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Tolerance of protein-hydrolyzed lactose-free A1 milk and A2 milk in lactose-tolerant and lactose-intolerant volunteers: A randomized crossover trial with 2 parallel groups

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ABSTRACT

Some studies have shown that only A2 β-casein–containing milk (A2 milk) causes fewer gut symptoms in milk-sensitive individuals compared with milk containing both A1 and A2 β-caseins (A1A2 milk). However, in most of the previous clinical studies, the role of lactose in symptom generation has been largely overlooked. Partial hydrolysis of β-caseins during milk processing has been noted to influence gastrointestinal symptoms of sensitive individuals, such as those with irritable bowel syndrome. Currently, there is no clear conclusion about the factors behind gut symptoms in milk-sensitive individuals, aside from lactose in those who are lactose intolerant, or milk protein allergy. Our study involved a 3-leg, 3-d randomized crossover trial examining the effects of heat-treated, homogenized A2 and hydrolyzed A1A2 milk on gastrointestinal symptoms, fecal calprotectin, and plasma inflammation markers in 36 self-reported milk-sensitive volunteers. During the result interpretation phase, the participants were categorized into groups according to their lactase enzyme genotype. There was no difference in the amount of perceived gut symptoms between A2 and A1A2 hydrolyzed milk in the lactose-tolerant group, while gut symptoms increased in the lactose-intolerant group along with the increasing lactose content. Calprotectin and high-sensitivity CRP did not increase during any of the intervention periods compared with the milk-free run-in period. Weak evidence of certain inflammatory cytokine changes was seen, but no significant results were obtained. In conclusion, protein-hydrolyzed lactose-free A1A2 milk was as tolerated as A2 milk in lactose-tolerant volunteers and better tolerated by lactose-intolerant volunteers.

Key words: A2 β-casein, crossover trial, gut symptoms, inflammation markers, lactose

INTRODUCTION

Milk and milk products are nutritionally and culturally major components of the Western diet. There is well established consensus of milk products as important providers of good quality proteins, calcium, and iodine (Blomhoff et al., 2023). However, the gastrointestinal symptoms perceived after cow milk consumption have interested researchers over decades, and recently the environmental impact of milk production has also raised attention. However, the world's milk consumption is on the rise (OECD and Food and Agriculture Organization of the United Nations, 2019; Miller et al., 2022). The prevalence of lactose intolerance in adults varies around the world (Catanzaro et al., 2021). In addition to lactose, factors such as the degree of processing (including both homogenization and heat treatments) have been investigated, but not conclusively validated, in relation to gastrointestinal perceptions (Nuora et al., 2018a,b).

Cow milk has 2 major subvariants of its β-casein protein, A1 and A2, due to a single nucleotide difference that changes the codon at position 67 (Kay et al., 2021; Cattaneo et al., 2023). A2 β-casein is more resistant to enzymatic cleavage during digestion, whereas A1 β-casein is more easily cleaved, resulting in the product peptide β-casomorphin-7 (**BCM-7**), a known μ-opioid receptor agonist. The extent of the enzymatic release of BCM-7 by gastrointestinal proteases in vitro thus depends on the genetic variant of β-casein (Jinsmaa and Yoshikawa, 1999; Cattaneo et al., 2023). Most of the commercial milks contain both A1 and A2 type β-caseins (**A1A2 milk**). Some studies have reported milk containing only

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

Table 1. β-Casein content in the study milks

Item	β-casein, g/100 g		
	A1	A2	Total
A2 milk	0.12	0.85	0.96
A1A2 hydrolyzed, lactose-free milk	0.11	0.14	0.25
A1A2 milk before hydrolysis[1]	0.42	0.95	1.37

[1]Not used as a study milk in the intervention.

Table 2. The peptide profiles of the study milks

Molecular weight (Da)	Percentage	
	A1A2 hydrolyzed milk	A2 milk
>10,000	49.62	84.35
5,000–10,000	23.52	6.31
2,500–5,000	5.56	—
1,000–2,500	8.39	—
<1,000	12.91	9.32

A2 casein (**A2 milk**) to cause fewer gut symptoms in so-called milk-sensitive individuals compared with milk with both A1 and A2 β-casein forms (He et al., 2017; Ramakrishnan et al., 2020; Kay et al., 2021). Because BCM-7 affects intestinal motility and inflammation (Robinson et al., 2025), the lesser gut symptoms of sensitive individuals from A2 compared with A1 milk have been hypothesized to result from the lower production of BCM-7 (Kay et al., 2021).

Avoiding milk can lead to nutritional challenges, especially in sensitive groups such as children. A Chinese study found that in a group of 75 children, A2 milk caused fewer symptoms than A1 milk (Sheng et al., 2019). However, the difference was more pronounced in children who were lactose intolerant compared with tolerant. In other milk-sensitive groups, symptoms have also been noted when subjects have consumed A2 milk (He et al., 2017; Milan et al., 2020; Ramakrishnan et al., 2020). In these studies, the reporting has highlighted differences in caseins and to lesser extent, if any, have considered the effect of lactose.

Currently, only 2 studies comparing milks with A1 and A2 β-caseins have also included lactose-free milk in the intervention. In the study of Milan et al. (2020), lactose-free milk seemed to fit better than A2 milk even for people who tolerate lactose but still get stomach symptoms from milk. With lactose intolerants, lactose-free milk has differed clearly from lactose-containing A1 and A2 milks in the breath hydrogen, which is explained by the fermentation of lactose by intestinal bacteria (Milan et al., 2020; Ramakrishnan et al., 2020). Although Ramakrishnan et al. (2020) did not actually report differences between A2 and lactose-free milk in their study, the research data shows that lactose-free milk with a 60:40 A1/A2 ratio caused less abdominal symptoms for lactose intolerants than A2 milk. Also, in the study of Milan et al. (2020), the digestive disorders, flatulence, and reflux symptoms were lower in lactose-free milk (reported as a control) than with A2 milk.

In addition to the casein form, protein hydrolysis during milk processing may contribute to the formation of breakdown intermediates in the gut and tolerance of milk proteins. The enzymatic hydrolysis of milk proteins (a mixture of both A1 and A2 caseins) can hydrolyze the majority of β-casein and does not affect the whey proteins when compared with unhydrolyzed milk (Laatikainen et al., 2020). Laatikainen et al. (2020) found that the partially hydrolyzed milk caused less symptoms in individuals with functional gastrointestinal disorders than the unhydrolyzed control milk. They discussed that the β-casein content of milk could be the primary cause of gastrointestinal symptoms, as they had ruled out the effect of lactose and whey protein. Because studies concerning both protein hydrolysis and A1 and A2 β-casein types are lacking, and thus far investigations on influence of homogenization or heat treatment are inconclusive, there is no conclusion, apart from the effect of lactose in lactose-intolerant individuals, about the factors behind gut symptoms in milk-sensitive individuals. To draw better conclusions about the effects of these protein variants and protein hydrolysis on milk-sensitive individuals, with the effect of lactose controlled, studies comparing the effects of protein-hydrolyzed lactose-free milk and lactose-free and lactose-containing A2 milk are urgently needed.

In this study, we conducted a 3-leg, 3-d randomized crossover study with 2 types of heat-treated homogenized milks: (1) A2 milk supplemented with lactase enzyme capsules, (2) A2 milk supplemented with placebo capsules, and (3) protein-hydrolyzed lactose-free milk supplemented with placebo capsules, and investigated their effects on perceived gastrointestinal symptoms, fecal calprotectin, and plasma inflammation markers in self-reported milk-sensitive volunteers. In the result interpretation phase, participants were divided into 2 groups based on their lactase enzyme genotype. The aim of this study was to compare the perceived gut symptoms and inflammation in subjects consuming A2 milk and lactose-free, protein-hydrolyzed A1A2 milk in a controlled setting, including both gene-tested lactose-tolerant and lactose-intolerant participants. To our knowledge, this is the first clinical trial investigating A2 milk versus lactose-free milk in a controlled setting that clearly distinguishes between subjects with genetic lactose intolerance and lactose tolerance.

Table 3. The demographics of the study participants as mean ± SE

			Genotype			
	All (n = 36)		T/T and C/T Lactose-tolerant (n = 23)		C/C Lactose-intolerant (n = 13)	
Item	Mean	SE	Mean	SE	Mean	SE
Age (yr)	32.6	2.1	33.6	2.8	30.8	3.0
Weight (kg)	68.3	2.0	70.1	2.4	65.1	3.5
BMI[1] (kg/m^2)	23.5	0.6	23.8	0.7	23.2	1.0

[1]BMI = body mass index.

