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ORIGINAL ARTICLE

Comparative effects of A1 versus A2 beta-casein on gastrointestinal measures: a blinded randomised cross-over pilot study

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BACKGROUND/OBJECTIVES: At present, there is debate about the gastrointestinal effects of A1-type beta-casein protein in cows' milk compared with the progenitor A2 type. *In vitro* and animal studies suggest that digestion of A1 but not A2 beta-casein affects gastrointestinal motility and inflammation through the release of beta-casomorphin-7. We aimed to evaluate differences in gastrointestinal effects in a human adult population between milk containing A1 versus A2 beta-casein.
SUBJECTS/METHODS: Forty-one females and males were recruited into this double-blinded, randomised 8-week cross-over study. Participants underwent a 2-week dairy washout (rice milk replaced dairy), followed by 2 weeks of milk (750 ml/day) that contained beta-casein of either A1 or A2 type before undergoing a second washout followed by a final 2 weeks of the alternative A1 or A2 type milk.
RESULTS: The A1 beta-casein milk led to significantly higher stool consistency values (Bristol Stool Scale) compared with the A2 beta-casein milk. There was also a significant positive association between abdominal pain and stool consistency on the A1 diet ($r = 0.520$, $P = 0.001$), but not the A2 diet ($r = -0.13$, $P = 0.43$). The difference between these two correlations (0.52 versus −0.13) was highly significant ($P < 0.001$). Furthermore, some individuals may be susceptible to A1 beta-casein, as evidenced by higher faecal calprotectin values and associated intolerance measures.
CONCLUSIONS: These preliminary results suggest differences in gastrointestinal responses in some adult humans consuming milk containing beta-casein of either the A1 or the A2 beta-casein type, but require confirmation in a larger study of participants with perceived intolerance to ordinary A1 beta-casein-containing milk.

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INTRODUCTION

Cows' milk contains ~32 g of protein per litre, of which ~80% is casein protein and ~20% is whey.[1] Beta-casein is the second most abundant casein type in cows' milk and comprises ~30% of total milk protein.[2] There are two families of beta-casein proteins, known as A1 and A2 beta-casein 'types'.[3] The A1 type variant arose in European herds from the original A2 type ~5000–10 000 years ago from a Proline[67] to Histidine[67] point mutation.[3] In countries that have dairy cows of northern European ancestry, the relative proportions of the co-dominant A1 to A2 beta-casein alleles are typically 1:1 in cows, which then produce the same ratio of A1 to A2 beta-casein in milk. This tends to be lower in breeds from Southern Europe. However, this ratio depends on the specific breeding history of the dominant breeds.[4] Once milk or milk products are consumed, the action of digestive enzymes in the gut on A1 beta-casein releases the bioactive opioid peptide beta-casomorphin-7 (BCM-7);[4–8] in contrast, A2 beta-casein releases much less and probably minimal amounts of BCM-7 under normal gut conditions.[7–10] BCM-7 is a mu-opioid receptor ligand,[8,11] and mu-opioid receptors are expressed widely throughout human physiology, including the gastrointestinal tract.[12]

Two animal studies have investigated the effects of A1 versus A2 beta-casein on gastrointestinal effects directly.[13,14] Barnett *et al.*[14] showed that feeding rodents milk containing A1 beta-casein resulted in significantly delayed gastrointestinal transit time compared with milk containing A2 beta-casein.[14] This delay could be eliminated by administration of the opioid blocker naloxone, which suggests that the gastrointestinal transit delay with A1 feeding is an opioid-mediated effect. They also demonstrated a significant 40% upregulation of dipeptidyl peptidase-4 in the jejunum of A1- relative to A2-fed rodents.[14] Dipeptidyl peptidase-4 not only breaks down BCM-7 quickly[15] but it also degrades the gut incretin hormones rapidly;[16] in humans, the incretin hormones modulate insulin and glucose metabolism,[17] gastric emptying[18] and antroduodenal motility.[19,20] Interestingly, Barnett *et al.*[14] also showed that A1 feeding relative to A2 feeding significantly increased the colonic activity of the inflammatory marker myeloperoxidase by ~65%, an effect also negated by the opioid blocker naloxone. Similarly, Haq *et al.*[13] showed in mice fed a milk-free basal diet supplemented with A1 relative to A2 beta-casein that MPO levels were increased significantly by 204%, whereas A2 beta-casein had no effect relative to controls.[13] Further, they showed significant increases in intestinal interleukin-4, immunoglobulin E and leukocyte infiltration with A1 compared with A2 feeding.[13] Intestinal inflammation disturbs colonic microbiota composition and enhances pathogen growth, which can affect stool composition and output.[21]

