Comparison of the impact of bovine milk β-casein variants on digestive comfort in females self-reporting dairy intolerance: a randomized controlled trial

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ABSTRACT

Background: Lactose malabsorption (LM) is a major cause of digestive discomfort from dairy products. Recently, a role for bovine β-casein A1 has been proposed.

Objectives: We examined whether there are distinct symptoms of digestive discomfort due to either lactose or differing bovine β-casein types.

Methods: Women (n = 40; age: 25.2 ± 0.5 y) with self-reported varying dairy tolerance underwent a 50-g lactose challenge. Based on postchallenge LM and digestive discomfort, participants were classified as either lactose intolerant (LI; n = 10, self-reported intolerant, diagnosed lactose intolerant), nonlactose dairy intolerant (NLDI; n = 20, self-reported intolerant, diagnosed lactose tolerant), or dairy tolerant (DT; n = 10, self-reported tolerant, diagnosed lactose tolerant). In a double-blinded randomized sequence, participants consumed 750 mL conventional milk (CON; containing A1 and A2 β-casein and lactose), a2 Milk (A2M; exclusively containing A2 β-casein with lactose), or lactose-free conventional milk (LF-CON; containing A1 and A2 β-casein without lactose). Subjective digestive symptoms and breath hydrogen (measuring LM) were recorded regularly over 3 h, and further ad hoc digestive symptoms over 12 h.

Results: LI subjects experienced prolonged digestive discomfort with CON milk. A2M reduced ($P < 0.05$) some symptoms (nausea: A2M 8 ± 3 mm compared with CON 15 ± 3mm; fecal urgency: A2M 4 ± 1 compared with CON 10 ± 3 mm), and attenuated the rise in breath hydrogen over 3 h, relative to CON milk (A2M 59 ± 23 compared with CON 98 ± 25 ppm at 150 min; $P < 0.01$). In contrast, NLDI subjects experienced rapid-onset, transient symptoms (abdominal distension, bloating, and flatulence) without increased breath hydrogen, irrespective of milk type.

Conclusions: In LI individuals, LM and digestive comfort with lactose-containing milks was improved with milk containing exclusively A2 β-casein. Furthermore, self-reported dairy intolerance without LM (NLDI) is characterized by early-onset digestive discomfort following milk ingestion, irrespective of lactose content or β-casein type. This trial was registered at www.anzctr.org.au as ACTRN12616001694404. *Am J Clin Nutr* 2019;0:1–12.

Keywords: lactose malabsorption, lactose intolerance, dairy intolerance, A2 β-casein, A1 β-casein, digestive comfort

Introduction

Digestive discomfort after dairy product consumption is frequently attributed to lactose. Lactose malabsorption (LM) affects ∼65% of adults worldwide, limiting their lactose digestion capacity due to insufficient lactase production (1). For these people, ingested malabsorbed lactose results in rapid bacterial fermentation (2) contributing to variable degrees of digestive discomfort (3), manifesting as a variety of symptoms over several hours (4). Yet, the severity of symptoms varies among malabsorbers (5), and can be modifiable. Studies have shown

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Supplemental Tables 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

Data described in the manuscript, code book, and analytic code will not be made available because approval has not been granted by subjects.

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Abbreviations used: A2M, milk containing exclusively A2 β-casein; BCM7, beta-casomorphin 7; BM, bowel movement; CON, conventional milk; DT, dairy tolerant; FAST, food and symptom time; IBS, irritable bowel syndrome; LF-CON, lactose-free conventional milk; LI, lactose intolerant; LM, lactose malabsorption; NLDI, nonlactose dairy intolerant; PBMC, peripheral blood mononuclear cell; SNP, single nucleotide polymorphism; SRM, selected reaction monitoring; UHT, ultra-high temperature; VAS, visual analogue scale.

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that lactose habituation (6, 7), prebiotic treatment (8), and delayed gastrointestinal transit (9–11) modify LM and associated digestive symptoms.

Self-reported digestive discomfort following dairy ingestion is also documented in individuals without LM (5, 12–15), for whom lactose-free diets are ineffective in alleviating discomfort associated with either lactose-containing or lactose-free dairy products (5). This suggests involvement of constituent dairy components other than lactose.

Of recent interest is the potential digestive impact of dairy proteins, in particular β-casein. Bovine β-casein exists in 2 predominant types: A1 or A2 (16). A single amino acid polymorphism of A1 β-casein results in greater liberation of the peptide beta-casomorphin 7 (BCM7) during digestion (17). Diets containing A1 β-casein fed to rodents have been shown to decrease gastrointestinal motility resulting in increased transit time (18); A1 β-casein has also resulted in increased intestinal fluid inflammatory marker concentrations, and intestinal leukocyte infiltration, suggesting gastrointestinal inflammatory mechanisms (19). In clinical studies, the consumption of milk containing exclusively A2 β-casein, compared with milk containing A1 β-casein, resulted in softer stools (20) and improved digestive comfort (21, 22). These improved digestive symptoms provided by milk containing exclusively A2 β-casein might be greater in lactose malabsorbers (21, 22), or those with self-described milk intolerance (17). Although these studies suggest that LM symptoms could be aggravated by A1 β-casein consumption (18, 19), assessment of LM was performed post hoc, using proxy measures, and subgroup analysis of those with self-described intolerances has failed to produce significant findings.

It remains unclear whether differing β-casein types influence digestive discomfort in individuals with lactose intolerance, and in individuals reporting dairy intolerance that cannot be attributed solely to LM (5, 12, 13).

Our first aim was to investigate whether the digestive symptoms experienced following milk ingestion by dairy-intolerant individuals were improved by the absence of either A1 β-casein or lactose in milk. Our second aim was to further characterize the symptoms of dairy intolerance in self-reported dairy-intolerant individuals, with or without LM. We hypothesized that individuals diagnosed with lactose intolerance would experience reduced severity of digestive symptoms following consumption of A1 β-casein–free milk relative to conventional milk (containing A1 and A2 β-casein), consistent with the previous literature (21, 22), and that conventional milk without lactose would not reduce symptoms associated with A1 β-casein ingestion. We further hypothesized that with milk ingestion, the digestive symptoms of nonlactose dairy intolerance would differ from lactose intolerance, and that A1 β-casein–free, but not lactose-free, milk would alleviate these symptoms relative to conventional A1 β-casein–containing milk.

Methods

Subject recruitment

A total of 59 community-dwelling healthy women aged 20–30 y from Auckland, New Zealand, were recruited to participate in the study (**Figure 1**) using digital and printed advertisements. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Southern Health and Disability Ethics Committee (New Zealand, 16/STH/175). Written informed consent was obtained from all subjects. The prospective clinical trial was registered at www.anzctr.org.au (ACTRN12616001694404).

The primary outcome measure was defined as the analysis of digestive symptom scores by a 10-cm visual analogue scale (VAS). The study was powered using published differences in total VAS scores of digestive comfort (21). Based on a 10-point scale, an SD of 0.8 is expected, which provides 80% power to detect a 1.1-point difference at α of 0.05. The dairy tolerant (DT) and lactose intolerant (LI) groups each had 10 subjects, whereas the nonlactose dairy intolerant (NLDI) group was increased to account for the likelihood of false positive reporting of perceived symptoms.

Secondary end points included: measurement of the breath metabolite hydrogen (H_2); plasma concentrations of glucose following lactose challenge; single nucleotide polymorphisms (SNPs) related to lactase persistence; and further assessment of gastrointestinal symptoms using a food and symptom time (FAST) diary. Additional prespecified secondary end points, not reported here, included metabolic responses (including insulin, triglyceride responses), immune responses (whole blood counts, circulating cytokines, inflammatory gene expression), breath volatile responses, MRI of gastrointestinal motility, and urinary creatinine and galactose.