MATERIALS AND METHODS

Characterization of Milks, and Enzyme and Placebo Capsules

The milk treatments supplemented with capsules were as follows: A2 milk with placebo capsules (A2), A2 milk with lactase enzyme capsules (A2 lactase), and A1A2 protein hydrolyzed milk with placebo enzyme capsules (A1A2 hydrolyzed, lactose-free). The A1A2 hydrolyzed, lactose-free milk was processed as described below and donated by Valio Ltd. (Finland), and A2 milk was purchased from Veco Zuivel B.V. (the Netherlands). Both milks were obtained as homogenized and ultra-high temperature heat-treated. Although processing was conducted at different dairies, our previous study has indicated that even large differences in heat treatments do not affect the gut symptoms perceived from milk ingestion (Nuora et al., 2018a).

The A1A2 hydrolyzed, lactose-free milk was produced according to Tikanmäki and Kallioinen, 2020 and Tossavainen and Sibakov, 2012. The production of lactose-free milk involves ultrafiltration and nanofiltration to remove lactose, followed by enzymatic hydrolysis of the remaining lactose. Proteins were hydrolyzed in a controlled way so that the degree of hydrolysis was 159 µg free tyrosine/mL as analyzed according to the modified method of Matsubara et al. (1958). Analysis was performed for samples, which were boiled for 4 min at 100°C and then centrifuged. Soluble tyrosine was determined for the supernatant after centrifugation (3,000 × *g*, 15 min at room temperature). According to capillary electrophoresis (Bonfatti et al., 2008), hydrolysis was directed predominantly on β-casein and κ-casein. According to the anion exchange gel filtration chromatographic method (Korbes et al., 1994), the concentration of β-casein was reduced by 60% to 90% as compared with the concentration in normal lactose-free milk.

Concentrations of A1 and A2 β-casein in our study milks were analyzed with an ultra-high performance liquid chromatography system coupled with a UV detector using the method of Bonfatti et al. (2008). The system was controlled with Empower software (version 3.6.1; Waters, Milford, MA). Analytes were resolved by C18 reversed phase column (Zorbax 300SB Rapid Resolution HD, 2.1 × 150 mm, 1.8 µm; Agilent Technologies, Santa Clara, CA) with a gradient run consisting of solvent A (0.1% trifluoroacetic acid [**TFA**] in H_2O) and B (0.1% TFA in acetonitrile). Flow rate was 0.2 mL/min, column temperature was 40°C, and injection volume was 3 µL. The β-casein content in the A2 and A1A2 milks is presented in Table 1.

The peptide profiles of the A1A2 hydrolyzed milk and A2 milk were analyzed using the method of Hong et al. (2012). An UPLC Premier system coupled with a UV detector was used, and the system was controlled with Empower software (Waters, Milford, MA). Analytes were resolved on a ACQUITY UPLC BEH125 SEC column (1.7 µm, 4.6 × 300 mm; Waters, Milford, MA) with an isocratic run consisting of 80% solvent A (0.1% TFA in H_20) and 20% B (0.1% TFA in acetonitrile). The flow rate was 0.2 mL/min, column temperature was 30°C, and injection volume was 2 µL. The UV wavelengths for detection were 214 and 280 nm. The distribution of the molecular weight of the peptides is shown in Table 2.

Hydrolyzed, lactose-free A1A2 milk contained 0.0 g lactose, 3.1 g carbohydrates, 3.3 g protein and 3.0 g fat per 100 mL. The commercial A2 milk contained, by the package label, 4.4 g sugars (i.e., lactose), 3.4 g protein, and 4.4 g fat per 100 mL. Due to the inaccessibility of lactose-free A2 milk, lactase enzyme capsules were introduced. The lactase enzyme capsules and placebo capsules were donated by Verman Plc (Finland). Lactase capsules were commercial dietary supplements and placebo capsules were obtained from the same producer without the enzyme. The capsules contained maltodextrin from corn, hydroxypropyl methylcellulose (capsule shell), stabilizer (magnesium salts of fatty acids), color (iron oxide), and lactase enzyme produced by *Aspergillus oryzae* microbe (5,000 Food Chemical Codex units per capsule, not in placebo capsules).

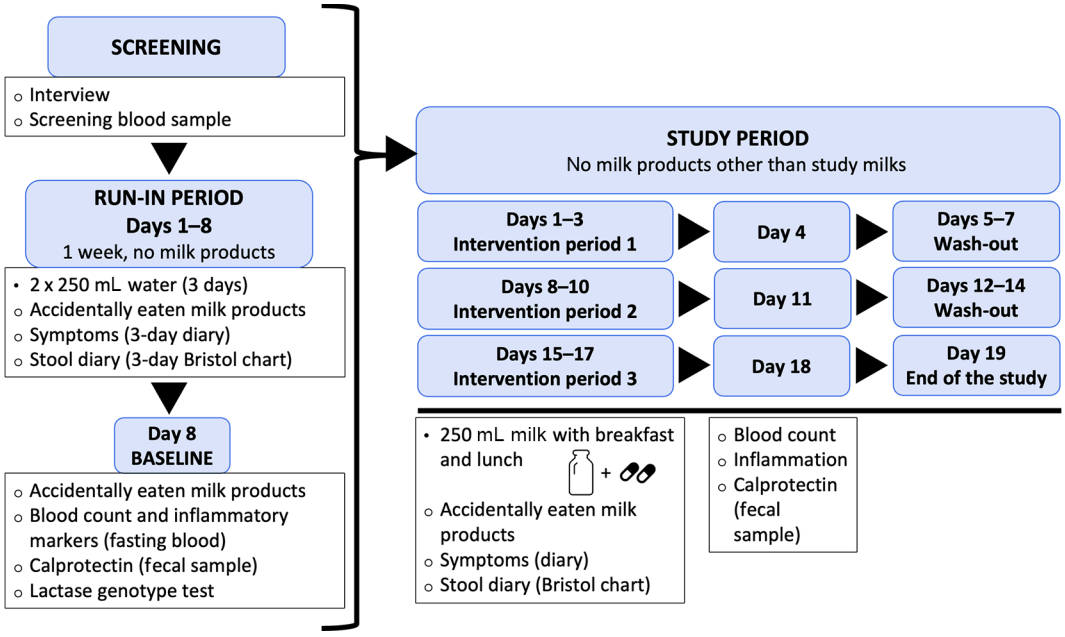

Figure 1. Overview of the study design.

Clinical Trial

The study was registered in the ClinicalTrials.gov (identifier: NCT05305391) and conducted at the facilities of the University of Turku (Turku, Finland). The study was approved by the Ethics Committee of the Hospital District of Southwest Finland (ETMK Dnro: 3/1801/2022).

Study Subjects. Subjects who self-reported to perceive symptoms from ingestion of milk products were recruited in Southwest Finland in 2022 from April to December. In total, 52 participants signed the informed consent to participate. Three of them did not meet the inclusion criteria and 11 withdrew consent before the study (Supplemental Figure S1, see Notes.) One volunteer withdrew consent after the first intervention period, and another was excluded during the result interpretation stage, as the fecal calprotectin analysis indicated severe intestinal inflammation already at the milk-free run-in phase. The participants were tested for lactase enzyme expression genotype, and during the data analysis, results were interpreted both for the entire group and separately for 2 subgroups: lactase producers (genotype T/T or C/T, n = 23) and lactase nonproducers (genotype C/C, n = 13).

Included volunteers were self-reported normal or overweight (body mass index 18.5–30) healthy adults (age 18–65 yr) who reported perceiving disturbing gut symptoms from regular milk and would commit to the research diet for the study period of 27 d. Subjects were excluded if they had milk allergy, medication that affected the gut (assessed by a medical doctor), a course of antibiotics within 3 mo prior to commencing the study, pregnancy or lactation, or diagnosed gastrointestinal disease. The suitability of volunteers was ensured with an interview. Participants meeting the preliminary inclusion criteria signed informed consent, underwent blood screening tests (blood count, thyroid, kidney, and liver function tests, and immunoglobulin A antibodies specific to transglutaminase for celiac diagnostics), and were admitted if the tests were in the normal range. The blood count and lipid levels were monitored during the study weeks. The demographics of the study participants are shown in Table 3. Research Electronic Data Capture (REDCap) tools hosted at the University of Turku were used for management of the participant flow and data collection (Harris et al., 2009, 2019).