BCM-7 has also been reported to alter human intestinal lymphocyte proliferation.[22,23] *In vitro*, BCM-7's effects on human colon goblet-like cells (HT29-MTX cells) include increasing mRNA

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concentration of the mucin MUC5AC, depending on mu-opioid receptor activation.[24] BCM-7 also induces rapid secretion of intestinal mucus through the activation of the enteric nervous system and opioid receptors.[25] More recently, bovine BCM-7 has been detected in the jejunal effluents in humans fed 30 g of casein in amounts compatible with a biological action,[5] which confirms the identification ~30 years earlier of immunoreactive BCM-7 materials in the aspirated small intestinal contents of healthy male adults following milk intake.[26] Bovine immunoreactive BCM-7 has also been detected in the blood of human infants fed cows' milk-based infant formula;[27,28] Kost *et al.*[27] showed with chromatographic characterisation that a material with the same molecular mass and polarity as BCM-7 was contained in the immunoreactive BCM-7 of those infants who were fed formula.[27]

As A1 beta-casein can result in the production of the opioid BCM-7[6–9] and because Barnett *et al.*[14] have shown opioid-related gastrointestinal effects with A1 but not with A2 beta-casein feeding (by comparing saline to naloxone), a physiologically plausible mechanism exists by which milk containing A1 beta-casein may be responsible for a range of gastrointestinal effects described above. However, no studies have assessed whether A1 relative to A2 beta-casein-containing milk imparts different gastrointestinal effects in human adults. The aim of this study was to compare the gastrointestinal effects of dietary A1 versus A2 beta-casein-containing milk in adults using subjective and objective measures of gastrointestinal performance.

MATERIALS AND METHODS

Study design and participants

This 8-week cross-over study saw 12 men and 29 women (19–68 years) from Perth, Western Australia, randomised to one of two groups for 2 weeks, following a 2-week dairy washout in which rice milk substituted dairy milk: (1) milk containing beta-casein of A1 type ($n = 21$); or (2) milk containing beta-casein of A2 type ($n = 20$) (Figure 1). Participants underwent a second 2-week dairy washout before crossing to the alternative milk intervention for another 2 weeks. Of the randomised participants at study entry, a subgroup ($n = 10$) had self-reported intolerance to commercial milk, containing a mix of A1 and A2 beta-casein. Exclusion criteria were as follows: (1) milk allergy; (2) diagnosed lactose intolerance; (3) pregnancy/ lactation; (4) cardiovascular events in the last 6 months; (5) opioid consumption; (6) antibiotic treatment in the previous 8 weeks; and (7) immunosuppressive medication or anti-inflammatory drugs in the 4 weeks before screening. Study recruitment and intervention was conducted from November 2011 to October 2012. Participants were randomised in the order of recruitment using a simple sequence generated from www.randomization.com by the researcher (SH). This study was approved by the Curtin University Human Research Ethics

