Cheddar Cheese Ripening Affects Plasma Nonesterified Fatty Acid and Serum Insulin Concentrations in Growing Pigs\textsuperscript{1–3}

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Abstract

Background: Meta-analyses of observational studies found cheese consumption to be inversely associated with risk of type 2 diabetes and metabolic syndrome. This may be attributed to the bioactive compounds produced during cheese ripening.

Objective: The objective of this study was to investigate by means of a porcine model how cheeses with different ripening times affect blood glucose, insulin, and lipid concentrations and fecal-fat excretion.

Methods: A parallel-arm randomized intervention study with 36 Landrace × Yorkshire × Duroc crossbred 3-mo-old female pigs was conducted. The pigs were fed a 21-d butter-rich run-in diet (143 g of butter/kg diet), followed by a 14-d intervention with 1 of 3 isocaloric diets: 4-mo ripened cheddar (4-MRC) diet, 14-mo ripened cheddar (14-MRC) diet, or 24-mo ripened cheddar (24-MRC) diet (350 g of cheese/kg diet). Serum cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, and insulin; plasma nonesterified fatty acids (NEFAs) and glucose; fecal-fat excretion; and body weight were measured.

Results: Plasma NEFAs were lower in the 24-MRC (201 ± 26 μEq/L) and in the 14-MRC (171 ± 19 μEq/L) diet groups than in the 4-MRC diet group (260 ± 27 μEq/L; \(P = 0.044\) and \(P = 0.001\)). Serum insulin was lower in the 24-MRC diet group (1.04 ± 0.09 mmol/L) than in the 4-MRC diet group (1.44 ± 0.14 mmol/L; \(P = 0.002\)), but intermediate and not different from either in the 14-MRC diet group (1.25 ± 0.11 mmol/L). Likewise, homeostasis model assessment of insulin resistance was lower in the 24-MRC diet group (0.030 ± 0.003) than in the 4-MRC diet group (0.041 ± 0.005; \(P < 0.01\)), but intermediate and not different from either in the 14-MRC diet group (0.036 ± 0.004).

Conclusions: Intake of long-term ripened cheddar improved indicators of insulin sensitivity in growing pigs compared with short-term ripened cheddar. This may also be important for human health. J Nutr 2015;145:1453–8.

Keywords: cheddar, cheese ripening duration, dairy, insulin sensitivity, glucose, blood lipids, NEFA, HOMA-IR, fecal-fat excretion, porcine model

Introduction

A recent study concluded that differences in food matrix may modify the effect of saturated fat on cardiovascular disease risk (1). Randomized controlled intervention studies in healthy and hypercholesterolemic adults have shown that consumption of full-fat cheese has a favorable effect on LDL cholesterol and/or total cholesterol (TC)\textsuperscript{8} concentrations compared with butter when matched for dairy fat content (2–5). A high intake of cheese might even have a neutral effect on LDL cholesterol and TC compared with a habitual diet with lower amounts of cheese, despite an increase in saturated fat intake (6). Recent meta-analyses of prospective cohort studies on dairy products and risk of type 2 diabetes mellitus (T2DM) also found cheese to be inversely associated with risk of T2DM (7, 8). In addition, observational studies have suggested cheese consumption to be

\(\text{TC} \) total cholesterol; \(\text{T2DM} \) type 2 diabetes mellitus; \(\text{4-MRC} \) 4-mo ripened cheddar; \(\text{14-MRC} \) 14-mo ripened cheddar; \(\text{24-MRC} \) 24-mo ripened cheddar.

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inversely associated with incidence or prevalence of metabolic syndrome (MetS) (9, 10), but not consistently (11). Furthermore, cheese consumption has been suggested to reduce the risk of impaired glucose tolerance (12). However, it is not known if these associations are causal because intervention studies on full-fat cheese consumption with primary endpoints being risk markers for the MetS remain to be conducted.