Study Diet. The participants followed their regular diet but were not allowed to consume milk-containing products other than the provided study milks during the study. They were given instructions on product categories and product types that contain or may contain milk and how to replace them. In case the subjects had no choice or noticed later that they had consumed milk by mistake, they were instructed to report the consumed milk-containing products to a separate food record with the product name, amount consumed, and date. The accidentally consumed milk products were not considered noteworthy if the consumed amount contained less than 2 g of milk protein or lactose, especially if the product was high in fat, as fat slows down gut transit time (Procházková et al., 2023)

Table 4. Accidentally consumed milk products reported (amounts in grams) by the participants during the run-in and milk periods[1]

Item	Run-in	A2 regular		A2 lactase		A1A2 hydrolyzed	
		On the previous washout	During the intervention	On the previous washout	During the intervention	On the previous washout	During the intervention
Milk, cream	200/30/100/50						
Quark (in a sweet bun)	10					10	
Cheese	125/15/40/206/100		20	20/100 (cottage cheese)		200/20	20
Cream cheese					50		
Milk protein as a vegetarian "tofu"			40				
Ice cream		30					
Chocolate	48			55			
Other							
Milk and whey powder	10						
Cream liqueur				50			
Tuna pie and a cake with cream						300	

[1]The amounts are estimations (in grams) of each product per participant. The incidents are separated by a slash (/) showing the variety in amounts that were accidentally consumed.

and because 12 g of lactose has been shown to be the lowest amount resulting in symptoms for most individuals with lactose intolerance or malabsorption (Shaukat, 2010). Less than 2 g of milk protein is below 3% of a regular daily intake of protein in men and women.

Study Design. This study was a randomized, double-blinded crossover study carried out in the Food Sciences Unit at the University of Turku, Finland. The study consisted of a milk-free run-in period and 3 study periods, each including 3 d of milk consumption (Figure 1). During the whole study (27 consecutive days in total) subjects were asked to avoid other products containing milk. The order of the 3 milk treatments was randomized for each participant with CompuSense20 software (CompuSense, Guelph, ON, Canada) by a researcher not involved in this study. Randomization was opened after all data were acquired. The first study week was a run-in (baseline) week when the participants were instructed to drink 250 mL of water or other dairy-free liquid twice a day on the first 3 d to avoid distortions in the results brought by increased hydration during the study periods. During these days, subjects also kept a gut symptom record and a fecal composition record with Bristol scale. On the day before the first study period, baseline measurements were taken: a fasting blood sample (blood count, high-sensitivity C-reactive protein (**hs-CRP**), inflammation markers) and a stool sample (calprotectin). During the 3-d intervention period, 250 mL of study milk was consumed twice a day in combination with 2 lactase or placebo capsules. On the fourth day, the same tests were taken as during the baseline. After that, the subject was in a washout period for 3 d before the next study period, but still avoided milk-containing products. There were 3 intervention periods in total. The participants were given, in total, six 250-mL bottles of milk and 12 enzyme or placebo capsules for each 3-d study period. The products were blinded into similar looking bottles by a study assistant. Participants returned empty bottles to the researchers.

Study Records. Participants filled in a gut symptom record, defecation record and a record for incidental milk product ingestion for 3 d in every study week. The participants were provided with written and oral instructions and kitchen scales to ensure accuracy. The same gut symptoms record was used as in a study by Laito et al. (2022). Shortly, participants recorded the experienced symptom (upper abdominal pain, lower abdominal pain, cramping, bloating, flatulence, abdominal gurgling, or "other" specified symptom) in 3-h time slots for each study day and a 6-h slot for 00–06 o'clock. The symptoms were summed up for each study week for each participant. Fecal record with the Bristol stool chart was used to record bowel movement frequency and stool quality with date and time (Heaton et al., 1992). The stool types were categorized into hard (types 1–2), normal (types

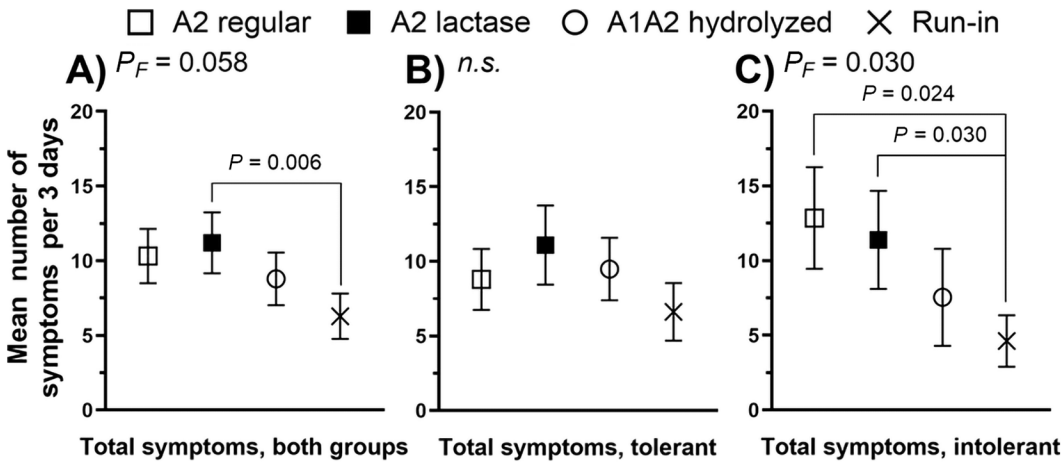

Figure 2. Mean of total symptoms during 3 intervention days in (A) both groups combined (n = 36), (B) the lactose-tolerant group (n = 23), and (C) the lactose-intolerant group (n = 13). Figures present mean values with SEM. All the participants started with the run-in period, and the milk periods were randomized. P_F = Friedman test *P*-value for between-intervention comparisons; *P* = Wilcoxon signed-rank test *P*-value for pairwise comparisons; n.s. = not significant.

3–5) and loose (types 6–7) stools. If the participant did not defecate, that day was marked as "no stool."

Blood and Stool Samples. Fasting blood samples were collected from the antecubital vein by a nurse in the Tyks Laboratories of the Turku University Hospital, Turku, Finland. Plasma was separated by centrifugation (2,200 × *g*, 15 min, room temperature). Blood count, hs-CRP, and markers of normal kidney, liver, and thyroid function were analyzed in the same facilities. We analyzed hs-CRP by immunonephelometric method (Siemens Prospec, Siemens Healthcare, Erlangen, Germany). Inflammation markers were analyzed from plasma at the University of Helsinki's Biomedicum Functional Genomics Unit by Olink Target 48 Cytokine panel (Olink Proteomics, Uppsala, Sweden). This panel uses a cDNA-based immunoassay and quantitative real-time PCR. All the samples passed quality control, but the following marker proteins gave assay warning indicating decreased precision and accuracy: C-X-C motif chemokine (CXCL) 12, IL-1B, IL-4, IL-8, IL-10, IL-17A, IL-17C, IL-18, IL-27, and matrix metalloproteinase (MMP)-12; and following had values under the lowest quantifiable level: Granulocyte-macrophage colony-stimulating factor (CSF2), IL-1B, IL-2, IL-4, IL-13, IL-17A, IL-17F, IL-27, IL-33, and thymic stromal lymphopoietin (TSLP). All measurements were analyzed statistically. Fecal calprotectin was analyzed at the Tyks Laboratories, Turku University Hospital (Turku, Finland) by using a fluoroenzyme immunoassay method (EliA Calprotectin 2, Phadia AB, Uppsala, Sweden) with a Phadia 250 instrument (Thermo Fisher Scientific, Waltham, MA). Participants were instructed to collect the first stool of the day in a collection tube and bring it to the study facility in a cooled bag. The samples were stored in −80°C before analysis.