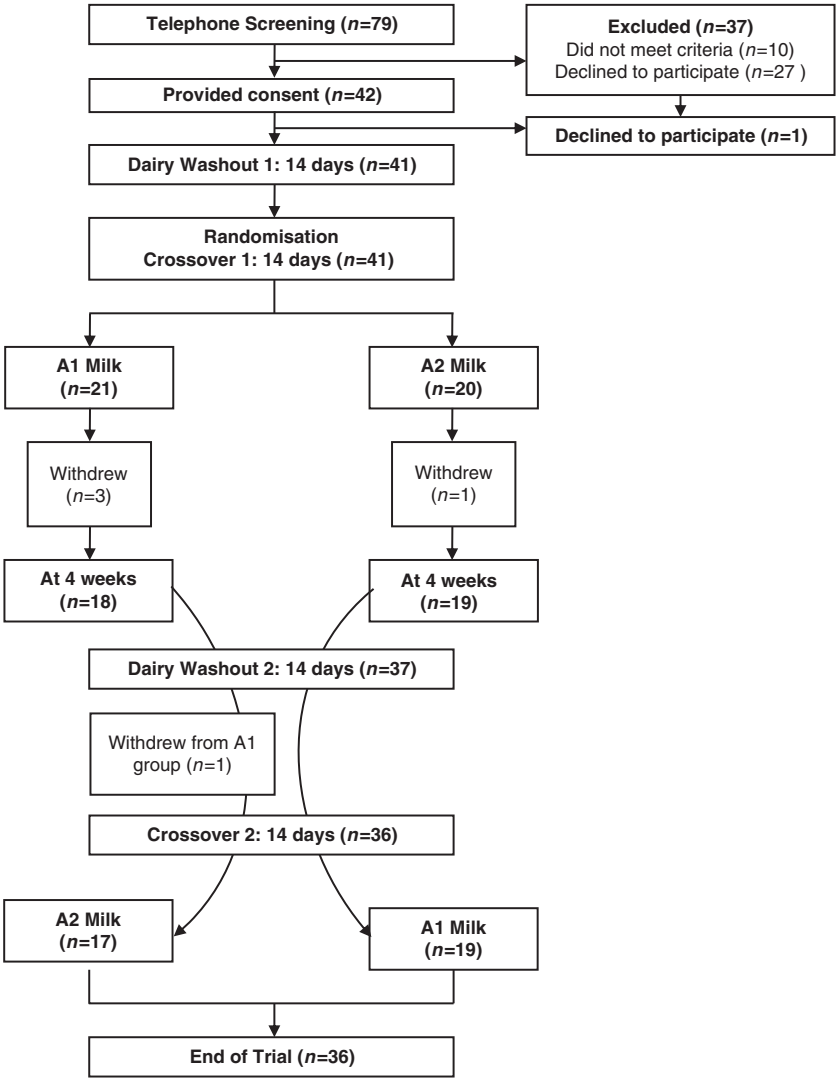

Figure 1. Participant flowchart.

Committee (HR 102/2011) and written informed consent was obtained from all participants.

Interventions

Washout rice milk. Participants replaced all dairy milk with supplied rice milk (So Natural Rice Milk, Freedom Foods, Taren Point, NSW, Australia) for both 2-week washouts and were instructed to avoid all other dairy. A dairy-free alternative list and information relating to hidden dairy sources was provided.

A1 and A2 beta-casein diets. During the 2-week A1 and A2 beta-casein interventions, participants were instructed to consume 750 ml/day of their allocated milk (containing ~7.5 g of *either* A1 or A2 beta-casein) over the day and to avoid all other dairy products. Both milk products were produced in November 2011, at Leppington Pastoral Company, NSW, Australia, by cows genotyped as homozygous for A1 beta-casein (A_1A_1) or A2 beta-casein (A_2A_2) based on genotyping tail hair follicle material, which was performed at Genomnz (AgResearch Invermay Agricultural Centre, Mosgiel, New Zealand). Milk was processed and packed in identical 1-l UHT plain packages (blinding participants and the investigator to each milk intervention) by Pactum Australia Pty Limited, Taren Point, NSW, Australia. The A1 and A2 milk were both standardised to the following nutrition profile per 100 ml: energy 189 kJ, total protein 3.1 g, total fat 2.5 g and lactose 5.2 g; no other known differences existed. Nano-liquid chromatography electrospray ionisation mass spectrometry analysis (Australian Proteome Analysis Facility, Macquarie University, Sydney, NSW, Australia) of the A1 and A2 milk showed that the A1-type beta-casein proportion of total beta-casein was $>99\%$ in the A1 milk and $\leqslant 0.5\%$ in the A2 milk. Participants recorded their daily milk intake on compliance calendars.

Assessments

Participants attended four clinical visits, including baseline visits after both dairy washouts and assessment visits after consuming the A1 and A2 diets.

Anthropometry, diet and physical activity measurements. At all visits, anthropometric measurements were collected in the School of Public Health Research Clinic at Curtin University. Height was measured without shoes to the nearest 0.5 cm using a stadiometer. Weight was measured using a digital scale (Omron, Kyoto, Japan). BMI was calculated as kg/m^2. During the first washout, at the start and during the milk interventions, participants kept a 3-day household measures food diary on two weekdays and one weekend day to monitor dietary intake. Data were analysed with Foodworks Professional 2007, Xyris Software, Kenmore Hills, QLD, Australia based on data from the AUSNUT database. At each visit, participants completed the IPAQ (International Physical Activity Questionnaire)[29] to monitor physical activity.