Inclusion criteria

All participants were without current or past history of gastrointestinal diseases including gastric reflux, inflammatory bowel disease, celiac disease, anosmia, or medication use likely to impact digestive function (e.g., stomach acid regulators). Irritable bowel syndrome (IBS) was not an exclusion criterion, because this is associated with intolerance to dairy (23–25). All subjects self-described as free from metabolic or cardiovascular disease. Participants were ineligible for participation if they had been diagnosed with milk allergy or had an alcohol intake >28 units/wk. Following informed consent, subjects provided background demographic information including self-reported IBS, objectively assessed using the Rome III criteria (26).

Participants were first screened for perceived intolerance to dairy using a symptom questionnaire validated as a prescreening tool for lactose tolerance with high sensitivity (0.82), but low specificity (0.35) (27). The severity of perceived symptoms when consuming milk was scored on a 100-mm VAS. Digestive severity was scored between 0 mm, corresponding to "no symptom," and 100 mm, corresponding to "the most severe symptom imaginable." Scores for diarrhea, flatulence, vomiting, abdominal cramping, and rumbling were summed to establish a total out of 500. A score >70 was indicative of LM, with a sensitivity of 0.77 and specificity of 0.67 (27). Subjects scoring >70 of 500 were provisionally classified as "intolerant," <70 as "tolerant."

Subjects were subsequently assessed for lactose tolerance by a standardized lactose challenge, consuming 50 g lactose in 250 mL water after an overnight fast (28), as described in more detail under Study procedures. Criteria were set to classify LI if a symptom score ≥70/500 (27) after lactose ingestion, plus

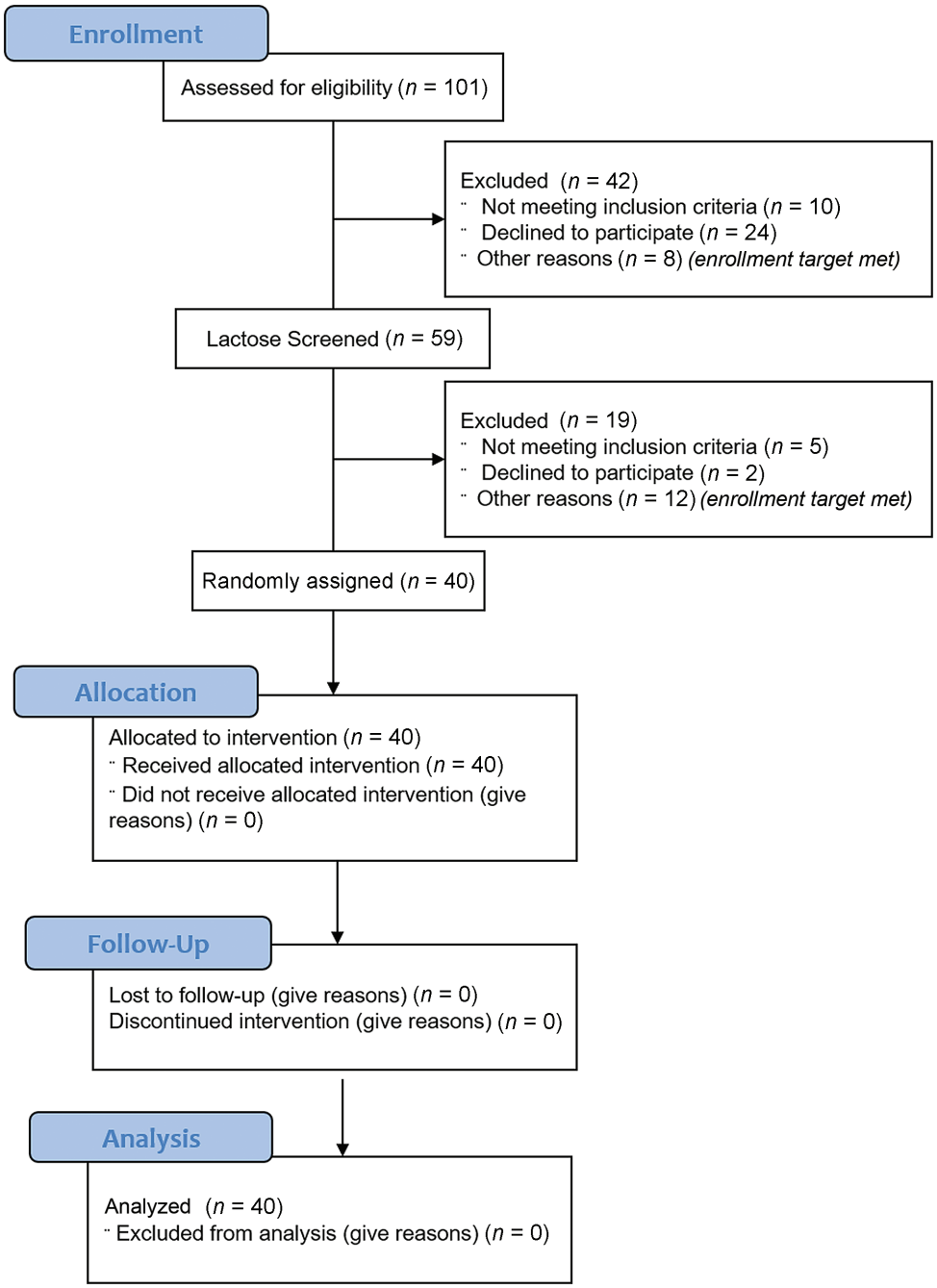

FIGURE 1 Consolidated Standards of Reporting Trials participant eligibility, enrollment, and randomization. Participants were randomly assigned to sequences of milk ingestion order blocked per subject group.

≥1 marker of LM—breath hydrogen rise (28) or homeostatic plasma glucose (4, 29); **Table 1**—was experienced. Both NLDI and DT criteria were established on the basis of experiencing minimal symptoms following lactose ingestion (≤70/500) and ≤1 marker of LM. NLDI subjects perceived considerable symptoms in response to dairy consumption (≥70/500) whereas DT subjects perceived minimal symptoms with dairy consumption (≤70/500). Recruitment was continued until 10 LI, 20 NLDI, and 10 DT individuals were identified and successfully enrolled into the study to ingest the milks. To account for the possibility that the perception questionnaire might identify a wide range of subjective symptomology and dairy avoidance behaviors, and the lack of published characterizations of this phenomenon, NLDI subjects were recruited into the study in a ratio of 2:1 relative to LI and DT subjects.

Experimental design

Subjects underwent a standardized lactose challenge and were subsequently block randomly assigned by tolerance group (LI, NLDI, DT) to a 3-treatment crossover intervention in an equal treatment allocation ratio. The sequence of treatment arms was randomly generated by www.randomizer.org, and subjects were allocated using concealed sealed envelopes before the first milk

TABLE 1 Subject classification criteria

Method	Criteria	Dairy tolerant	Lactose intolerant	Nonlactose dairy intolerant
Perceived symptoms (27)	Sum of score[1]	≤70	≥70	≤70
Symptoms[2]	Sum of score[1]	≤70 plus ≤1 biological marker	≥70 plus ≥1 biological marker	≤70 plus ≤1 biological marker
Hydrogen breath test[2] (28)	Increase	Δ ≤25 ppm	Δ ≥25 ppm	Δ ≤25 ppm
Lactose tolerance test[2] (4, 29)	No increase	≥1.11 mmol/ L	≤1.11 mmol/ L	≥1.11 mmol/ L

[1]Sum of visual analogue scale (100 mm each, total 500) for diarrhea, abdominal cramping, abdominal rumbling, flatulence, and vomiting (27).
[2]Following standardized lactose challenge (50 g lactose).

tolerance test. Investigators and participants were blinded to the identity of treatments and for the duration of the data analysis. No sensory masking of products was employed.