Ripening has been proposed as a possible cause for the different effect of cheese on metabolic health compared with unripened dairy products such as butter. A study in genetic obese diabetic mice found significantly reduced hepatic lipid content and improved glucose tolerance in mice fed 35-d ripened cheese compared with mice fed 0- or 15-d ripened cheese (13). This finding supports the hypothesis that bioactive components are produced and compositional changes occur in the cheese matrix during ripening, with implications for metabolic impact after ingestion. Changes occurring during cheese ripening include proteolysis of caseins to smaller peptides and amino acids, and lipolysis of TGs to nonesterified FAs (NEFAs) (14). In addition, potentially favorable changes may occur in the cheese microflora during ripening. It is, however, still unknown if the compositional changes that occur during cheese ripening affect glucose and lipid metabolism. Furthermore, it is unclear how cheese ripening duration may affect mechanisms associated with the cheese mineral content, i.e., calcium and phosphate that are responsible for the reduced fat digestibility frequently observed (15–18). The present study used a porcine model for human gastrointestinal and cardiovascular metabolism to investigate how different ripening durations affect blood lipids, glucose and insulin concentrations, body weight, and fecal-fat excretion.

Methods

Animals
The study protocol complied with the Danish Ministry of Justice Law No. 1306 (23 November 2007) concerning experiments with animals and care of experimental animals. The animals included in the study were 36 specific pathogen-free Landrace and Yorkshire; 3 mo. A porcine model was chosen because pigs, like humans, are omnivorous and eat bolus meals. Pigs are frequently used as an animal model for human digestion and cardiometabolic response because of digestive similarities, bolus secretion of bile acids, and comparable responses to dietary fat and cholesterol on blood lipid balance (19–21). The pigs were kept together in large pens on bedding before study commencement. During the study periods they were kept individually in pens without bedding.

Design
A randomized controlled parallel-arm design with 3 intervention diets was used. A total of 36 pigs were included with 12 pigs in each intervention group. The sample size calculation was based on an expected mean difference in serum LDL cholesterol of 0.3 mmol/L and a 0.25 SD between the 2 least different diet groups. Assuming a power of 80% and a significance of 0.05, 12 pigs in each group would be needed to detect the expected difference. There was a 21-d run-in period on a dairy fat (butter)–rich diet to habituate the pigs to a diet containing dairy saturated fat. After run-in, pigs were allocated to 1 of 3 intervention diets by means of stratified randomization according to initial TC concentration. The intervention period lasted for 14 d. For logistic reasons there was a slight aberration from the parallel-arm study design because the 36 pigs were divided into 2 groups of 18 pigs; each group was administered the diets in partly overlapping time periods. The first group started the run-in period 2 wk before the second group.

Fasting venipuncture blood samples were drawn 3 times: before the run-in period (solely used for the stratified randomization), before the intervention period (preintervention), and after the intervention period (postintervention). Body weight was measured on the same occasions and before the feces collection. Feces were collected the last 48 h of the intervention period, and the pigs were put in stainless steel metabolic cages 24 h before the collection period.

Diets
The 3 intervention diets were isocaloric and contained high amounts of 1 of 3 cheddar cheeses with different ripening durations: 4-mo ripened cheddar (4-MRC), 14-mo ripened cheddar (14-MRC), and 24-mo ripened cheddar (24-MRC). The 4-MRC was the shortest possible ripening duration for cheddar, because it ripens very slowly; therefore, the 4-MRC diet served as the control. As a consequence of ripening, the content of peptides was higher in the 14-MRC diet than in the 4-MRC and 24-MRC diets (Figure 1), whereas the amino acid content was higher in the 24-MRC diet than in the 4-MRC and 14-MRC diets (Figure 2). To ensure complete consumption, the diets were fed in amounts corresponding to 90% of the habitual energy intake of the pigs. Habitual energy intake was assessed from body weight and growth curves (22) before the run-in diet and again before the intervention diet to match the energy supply to the 21-d increase in body weight. For all 3 diets equal amounts of cheese (350-g/kg diet) were included, corresponding to a daily consumption of ~700 g of cheese in a 60-kg pig. Because of minor differences in fat and protein content of the 3 cheeses (Supplemental Table 1), there were small differences in the energy, fat, and protein contents of the 3 cheddar diets (Supplemental Table 2). We did not adjust diets for differences between cheeses because these were considered to reflect normal variations in milk used in cheese production. FA composition was similar in all diets (Supplemental Table 3).