Statistical Analysis

The group size was based on the power calculation (Schoenfeld, 2015) with IL-4 (Sheng et al., 2019). According to the power calculation, 18 participants would generate 80% power to find a statistical difference for cytokine IL-4 at a 2-sided significance level of 0.05. The target size of the study was set to 40 with the aim to recruit both lactose-tolerant and lactose-intolerant volunteers. We used IBM SPSS Statistics software (version 29.0.2.0, IBM Corp. Armonk, NY) for the statistical analyses. Normality of the data for gut symptoms, bowel movements, and stool types was assessed using the Shapiro–Wilk test. Statistically significant differences ($P < 0.05$) between the intervention periods were evaluated using either repeated measures ANOVA (**rANOVA**; for parametric data) or Friedman test (for nonparametric data). For nonparametric data, differences between groups were evaluated by Wilcoxon signed-rank test. Bonferroni adjustment was applied for the pairwise comparison significance values for both parametric and nonparametric data. The hs-CRP and calprotectin values were presented as medians with interquartile ranges and individual points, where the values below the limit of detection (**LOD**; 0.20 and 20.0, respectively) were shown as imputed values, LOD/√2 (Richardson, 2003). The values were then further categorized into 3 groups (under LOD, under the clinical reference, over the clinical reference) and analyzed with a Fisher's exact test. The

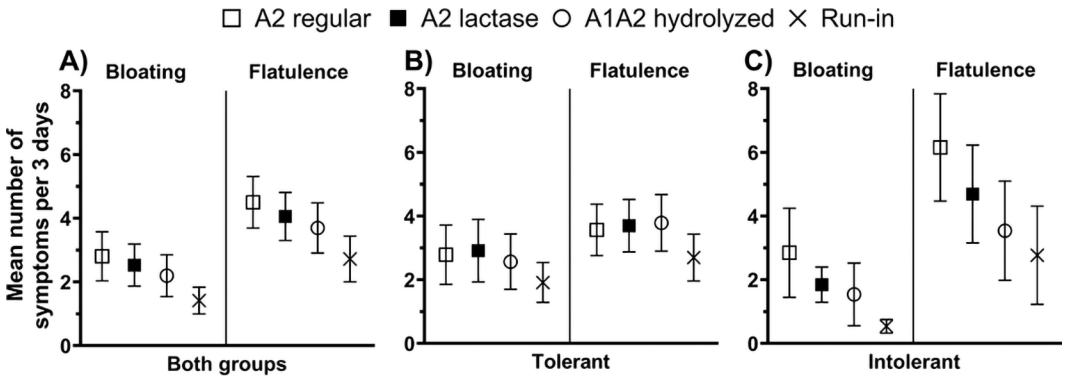

Figure 3. Mean number of bloating and flatulence symptoms during 3 intervention days in (A) both groups combined (n = 36), (B) the lactose-tolerant group (n = 23), and (C) the lactose-intolerant group (n = 13). Figures present mean values with SEM. There were no statistically significant differences (Friedman test). All the participants started with the run-in period, and the milk periods were randomized.

quantified inflammatory markers from Olink panel were analyzed log2 transformed with OlinkAnalyze package (Nevola et al., 2025) in R (v. 4.2.3; R Core Team, 2023; Vienna, Austria). The differences in inflammatory markers between the 3 milk periods were compared with linear mixed model, subject as a random effect. A post hoc test was applied to those markers that showed $P < 0.05$ without adjusting. The milk periods were then compared separately to the run-in period with Wilcoxon signed-rank exact test. The *P*-values were adjusted according to the Benjamini–Hochberg method (Benjamini–Hochberg adjusted *P*-value; **q**). All the analyses were performed separately to (1) both lactose tolerance groups together (n = 36), (2) lactose-tolerant (n = 23), and (3) lactose-intolerant (n = 13) groups. As there were few missing samples due to problems in blood sampling, the accurate number of participants for each assay is reported. The results are presented as the mean ± SE within the text and mean ± SEM in the figures (GraphPad Prism v10.4.1 build no. 627, GraphPad Software, Boston, MA; https://www.graphpad.com).

RESULTS

Diet of the Study Subjects

Compliance with the intervention diet was considered successful. According to the records, it was challenging for the participants to refrain from milk products for the requested 27-d period even though they were given written and oral instructions. We anticipated this, as milk products are a substantial part of a regular Finnish diet and fairly difficult to avoid. Most of the accidental milk product ingestions happened during the first, run-in (baseline) week. Out of the total 108 milk intervention periods, there were only 4 noteworthy incidents (Table 4). The incidents considered inconsequential are shown in Supplemental Table S1 (see Notes).

Gut Symptoms

Despite the fact that only self-reported milk-sensitive participants were recruited, some of them reported various gut symptoms and some reported only few or no symptoms during the intervention periods. During the run-in period, 78% of the participants reported symptoms (in total, 226 records), 81% during the A2 regular (371 records), 86% during the A2 lactase (403 records), and 89% during the hydrolyzed A1A2 milk period (316 records). When considering all participants, the mean of total symptoms per 3-d intervention period tended to differ ($P = 0.058$, Friedman test; Figure 2A) so that during the run-in period the occurrence of symptoms was lower (6.3 ± 1.5) compared with the A2-lactase milk period (11.2 ± 2.0, $P = 0.006$). Among the lactose-tolerant group, no significant differences between the milk periods were observed ($P = 0.149$, Friedman test; Figure 2B). In the lactose-intolerant group ($P = 0.030$, Friedman test; Figure 2C), participants reported more symptoms with A2 regular (13.0 ± 3.5, $P = 0.024$) and A2 lactase milks (11.4 ± 3.3, $P = 0.030$) compared with the run-in period (5.7 ± 2.6). These results most likely reflect the increasing lactose amounts in A2 lactase and A2 regular milks.

Over 60% of the reported symptoms in every intervention period were from bloating and flatulence, but no significant differences were found between the intervention periods ($P \geq 0.138$, Friedman test; Figure 3A). However, we observed different patterns in the occurrence of these symptoms between the lactose-tolerant (Figure 3B) and lactose-intolerant groups (Figure 3C). In the lactose-tolerant group, we did not observe clear tendency between the intervention periods, whereas in the lactose-intolerant

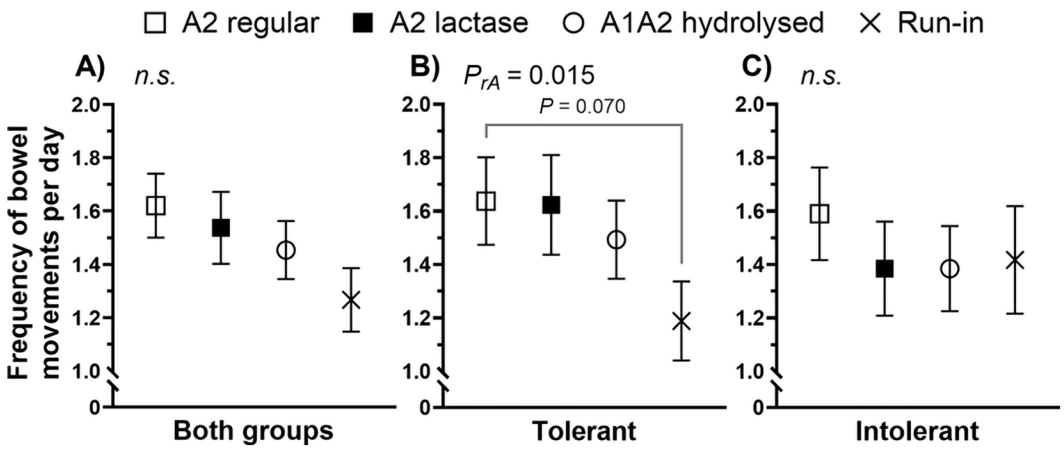

Figure 4. Bowel movement frequency during the 3 intervention days in (A) both groups combined (n = 36), (B) the lactose-tolerant group (n = 23), and (C) the lactose-intolerant group (n = 13). Figures present mean values with SEM. All the participants started with the run-in period, and the milk periods were randomized. P_{rA} = rANOVA test *P*-value for between-intervention comparisons; *P* = Bonferroni adjusted *P*-value for pairwise comparisons; n.s. = not significant.

group, the number of symptoms tended to increase with the amount of lactose in diet.

Bowel Movement and Stool Types

Within all participants, the milk periods did not differ in terms of the bowel movement frequency per day ($P = 0.166$, Friedman test; Figure 4A). Within the tolerant group, we observed a difference between the periods ($P = 0.015$, rANOVA), with a tendency ($P = 0.070$) for higher frequency for the A2 regular period (1.64 ± 0.16) compared with the run-in period (1.19 ± 0.15, Figure 4B). In the lactose-intolerant group, we found no significant differences in the bowel movement frequencies between the intervention periods ($P = 0.619$, rANOVA, Figure 4C).

