

Ripened Dairy Products Differentially Affect Hepatic Lipid Content and Adipose Tissue Oxidative Stress Markers in Obese and Type 2 Diabetic Mice

Lucie Geurts,^{†,§} Amandine Everard,^{†,§} Pascale le Ruyet,[‡] Nathalie M. Delzenne,[†] and Patrice D. Cani^{*,†}

[†]Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, Av. E. Mounier, 73 Box B1.73.11, B-1200 Brussels, Belgium

[‡]Lactalis, RetD Lactalis, Laval, France

ABSTRACT: Growing evidence suggests that the consumption of dairy products may contribute to a reduced incidence of cardiovascular risk factors, such as obesity, dyslipidemia, and type 2 diabetes. The fatty acid composition in milk fat, the duration of ripening, and the complexity of the food matrices are important factors that may interfere with the physiological impact. In this study, we treated genetic obese and type 2 diabetic mice (*db/db*) for 4 weeks with different dairy (cheese-based) products, differing by the duration of ripening (0, 15, or 35 days). We found that 35 days ripened product significantly improved glucose tolerance, an effect associated with a decreased adipose tissue lipid peroxide markers (TBARS and NAPDH-oxidase mRNA expression), without affecting body weight, food intake, and fat mass. Both fermented matrices significantly decreased the hepatic lipid content, without modifying plasma triglycerides or plasma total cholesterol. These data suggest that dairy products issued from longer ripening positively impact glucose tolerance, hepatic steatosis, and adipose tissue oxidative stress. Further investigations are warranted to decipher the interactions between milk products fermentation, lipids, and host metabolism.

KEYWORDS: *glucose tolerance, hepatic steatosis, type 2 diabetes, cheese, oxidative stress*

■ INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has emerged as a serious common feature associated with obesity and associated disorders.¹ A dietary excess of saturated fat has been shown to contribute significantly to hepatic lipid accumulation.^{1,2} Given that milk fat represents a well-known rich source of saturated fatty acids, dairy products are recommended to be consumed sparingly. Besides this knowledge, recent epidemiological studies have challenged this concept.^{3–5} Several studies have shown that increased consumption of milk and other dairy products is associated with a reduced incidence of cardiovascular risk factors, such as obesity, insulin resistance, dyslipidemia, and type 2 diabetes.^{3–5} Although numerous attempts to find biologically active dairy products have been unsuccessful, several investigations have identified putative interesting compounds. For instance, negative correlations between vitamin D levels and the risk of hypertension, myocardial infarction, and stroke have been reported in several observational studies.^{5,6} However, on the basis of the known deleterious impact of saturated fatty acids on lipid profile milk phospholipids, one would expect adverse effects on plasma and liver lipid metabolism. Conversely, this hypothesis has been recently questioned in a study showing that despite the presence of saturated fatty acids in phospholipids from dairy milk, dietary supplementation with phospholipid-rich dairy milk extract was found to have favorable effects on plasma and liver lipid metabolism.⁷ Thus, the variation of fatty acid composition in milk fat, the type and the duration of maturation, and the complexity of the food matrices are important factors that may interfere with the physiological impact of dairy products ingestion.

In the present study, we hypothesized that the ripening duration of dairy products such as cheese differentially affects glucose and lipid metabolism. Thus, the objective of this study was to determine the influence of administration of 4 weeks of treatment with dairy (cheese-based) products on glucose tolerance, hepatic lipid content, and profiles on the liver. We further assayed lipoperoxides (thiobarbituric acid-reactive substances, TBARS) in the adipose tissues of genetic obese and diabetic mice.