Study procedures

The lactose tolerance test and all subsequent milk tolerance tests were conducted in the Nutrition and Mobility Clinic at the Liggins Institute, University of Auckland, between January and May 2017. For the week prior to the lactose tolerance test, and prior to each of the subsequent 3 milk tolerance tests, subjects were instructed to abstain from consuming all lactose-containing dairy products and foods. Further, on the day before each intervention, subjects were requested to abstain from vigorous physical exercise, to avoid foods rich in fat, dietary fiber, lactose, fructose, or artificial sweeteners and to avoid the use of laxatives or antacids. They were also provided with a standardized low-fat and low-dietary-fiber meal to be consumed in the evening prior to the experimental day, with instruction to remain fasted from 22:00 that night. Subjects were not scheduled for visits during periods of active menstruation.

Upon arrival, a venous cannula was inserted for fasting blood sample collection. Fasting breath samples were collected and gastrointestinal symptomology completed by VAS questionnaire. VAS symptoms assessed included belching, gastric reflux, nausea, abdominal distension, abdominal pain, abdominal cramps, abdominal rumbling, bloating, flatulence, fecal urgency, diarrhea, and vomiting, aiming to be inclusive of the varying range of symptoms reported in the literature (30). Subjects then underwent the lactose tolerance test or milk tolerance test as described.

Lactose tolerance test

Lactose tolerance was assessed by a standardized lactose challenge, consisting of 50 g lactose in 250 mL water (28). Following the lactose challenge, blood samples were taken every 30 min for 2 h, whereas digestive symptom scores (by VAS) were recorded every 30 min for 3 h and breath every 15 min for the first 90 min, then every 30 min until 3 h had elapsed since the challenge.

Milk tolerance test

Conventional milk (CON), milk containing exclusively A2 β-casein (A2M; a2 Milk; a2 Milk Company Limited), and lactose-free conventional milk (LF-CON) were provided to subjects over the course of the 3 visits. The composition of each milk is shown in **Table 2**. Milks were: ultra-high temperature (UHT) processed (CON; UHT Blue Top Longlife Milk; Anchor); A2M (a2 Milk Full Cream Milk; a2 Milk Company Limited); and LF-CON (Free From Lactose Full Cream Milk; Progressive Enterprises Limited). All were chilled (4°C) for 12 h and served in plasticware with the milks exposed to room temperature for 10 min prior to ingestion. A serving size of 750 mL milk was chosen based on daily doses provided in previous studies (20), and to exceed a serving size tolerable for those with lactose intolerance (3), for example, 250 mL (30) or 500 mL (5). Further, this quantity provided a lactose dose (30 ± 1 g) more similar to the lactose tolerance test while remaining an achievable quantity to consume in a single sitting.

The allocated milk was consumed within 10 min. Subjects were asked to indicate their perceived identity of each milk, because no masking was used. Then, at 30-min intervals for 3 h postingestion, digestive symptom scores were measured using the VAS. Also, breath samples were collected every 15 min for 90 min, then hourly between 2 and 3 h.

Further digestive symptoms and bowel motions were evaluated ad hoc for 12 h using the FAST diary (31). The severity of digestive symptoms was scored on a 5-point Likert scale: "not bad at all," "a little bad," "somewhat bad," "quite bad," and "very bad" to assess abdominal pain, abdominal swelling/distension, abdominal fullness, abdominal bloating, and bowel motions. In addition to the severity score, symptoms were scored along a 24-h scale to identify when a particular symptom began and

TABLE 2 Nutritional composition per serving milk (750 mL)[1]

Nutrient	A2M	CON milk	LF-CON milk
Energy, kJ	2063	2010	1935
Protein, g	24.8	26.3	25.5
Total fat, g	26.3	25.5	25.5
Saturated fat, g	18.0	17.3	18.0
Total carbohydrate, g	37.5	36.0	33.0
Lactose, g	35.3	36.0	Not detected
Galactose, g	—	—	17.25
Sodium, mg	247	300	292
Potassium, mg	1102	—	—
Calcium, mg	817	915	900
A1 β-casein,[2] % total β-casein	0 ± 0	21.6 ± 1.2	23.5 ± 0.2
Lactose,[2] g	30.9 ± 1.7	31.0 ± 10.7	0.1 ± 0.0

[1]Unless otherwise stated, values are as provided on the nutrition information panel (NIP), the New Zealand and Australian equivalent to a nutritional facts label on food packaging. A2M, milk containing exclusively A2 β-casein; CON, conventional milk containing both A1 and A2 β-casein; LF-CON, lactose-free conventional milk.
[2]A1 β-casein and lactose values as measured by LC-MS. Mean ± SD for 3 replicates.

ended. Bowel motions were assessed using the Bristol Stool Scale (32), and the severity of straining, abdominal pain prior to the bowel motion, and urgency were assessed using Likert scales; relief or worsening of abdominal pain after each bowel motion was also scored ("yes," "no," or "not applicable").

Analysis methodology

Breath hydrogen.

Breath samples were collected using AlveoSampler Breath Test Kits and analyzed by a BreathTracker H2+ (Quintron). Data were collected as carbon dioxide-corrected hydrogen concentrations (ppm) as a measure of LM.

Biochemical analysis.

Venous bloods were collected in EDTA vacutainers (Becton Dickinson & Company), and plasma was removed after centrifugation at 2000 × *g* for 15 min at 4°C and frozen at −80°C prior to analyses.

Plasma glucose was measured using a Cobas c311 clinical chemistry analyzer (Roche Diagnostics).

Lactase persistence genotyping.

To provide further evidence of a correct diagnosis of lactose intolerance in study participants, lactase persistence genotype was determined by RFLP (restriction fragment length polymorphism) of PCR-amplified DNA of the lactase (*LCT*) gene. Peripheral blood mononuclear cells (PBMCs) were isolated from fasted whole blood collected in EDTA-containing blood collection tubes using a Ficoll gradient (Histopaque 1077; Sigma-Aldrich), according to the manufacturer's protocol. Genomic DNA was isolated from PBMCs with an AllPrep DNA/RNA Mini Kit (Qiagen) as per the manufacturer's protocol. Genomic DNA containing rs4988235 (C/T-13,910) was PCR amplified using forward primer 5-GGACATACTAGAATTCACTGCAA and reverse primer 5-GGTTGAAGCGAAGATGGGACG (33). rs182549 (G/A-22,018) was amplified using forward primer 5-TAGCTGGGACCACAAGCACC and reverse primer 5-GAAGTCAGAATACCCCTACCC; PCR was carried out on 100 ng genomic DNA using an Invitrogen PCR SuperMix kit (Thermo Fisher Scientific). Each reaction was denatured at 95°C for 3 min followed by 35 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s with a final extension of 2 min at 72°C.

The amplification product for C/T-13,910 was digested with *Bsm*F1 (New England Biolabs) for 3 h at 65°C. Digestion resulted in 2 fragments (386 and 34 bp) for the C allele, and 3 fragments (238, 148, and 34 bp) for the T allele. The amplification product for G/A-22,018 (252 bp) was digested with *Hha*1 (New England Biolabs) at 37°C. Digestion resulted in 2 fragments (167 and 85 bp) for the G allele, whereas the A allele remained undigested. PCR and digested PCR products were separated by electrophoresis on a 5% MetaPhor agarose gel (Lonza) and visualized using ethidium bromide.

Milk compositional analysis.