The diets were planned to reflect the macronutrient composition of the average diet in the Danish population. However, because of the fat tolerance limit of the pigs, the fat content was slightly lower and the protein content was slightly higher than that of the average diet in the population (23). The high cheese content was not comparable with the average intake in the Danish population.

The pigs had free access to water during the intervention, except when they were feed deprived overnight before sampling days when water intake was restricted to 1 L.

Laboratory analyses

Cheeses. Cheese samples were analyzed for fat content by the Gerber method (International Organization for Standardization: 3433) and protein content was estimated according to the Kjeldahl method (International Dairy Federation 20B:1993). Energy content was measured using a bomb calorimeter (International Organization for Standardization: 9831; Parr 6300 Oxygen Bomb Calorimeter, Parr Instrument Co.).

![Figure 1](https://example.com/figure1.png)  
*Figure 1* Amino acid concentrations in the 4-MRC, 14-MRC, and 24-MRC cheeses used in the 3 intervention diets fed to 3-mo-old female pigs for 14 d (n = 2). 4-MRC, 4-mo ripened cheddar; 14-MRC, 14-mo ripened cheddar; 24-MRC, 24-mo ripened cheddar.
Sodium content was assessed as described in International Dairy Federation standard 88A:1988 and pH as described by Ardó et al. (24). Calcium content was measured by atom absorption spectrometry (Model S2AA System, Thermo Electron Corp., Ltd.) after incineration at 450°C and hydrochloric acid/nitric acid treatment. Extent of cheese ripening was determined as described by Rehn et al. (25), and reverse-phase HPLC and HPLC were used to determine peptides and free amino acids, respectively (26).

Diets. Samples of the intervention diets were homogenized and energy content was determined as described previously. Fat content was measured using a modified Bligh and Dyer method (27, 28). FA composition was determined as described by Bendsen et al. (18) and acid-insoluble ash was determined as described by McCarthy et al. (29, 30).

Feces. After collection feces were immediately frozen at −80°C (LDL, HDL, and TGs) and −20°C (TC, NEFAs, insulin, and glucose). Serum TC, HDL cholesterol, TGs, and LDL cholesterol were determined according to standard procedures (Siemens Diagnostics Clinical Methods for ADVIA 1650). Plasma NEFAs were determined using the Wako, NEFA C acyl-coenzyme A synthetase acyl-coenzyme A oxidase assay method. All analyses were performed using an autoanalyser, ADVIA 1650 Chemistry System (Siemens Medical Solutions). Intra- and interassay precision were <4% CV and accuracy was within 5% for all analyses, for both low and high control samples. Plasma glucose was measured by an enzymatic method (Hexokinase) using an ABX Pentra Glucose HK CP kit on an ABX Pentra 400 analyser (HORIBA ABX). Serum insulin was measured by ELISA assay with species-specific antibodies (Shibayagi Co., Ltd.), according to manufacturer’s procedures. Inter- and intra-assay precision was 5% (CV, n = 3, n = 3, and n = 5). The HOMA-IR was calculated using HOMA-IR = [fasting serum insulin (µIU/mL) × fasting plasma glucose (mmol/L)]/22.5.

Body weight. Fasting body weight was measured on a Meier-Brakken body weight scale (MB WA 100, Meier-Brakken GmbH & Co. KG).