Percentual distribution of different stool types during the intervention periods is presented in Figure 5. We found no significant differences between the mean frequencies of different stool types between the milk periods ($P > 0.113$ Friedman test/rANOVA). However, the proportions of the stool types indicated trends accordingly: normal stool was highest during the run-in period (74.5%) followed by the A1A2 hydrolyzed (68.1%), A2 lactase (67.8%), and A2 regular (64.9%) periods. The occurrence of hard and loose stool was lowest during the run-in period (6.7% and 8.1%, respectively), whereas the proportion of no stool-category was highest during the run-in (10.7%). In the lactose-tolerant group, the proportion of normal stool was highest during the run-in period (76.6%), and all the milk periods lowered the level in a roughly similar manner (65.8%–66.7%). In the lactose-intolerant group, however, the normal stool proportion was similar during the run-in, A1A2 and A2 lactase periods (70.0–70.9%), whereas the regular A2 milk with highest lactose content resulted in lower normal stool level (63.1%).

Inflammation Markers and High-Sensitivity CRP

Fecal calprotectin is a sensitive marker of inflammation in the gastrointestinal tract, with levels below 100 µg/g regarded as normal. None of the studied milks increased the calprotectin levels over the reference values in the participants, and median levels remained below the LOD in all groups (2-sided Fisher's exact test $P > 0.4$ in both groups and when separated by tolerance, Figure 6A–C). We observed a similar tendency for hs-CRP, as there were no differences between the periods and the median values did not increase noticeably during any of the milk intervention periods compared with the run-in period (2-sided Fisher's exact test $P > 0.6$ in both groups and when separated by tolerance, Figure 6D–F). Only a few and different participants had values exceeding the clinical references during different periods, indicating that milk consumption did not cause physiologically significant inflammation (Figure 6A–F individual points).

A total of 45 different inflammatory biomarkers were measured. The quantified results (pg/mL) for both tolerance groups together are shown in Table 5. Results for tolerant and intolerant groups separately are presented in Supplemental Tables S2 and S3, respectively (see Notes). Point-range plots for every inflammatory marker, including combined data for both groups and separate data for lactose-tolerant and lactose-intolerant groups, are presented in Supplemental Figures S2, S3, and S4, respectively (see Notes).

After adjusting the *P*-values, no significant differences were seen. Still, the original *P*-values suggest possible

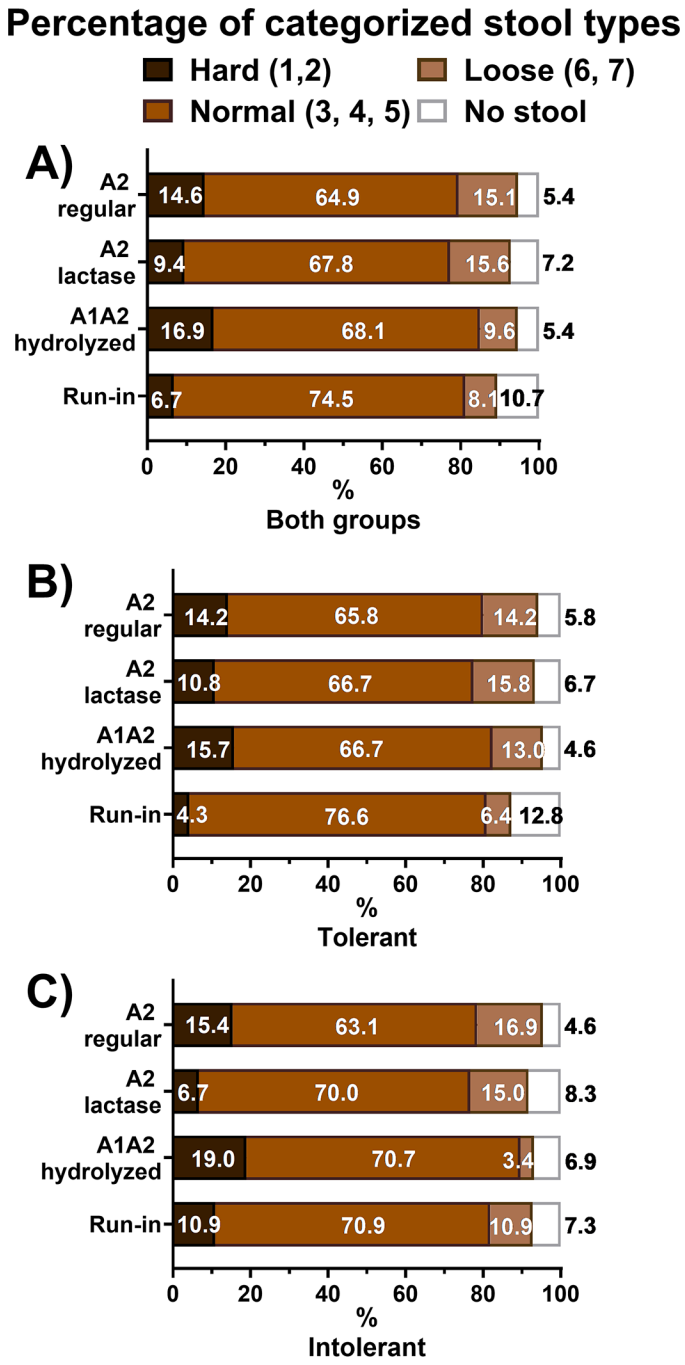

Figure 5. The percentage of each categorized Bristol stool types during intervention periods in (A) both groups combined (n = 36), (B) the lactose-tolerant group (n = 23), and (C) the lactose-intolerant group (n = 13). There were no statistically significant differences between the mean frequencies of different stool types between the milk periods (Friedman test/rANOVA). All the participants started with the run-in period, and the milk periods were randomized.

differences between milk periods for the proinflammatory markers C-C motif chemokine (CCL) 3 ($P = 0.039$, q = 0.434; Nightingale et al., 2017), vascular endothelial growth factor A (VEGF-A; $P = 0.029$, q = 0.434 Scaldaferri et al., 2009), IL-33 ($P = 0.039$, q = 0.434; Kaur et al., 2023), and MMP-1 ($P = 0.035$, q = 0.434; Meijer et al., 2007), and weak indication for CCL4 ($P = 0.051$, q = 0.434), monocyte chemotactic protein (MCP) 4 ($P = 0.058$, q = 0.434), and MCP-1 ($P = 0.081$, q = 0.488) when both tolerance groups were analyzed together. Interestingly, for all the above-mentioned biomarkers except IL-33, there was a trend for higher level after A2 lactase period compared with A1A2 period. In case of IL-33, the level was higher after A2 regular period compared with A1A2 period, which was due to the strong trend in the lactose-intolerant group, possibly caused by the high lactose content in the A2 regular milk (Supplemental Table S3, Supplemental Figure S4). IL-33 is not directly related to lactose malabsorption but is known to regulate gut immunity by maintaining barrier integrity, controlling immune responses, and promoting tissue repair after injury (Kaur et al., 2023).

We did not observe significant differences in markers between the milk periods in the lactose-tolerant group, even though it consisted of more participants than the lactose-intolerant group (Supplemental Table S2, Supplemental Figure S3). Only pairwise comparisons to run-in period showed tendencies in IL1B, IL-4, lectin-like oxidized LDL receptor 1 (LOX-1), and oncostatin-M (OSM). IL1B, a proinflammatory cytokine related to intestinal permeability (Al-Sadi and Ma, 2007; Rudzki et al., 2017), had higher levels after A2 milk (regular and lactase) compared with the run-in (A2 lactase $P = 0.029$, q = 0.326; A2 regular $P = 0.0074$, q = 0.335; Supplemental Table S2, Supplemental Figure S3). IL-4 ($P = 0.014$, q = 0.217), LOX-1 ($P = 0.0093$, q = 0.209) and OSM ($P = 0.0066$, q = 0.209) levels were higher after the A2 lactase period compared with the run-in. IL-4 is a signaling cytokine in type 2 immune response (Haase and Voehringer, 2021), LOX-1 is a receptor for oxidized LDL and potentially an indicator for endothelial dysfunction (Lubrano and Balzan, 2016), and OSM is a proinflammatory cytokine that activates endothelial cells and is linked to inflammatory bowel disease (Mestrovic et al., 2024).

