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Cheese intake lowers plasma cholesterol concentrations without increasing bile acid excretion

Julie B. Hjerpsted, Lars O. Dragsted, Tine Tholstrup*

Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, 1958, Frederiksberg, Denmark

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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.

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

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CVD, cardiovascular disease; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; HDL, high-density lipoprotein; LCA, lithocholic acid; LDL, low-density lipoprotein; UDCA, ursodeoxycholic acid.

* Corresponding author.
E-mail address: tth@life.ku.dk (T. Tholstrup).

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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 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

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%)