Statistical methods

Statistical analyses of blood lipids and body weight were performed using ANCOVA on postintervention measurements. ANCOVA models included the treatment (diet) as well as baseline (preintervention) adjustment and adjustment for the fact that the study was performed in 2 periods. For all outcomes an approximate F-test was initially performed to test if diets were different. If the global test was significant, pairwise comparisons between diets were subsequently performed using post hoc t tests. In addition, the influence of body weight change was investigated in the analyses of blood lipids using ANCOVA models that also included adjustment for body weight change. Model assumptions were checked by means of residual plots and normal probability plots. Furthermore, the presence of outliers was investigated using Cook’s distance. Statistical analyses were performed using SAS 9.4 (SAS Institute, Inc.). Values were considered significantly different for P < 0.05. All values in tables are means ± SEMs, unless otherwise stated.

Results

The study was completed as planned and no side effects of the diets were observed. Preintervention values did not differ between the 3 intervention groups for any of the measures (P > 0.05). One influential outlier on blood lipids was found in the 4-MRC diet group and was removed from statistical analyses of these.

Blood glucose and insulin concentrations and HOMA-IR

No difference in plasma glucose concentrations between diet groups was found (Table 1). When compared with the 4-MRC diet group, the serum insulin concentration was 30% lower (−0.44 ± 0.13 µIU/L, P = 0.002) in the 24-MRC diet group, but intermediate and not different from either in the 14-MRC diet group.

| TABLE 1 Plasma glucose and serum lipid concentrations in 3-mo-old female pigs before and after being fed the 4-MRC, 14-MRC, and 24-MRC diets for 14 d |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Preintervention | Postintervention | Preintervention | Postintervention | Preintervention | Postintervention | Pdiet          |
|                  |                 |                 |                 |                 |                 |                 |                |
| 4-MRC diet       |                 |                 |                 |                 |                 |                 |                |
| P-glucose, mmol/L| 4.53 ± 0.14     | 4.46 ± 0.08     | 4.58 ± 0.07     | 4.50 ± 0.17     | 4.59 ± 0.13     | 4.52 ± 0.07     | NS             |
| S-TC, mmol/L     | 3.28 ± 0.13     | 3.75 ± 0.13     | 3.28 ± 0.12     | 3.72 ± 0.14     | 3.36 ± 0.11     | 3.94 ± 0.15     | NS             |
| S-LDL-C, mmol/L  | 1.65 ± 0.11     | 2.03 ± 0.11     | 1.63 ± 0.09     | 2.07 ± 0.12     | 1.72 ± 0.10     | 2.21 ± 0.14     | NS             |
| S-HDL-C, mmol/L  | 1.39 ± 0.05     | 1.45 ± 0.04     | 1.39 ± 0.04     | 1.42 ± 0.08     | 1.37 ± 0.05     | 1.45 ± 0.05     | NS             |
| S-TG, mmol/L     | 0.47 ± 0.02     | 0.49 ± 0.02     | 0.49 ± 0.02     | 0.49 ± 0.02     | 0.49 ± 0.02     | 0.49 ± 0.03     | NS             |
| S-LDL-C-S-HDL-C  | 1.19 ± 0.07     | 1.40 ± 0.09     | 1.18 ± 0.06     | 1.53 ± 0.14     | 1.27 ± 0.07     | 1.53 ± 0.09     | NS             |
| S-TCS-HDL-C      | 2.37 ± 0.08     | 2.57 ± 0.07     | 2.37 ± 0.06     | 2.70 ± 0.15     | 2.57 ± 0.07     | 2.72 ± 0.07     | NS             |

*All values are shown as means ± SEMs, n = 12. HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; P, plasma; S, serum; TC, total cholesterol; 4-MRC, 4-mo ripened cheddar; 14-MRC, 14-mo ripened cheddar; 24-MRC, 24-mo ripened cheddar.
diet group (−0.20 ± 0.13 μg/L, P = 0.13; Figure 3A). Likewise, HOMA-IR was lower in the 24-MRC diet group than in the 4-MRC diet group (P = 0.01), but intermediate and not different from either in the 14-MRC diet group (Figure 3B).