Within the intolerant group, IL-7, which has also been associated with gut inflammation (Belarif et al., 2019), behaved similarly to IL-33 ($P = 0.03$, q = 0.255, and $P = 0.004$, q = 0.182, respectively), whereas the other proinflammatory cytokines, IL-17A ($P = 0.016$, q = 0.247; McGeachy et al., 2019), CXCL11 ($P = 0.033$, q = 0.247), CCL3 ($P = 0.038$, q = 0.247), MCP-2 (0.026, q = 0.247), and MCP-4 ($P = 0.033$, q = 0.247; Nightingale et al., 2017), had a tendency for higher levels after A2 lactase period compared with A1A2 period (Supplemental Table S3, Supplemental Figure S4). We found weak indication of differences in milk periods with markers MMP-1 ($P = 0.078$, q = 0.255), CCL4 ($P = 0.067$, q =

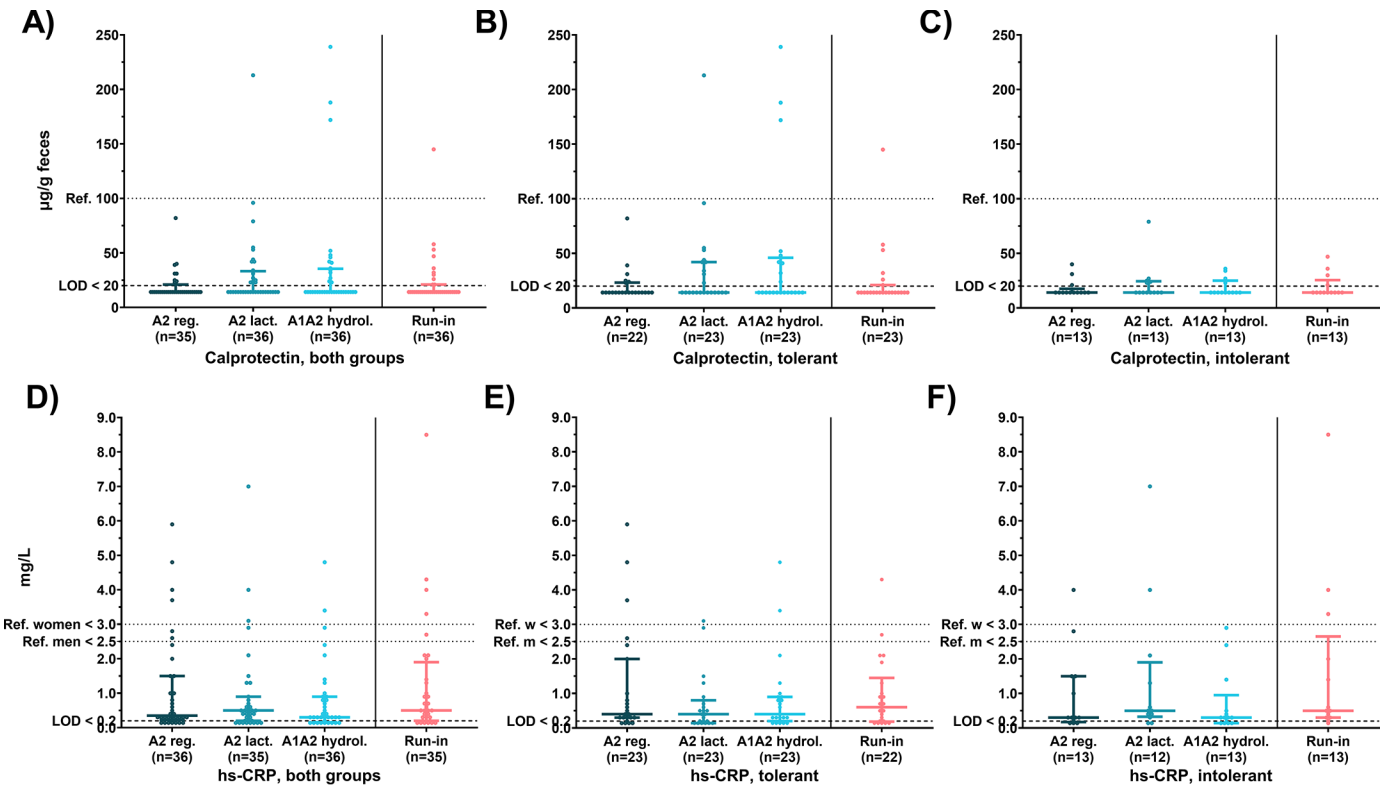

Figure 6. Median values for calprotectin (A, B, C) and hs-CRP (D, E, F) levels after each intervention period. (A and D) both groups combined (n = 36), (B and E) the lactose-tolerant group (n = 23), and (C and F) the lactose-intolerant group (n = 13). Figures present the median with interquartile range and individual data points. There were no differences between the intervention periods (Fisher's test). All the participants started with the run-in period, and the milk periods were randomized. Certain values were missing due to problems in blood sampling. hs-CRP = high-sensitivity C-reactive protein; A2 reg. = A2 regular; A2 lact. = A2 lactase; A1A2 hydrol. = A1A2 hydrolized; Ref = reference value (w, for women; m, for men); LOD = limit of detection.

0.255), pro-epidermal growth factor (EGF; P = 0.077, q = 0.255), VEGF-A (P = 0.074, q = 0.255), granulocyte colony-stimulating factor (G-CSF; P = 0.073, q = 0.255), IL-8 (P = 0.079, q = 0.255), and IFN-γ (P = 0.06, q = 0.255).

The results comparing the milk periods to run-in are shown in footnotes of Table 5 (for both groups together) and in Supplemental Tables S2 and S3 (for tolerant and intolerant groups separately). Although not significant, the high levels after A2 lactase milk period tended to differ the most from run-in period, especially within intolerants.

Concerning anti-inflammatory cytokines, we were not able to show significance in any comparison. Still, the levels of IL-10, the most important anti-inflammatory cytokine, were higher in all milk periods compared with the run-in (Table 5, Supplemental Tables S2 and S3, Supplemental Figure S2–S4). The behavior was similar: run-in < A2 regular < A2 lactase < A1A2 hydrolyzed in the tolerant and combined groups data, and run-in < A1A2 hydrolyzed < A2 regular < A2 lactase in the intolerant group data (differences not significant; P > 0.2, q >0.6).

DISCUSSION

In total, results of 36 participants who reported receiving gut symptoms from regular milk were analyzed. We interpreted the results for the entire group, as well as separately for the lactose-tolerant (n = 23) and lactose-intolerant (n = 13) groups, based on participants' lactase enzyme genotype. The lactose-tolerant and lactose-intolerant groups showed clearly different behaviors, emphasizing the importance of distinguishing these groups when studying milk-related gut symptoms and inflammation.

We did not observe significant differences in the perceived gut symptoms, bowel movements, or stool types between A1A2 hydrolyzed lactose-free milk and A2 milk when considering all participants together or only the lactose-tolerant group, despite adequate number of par-

Table 5. Inflammatory marker concentrations (pg/mL) during the intervention periods in both groups combined[1]

