14.5 for CDCA, 12.3 for DCA, 12.0 for HDCA, 28.3 for LCA and 19.1 for UDCA. One subject had samples below the detection limit of CDCA and two subjects had samples below the detection limit of HDCA. Bile acid results for each subject from the two consecutive days were averaged before the statistical analyses.

Calcium content in fecal samples collected in the first 24 h of the 48 h collections in each dietary period was analyzed using atomic absorption spectroscopy (Spectra AA 200 Varian, Varian Techtron Pty. Limited, Mulgrave Victoria, Australia). Before analysis samples were freeze dried, homogenized, and destructed (DigiPREP MS, SCP Science, Quebec, Canada) with 67−70% HNO$_3$ (Plasma Pure, SCP

Science, Quebec, Canada) and 30% H_2O_2 (SupraPur, SCP Science, Quebec, Canada). Samples were diluted by a solution of 0.5% lanthanum oxide (Lanthanum(III) oxide, Merck, Darmstadt, Germany) and 1% HNO_3 (Plasma Pure, SCP Science, Quebec, Canada). The calcium standard for the standard curve (1000 mg Ca/L, Titrisol, Merck, Darmstadt, Germany) was diluted by the same solution. A reference diet (Standard Reference Material, Typical Diet 1548a, National Institute of Standards and Technology, Gaithersburg, MD) was analyzed together with the fecal samples. CV% of the reference diet was 2.4. The analyses were performed in duplicates.

Fasting blood samples were taken in duplicates on consecutive days after each intervention period. The results from all 53 participants and the method of the blood samples analyses have been described in details elsewhere [12]. In brief, blood samples were collected in dry tubes and kept at room temperature for 30 min to coagulate. Hereafter, they were centrifuged at 2200 g for 10 min at 20 °C and stored at −80 °C until analyzed. A standardized enzymatic colorimetric procedure (ABX Pentra LDL Direct CP, ref. no. A11A01638 and HDL Direct CP, ref. no. A11A01636, Horiba ABX, Montpellier, France) was used to measure low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol. Total cholesterol and triacylglycerol concentrations were measured by enzymatic procedures (Cholesterol CP, ref. no. A11A011634 and Triglycerides CP, ref. no. A11A01640, Horiba). The analyses were performed on an ABX Pentra 400 Chemistry Analyzer (Horiba). The values from the two consecutive days from each intervention period were averaged before the statistical analyses. Self-reported compliance which was 99.8% during the butter period and 99.6% during the cheese period. In addition, we previously reported that feces were collected the last 2 d of each intervention period from 23 subjects and that fecal fat excretion did not differ between the cheese and butter periods (P = 0.1035).

Statistical analyses were performed using PROC MIXED in Statistical Analysis System (SAS), version 9.2 (SAS Institute, Cary, NC). A linear mixed model incorporating systematic effects of period and treatment and their interaction was used to assess the effects of treatment for all data except percent bound DCA as described below. Age, gender, period, weight and smoking were included as explanatory variables, if significant, to adjust for possible differences between subjects. Non-significant-effects were removed one by one from the model. Subject was included as random effect to account for the inter-subject variability. Normal probability plots and residual plots were used to validate the model together with statistical tests for normality (Shapiro–Wilk, Kolmogorov–Smirnov, Cramer-von Mises and Anderson-Darling). Outcome variables were logarithmically transformed before analysis, if necessary. Percent bound DCA data were not normally distributed and therefore Wilcoxon signed rank test was used for these data. All p-values were evaluated at a 5% significant level. Values presented in the text are means ± SD.

3. Results

No difference was observed in weight of the feces during the cheese and butter period (data not shown; p = 0.39). Fecal pH tended to increase with cheese intake (6.8 ± 0.5) compared to butter intake (6.6 ± 0.5), although this was not significantly different (p = 0.08).