Triplicate samples were assessed for A1 β-casein following enzymatic digestion in a mixture containing individually labeled internal standards (Biomatik) for A1 β-casein. Peptide digests were quantified on a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific) using a selected reaction monitoring (SRM) method. LC separation was performed using a Hypersil Gold (Thermo Scientific) C18 column (2.1 × 100 mm, 1.9 μm).

Lactose was analyzed following separation from milk proteins by acid precipitation and dilution in 200 μM $NaHCO_3$ buffer. LC separation was performed on a Hypercarb 3-μm, 2.1 × 50-mm column (Thermo Scientific) using a Dionex Ultimate 3000 UHPLC (Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer by a heated electrospray interface. The mass spectrometer was operated in SRM mode measuring the transitions *m*/*z* 365.1 to 305.1 and 365.1 to 245.0. For quantitative analysis, the Quan Browser of Xcalibur (Thermo Scientific) was used to integrate peaks of the extracted ions. Calibration curves were constructed using pure lactose (D-lactose monohydrate; Serva Electrophoresis GmbH) as the analytical standard.

Statistical analysis.

Statistical analyses were performed with SPSS version 25 (IBM Corporation). Continuous data are presented as mean ± SEM. Ordinal data are presented as median ± IQR. Continuous variables were analyzed using a mixed generalized linear model; a Huynh–Feldt covariance structure was used, with fixed factors milk, time, and tolerance group. Sidak-adjusted post hoc tests were used for all multiple comparisons. Where no 3-factor interaction existed, data were pooled over the non-interacting factor for presentation. Frequencies were weighted for unequal group distributions, and frequency distributions were analyzed using Pearson chi-square test and Bonferroni adjustments made for multiple comparisons. Ordinal variables were analyzed using the Kruskal–Wallis test; α was set at $P < 0.05$.

Results

Demographics

The female participants' age, anthropological measures, fasting glucose, and blood lipids did not differ between groups (**Table 3**). There were disproportionately fewer Caucasians in the LI group ($P = 0.003$) and more Caucasians (100%) and fewer Asians (0%) in the NLDI group ($P < 0.001$ and $P = 0.019$, respectively). There were also more self-reported and ROME III criteria IBS subjects in the NLDI group ($P = 0.003$ and $P = 0.039$, respectively).

On the basis of the screening criteria, LI and NLDI subjects had greater perceived adverse symptoms with dairy than the DT subjects. In response to the lactose challenge, the LI subjects reported higher pooled symptom scores than both NLDI and DT subjects (Table 3). This was accompanied by greater breath hydrogen and an attenuated rise in plasma glucose. Neither

TABLE 3 Baseline subject characteristics[1]

Attribute	LI ($n = 10$)	NLDI ($n = 20$)	DT ($n = 10$)
Age, y	26.6 ± 0.8	26.0 ± 0.7	25.1 ± 0.5
Ethnicity			
Caucasian, *n* (%)	2 (20)**	20 (100)***	5 (50)
Asian, *n* (%)	5 (50)	0 (0)*	3 (30)
South Asian, *n* (%)	2 (20)	0 (0)	2 (20)
Maori, *n* (%)	1 (10)	0 (0)	0 (0)
Genotype[2]			
CC/GG, *n* (%)	9 (90)***	1 (5)	4 (40)
CT/GA, *n* (%)	1 (10)	7 (35)	2 (20)
TT/AA, *n* (%)	0 (0)	12 (60)**	4 (40)
Self-report IBS, *n* (%)	0 (0)	7 (35)**	0 (0)
Rome III IBS, *n* (%)	3 (30)	15 (75)*	3 (30)
IBS-C, *n* (%)	1 (10)	0 (0)	1 (10)
IBS-D, *n* (%)	0 (0)	8 (40)	1 (10)
IBS-M, *n* (%)	2 (20)	7 (35)	1 (10)
Height, cm	161.0 ± 2.1	166.4 ± 1.5	162.3 ± 1.6
Weight, kg	59.5 ± 2.9	62.4 ± 1.8	64.5 ± 2.7
BMI, kg/m^2	22.9 ± 0.9	22.5 ± 0.5	24.5 ± 1.1
Perceived VAS,[3,4] mm	132 ± 35^a	191 ± 18^a	26 ± 8^b
Lactose VAS,[3,4] mm	166 ± 29^a	12 ± 3^b	14 ± 4^b
Lactose H_2 Δ,[3] ppm	185 ± 33^a	14 ± 8^b	71 ± 29^b
Lactose glucose Δ,[3] mmol/L	0.61 ± 0.09^a	1.88 ± 0.24^b	1.86 ± 0.38^b

[1]Values presented as mean ± SEM over all treatments or count (percentage) as indicated. Main effects and interactions were analyzed by mixed generalized linear model with Sidak corrected post hocs. There were no differences between group baseline values between treatment days. *,**,*** Greater frequency than expected: *$P <$ 0.05, ** $P < 0.01$, *** $P < 0.001$. For differences between the LI, NLDI, and DT groups, means without a common letter differ, $P < 0.05$. DT, dairy tolerant; IBS, irritable bowel syndrome; LI, lactose intolerant; NLDI, nonlactose dairy intolerant; VAS, visual analogue scale.

[2]rs4988235 (C/T −13,910) genotype/rs182549 (G/A −22,018) genotype. The presence of the T −13,910 and A −22,018 variants is associated with persistent levels of lactase activity (33).

[3]Lactose tolerance screening: perceived intolerance questionnaire followed by a standardized lactose challenge (50 g lactose).

[4]Combined VAS symptom score for abdominal cramps, abdominal rumbling, flatulence, vomiting, and diarrhea (27).

NLDI nor DT experienced significantly elevated breath hydrogen following the lactose challenge.

Analysis of C/T −13,910 and G/A −22,018 genotype demonstrated that the LI subjects were more likely to have the lactase nonpersistent genotypes C/C −13,910 and G/G −22,018 (9/10; $P <0.001$), whereas NLDI subjects were more likely to have the lactase persistent genotype (12/20; $P = 0.006$). DT subjects were equally distributed among lactase persistent and nonpersistent genotypes.

Digestive symptoms in response to milk β-casein types and lactose

There was a difference in perception of which milk was consumed, based on the frequency of reporting ($P = 0.029$). Specifically, subjects were more likely to perceive LF-CON to be LF-CON and not CON milk, and to perceive A2M to be CON milk.

Because the symptom responses were not simultaneously dependent on milk, group, and time (milk × group × time interaction), data have been reported for the symptom differences between milks across subjects (milk × group interaction, independent of time) and the differences between subjects over time (group × time interaction independent of milk).

There were no differences in symptom severity for abdominal cramps, rumbling, distension, belching, or bloating between milks (main milk effect $P > 0.05$), or between subject groups (main group effect and milk × group interaction, $P > 0.05$ each, respectively; **Supplemental Table 1**).

LI subjects experienced less nausea and fecal urgency with A2M and LF-CON, compared with CON milk, irrespective of time (**Figure 2**A, B).

Flatulence was reduced in LI subjects after the LF-CON milk relative to both A2M and CON milks (Figure 2C). LI subjects also experienced less gastric reflux with LF-CON milk relative to both lactose-containing milks (A2M and CON) (Figure 2D). Digestive comfort in LI subjects was reported to be lower after either A2M or CON milk compared with LF-CON milk (Figure 2E).

LI and NLDI subjects reported differing severity of digestive symptoms between the milks. Neither NLDI nor DT subjects reported any differences in symptom severity between milks across nausea, fecal urgency, or digestive comfort, unlike LI subjects (Figure 2). Yet, in contrast to LI subjects, NLDI subjects did not report reduced flatulence with LF-CON milk relative to CON or A2M, but they had more flatulence with A2M than CON milk (Figure 2C; $P = 0.391$ between LF-CON and CON; $P = 0.346$ LF-CON compared with A2M; $P = 0.010$ A2M compared with CON).