■ MATERIALS AND METHODS

Mice. A set of 6 week old male *db/db* mice C57BL/J background (BKS.Cg-Dock7^m +/+ Lepr^{db}/J) (Jackson-Laboratory, Bar Harbor, ME) (30 mice, *n* = 10/group) were housed in groups of two or four mice/cage, with free access to food and water. After 1 week of acclimatization, all of the mice were fed a control diet (A04, SAFE, Villemoisson-sur-Orge, France), supplemented with 100 g/kg of lyophilized cheeses (camembert technology) made with pasteurized milk with different days of ripening, 0 days (CT), 15 days (15d), or 35 days (35d) and pelleted by the company (SAFE); all of the cheeses were provided by Lactalis (Retiers, France). The nutritional composition of the A04 diet was as follows: protein, 16.1 g/100 g; carbohydrate, 60 g/100 g; starch, 45.8 g/100 g; sucrose, 2 g/100 g; cellulose, 3.9 g/100 g; fat, 3.1 g/100 g; and vitamin mix, 4.1 g/100 g. The nutritional composition of the different lyophilized matrices is shown in Table 1.

Treatment continued for 4 weeks, and mice food and water intake were recorded once a week. All mouse experiments were approved by

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Table 1. Nutritional Composition of the Lyophilized Matrices

	CT	15d	35d
total fat (g/100 g)	45.53	47.77	46.69
total free fatty acids (g/100 g)	0.207	3.28	5.63
total protein (g/100 g)	38.92	35.67	33.04
total free amino acids (g/100 g)	0.114	1.60	2.43

and performed in accordance with the guidelines of the local ethics committee. Housing conditions were specified by the Belgian Law of April 6, 2010, regarding the protection of laboratory animals (agreement number LA1230314).

Oral Glucose Tolerance Test. Oral glucose tolerance tests were performed after 4 weeks of treatment. Food was removed 2 h after the onset of the daylight cycle, and mice were treated after a 6 h fasting period as previously described.^{8,9} Glucose was orally administered (1 g of glucose per kg of body weight, 50% glucose solution), and blood glucose was determined through a glucose meter (Roche Diagnostics) on 3.5 μ L of blood collected from the tip of the tail vein before glucose load (−30 and 0 min) and after glucose load (15, 30, 60, 90, and 120 min). A 120 μ L amount of blood was sampled 30 min before and 15 min following the glucose load to assess plasma insulin concentration using an enzyme-linked immunosorbent assay kit (Merckodia, Uppsala, Sweden) following the manufacturer's instructions.

Tissue Sampling. Three days after the oral glucose tolerance test, mice were anesthetized with isoflurane gas (Forene, Abbott, Queenborough, Kent, England) after a 6 h fasting period. Portal vein blood samples were harvested for further analysis. Mice were sacrificed by cervical dislocation. Epididymal, subcutaneous (inguinal and dorsolumbar), and visceral (mesenteric) adipose deposits were precisely dissected and weighed. The liver and adipose tissues were immediately immersed in liquid nitrogen and stored at -80°C for further analysis.

Biochemical Analyses. Total lipids were measured in the liver tissue after an extraction with chloroform–methanol (Lab-Scan) according to Folch et al.¹⁰ Tissue lysate was prepared in phosphate buffer by using Ultra-Turrax (IKA, T10 basic, Boutersem, Belgique) until complete tissue lysis. Lipids were extracted by mixing 125 μ L of lysates with 1 mL of chloroform:methanol (2:1). The chloroform phase was evaporated under nitrogen flux, and the dried residue was weighed to determine the total lipid content.

Plasma triglycerides and total cholesterol concentrations were measured using kits coupling enzymatic reaction and spectrophotometric detection of reaction endproducts (DiaSys, Brussels, Belgium). Lipid peroxidation was evaluated by measuring TBARS. The tissue lysate was prepared in saline by using Ultra-Turrax (IKA, T10 basic) until complete tissue lysis. Aldehydes contained in tissue lysate reacted with thiobarbituric acid (Fluka) forming an aldehyde–thiobarbituric acid complex, which can be spectrophotometrically detected.^{8,9,11}