		Milk period									
		Run-in, n = 35		A2 regular, n = 36		A2 lactase, n = 35		A1A2 hydr., n = 35			
Protein	Gene name	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P*	q
C-C motif chemokine 3 (CCL3)	*CCL3*	4.22	0.18	4.58	0.27	5.10	0.37	4.45	0.32	**0.039**	0.434
C-C motif chemokine 4 (CCL4)	*CCL4*	58.03	4.47	61.67	4.61	67.45	4.85	59.65	3.74	0.051	0.434
C-C motif chemokine 19 (CCL19)	*CCL19*	136.29	18.68	147.82	17.64	162.03	22.06	152.19	20.71		
C-X-C motif chemokine 9 (CXCL9)	*CXCL9*	56.46	9.35	79.76	23.50	108.19	40.55	114.95	40.22		
C-X-C motif chemokine 10 (CXCL10)	*CXCL10*	95.86	10.27	126.17	27.01	143.76	36.20	146.57	32.54		
C-X-C motif chemokine 11 (CXCL11)	*CXCL11*	64.62	13.94	80.66	16.07	88.81	17.95	73.43	15.72		
Eotaxin (CCL11)	*CCL11*	80.44	7.02	85.31	7.15	91.13	6.37	84.31	7.69		
Fms-related tyrosine kinase 3 ligand (Flt3L)	*FLT3LG*	137.86	8.75	147.73	10.82	158.94	11.14	143.37	8.59		
Granulocyte colony-stimulating factor (G-CSF)	*CSF3*	104.08	6.54	118.37	8.19	113.55	7.18	119.20	11.43		
Granulocyte-macrophage colony-stimulating factor (CSF2)[2]	*CSF2*	0.21	0.02	0.23	0.02	0.24	0.01	0.23	0.02		
Hepatocyte growth factor (HGF)	*HGF*	240.08	9.24	248.97	9.86	269.72	11.24	267.53	18.38		
Interferon gamma (IFN-γ)	*IFNG*	0.17	0.02	0.25	0.04	0.31	0.08	0.76	0.39		
Interleukin-1β (IL1B)[2,3]	*IL1B*	0.14	0.02	0.18	0.02	0.17 a	0.03 a	0.17	0.04		
Interleukin-2 (IL-2)[2]	*IL2*	1.28×10^{-2}	1.20×10^{-2}	1.19×10^{-2}	0.13×10^{-2}	1.20×10^{-2}	0.12×10^{-2}	1.57×10^{-2}	0.21×10^{-2}		
Interleukin-4 (IL-4)[2,3]	*IL4*	1.08×10^{-2} b	0.04×10^{-2} b	1.09×10^{-2}	0.05×10^{-2}	1.22×10^{-2}	0.11×10^{-2}	1.08×10^{-2}	0.05×10^{-2}		
Interleukin-6 (IL-6)	*IL6*	2.23	0.23	2.51	0.33	2.64	0.45	2.56	0.26		
Interleukin-7 (IL-7)	*IL7*	2.41	0.44	2.69	0.63	3.01	0.85	2.29	0.56		
Interleukin-8 (IL-8)[3]	*CXCL8*	5.18	0.52	5.24	0.47	5.38	0.48	4.91	0.46		
Interleukin-10 (IL-10)[3]	*IL10*	8.32	0.62	9.15	0.79	9.71	0.77	10.01	1.07		
Interleukin-13 (IL-13)[2]	*IL13*	1.69	0.68	1.82	0.74	1.98	0.81	1.80	0.79		
Interleukin-15 (IL-15)	*IL15*	15.81	0.90	16.65	1.11	17.87	0.92	16.50	0.83		
Interleukin-17A (IL-17A)[2,3]	*IL17A*	0.60	0.12	0.48	0.06	0.54	0.09	0.47	0.07		
Interleukin-17C (IL-17C)[3]	*IL17C*	29.54	6.30	19.64	1.83	26.53	3.97	20.76	1.91		
Interleukin-17F (IL-17F)[2]	*IL17F*	1.17	0.30	1.21	0.31	1.24	0.31	1.23	0.30		
Interleukin-18 (IL-18)[3]	*IL18*	267.04	15.61	274.50	12.98	276.44	16.54	257.27	11.31		
Interleukin-27 (IL-27)[2,3]	*IL27*	11.54	1.26	12.47	1.37	13.08	1.25	13.32	1.66		
Interleukin-33 (IL-33)[2]	*IL33*	0.18	0.02	0.21	0.02	0.19	0.02	0.17	0.02	**0.034**	0.434
Lectin-like oxidized LDL receptor 1 (LOX-1)	*OLR1*	36.74	2.03	40.28	2.18	43.13	2.25	41.69	2.48		
Macrophage colony-stimulating factor 1 (CSF-1)	*CSF1*	123.58	3.15	126.42	3.02	130.58	4.30	126.27	3.23		
Matrix metalloproteinase-1 (MMP-1)	*MMP1*	704.77	80.18	669.02	60.24	768.86	95.75	560.07	54.88	**0.035**	0.434
Matrix metalloproteinase-12 (MMP-12)[3]	*MMP12*	185.42	11.40	189.81	12.99	205.24	13.34	191.84	11.26		
Monocyte chemotactic protein 1 (MCP-1)	*CCL2*	285.66	39.39	304.02	36.22	324.10	37.99	294.43	37.24	0.081	0.488
Monocyte chemotactic protein 2 (MCP-2)	*CCL8*	24.86	1.61	28.74	2.38	29.91	2.57	26.91	2.19		
Monocyte chemotactic protein 3 (MCP-3)	*CCL7*	0.33	0.02	0.40	0.05	0.43	0.07	0.36	0.03		

Continued

Table 5 (Continued). Inflammatory marker concentrations (pg/mL) during the intervention periods in both groups combined[1]

		Milk period									
		Run-in, n = 35		A2 regular, n = 36		A2 lactase, n = 35		A1A2 hydr., n = 35			
Protein	Gene name	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P*	q
Monocyte chemotactic protein 4 (MCP-4)	*CCL13*	66.14	4.08	74.11	5.24	76.84	6.05	65.71	4.56	0.058	0.434
Oncostatin-M (OSM)	*OSM*	1.45	0.15	1.70	0.16	1.87	0.18	1.84	0.25		
Pro-epidermal growth factor (EGF)	*EGF*	64.33	6.53	65.72	5.74	82.79	15.45	54.09	4.23		
Stromal cell-derived factor 1 (CXCL12)[3]	*CXCL12*	208.07	10.81	205.62	6.43	216.27	10.72	197.09	9.30		
Thymic stromal lymphopoietin (TSLP)[2]	*TSLP*	0.21 a	0.05 a	0.25 a	10.62 a	0.34 b	0.11 b	0.48 b	0.25 b		
Transforming growth factor α (TGF-α)	*TGFA*	5.30	0.24	5.54	0.27	6.00	0.36	5.56	0.29		
Tumor necrosis factor (TNF)	*TNF*	14.67	0.51	16.42	0.74	17.98	1.66	17.04	1.49		
TNF-β (TNFB)	*LTA*	9.26	0.40	10.11	0.43	10.53	0.94	10.05	0.76		
TNF-related apoptosis-inducing ligand (TRAIL)	*TNFSF10*	651.52	145.73	625.24	92.15	649.86	121.02	578.20	59.15		
TNF (Ligand) superfamily, member 12 (TWEAK)	*TNFSF12*	542.94	25.06	573.64	27.47	603.68	29.04	549.36	26.47		
Vascular endothelial growth factor A (VEGF-A)	*VEGFA*	264.71	10.76	284.11	10.62	312.11	19.85	269.62	10.55	**0.029**	0.434

[1]The results are from both lactose-tolerant and lactose-intolerant groups combined (n = 35) due to problems in blood sampling; n = 36 in A2 regular. Lowercase letter a indicates n = 34 and lowercase letter b indicates n = 33 due to values under the detection limit or failed assay. The statistical significance from linear mixed model presents differences between the 3 milk periods. Significant original *P*-values are shown in bold. Posthoc results of the comparison between the milk periods: CCL3: A1A2 hydr. vs. A2 lact. q = 0.036; A2 lact. vs. A2 reg. q = 0.172; A1A2 hydr. vs. A2 reg. q = 0.743; CCL4: A1A2 hydr. vs. A2 lact. q = 0.052; A2 lact. vs. A2 reg. q = 0.162; A1A2 hydr. vs. A2 reg. q = 0.847; IL-33: A1A2 hydr. vs. A2 reg. q = 0.026; A2 lact. vs. A2 reg. q = 0.335; A1A2 hydr. vs. A2 lact. q = 0.440; MMP-1: A1A2 hydr. vs. A2 lact. q = 0.032; A1A2 hydr. vs. A2 reg. q = 0.161; A2lact. vs. A2 reg. q = 0.734; MCP-1: A1A2 hydr. vs. A2 lact. q = 0.067; A2 lact. vs. A2 reg. q = 0.349; A1A2 hydr. vs. A2 reg. q = 0.645; MCP-4: A1A2 hydr. vs. A2 lact. q = 0.085; A1A2 hydr. vs. A2 reg. q = 0.105; A2lact. vs. A2 reg. q = 0.992; VEGF-A: A1A2 hydr. vs. A2 lact. q = 0.021; A2 lact. vs. A2 reg. q = 0.320; A1A2 hydr. vs. A2 reg. q = 0.396. Pairwise comparison with Wilcoxon signed exact test for each milk period with the run-in ($P < 0.1$): CCL3: A2 lact. $P = 0.010$, q = 0.126; CCL4: A2 lact. $P = 0.011$, q = 0.126; CCL19: A2 lact. $P = 0.072$, q = 0.217; CXCL11: A2 lact. $P = 0.084$, q = 0.237; CCL11: A2 lact. $P = 0.039$, q = 0.195; HGF: A2 lact. $P = 0.091$, q = 0.240; IFN-γ: A2 lact. $P = 0.019$, q = 0.140; IL-1B: A2 lact. $P = 0.032$, q = 0.181; A2 reg. $P = 0.0087$, q = 0.377; IL-27: A2 lact. $P = 0.070$, q = 0.217; IL-33: A1A2 hydr. $P = 0.067$, q = 0.846; LOX-1: A1A2 hydr. $P = 0.012$, q = 0.463; A2 lact. $P = 0.0013$, q = 0.058; A2 reg. $P = 0.087$, q = 0.461; MMP-1: A1A2 hydr. $P = 0.075$, q = 0.846; MMP-12: A2 lact. $P = 0.048$, q = 0.198; MCP-1: A2 lact. $P = 0.057$, q = 0.214; MCP-2: A2 lact. $P = 0.027$, q = 0.176; A2 reg. $P = 0.036$, q = 0.401; OSM: A1A2 hydr. $P = 0.021$, q = 0.463; A2 lact. $P = 0.011$, q = 0.126; TNF: A2 lact. $P = 0.044$, q = 0.198; A2 reg. $P = 0.017$, q = 0.377; TNFB: A2 reg. $P = 0.025$, q = 0.377; TWEAK: $P = 0.072$, q = 0.217; VEGF-A: A2 lact. $P = 0.015$, q = 0.138. q = Benjamini–Hochberg adjusted *P*-value; hydr. = hydrolyzed; lact. = with lactase capsule; reg. = without lactase enzyme.