Digestive symptom differences between dairy intolerant individuals

Regardless of the type of milk ingested, LI and NLDI subjects experienced greater discomfort and symptom severity than DT subjects, but the specific symptoms reported, their onset, and duration differed. Whereas LI subjects experienced commonly reported symptoms with lactose intolerance following milk ingestion (i.e., abdominal cramping, rumbling) occurring later in digestion, NLDI subjects reported more early symptoms, which included distension and bloating (**Figure 3**), but these symptoms reduced as digestion progressed. In contrast, DT subjects did not report severe or sustained symptoms, and maintained digestive comfort scores comparable to fasting (Figure 3).

Both LI and NLDI subjects had poorer digestive comfort from 30 min after milk consumption. However, whereas LI subjects continued to experience poor digestive comfort until 180 min, NLDI subjects' digestive comfort was no longer significantly reduced from fasting scores at 90 min and continued to improve toward fasting scores from 120 min onward (**Supplemental Table 2**; $P = 0.041$ for 30 compared with 120 min in NLDI subjects; Figure 3A).

After milk consumption, LI and NLDI subjects experienced reduced digestive comfort and more severe abdominal cramps, rumbling, distension, bloating, belching, and flatulence, an effect seen after consumption of all milks, unlike DT subjects (Supplemental Table 2). However, the change in severity of nausea and gastric reflux over time was not different between subject groups.

Abdominal cramps (Figure 3B) and rumbling (Supplemental Table 2) were most severe in LI subjects and progressed following milk consumption. Neither DT nor NLDI subjects

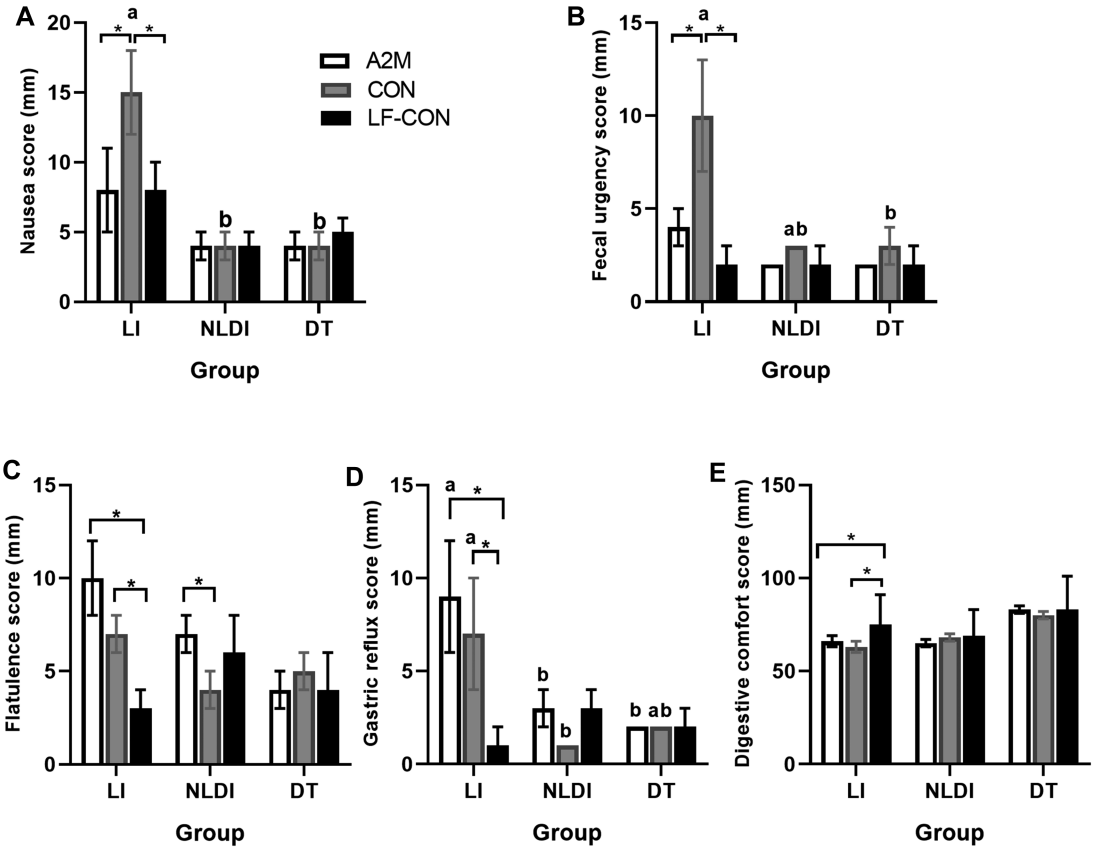

FIGURE 2 Subjective VAS scores between groups after milk ingestion. Nausea (A), fecal urgency (B), flatulence (C), gastric reflux (D), and digestive comfort (E) for: A2M (milk containing exclusively A2 β-casein); CON (conventional milk); and LF-CON (lactose-free conventional milk) across DT ($n = 10$), LI ($n = 10$), and NLDI ($n = 20$) subjects, with times pooled. Data were compared by generalized linear mixed model using group, time, and milk as factors, where time and milk were repeated. There were group × milk interactions for nausea, fecal urgency, digestive comfort, flatulence, and gastric reflux ($P < 0.05$ each, respectively). *Difference between indicated milks, $P < 0.05$. For LI, NLDI, and DT groups within milk, matching colored bars without a common letter are significantly different, $P < 0.05$. Data are mean ± SEM. DT, dairy tolerant; LI, lactose intolerant; NLDI, nonlactose dairy intolerant; VAS, visual analogue scale.

reported cramping, but NLDI subjects reported rumbling at 30 min only. Similarly, fecal urgency was not severe in DT or NLDI subjects but increased in severity for LI subjects as time progressed.

Similar to overall digestive comfort, abdominal distension and bloating were more severe in NLDI subjects early on, whereas in LI subjects these symptoms persisted (Figure 3C,D). Although there were no between-group differences at any single time point, NLDI subjects experienced increased severity of distension and bloating from 30 min until 60 and 120 min, respectively. In LI subjects, these symptoms remained more severe than fasting for the duration of the intervention, whereas in NLDI subjects, these symptoms reduced to baseline scores from 90 and 150 min, respectively. Belching, an additional upper gastrointestinal symptom, was experienced for 120 min following milk ingestion in NLDI subjects, but unlike other symptoms, belching was experienced by LI subjects only transiently at 30 min following milk ingestion (Figure 3E).

Flatulence scores were higher than baseline and more frequent among LI subjects from 150 min onward, and this was more severe than in NLDI subjects (Figure 3F). In contrast to both LI and DT subjects, NLDI subjects experienced early flatulence after drinking, which resolved by 150 min.

Objective measures of digestive changes

Breath hydrogen concentration was greater in LI subjects following CON milk than following the ingestion of either A2M or LF-CON milk (interaction group × milk × time $P = 0.049$; $P = 0.001$ CON compared with A2M at 150 min; $P < 0.001$ CON compared with LF-CON at 150 min; **Figure 4**A). Breath hydrogen concentration remained higher in these subjects following A2M milk relative to LF-CON, 90 min onward ($P < 0.05$ compared with fasting, each time point respectively). Neither NLDI nor DT subjects had elevated breath hydrogen following milk consumption, unlike LI subjects (Figure 4B,C).

Persistence of symptoms and frequency of bowel movements over 12 h

Symptoms over the course of 12 h were reported ad hoc using the FAST diary. The frequency of reporting abdominal pain, fullness, bloating, and distension was different between groups and type of milk consumed ($P < 0.001$ each, respectively; **Table 4**). However, the severity of these symptoms was not different across subjects or depending on the type of milk consumed (**Table 5**).