**Blood lipids, fecal-fat excretion, and body weight.** When compared with the 4-MRC diet group NEFA concentrations were 34% lower (89.8 ± 25.6 μEq/L, P = 0.001) in the 14-MRC diet group and 23% lower (58.6 ± 27.9 μEq/L, P = 0.044) in the 24-MRC diet group (Figure 4). NEFA concentration did not significantly differ between the 14-MRC or 24-MRC diet group. There were no differences in TC, LDL cholesterol, HDL cholesterol, or TG concentrations or in the lipid ratios between diet groups (Table 1), and there were no differences in fecal-fat excretion (pooled mean: 30.5 ± 1.6 g/d) or increase in body weight (pooled mean: 29.9 ± 0.5 kg). Body weight had no effect on any of the blood lipids.

**Discussion**

This study aimed to investigate whether cheese ripening duration is of importance with regard to blood lipid and insulin responses to cheese consumption in a porcine model. It also investigated whether ripening duration could affect the digestibility of cheese fat.

The main findings were that the long-term ripened cheddars (14-MRC and 24-MRC) suppressed serum NEFA concentrations in pigs compared with short-term ripened cheddar (4-MRC) with the effect being strongest in the 14-MRC diet group. Long-term ripened cheddars improved insulin sensitivity in pigs compared with short-term ripened cheddar, but the effect was only significant with the 24-MRC diet. The lower serum insulin response in pigs fed the 24-MRC diet might be caused by the reduced plasma NEFA concentration, because elevated NEFAs have previously been suggested to increase insulin secretion (32). However, this is not the most plausible explanatory mechanism, because the effects of ripening on NEFAs seemed to be biphasic. When compared with the 4-MRC diet group NEFA concentration was lowest in the 14-MRC diet group, whereas the insulin concentration was lowest in the 24-MRC diet group. The reduced NEFA concentration in pigs fed the 14-MRC diet did not cause a significantly lower insulin concentration.

As far as we know only 1 animal study, in mice, has investigated the metabolic effects of consumption of cheese with different ripening durations (13). In accordance with the present findings, this study observed that cheese ripening duration could affect glucose metabolism. The study found that a 4-wk diet containing camembert cheese ripened for 35 d caused significant reductions in hepatic lipid content and improvements in glucose tolerance, independently of insulin secretion, compared with diets containing camembert ripened for 0 or 15 d (13). However, we observed the main effect of our 2-wk cheese intervention to be on NEFA and insulin concentrations, without impact on glucose concentration, hence, affecting insulin sensitivity. It would be expected that fasting insulin increases before fasting glucose concentration, as observed in our study, because glucose concentration is strictly maintained within the normal range by insulin at normal physiologic conditions. The difference in the observations of the 2 studies of the response to consumption of the ripened cheeses could be caused by differences between the mouse and pig species in glucose metabolism. It is, however, more likely that the different effects were caused by the different types of cheese. Cheddar cheese, as used in our study, is ripened for a long time with lactic acid bacteria activity in the anaerobic interior of the cheese, whereas ripening of a camembert cheese...
is highly influenced by white mold growing on the surface. Cheddar ripening causes gradual proteolysis of casein by chymosin from coagulant, plasm in from milk, and proteolytic enzymes from starter and nonstarter lactic acid bacteria to smaller caseins, peptides, and amino acids (33). Only slight lipolysis of TGs to NEFAs occurs during cheddar ripening. By contrast, camembert ripening causes substantial lipolysis of TGs and primary proteolysis of casein; however, because of the short ripening time the formation of peptides and release of free amino acids is limited (34). The cheddar cheeses used in our study had a total amount of free amino acids ranging from 2.5% to 7.5%, whereas the ripest camembert cheese used in the mouse study only had a free amino acid content of 2.4% (13). It is possible that cheddar and camembert cheeses have distinctly different effects on glucose metabolism after consumption because of differences in their ripening products, i.e., peptides and free amino acids vs. NEFAs.