Gut inflammation. Faecal calprotectin is a non-invasive marker of gastrointestinal inflammation.[30,31] Participants collected faecal samples at home on the morning of each of the two assessment visits using kits provided. Several heterogeneous stool portions were collected from the day's first stool passed onto the provided collection tray. Specimens were stored at Curtin University at $-80\,^{\circ}C$ before being sent to Dorevitch Laboratories (Heidelberg, Victoria, Australia) for assessment. Faecal calprotectin was measured by a single-step enzyme-linked immunosorbent asssay using antibodies against six epitopes found on the calprotectin molecule.

Gastrointestinal symptom recording. Participants recorded symptoms of bloating, abdominal pain, flatus and difficulty in voiding as they occurred in a Symptom Report Diary according to a severity scale (0 = none; 1 = mild; 2 = moderate; 3 = severe) on all days during both interventions and during dairy washouts. The validated Bristol Stool Scale (BSS) participant-recording system[32] was used to assess bowel frequency (number of bowel motions/day) and stool consistency (1 = separate hard lumps like nuts; 2 = sausage-shaped but lumpy; 3 = like a sausage or snake but with cracks on its surface; 4 = like a sausage or snake, smooth and soft; 5 = soft blobs with clear-cut edges; 6 = fluffy pieces with ragged edges, a mushy stool; 7 = watery, no solid pieces).

Sample size. There are no data available on the effects of A1 relative to A2 beta-casein-containing milk on gastrointestinal symptoms in humans, and as such this study must be considered a pilot study so that powering of future studies can be performed.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics Version 20 (IBM Corp., Chicago, IL, USA). Output data were first tested for normality (Kolmogorov–Smirnov test), and depending on outcomes they were analysed using either parametric paired *t*-tests (physical activity and mean 2-week Bristol Stool analyses) or non-parametric Wilcoxon signed-rank test (faecal calprotectin, bloating, abdominal pain, flatus and voiding difficulty). Parametric analyses are presented as means ± s.e.m., whereas non-parametric analyses are presented as means for descriptive purposes together with non-parametric statistics as appropriate to the specific comparison. Linear associations between measures are reported as Pearson's *r*.

RESULTS

Baseline characteristics

Baseline data following the first washout are presented as means ± s.d. and range (Table 1). There were no between-treatment group differences before the study commencement or at the start of intervention 1.

Study attrition, adherence and changes in milk, calcium, energy and fibre intake

Four (9.8%) participants withdrew from the study (one from the A2 and three from the A1 diets) and one failed to provide a symptom diary (Figure 1). Two withdrawals were from the self-identified milk-intolerant subgroup. Mean compliance with the A1 and A2 diets was 96.2% (±5.3) and 96.4% (±6.6), respectively. Greater than 100% compliance stems from some participants consuming extra study milk in tea/coffee/food. There were no significant between-group differences for milk, energy, fibre or calcium intakes during the intervention.

Stool consistency and bowel frequency

Stool consistency was assessed using the BSS (Table 2). BSS was analysed as 2-week mean values for each participant on the A1 and A2 diets. Stool consistency values on the BSS were significantly higher on the A1 diet compared with the A2 diet when all participants were assessed, and this result was retained when self-identified milk tolerants were considered alone (Table 2). This result was stronger (both size effect and significance) for women alone (Table 2). There were no significant treatment order effects (data not shown). There were no significant differences between the A1 and A2 diets for bowel frequency, although a notable feature was considerable within-group variation, ranging from 0.43 to 3.6 under A1 and from 0.36 to 4.5 under A2 (data not shown).

Subjective measures of intolerance symptoms

Bloating, abdominal pain, flatus and voiding difficulty, as reported by all participants, were analysed as measures of digestive discomfort. Although all mean values were numerically higher on the A1 diet, none were statistically significant. For those who self-identified as milk intolerant ($n = 8$), the mean A1 values were considerably higher than A2 values for bloating (61% higher), abdominal pain (38% higher) and voiding difficulty (83% higher). However, given the small participant numbers in the self-identified milk-intolerant group, it was not possible to demonstrate statistically significant differences. In relation to these subjective measures, there was evidence of an order of treatment effect. For cases where the A1 diet was consumed first, bloating and flatus were both significantly higher on the A1 than on the A2 diet ($P = 0.05$ and 0.048, respectively). For participants who

consumed the A1 diet second, there were no significant differences between the diets in any of these measures.