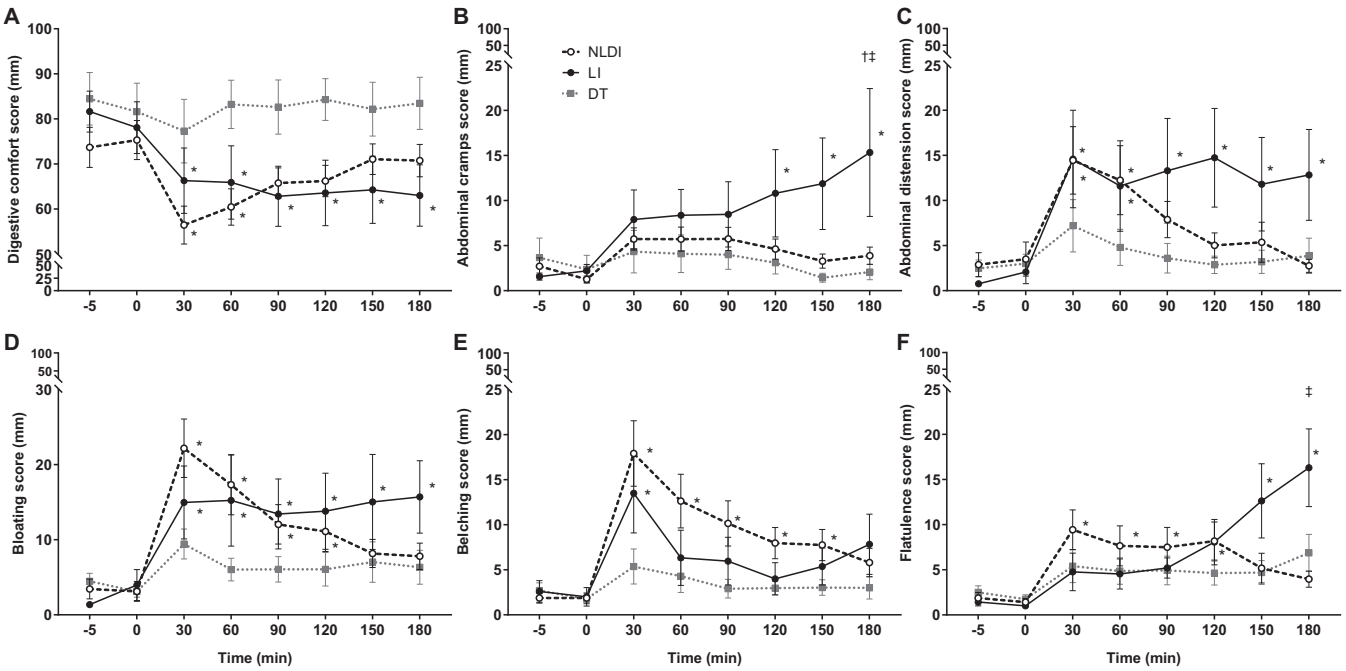

FIGURE 3 Subjective VAS scores that differed between groups after drinking milk. Digestive comfort (A), abdominal cramps (B), abdominal distension (C), bloating (D), belching (E), and flatulence (F) for DT ($n = 10$), LI ($n = 10$), and NLDI ($n = 20$) subjects, with milk types pooled to show effects independent of milk type. Data were compared by generalized linear mixed model using group, time, and milk as factors, where time and milk were repeated. There were group × time interactions for digestive comfort, abdominal cramps, abdominal distension, bloating, belching, and flatulence ($P < 0.05$ each, respectively). Data are mean ± SEM. *Individual group differs from baseline, $P < 0.05$; ‡LI differs from DT, $P < 0.05$; †LI differs from NLDI, $P < 0.05$. DT, dairy tolerant; LI, lactose intolerant; NLDI, nonlactose dairy intolerant; VAS, visual analogue scale.

Abdominal pain after 12 h was reported more frequently than expected (on the basis of chi-square analysis) in LI subjects when they drank CON milk, after LF-CON milk for NLDI subjects, and in DT subjects when they drank A2M ($P < 0.001$ each, respectively; Table 4). Feelings of abdominal fullness, bloating, and distension were reported more often than expected by LI subjects after CON milk and by DT subjects after A2M. LI subjects reported instances of abdominal fullness, bloating, and distension more frequently than expected after CON milk. These subjects reported more instances than expected of bloating and

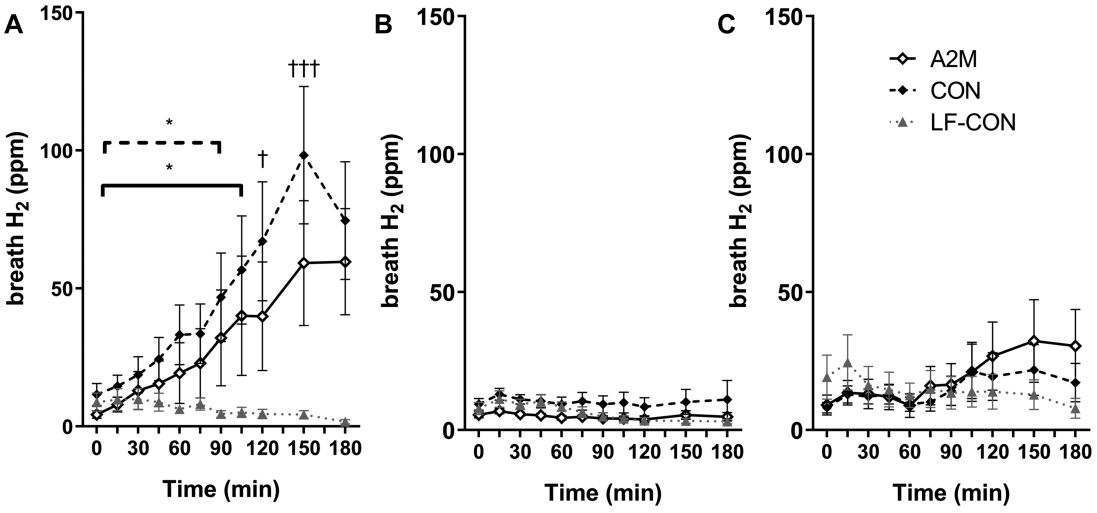

FIGURE 4 Lactose malabsorption assessed by exhaled breath hydrogen following milk consumption in (A) lactose intolerant ($n = 10$), (B) nonlactose dairy intolerant ($n = 20$), and (C) dairy tolerant ($n = 10$) subjects. Data were compared by generalized linear mixed model using group, time, and milk as factors, where time and milk were repeated. There was a group × time × milk interaction ($P < 0.05$ each). *Comparison between indicated time points differs: *$P < 0.05$; †,††† comparison between milks differs: †$P < 0.05$, †††$P < 0.001$. A2M, milk containing exclusively A2 β-casein; CON, conventional milk; LF-CON, lactose-free conventional milk.

TABLE 4 Symptom frequency reported ad hoc by food and symptom time diary over 12 h[1]

Symptom	Group	A2M	CON	LF-CON	*P* value[2]
Abdominal pain	LI	6	12***	2	<0.001
	NLDI	8	13	9***	
	DT	5***	3	0	
Abdominal fullness	LI	3	6***	0	<0.001
	NLDI	12*	12**	4***	
	DT	6***	1	0	
Abdominal bloating	LI	8	8**	5*	<0.001
	NLDI	9	8	5	
	DT	7***	2	0	
Abdominal distension	LI	4	7***	4***	<0.001
	NLDI	7***	3	1	
	DT	5***	2	0	
BM 3 h	LI	1	4	2	0.839
	NLDI	2	4	1	
	DT	0	1	0	
BM 12 h	LI	10	18	4	0.024
	NLDI	8	9	12*	
	DT	8	9	4	
Loose BMs (diarrhea; BSS score >6)	LI	1	7***	0	<0.001
	NLDI	0	4	4	
	DT	5***	2***	2	

[1]Values presented as count (*n*) over 12 h unless otherwise specified. A2M, milk containing exclusively A2 β-casein; BM, bowel movement; BSS, Bristol Stool Scale; CON, conventional milk; DT, dairy tolerant; LF-CON: lactose-free conventional milk; LI: lactose intolerant; NLDI: nonlactose dairy intolerant.