[2]Values were under the lowest quantifiable level of the panel.

[3]Compromised precision and accuracy in the panel.

ticipants according to the power calculations. However, in the lactose-tolerant group, all the study milks tended to increase the bowel movement frequency and decrease the proportion of normal stool compared with the run-in period, but these changes were not found significant. Previously, lactose-containing A1A1 milk has caused slightly higher stool consistency compared with lactose-containing A2A2 milk in self-reported lactose-tolerant participants (Ho et al., 2014). The results suggest that the gut symptoms experienced from milk by lactose-tolerant individuals are related to factors other than β-casein or lactose concentrations. In some studies, partial hydrolysis of milk proteins has been reported to decrease gut symptoms in subjects with gastrointestinal disorders (Turpeinen et al., 2016; Laatikainen et al., 2020). In these studies, the intervention periods were longer (10 d) compared with current study (3 d), which may have affected the results. Additionally, the concentration of A1 β-casein, which is proposed to increase gastrointestinal symptoms and inflammation in regular milk (Jianqin et al., 2016; He et al., 2017; Sheng et al., 2019; Ramakrishnan et al., 2023), was similar in the A1A2 hydrolyzed and A2 milks used in our study. In a recent study by Ramakrishnan et al. (2024), however, even the differing A1/A2 β-casein contents in the study milks ,which contained similar lactose amounts, did not affect daily symptoms of lactose maldigesters during a 2-wk intervention period, except for urgency to defecate.

In the lactose-intolerant group of our study, gut symptoms intensified with higher lactose concentrations in the ingested milk. Also, the proportion of normal stool suggested a reaction to lactose content, being at the lowest level in the A2 regular milk. Previous research has demonstrated that lactose-free A1A2 milk is better tolerated than A2 milk within lactose-intolerant individuals (Milan et al., 2020; Ramakrishnan et al., 2020), highlighting the role of lactose in digestive discomfort in individuals with lactose intolerance.

We did not observe significant differences in inflammatory marker levels, including calprotectin and hs-CRP, between the milk periods. Calprotectin and hs-CRP levels did not increase in any of the milk groups compared with the run-in period. No significant differences were found in the Olink inflammation panel markers either, although the original *P*-values suggested a weak indication of differences between the milk groups. Interestingly, there was a tendency for elevated inflammatory marker levels after the A2 lactase period. This outcome is difficult to explain because adding lactase enzyme would not be expected to increase inflammation. There is evidence of sensitization to lactase derived from *Aspergillus oryzae* when in contact with the skin or lungs, but this remains unclear in our study, as the enzyme was consumed inside a capsule (Bernstein et al., 1999; Lohrenz and Kanani, 2023).

No differences between milks in inflammation markers were observed in the lactose-tolerant group, whereas in the lactose-intolerant group, IL-33 and IL-7 showed the highest levels after the A2 regular milk period, which contained the most lactose, although the results were not significant. Other proinflammatory cytokines (IL-17A, CXCL11, CCL3, MCP-2, and MCP-4) had a nonsignificant tendency for higher levels after the A2 lactase period compared with the A1A2 period. Also, other studies have reported changes in gut symptoms due to short-term study milk ingestion without effects on inflammatory markers with protein-hydrolyzed (Laatikainen et al., 2020) or modified A1/A2 β-casein milks (Ramakrishnan et al., 2024). In the current study, the amount of A1 β-casein, the presumed cause of inflammatory responses, was also low in both study milks. In addition, in systematic reviews, no proinflammatory effects have been found after milk or dairy consumption (Labonté et al., 2013; Ulven et al., 2019). Dairy products, especially fermented, are considered anti-inflammatory (Bordoni et al., 2017). More studies are needed in the area of inflammation and milk consumption.

The strengths of our study include randomized crossover trial design, extensive characterization of the study milks, and division of the participants according to their genotypic lactose digestion. The usage of clinical markers and wide panel of quantified inflammatory markers, in addition to the self-reported symptoms, are considered strengths. The participants were largely committed and we were able to provide comprehensive materials and instructions for them to successfully adhere to the study diet.

Naturally, our study has also limitations. Our study would have benefited from the inclusion of nonhydrolyzed A1A2 lactose-free milk, and nonhydrolyzed A1A2 milk with and without lactase capsules. However, because this study was already 27 d long with a culturally challenging milk product-free diet and with 5 blood draws, we limited the study to 3 milk periods. Lactase enzyme capsules were introduced due to the inaccessibility of lactose-free A2 milk. Although it is possible that the absorption, activity, and efficiency of the lactase capsules might have varied between individuals, this is unlikely, as the degree of variation during the lactase capsule periods were similar to the variation during the lactose-free milk period. The study would have benefited from larger volunteer group sizes, especially as the results of the gastrointestinal symptoms had substantial variability. Self-reporting can be considered a

limitation, as it depends on the motivation to record, and also on the possibility of differential characterization of the discomfort between volunteers, despite careful instructions. The use of breath hydrogen tests after an oral lactose load could have shed more light on the lactose fermentation rates, and possibly indicated if some of our genetically lactose intolerant volunteers were lactose tolerant (e.g., via the influence of their gut microbiota). Regarding microbiota, fecal microbiota was not measured in this study due to doubts about the 3-d intervention changing gut microbiota and the available resources for metagenomics.

Milk is very important part in human nutrition globally, and thus it is crucial to understand why some people face problems that cannot be explained by lactose content. Milk hydrolysis is widely available and more cost effective than breeding of A2 cows (Żbik et al., 2024). Also, the hype related to A2 milk might be somewhat marketing-oriented, as many of the studies highlight the negative effects of A1 β-casein compared with A2 and neglect the role of lactose (Sun et al., 2024). The pressure toward more environmentally friendly and animal friendly milk might lead to recombinant or cell-cultured milk production in the future (Kwon et al., 2024; Piazenski et al., 2024). For this reason, it is important to understand the potential health-beneficial or neutral proteins and produce proteins that are suitable for everyone. More studies are needed to understand why some people can tolerate milk proteins and others do not, as this knowledge is crucial for the future of the food industry. Future research could also consider different populations and dietary contexts (Brooke-Taylor et al., 2017) and testing IgG-mediated milk sensitivities (Pratelli et al., 2024; Mullin et al., 2010), in addition to including the role of lactose in the study design. Our suggestion for future studies is to provide the participants substitutive products during the intervention so that they would comply even better with the diet. However, the current approach with instruction of suggestions allowed participants to make their own substitutive choices. Also, if possible, lactose-free products should be prioritized over the use of lactase enzyme.

Our study found that lactose-free hydrolyzed A1A2 milk is as well tolerated as A2 milk among subjects who perceive gut symptoms from milk. The lactose-tolerant group should be investigated more as it seems that the source of symptoms is something else than β-casein or lactose content. Metabolomics and microbiota sequencing could be conducted in future studies to gain more insight into the possible reasons behind the gut symptoms in the tolerant group, and also for higher understanding of the biological relevance of the inflammatory markers (Ulven et al., 2019).

NOTES

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Nonstandard abbreviations used: A1A2 hydrol. = A1A2 hydrolized; A1A2 milk = milk containing both A1 and A2 β-caseins; A2 lact. = A2 lactase; A2 milk = A2 β-casein–containing milk; A2 reg. = A2 regular; BCM-7 = β-casomorphin-7; BMI = body mass index; hs-CRP = high-sensitivity C-reactive protein; hydr. = hydrolyzed; lact. = with lactase capsule; LOD = limit of detection; n.s. = not significant; P_F = Friedman test *P*-value for between intervention comparisons; P_{rA} = rANOVA test *P*-value for between intervention comparisons; q = Benjamini–Hochberg adjusted *P*-value; rANOVA = repeated measures ANOVA; Ref = reference value; reg. = without lactase enzyme; TFA = trifluoroacetic acid.

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