Adipose Tissue Morphometry and Staining. The mean relative proportion of adipocytes was estimated by a point-counting technique, on paraffin-embedded, HES (hematoxylin eosine staining) counter-stained sections of subcutaneous tissue. The number of adipocytes per microscopic field (density) was determined as previously described.^{9,12}

RNA Preparation and Real-Time qPCR Analysis. Total RNA was prepared from tissues using TriPure reagent (Roche). Quantitation and integrity analysis of total RNA were performed by running 1 μ L of each sample on an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent). cDNA was prepared by reverse transcription of 1 μ g of total RNA using a Reverse Transcription System kit (Promega, Leiden, The Netherlands). Real-time PCRs were performed with the StepOnePlus real-time PCR system and software (Applied Biosystems, Den IJssel, The Netherlands) using Mesa Fast qPCR (Eurogentec, Seraing, Belgium) for detection according to the manufacturer's instructions. RPL19 RNA was chosen as the house-keeping gene. Primer sequences were previously described.^{13–15} All samples were run in duplicate in a single 96-well reaction plate, and data were analyzed according to the $2^{-\Delta\text{CT}}$ method. The identity and

purity of the amplified product were checked through analysis of the melting curve carried out at the end of amplification.

Statistical Analyses. The data are expressed as the mean \pm SEMs. Differences between groups were assessed using one way analysis of variance (ANOVA), followed by posthoc Tukey test. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). The results were considered statistically significant at $P < 0.05$.

RESULTS

Thirty-Five Days Ripened Cheese Matrices Feeding Improves Glucose Tolerance. Fasted plasma glucose levels were equivalent between groups (Figure 1A). Interestingly,

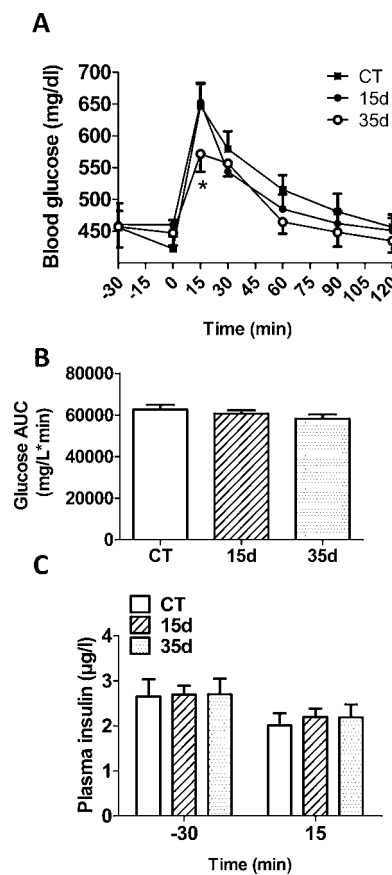


Figure 1. Glucose tolerance and plasma insulin in obese and diabetic mice consuming ripened dairy products. (A) Blood glucose profile, (B) area under the curve following 1 g/kg glucose oral challenge, and (C) plasma insulin levels measured in freely moving mice fed a CT, 15 day, or 35 day diet. Means \pm SEMs, $n = 10$ mice/group; * $P < 0.05$ vs CT and 15 days determined by a two-tailed Student's t test.

mice fed with the 35 days ripened products exhibited an improved glucose tolerance as compared to the two other groups. Glycemia was significantly decreased 15 min after the oral glucose load and tended to remain lower during the 2 h period (Figure 1A). Although this effect, the area under the curve was not significantly different between groups (Figure 1B). Plasma insulin levels measured in fasted and 15 min after the oral glucose challenge were not affected by the treatments (Figure 1C).

Stage of Cheese Ripening Did Not Change on Body Weight, Fat Mass, and Food Intake. The different diet did not affect the body weight, adipose tissue weight, and food intake at completion of the experimental period (Figure 2A–

C). The adipocyte size was not affected by the treatment (Figure 2D).