[2]Frequency was compared by Pearson χ^2 test weighted for total subject numbers with adjusted residual post hoc values Bonferroni corrected for multiple comparisons. *P* value relates to the symptom interaction value (group x milk). *,**,***Frequency of post hoc value differs significantly from expected: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. DT: $n = 10$; LI: $n = 10$; NLDI: $n = 20$.

distension after LF-CON milk. NLDI subjects similarly reported more instances than expected of abdominal fullness after CON milk, yet they also reported fullness more frequently after LF-CON. Both NLDI and DT subjects reported more instances than expected of abdominal fullness and distension after A2M.

Bristol Stool Scale scores and symptoms with bowel movements

Bowel movements (BMs) were reported on only 15 occasions during the first 3-h clinical session, with 82 BMs reported over the 12 h. BM frequency was not different across tolerance groups or type of milk ingested (Table 4). More BM events occurred in the NLDI subjects after LF-CON milk ($P = 0.020$). Over 12 h, the median stool score did not differ between milks or tolerance groups (Table 5). Due to the low frequency of BMs over 3 h, these stool scores could not be compared.

When stool scores were assessed on the basis of loose stools ($n = 25$ events), diarrhea was more frequent in certain tolerance groups depending on the milk consumed ($P < 0.001$). LI subjects had more diarrhea after CON milk ($P < 0.001$). DT subjects had more diarrhea after the A2M than the other groups, and more than after CON milk ($P < 0.001$ each, respectively). Of the DT subjects that had diarrhea ($n = 5$ subjects), 3 experienced some LM. These 3 subjects accounted for 80% of the diarrhea events experienced with A2M.

Of the BMs that occurred during the 12 h, milk type did not affect the severity of BM-related discomfort (Table 5). LI subjects had more pain before a BM than DT subjects, whereas both LI and NLDI subjects strained more with BMs than did DT subjects.

Discussion

Dairy avoidance is increasingly common, often due to a presumption of lactose intolerance (34). However, there is evidence that the protein fraction of dairy products also contributes to adverse digestive symptoms (35), with possible adverse interactions with LM (21, 22). This study demonstrated that LI individuals, identified using a standardized lactose challenge, experienced reduced symptoms, specifically nausea and fecal urgency, following the consumption of A2M, relative to CON milk (containing both A1 and A2 β-caseins). LM, measured by breath hydrogen, was also attenuated by A2M, despite the latter having the same lactose content as CON milk. Yet dairy intolerance symptoms were shown not to be solely dependent upon LM, because we identified NLDI females who were asymptomatic to lactose ingestion, with no adverse GI symptoms or LM, but experienced digestive discomfort following milk ingestion. These adverse digestive symptoms were early-onset bloating, abdominal distension, and flatulence. In this study, there were no clear differences in the type and severity of discomfort experienced in NLDI subjects across all milk challenges.

Previous research has described reduced digestive symptoms with A2 relative to A1 β-casein. In the first acute study, He et al. (22) reported more severe acute symptoms following consumption of conventional milk compared with A2M at 1 and 3 h postingestion. Many symptoms persisted until 12 h (e.g., bloating, abdominal pain), and were greater in lactose malabsorbers, identified through urinary galactose (22). Two longer-term clinical studies similarly reported reduced symptoms with diets including milk containing exclusively A2 β-casein relative to milk containing A1 β-casein, an effect amplified in dairy intolerant (20) or LI subjects (21). Symptoms (i.e.,

TABLE 5 Symptom scores reported ad hoc by food and symptom time diary over 12 h[1]

Symptom[2]	Group	A2M	CON	LF-CON	*P* value[3] Factor	
					Group	Milk
Abdominal pain (1–5)	LI	2 (0, 2)	1.5 (0, 3.8)	0 (0, 1)	0.441	0.135
	NLDI	2 (0, 3)	1 (0, 2)	1 (0, 2)		
	DT	0 (0, 1.8)	0 (0, 1)	0 (0, 0)		
Abdominal fullness (1–5)	LI	0 (0, 2)	2 (0, 3.5)	0 (0, 0.8)	0.157	0.997
	NLDI	2 (1, 3)	1 (1, 3)	2 (0.3, 3)		
	DT	1 (0, 2)	0 (0, 1)	0 (0, 0)		
Abdominal bloating (1–5)	LI	1 (0, 3)	2 (0, 3)	0 (0, 1.8)	0.340	0.162
	NLDI	2 (1, 3)	1 (0, 2)	2 (0, 2.8)		
	DT	1 (0, 2)	0.5 (0, 1)	0 (0, 0)		
Abdominal distension (1–5)	LI	0 (0, 1.3)	1.5 (0, 3)	0 (0, 1.3)	0.435	0.223
	NLDI	1 (0, 2.5)	0 (0, 1)	0 (0, 1)		
	DT	0 (0, 1)	0 (0, 1.8)	0 (0, 0)		
BM 12-h score (1–7)	LI	5 (5, 5)	4.5 (3, 6)	4.5 (3.8, 5)	0.829	0.972
	NLDI	3.3 (3, 4.3)	5 (5, 6)	5 (3.8, 6)		
	DT	6 (5, 6)	3 (3, 5)	4.8 (3.4, 6)		
BM strain (1–5)	LI[a]	1 (1, 2.8)	1.5 (1, 2.8)	1.5 (1, 2.3)	0.008	0.474
	NLDI[a]	2 (1.8, 2)	2 (1, 2)	2 (1, 2)		
	DT[b]	1 (1, 1)	1 (1, 1)	1 (1, 1.3)		
Abdominal pain before BM (0–5)	LI[a]	1 (0, 2.8)	3 (2, 4)	3 (2.3, 3.5)	0.001	0.904
	NLDI[a,b]	0.5 (0, 2.3)	2 (1, 2)	2 (0, 2.3)		
	DT[b]	0.5 (0, 1.3)	0 (0, 1)	0 (0, 0.3)		
BM urgency (1–5)	LI	2 (1, 2)	2 (1, 2.8)	2 (1.8, 2)	0.413	0.069
	NLDI	2 (2, 3)	2 (2, 2)	2 (1, 2)		
	DT	2 (1.8, 2.6)	2 (2, 2)	2.5 (1.8, 3.3)		
Pain relief following BM (1–3)	LI	2.5 (1, 3)	1 (1, 2)	2 (1.8, 2.3)	0.171	0.937
	NLDI	2.5 (1.8, 3)	1 (1, 3)	2 (1, 3)		
	DT	2 (1, 3)	3 (1, 3)	3 (2.5, 3)		
Pain worsening following BM (1–3)	LI[a]	2 (2, 2.8)	2 (2, 2)	2 (1.8, 2.3)	0.013	0.302
	NLDI[a,b]	2.5 (1.8, 3)	2 (2, 2.3)	2 (2, 3)		
	DT[a]	2.5 (2, 3)	3 (2, 3)	3 (2.8, 3)		

[1] Values presented as median (IQR) over 12 h. DT: $n = 10$; LI: $n = 10$; NLDI: $n = 20$. A2M, milk containing exclusively A2 β-casein; BM, bowel movement; BSS, Bristol Stool Scale; CON, conventional milk; DT, dairy tolerant; LF-CON, lactose-free conventional milk; LI, lactose intolerant; NLDI, nonlactose dairy intolerant.