Peptides and amino acids produced during ripening have previously been suggested to have bioactive properties (35). The peptide content was 8% higher in the 14-MRC than in the 24-MRC, whereas the 24-MRC had a 54% higher content of free amino acids compared with the 14-MRC. Because ripening was the major difference between the 3 cheddar diets provided for the pigs in our study a longer ripening duration, with higher amino acid and peptide content, must necessarily be responsible for the lower plasma NEFA and serum insulin concentrations caused by the long-term ripened cheeses. The specific mechanism underpinning the effect of ripening on suppression of NEFAs and insulin is unclear. The responses observed in pigs fed the 2 long-term ripened cheeses could be mediated through different pathways. The content of peptides was highest in the 14-MRC diet (Supplemental Table 1, Figure 2) and serum NEFAs was lowest in pigs fed the 14-MRC diet (Figure 4). The content of free amino acids (Figure 1, Supplemental Table 1) was highest in the 24-MRC diet, which could be responsible for the lower insulin concentration and lower insulin sensitivity in pigs on this diet (Figure 3). However, we can only speculate on the underlying mechanisms, and these should be the focus of future studies on metabolic effects of cheese ripening.

Three human intervention studies have investigated insulin and glucose response after cheese intake compared with butter and/or milk (3, 4, 6). One of these studies found higher fasting plasma glucose concentrations after cheese consumption compared with the baseline habitual diet and compared with consumption of butter (6). Another found higher postprandial glucose concentrations after cheese intake (4), whereas the last study observed no effect of cheese on glucose or insulin concentrations (3). These human studies used shortly ripened semihard cheeses. The ripening time of cheddar cheese, as used in our study, varies considerably. We found that consumption of the short-term ripened cheddar caused higher serum insulin concentrations compared with long-term ripened cheddar, which is in line with effects observed in previous human studies with short-term ripened semihard cheese, although we did not find differences in plasma glucose concentrations by ripening. We propose that the metabolic responses to cheese consumption may depend on the ripening duration of the cheese. It is therefore crucial to report cheese ripening duration in future studies examining the impact of cheese consumption on T2DM risk. A recent dose-response meta-analysis on dairy consumption and risk of T2DM found cheese to be inversely associated with T2DM risk, with an RR of 0.8 per 30 g/d of cheese (7), and observational studies have also shown cheese consumption to reduce the risk of MetS (9, 10). These studies did not report the types of cheese consumed or the relative distribution of short-term vs. long-term ripened cheeses, and our results suggest that the latter is crucial in the metabolic effects of cheese consumption.

We found no effect of ripening duration on blood lipid concentrations, except for plasma NEFAs, and we did not observe an effect on fecal excretion of fat. Hence, ripening duration does not affect the fat digestibility reducing capacity of calcium in the cheese.

In conclusion, consumption of long-term ripened cheddar cheese improved indicators of insulin sensitivity in 3-mo-old female domestic pigs compared with short-term ripened cheddar. Specifically, consumption of 24-MRC caused lower plasma NEFA and serum insulin concentrations and lower HOMA-IR in pigs compared with consumption of 4-MRC. Consumption of the 14-MRC caused a lower plasma NEFA concentration in pigs whereas serum insulin concentration and HOMA-IR were intermediate and not significantly different compared with consumption of the 4-MRC. The difference in ripening duration did not affect fat digestibility, body weight, or the concentrations of serum TC, HDL cholesterol, LDL cholesterol, or TG, or plasma glucose. Studies, preferably in humans, investigating the underlying mechanism behind the lower plasma NEFA and improved insulin sensitivity after consumption of long-term ripened cheese should be conducted.

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References


22. Andersen PE, Just A. Tabeller over foderstoffers sammensætning m.m. kvæg-svin. [Tables of feed composition etc. cattle and pigs.] Copenhagen (Denmark): Landhusholdningsselskabets Forlag; 1983 (in Danish).