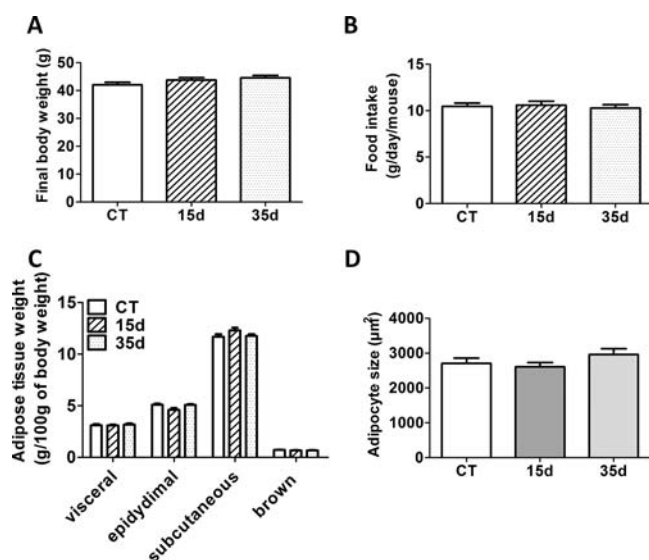


Figure 2. Body weight, fat mass, food intake, and adipocyte size in obese and diabetic mice consuming ripened dairy products. (A) Body weight (g); (B) food intake; (C) visceral (mesenteric), epididymal, subcutaneous (inguinal and dorsolumbar), and brown adipose tissues weight; and (D) mean adipocyte size of mice fed a CT, 15 day, or 35 day diet. Means \pm SEMs, $n = 10$ mice/group.

Thirty-Five Days Ripened Cheese Matrices Feeding Reduces Adipose Tissue Oxidative Stress Markers.

Oxidative stress has been associated with fat feeding and metabolic disturbances.^{16,17} Here, we found that the 35d group displayed a significant decrease in subcutaneous adipose tissue TBARS content, suggesting a lower lipid peroxidation (Figure 3A). To shed light on the mechanism whereby this

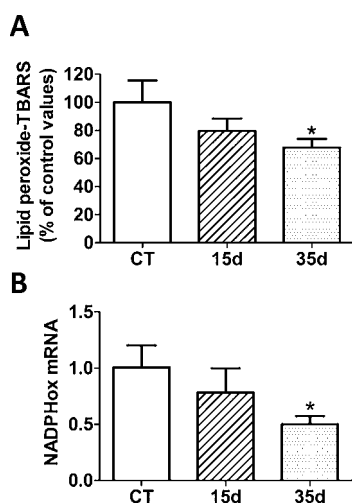


Figure 3. Adipose tissue oxidative stress markers in obese and diabetic mice consuming ripened dairy products. Lipid peroxidation markers: (A) TBARS, (B) NADPH-oxidase mRNA expression levels measured in mice fed a CT, 15 day, or 35 day diet. Means \pm SEMs, $n = 10$ mice/group; * $P < 0.05$ vs CT according to posthoc ANOVA one-way statistical analysis and two-tailed Student's t test.

phenomenon occurs, we investigated NADPH oxidase, a key enzyme involved in the production of reactive species. In

accordance with the observation that 35 days fermented matrix reduced reactive oxygen species, we observed a 2-fold decreased NADPH-oxidase mRNA expression (Figure 3B). These results further support the link between this enzyme, TBARS content, and the perturbation of glucose homeostasis in different tissues, including the adipose tissue.¹⁵

Ripened Cheese Matrices Feeding Reduces Steatosis.