[2] Values scored using ordinal scales (low to high). Five-point Likert scale (for abdominal pain, fullness, bloating, and distension) from 1 to 5: "not bad at all," "a little bad," "somewhat bad," "quite bad," and "very bad"; 7-point Likert scale (BSS) from 1 to 7 signifying "hard to pass" to "entirely liquid"; 5-point Likert scale (BM strain) from 1 to 5: "not at all," "slightly strain," "moderately strain," "significantly strain," "unable to empty bowel"; 5-point Likert scale (abdominal pain) from 0 to 5: "no abdominal pain," "not bad at all," "a little bad," "somewhat bad," "quite bad," and "very bad"; 5-point Likert scale (BM urgency) from 1 to 5: "not at all, "a little urgency," "I have to hurry," "I have to go immediately," "I am incontinent"; 3-point Likert scale from 1 to 3: "yes" (1), "no" (2), "not applicable" (3).

[3] Scores were compared by Kruskal–Wallis test. *P* value relates to the symptom interaction value (group x milk). For differences between the LI, NLDI, and DT groups, groups without a common letter differ significantly, $P < 0.05$.

bloating, abdominal pain, flatulence) tended to be more severe following habitual consumption of milk containing A1 β-casein relative to exclusively A2 β-casein, but these findings were either nonsignificant (20), or only significant when assessing the distribution of scores or accounting for sequence allocation (21). Although all 3 studies suggested that symptoms from conventional milk containing A1 β-casein were worse in self-described milk intolerance (20) or lactose intolerance (21, 22), LM was detected using nonstandardized methods, and the heterogeneous and uncharacterized groups of dairy intolerant subjects limited direct comparisons (21, 22). Therefore, we have demonstrated that in LI individuals diagnosed by standardized methods, A2M reduced nausea and fecal urgency on a par with LF-CON. These differences were reported for 3 h and did not persist to 12 h. Our data demonstrate that A2M reduces some acute symptoms of lactose intolerance, even in the presence of lactose. However, it must be noted that acute exposure to LF-CON was well tolerated in LM individuals.

In the current study, digestive symptom improvements in LI individuals were accompanied by reduced breath hydrogen production following A2M consumption, relative to CON milk. Slower GI transit (36, 37), particularly slowed lactose transit (10, 11), is known to reduce LM (38) and symptom severity (36). Yet, evidence for an A2 β-casein–dependent action to delay small intestinal transit is absent. Previously, Jianqin et al. (21) reported longer whole gastrointestinal and colonic transit times with CON milk, consistent with rodent analysis (18). These findings align with harder stool consistency reported with CON milk (20, 21), consistent with longer transit times (32, 39). The current study reports no such differences in stool consistency between

CON and A2M; however, there was limited statistical power in the number of BM events experienced. These previous studies showed that A2M accelerated GI transit, speculated to be due to the absence of BCM7 production (18, 20, 21). However, given that BCM7 was not measured in either the gastrointestinal digesta or in circulation, the possible relation between BCM7 and breath hydrogen production remains undetermined. Further studies to elucidate the mechanisms of A2M reduction in symptoms and LM should also consider additional possible compositional or physicochemical property differences between milks.

Although A2M might offer some advantages for LI subjects, our data conversely show that A2M caused increased feelings of distension and fullness in DT and NLDI subjects, whereas DT subjects had more diarrhea than expected (over 12 h) and NLDI subjects more flatulence after A2M compared with CON milk. Although these DT subjects also showed some delayed malabsorption, LM incompletely explains this, given no greater diarrhea incidence with CON or LF-CON milks. Along with similar feelings of fullness in DT and NLDI subjects, and with the flatulence seen in NLDI subjects, these findings suggest possible interactions between A1 β-casein and lactose in those with mild or absent malabsorption, occurring through as yet unexplained mechanisms. Hence, A2M might offer digestive advantages or disadvantages depending on the type of dairy intolerance experienced, an important consideration in the context of research or recommendations.

Whereas lactose intolerance is well characterized, allowing for the development of screening questionnaires based on specific symptoms (27), the symptoms of potential sensitivity to β-casein have been rarely reported. Previous literature has indicated that not all milk intolerance is attributable to lactose (5, 12–15). This study aimed to characterize the symptoms of non-lactose-mediated digestive discomfort assessed in a group of subjects with self-reported perceived symptoms following dairy consumption, but without LM or discomfort following a standardized lactose hydrogen breath test. Although tolerant to lactose, these NLDI individuals experienced digestive discomfort with ingestion, irrespective of the presence or absence of either A1 β-casein or lactose. For NLDI individuals subjective symptoms were characterized by early-onset distension, bloating, and flatulence, which largely resolved within 2 h following milk ingestion.

This study did not identify the trigger for milk-induced discomfort in the NLDI individuals. Without the use of either isolated milk protein fractions or a nonmilk placebo, it remains uncertain if the primary determinant is bovine milk as a whole, or a particular constituent of it. Further, the mechanisms triggering symptoms in NLDI remain unclear. The early onset, rapid resolution, and range of symptoms (i.e., belching, bloating, distension, flatulence) are consistent with altered gastrointestinal transit (36). These NLDI subjects could share perceptual differences to intolerances (24, 25) or discomfort in line with other functional gastrointestinal disorders. It has been reported that IBS is associated with visceral hypersensitivity (40), and greater feelings of bloating and fullness (41), particularly following meal consumption (42–44). Indeed, perceived pain from milk was highest in NLDI subjects, and 75% met the Rome III criteria for IBS, although only 35% self-identified as such. Because neither lactose nor A1 β-casein were confirmed as the cause of digestive symptoms in NLDI subjects, additional work is required to determine the causative trigger and biological mechanism of this intolerance, and overlap with other etiologies.

Although lactose malabsorbers were separated from absorbers, the 3-h screening time might have missed instances of LM. Indeed, the DT group reported some later instances of diarrhea, particularly after A2M, suggesting possible delayed malabsorption. Genotyping indicated 40% of DT and only 5% of NLDI subjects lacked the SNPs associated with lactase persistence. Although the measured SNPs could be biased toward Caucasian populations (1), this mismatch between genetic lactase nonpersistence and LM/symptomology with lactose or milk, as previously observed (25), highlights the multifaceted etiology and pathophysiology of lactose (and/or dairy) intolerance, supporting the influence of additional factors such as diet (7) and the microbiota (8). Further, the exclusively Caucasian demographic of the NLDI subjects likely reflects an inherently lactase-persistent group, one that could have distinct intolerance mechanisms, and also demonstrates the current inability to tease apart a nonlactate dairy intolerance that can coincide with LM, if these etiologies are distinct. Additionally, these later-onset symptoms, although monitored by the FAST diary, were not adequately captured for statistical comparisons and the data could be confounded by subsequent meals.

In summary, we have demonstrated that A2M reduces LM and some acute symptoms of intolerance in clinically confirmed LI individuals. Furthermore, individuals without LM but with self-described dairy intolerance (NLDI subjects) did not experience an acute alleviation of dairy symptoms with A2M, but had increased flatulence, whereas DT individuals showed increased incidence of diarrhea in response to A2M. We propose that NLDI individuals exhibit other forms of dairy intolerance, distinct from LM, which we have termed nonlactose dairy intolerance. This is characterized by early-onset feelings of distension, bloating, and flatulence following milk ingestion. Further work is required to ascertain the underlying mechanisms of both this nonlactose dairy intolerance, and of the acute reduction of LM and intolerance symptoms in LI individuals in response to A2M.

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