Results from the 3-day dietary records (Table 2) showed that the energy and fat intake was not significantly different during the two periods. The cheese period resulted in higher intakes of protein (p < 0.0001) and lower intakes of carbohydrates (p < 0.05) compared to the butter period. Finally, the calcium intake during the cheese period was almost three times as high compared to the butter period (p < 0.0001). The cheese period resulted in a 55%

Table 2
Average daily consumption of energy, macronutrients and calcium during the run-in, cheese and butter period for the 23 subjects handing in fecal samples.[a]

	Run-in[b]	Cheese	Butter
Total energy (kJ)	9312 ± 3037	9410 ± 2058	9721 ± 2431
Fat (% of energy)	31.2 ± 5.6	33.5 ± 5.7	35.5 ± 6.7
Fat (g)	77.8 ± 27.8	83.1 ± 15.2	90.5 ± 17.8
Saturated fat (g)	26.8 ± 9.8	37.0 ± 6.0	39.5 ± 6.9
Monounsaturated fat (g)	24.9 ± 11.2	26.1 ± 7.5	29.4 ± 7.6
Polyunsaturated fat (g)	12.2 ± 6.0	10.9 ± 4.7	11.5 ± 3.5
Protein (% of energy)	16.5 ± 3.4	19.2 ± 2.9**	14.0 ± 3.4
Carbohydrate (% of energy)	47.7 ± 8.1	44.8 ± 8.8*	48.3 ± 7.5
Calcium (mg)	969.1 ± 319.4	1220 ± 180**	451 ± 152

*Different from butter period (p < 0.05) (comparison between cheese and butter period).
**Different from butter period (p < 0.0001) (comparison between cheese and butter period).
[a] All values are expressed as means ± SD.
[b] n = 22 because one subject had dietary record considered nonsufficient during this period.

higher excretion of calcium in feces (955.1 ± 685.0 mg/24 h) compared to the butter period (432.6 ± 305.8 mg/24 h) (p < 0.001).

The bile acids observed in measurable amounts in the samples were the primary bile acids CA and CDCA and the secondary bile acids DCA, LCA, HDCA and UDCA. No difference was observed between the two periods of cheese and butter intake regarding bile acids excreted in the 24 h fecal collections (Table 3). Large interindividual and day-to-day variations were observed in the amounts of bile acids excreted in feces for each individual whereas the pooled calibration samples were showing limited variation.

The difference in percent of bound bile acids in fecal solids was also not significantly different between the two periods (Table 4). Thus, no difference was observed in amounts or types of bile acids when comparing the cheese and butter period. However, the cheese period resulted in lower total-, LDL- and HDL cholesterol concentrations in plasma compared to the butter period (p < 0.01, p < 0.05 and p < 0.05, respectively; Table 5). There was a positive correlation between change in Ca intake and change in bile acid excretion between the two treatments (r^2 = 0.34), however, the correlation between changes in Ca or bile acids with change in cholesterol was weakly negative (r^2 = 0.09).

4. Discussion

In this study the effect of cheese and butter intake on calcium and bile acid excretion was evaluated. The amount of fat ingested from the two dairy products was similar, however the calcium content in these food items was highly different, which was substantiated from the dietary records. We found no difference in fecal weight or fecal pH, although other studies suggested that fecal pH

Table 3
Total fecal bile acids after cheese and butter intake in men and women.[a]

Bile acid	Cheese	Butter
	(µmol/24 h)	
Total bile acids	272.7 ± 258.2	224.8 ± 225.8
Total primary bile acids	9.6 ± 19.1	10.5 ± 19.1
Total secondary bile acids	263.1 ± 250.2	214.3 ± 214.1
Cholic acid	4.6 ± 10.7	4.9 ± 9.7
Chenodeoxycholic acid	5.0 ± 8.5	5.6 ± 9.8
Deoxycholic acid	120.8 ± 144.0	107.4 ± 138.4
Hyodeoxycholic acid	22.5 ± 25.9	20.7 ± 24.1
Lithocholic acid	116.3 ± 88.8	83.3 ± 56.0
Ursodeoxycholic acid	3.4 ± 4.3	2.9 ± 3.0

[a] Values are means ± SD, n = 23.

Table 4
Percentage of bile acids bound in fecal solids after cheese and butter intake in men and women.[a]

Bile acid	Cheese	Butter
	(%/24 h)	
Total bile acids	94.7 ± 2.5	94.5 ± 4.37
Total primary bile acids	75.1 ± 17.9	79.6 ± 17.4
Total secondary bile acids	95.3 ± 2.4	95.3 ± 3.8
Cholic acid	59.7 ± 21.1	66.5 ± 18.8
Chenodeoxycholic acid[b]	91.0 ± 6.6	87.6 ± 18.7
Deoxycholic acid	92.4 ± 3.5	89.0 ± 11.8
Hyodeoxycholic acid[c]	76.4 ± 15.6	82.4 ± 13.2
Lithocholic acid	99.6 ± 0.3	99.5 ± 0.5
Ursodeoxycholic acid	84.1 ± 8.9	85.3 ± 11.0

[a] Values are means ± SD. n = 19.
[b] n = 18 because some samples were below the detection limit.
[c] n = 17 because some samples were below the detection limit.