We found that both 15d and 35d fermented milk matrices significantly decreased hepatic lipid content by about 27 and 42%, respectively (Figure 4A). This effect partly may be

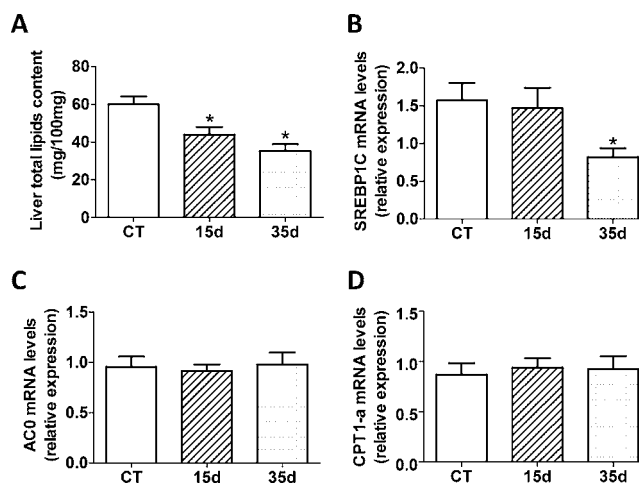


Figure 4. Hepatic lipid content and mRNA expression of genes involved in lipogenesis and lipid oxidation markers in obese and diabetic mice consuming ripened dairy products. (A) Liver lipids content; (B) SREBP-1c, (C) ACO, and (D) CPT1-a mRNA expression levels in the liver of mice fed a CT, 15 day, or 35 day diet. Means \pm SEMs, $n = 10$ mice/group; * $P < 0.05$ vs CT according to posthoc ANOVA one-way statistical analysis.

explained by a decreased expression of the key factor controlling hepatic lipogenesis, namely, sterol regulatory element-binding protein 1c (SREBP-1c).¹⁸ Although, we found a 2-fold decrease in SREBP-1c mRNA expression in the 35d group than CT mice, this parameter was not affected in the 15d group of mice (Figure 4B). Moreover, fatty acid synthase (FAS) mRNA expression decreased by about 25% in the 35d group than CT mice but did not reach significance ($P = 0.1$) (FAS mRNA relative expression levels: CT, 1.07 ± 0.12 ; 15d, 0.97 ± 0.17 ; and 35d, 0.80 ± 0.08), thereby suggesting that another mechanism participates in this effect. Therefore, we measured the mRNA expression of the rate-limiting enzyme Acyl CoA oxidase (ACO) involved in peroxisomal lipid oxidation and the mRNA expression of the protein CPT1a, which transports fatty acids for oxidation in mitochondria.¹⁹ We did not find any modification of these markers following the treatments (Figure 4C,D). Although the dietary treatments reduced the hepatic lipid content, we did not find any significant change of the plasma lipids (triglycerides and total cholesterol): plasma triglycerides (mmol/L): CT, 1.32 ± 0.11 ; 15d, 1.27 ± 0.12 ; and 35d, 1.11 ± 0.09 ($P > 0.05$); and plasma total cholesterol (mmol/L): CT, 3.27 ± 0.13 ; 15d, 3.23 ± 0.14 ; and 35d, 3.07 ± 0.08 ($P > 0.05$).

DISCUSSION

In the present study, we found that the duration of ripening of cheese matrices is an important factor interfering with the

physiological impact of dairy products ingestion. First, we found that 35 days of ripening significantly improved glucose tolerance, without affecting insulin secretion. Second, we observed that the same diet significantly decreased adipose tissue lipid peroxide markers (TBARS and NADPH-oxidase mRNA expression). Third, both fermented matrices significantly decreased hepatic lipid content with a more pronounced effect in mice fed the 35 days matured products. These data indicate that the ripening of dairy products (i.e., cheese matrices) may differentially and positively impact glucose and lipid homeostasis in obese and type 2 diabetic mice. It is worth mentioning that this study has been performed in genetic obese and type 2 diabetic mice exhibiting a severe phenotype (obesity, diabetes, hepatic steatosis, and pronounced oxidative stress) as previously characterized.^{20–22} Unless this marked metabolic disorders, we found that 4 weeks treatment with 35 days fermented dairy products positively affect some of these metabolic features. In addition, although dairy products contain predominantly saturated fatty acids, we found that ripened cheese modestly improved glucose tolerance and hepatic steatosis. Thus, these findings challenge the concept that saturated fatty acid ingestion is mainly involved in the onset of metabolic disorders.