Cross-correlations by treatment

There were strong cross-correlations between the four subjective intolerance measures on both diets (Table 3). The flatus with bloating correlation on the A1 diet was significantly higher than the correlation on the A2 diet ($r = 0.63$ versus $r = 0.25$, $P = 0.02$).

There was also a significant positive association between abdominal pain and stool consistency on the A1 diet ($r = 0.520$, $P = 0.001$), providing evidence that greater pain on the A1 diet is associated with softer stool. In contrast, there was no relationship between these two measures on the A2 diet ($r = -0.13$, $P = 0.43$). The difference between these two correlations (0.52 versus −0.13) was highly significant ($P < 0.001$).

Faecal calprotectin

There were no overall differences in faecal calprotectin (FC) between the A1 and A2 diets (mean values of 41.6 versus 35.8 μg/g and median values of 15 versus 14 μg/g). Most cases fell within the normal cutoff (< 50 μg/g). However, eight cases stood out from the others (Table 4). Five of these standout cases had FC values of $\geqslant 50$ μg/g for both the A1 and A2 diets, and all of these had the A1 diet first. Another three standout cases had FC values > 100 μg/g on the A1 diet, but < 50 μg/g on A2 (Table 4). The five cases with high FC values on both diets also had a general tendency to have high values for the four subjective intolerance measures relative to median values on both diets (Table 4). Interestingly, those with high FC values on the A1 diet but not on the A2 diet tended to have high subjective intolerance measures for the A1 diet but not the A2 diet.

There were strong and statistically significant correlations between FC and subjective intolerance measures when participants were on the A1 diet (Table 5). There was also a particularly strong association with a composite index comprising these four measures summed. When participants were on the A2 diet, these relationships were absent in relation to bloating and abdominal pain and considerably weaker on the composite measure, but still present in relation to flatus and voiding difficulty. The difference in the correlation measures between the A1 and A2 diets was significant for abdominal pain (0.46 vs 0.03; $P = 0.02$) and bloating (0.36 vs −0.02; $P = 0.05$).

DISCUSSION

In this study, the BSS measure of stool consistency was significantly higher on the A1 versus A2 beta-casein diet, and this finding was retained when self-identified milk intolerants were excluded. The appropriate interpretation to be placed on these BSS results requires careful consideration.

It has been shown that extremes in stool formation may reflect gastrointestinal transit time,[33,34] where softer stools reflect faster transit time. However, Davies *et al.*[35] have shown that BSS may not always reflect the speed of gut transit excursions.[35] Importantly, Barnett *et al.*[14] have shown clearly that A1 beta-casein feeding delays gut transit through an opioid pathway in rats[14] and confirmed earlier rodent study results[13] that A1 compared with A2 beta-casein feeding increases gut inflammation significantly, as evidenced by myeloperoxidase levels. Together, these studies are suggestive of the fact that the significantly higher BSS values

Table 1. Baseline characteristics of all participants (mean ± s.d. (range))

Characteristic	*All*	*Self-described as milk tolerant (n = 27) or not described (n = 1)*	*Self-described as milk intolerant (n = 8)*
Starting anthropometric characteristics			
Age (years)	45.5 ± 15.7 (19–68)	44.1 ± 15 (21–68)	50.2 ± 18.1 (19–66)
Height (cm)	165.8 ± 7 (149–182)	166.4 ± (149–182)	163.6 ± 6.7 (154–175.5)
Weight (kg)	69.2 ± 14.8 (47.2–110.8)	68.2 ± 14.4 (47.2–110.8)	72.6 ± 16.8 (51.2–98.7)
BMI (kg/m^2)	25.2 ± 5.2 (16.5–43)	24.7 ± 5.3 (16.5–43)	26.9 ± 5 (20.6–36.5)
Systolic BP (mm Hg)	117.3 ± 16.9 (82–157)	117.6 ± 15.5 (93–152)	116.2 ± 22.4 (82–157)
Diastolic BP (mm Hg)	73.5 ± 9.6 (53–95)	74.2 ± 8.8 (63–95)	71.1 ± 12.5 (53–93)
Usual dietary characteristics			
Energy (kJ/day)	7982 ± 2482 (2901–13 842)	8278 ± 2425 (2901–13842)	7058 ± 2757 (3460–12 564)
Fibre (g/day)	24 ± 11 (7–55)	24 ± 9 (7–49)	26 ± 16 (9–55)
Milk (ml/day)	144 ± 176 (0–633)	170 ± 190 (0–633)	57 ± 78 (0–185)[a]
Calcium (mg/day)	842 ± 401 (218–1639)	853 ± 380 (218–1474)	808 ± 492 (347–1639)
Usual physical activity			
Physical activity (Met-min/week)	2330 ± 3025 (0–13608)	2100 ± 2461 (0–11 304)	3137 ± 4631 (132–13 608)

Abbreviations: BMI, body mass index; BP, blood pressure. Anthropometric characteristics $n = 36$ (11 male, 25 female); dietary characteristics $n = 35$ (11 male, 24 female). [a]$P = 0.019$ between tolerant and intolerant.