Table 5
Blood lipids during the cheese and butter period for the 23 men and women handing in fecal samples.[a]

	Cheese	Butter
	(mmol/l)	
Total cholesterol	5.1 ± 0.8[**]	5.4 ± 0.9
LDL cholesterol	3.1 ± 0.7[*]	3.3 ± 0.8
HDL cholesterol	1.4 ± 0.3[*]	1.5 ± 0.3
Triacylglycerol	1.1 ± 0.4	1.1 ± 0.4

Statistical differences are based on linear mixed model.
[*] Different from butter period ($p < 0.05$).
[**] Different from butter period ($p < 0.01$).
[a] All values are expressed as means ± SD.

may increase with calcium supplementation [20,21] and with calcium intake from dairy products [22]. Increased pH has been suggested to be associated with increased solubility of bile acids leading to increased toxicity and colon cancer risk [23], however in a study in adenoma patients and controls this was not substantiated and no association was found between dietary intake of calcium and pH [24].

The main purpose of our study was to investigate if the higher calcium content in cheese could be responsible for a higher excretion of calcium and bile acids in feces resulting in a lowering of cholesterol in plasma. While calcium excretion was significantly elevated this did not appear to significantly affect bile acid precipitation in feces or total bile acid excretion. Still, cheese intake resulted in lower cholesterol concentrations compared to butter intake.

We investigated the effect of calcium in the form of cheese while others have investigated the effect of calcium as a dietary supplement [20,21]. Lupton et al. reported no effect in total bile acids output after 16 weeks of calcium supplementation in 22 subjects with a history of resected adenocarcinoma of the colon. However, they found the primary bile acids in dry stool to decrease with calcium supplementation [21]. Alder et al. conducted a similar study where calcium supplementation increased DCA in fecal water compared to baseline measures [20]. In addition, they found an increase in total bile acids and LCA in dry stool with calcium supplementation compared to baseline measures. These studies had some limitations as they compared with baseline measures instead of e.g. placebo tablets. In addition, their focus was colon cancer and therefore cholesterol concentrations were not measured making the comparison with our objective difficult. However, they found some changes in bile acid concentrations with calcium supplementation which suggests that calcium should be able to change bile acid excretions. Nevertheless, calcium supplementation has been found to lower serum total cholesterol concentrations and to

increase excretion of both total and secondary bile acids in feces [25]. The reason for the lowering of the cholesterol concentrations was ascribed to bile acids bindings to insoluble calcium phosphate.

Calcium supplementations may affect fecal bile acids differently than calcium provided as a natural part of the cheese matrix. A limited number of studies have investigated the effect of dairy products on fecal bile acids. Govers et al. compared milk products with high calcium content (1200 mg/d) with placebo milk products with low calcium content (120 mg/d) in 13 males [22]. They found the concentrations of secondary bile acids (DCA and LCA) in fecal water to decrease with calcium intake from milk products. In addition, they found the total amount of fecal bile acids to increase with calcium intake supporting the hypothesis that calcium may bind to bile acids in the intestine. More recently, Bendsen et al. found similar results to ours, when comparing the excretion of bile acids from a diet with high calcium content from dairy products to a diet with low calcium content [26]. They found no differences in total amounts of bile acids excreted but they found that the excretion of conjugated bile acids were greater with a high calcium diet compared with a low calcium diet. In their study, the diet high in calcium consisted of low-fat dairy products and therefore, it could be speculated that their result would have been different if the diet had consisted of full-fat dairy products as in the present study. This is of interest because increasing fat intake increases the fecal bile acid output [27]. Results from a study taking the fat intake into account, showed no increase in bile acid excretion with increased dairy fat intake but an increased excretion with increased calcium intake from dairy [28]. Also, in that study a diet rich in dairy calcium significantly decreased the total- and LDL cholesterol concentrations. Thus, the study supports the hypothesis that dairy calcium increases fecal bile acid output and thereby decreases cholesterol concentrations which was also suggested as a possible mechanism by the authors. However, we were not able to support the hypothesis that calcium from cheese increases excretion of fecal bile acids, as results did not reach statistical significance. Other mechanisms responsible for the lowering of cholesterol concentrations with cheese intake compared to butter intake may have been involved in the current study. A high calcium intake has also been found to increase fecal fat excretion [22,26,28,29]. The reason for this has been proposed to be binding of calcium to fatty acids in the intestine resulting in the formation of insoluble soaps [30]. However, this did not appear to be a possible explanation in our study as we found no significant difference in fecal fat excretion between the cheese and butter period from the same 23 subjects included in this study [12]. The positive correlation between change in Ca intake and change in bile acid excretion between the two treatments ($r^2 = 0.34$) would give the impression of an effect, however it is driven mainly by two data points and the weak correlation between changes in Ca or bile acids with change in cholesterol does not support an inverse relationship in this limited data set. Therefore, the possible mechanisms explaining why cheese does not increase cholesterol concentrations to the same extent as butter (with equal amounts of fat) remains unresolved.