We observed a modest but significant improvement of the glycemic response following the oral glucose load. This effect can be partly attributed to two different mechanisms such as the decreased hepatic lipid content and the reduced oxidative stress in the adipose tissue. Previous studies have already demonstrated that such relationship exists and could be in part associated with obesity and type 2 diabetes phenotypes.^{16,17} From a mechanistic point of view, the relationship between steatosis and glucose intolerance or type 2 diabetes is thought to result predominantly from hyperinsulinemia. The steatotic liver develops resistance to insulin in terms of inhibition of hepatic glucose production and stimulation of glycogen synthesis. Although several mechanistic proofs support the role of fatty liver in the pathogenesis of increased hepatic glucose production and altered glucose metabolism, recent studies have also provided evidence that in hepatic steatosis there is an accumulation of nonesterified fatty acids and triacylglycerides metabolites (fatty acyl CoA, diacylglycerol, and ceramides). Some of these lipids have been shown to inhibit insulin signal transduction, thereby promoting insulin resistance.^{23,24}

Although we did not find any modification of the plasma insulin, we observed a significant decrease in hepatic lipid content. Interestingly, it has been previously demonstrated that mobilizing a relatively small pool of intrahepatic lipid is able to reverse hepatic insulin resistance and to normalize glucose homeostasis, independently of any changes in insulin-stimulated peripheral glucose metabolism.²⁵ Given that we did not find any modification of the body weight and the fat mass following the dietary treatment, we may not explain the improved glucose and lipid metabolism observed in the 35d group of mice by these parameters.

Different mechanisms could be responsible for the beneficial effect of prolonged ripening on glucose tolerance, hepatic steatosis, and adipose tissue oxidative stress. For instance, it has been proposed that the composition and the origin of phospholipids were able to differentially affect glucose and lipid homeostasis.^{7,26,27} Therefore, we may not rule out the existence of such effect in our study. In addition, evidence suggests a role for tissues fatty acid profiles and the

susceptibility to oxidative stress.^{14,28} However, according to the hepatic fatty acid profile observed in this study (data not shown), we could not attribute these effects to specific changes in one or more key fatty acid family (e.g., saturated vs unsaturated). Thus, this challenges the concept that the hepatic fatty acid profile directly impacts whole-body metabolism.

Importantly, the biochemical compositions of dairy fermented products (cheese) are the result of specific microbial community interactions.^{29,30} Ripening processes are associated with numerous changes. For instance, the microbial composition, the production of specific microbial metabolites, and proteolysis from the casein matrix are all specific events that are dependent on the ripening duration. Therefore, it is likely that these neglected factors markedly affect the host metabolism. In accordance with this hypothesis, we found that ripening profoundly affects the free amino acids content (Table 1). For instance, it has been previously proposed that milk proteins (whey or casein) reduce the postprandial blood glucose response of mixed meals and glucose^{31–33} via several mechanisms including insulin secretion or energy homeostasis (thermogenesis and food intake).³³

In addition, we and others have found that the gut microbiota participate to the control of hepatic lipid composition and adipose tissue oxidative stress.^{9,13,34–36} Besides, we did not investigate this particular mechanism in the present study, but we speculate that this potential interaction between microbes and/or their metabolites as well as the host metabolism contributes to the phenotype observed. However, the exact mechanisms responsible for the improved phenotype observed here remain to be elucidated yet.

In conclusion, our data suggest that feeding dairy products issued from longer ripening positively impact glucose tolerance, hepatic steatosis, and adipose tissue oxidative stress in obese and diabetic mice. Specific investigations are still required to decipher this complex interaction between dairy products fermentation, dietary lipids, food matrices, and host metabolism.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +32 2 764 73 97. Fax: +32 2 764 73 59. E-mail: patrice.cani@uclouvain.be.

Author Contributions

[§]These authors contributed equally to this work.

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Notes

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