Other possible mechanisms explaining this effect has been reviewed in a paper recently accepted [31]. Here it is speculated that the high protein content of cheese, the matrix of cheese, or the fact that cheese is a fermented product may affect cholesterol concentrations. Also, calcium was mentioned as a possible mechanism, but it seems more unlikely to be a sole explanation unless some unidentified mechanisms by which calcium can affect cholesterol concentrations are involved.

Strength of the current study is that the full fecal collections were homogenized before removing sample for later analysis; this precludes that sampling errors were causing the large individual and day-to-day fluctuations observed. Use of a pooled fecal sample

analyzed in triplicate with each sample batch and a stable isotope-labeled internal standard for each bile acid also increases the analytical validity of our findings. A limitation of the current study is that a sample of fecal water should have been collected straight after the 24 h collection. In addition, we observed large inter-individual and day-to-day variations in the amounts of bile acids excreted in feces whereas the repeated analyses of pooled samples were showing limited variation, suggesting that large fluctuations in bile acid excretion rather than analytical variation decreased the power in this study. The major limitation may have been the large fluctuations in bile acids from sample to sample in this study, even from the same individual. This fluctuation was larger than we have seen before. Our initial comparison with other studies indicated that we were amply powered [21], however more samples per volunteer at the end of the study would have improved precision and thereby power more in this study than an increase in the number of participants. Collection of fecal samples for more than two days would have resulted in better estimates of the average bile acid loss and perhaps significant differences in the amounts of bile acids excreted. In regard to concentration of total bile acid levels it is similar to those published by others [26,32], where we report 10–30% higher values. There is, however, also studies reporting higher levels, up to 50% higher than ours [33]. Thus, we are confident that we observe bile acid levels within the normal range, still we find no indication of total, bound or free bile acid co-variation with dairy calcium intake in this study. However, levels of the individual bile acids vary considerably between studies and seem to be highly matrix dependent and this is probably a major cause of variation in total bile acid excretion. A fully controlled diet could therefore have decreased other matrix factors thereby improving the possibility to show a possible effect of Ca intake on bile acid excretion.

We cannot exclude the possibility that measurement of the bile acids at baseline could possibly have accounted for part of this variation as individual differences in bile acid metabolism. In a crossover design, individual differences should have minor effects on the outcome measures so we regard this possibility as low and the scope of the study was to compare the effects of cheese and butter intake and therefore baseline measures of bile acids was not assessed.

5. Conclusion

The findings of the present study do not support the hypothesis that the high calcium content of cheese increases fecal bile acid output. In future studies it may be advised to give a fully controlled diet and to collect fecal samples for more than two days to further investigate the effect of specific foods on fecal bile acid levels. The mechanisms responsible for the lowering of cholesterol concentrations with cheese intake compared to butter intake remains unresolved.

Statement from authors'

Julie Hjerpsted participated in designing the intervention, conducted the intervention, performed the calcium analyses, performed the statistical analysis and wrote the paper.

Tine Tholstrup designed the intervention and collaborated in writing the manuscript.

Lars Ove Dragsted was responsible for the bile acid analyses and collaborated in writing the manuscript.

All authors have read and approved the final version of the manuscript.

The sponsors did not have any influence on the manuscript or its conclusions.

The authors declare that they have no conflict of interest.

All study subjects signed an informed consent prior to their inclusion and the study was approved by the Danish National Committee on Biomedical Research Ethics.

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