Table 2. Bristol Stool Scale analyses of stool consistency (mean ± s.e.m.)

Group	*A1*	*A2*	*Difference A1 − A2*	P-*value for paired* t-*test*
All participants ($n = 36$)	3.87 (0.11)	3.56 (0.15)	0.31 (0.14)	0.04
Women only ($n = 25$)	3.93 (0.15)	3.50 (0.16)	0.43 (0.16)	0.01
Men only ($n = 11$)	3.72 (0.15)	3.70 (0.31)	0.02 (0.28)	0.95
Self-described as milk tolerant ($n = 28$)	3.82 (0.12)	3.47 (0.16)	0.35 (0.17)	0.04
Self-described as milk intolerant ($n = 8$)	4.02 (0.28)	3.87 (0.34)	0.16 (0.29)	0.63

on A1 compared with A2 beta-casein diets are caused by proinflammatory factors. This is reinforced by prior evidence that intestinal inflammation is associated with malabsorption of fluids, nutrients and electrolytes.[36,37] This explanation is also consistent with the significant and positive association between abdominal pain and stool consistency on the A1 diet.

Cows' milk is cited commonly as a cause of symptoms such as bloating, abdominal distension, flatulence and disturbed voiding (that is, digestive discomfort), and in the majority of cases lactose may not be the mediator.[38–40] Given prior evidence that A1 beta-casein feeding can delay intestinal transit,[14] an alternative explanation is that A1 beta-casein could create greater opportunities for food fermentation and hence digestive discomfort within the gastrointestinal system. Although the differences in digestive discomfort measures between the two diets were not statistically significant for this predominantly milk-drinking cohort of people, the effect sizes suggest that this may be possible. However, a much larger study of susceptible people is needed to either confirm or refute this hypothesis.

Table 3. Correlations (Pearson's *r*) for subjective measures of intolerance on the A1 and A2 diets

Characteristic	*Bloating*	*Abdominal pain*	*Flatus*
A1 diet			
Bloating	1.00		
Abdominal pain	0.61***	1.00	
Flatus	0.63***	0.44**	1.00
Voiding difficulty	0.51**	0.15	0.27‡
A2 diet			
Bloating	1.00		
Abdominal pain	0.61***	1.00	
Flatus	0.25	0.14	1.00
Voiding difficulty	0.39*	0.24	0.29†

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; †$P = 0.08$; ‡$P = 0.11$.

The current pilot study shows three cases with abnormally high FC values following 14 days of exposure to the A1 but not A2 beta-casein diet. These case study FC results are consistent with prior research regarding the pro-inflammatory characteristics of A1 beta-casein.[13,14] However, in themselves, these cases are insufficient to provide any conclusion. As with intolerance symptoms, the present study protocol could have mitigated against high FC rates as a consequence of susceptible people being either unwilling to enrol or predisposed to study non-completion.

Considering all cases, it is apparent that there is overall evidence for cross-correlation between subjective measures of intolerance. There is also evidence for correlation between FC values and subjective intolerance measures and also between these measures of digestive discomfort and stool consistency. This provides support for a finding that perceived symptoms of digestive discomfort have a physiological basis. It is both notable and intriguing that there is overall suggestion for these relationships being stronger on the A1 diet.

CONCLUSION

Our pilot study demonstrated that consuming the A1 beta-casein milk led to significantly higher BSS stool consistency values compared with the A2 beta-casein milk among a normal milk-drinking population. This finding may be linked to the known digestive release of BCM-7 from milk containing A1 beta-casein. FC values correlated highly with subjective measures of digestive discomfort on the A1 diet but less so on the A2 diet. We also showed for the A1 diet that greater abdominal pain is associated with softer stool. Furthermore, some individuals may be susceptible to A1 beta-casein as evidenced by higher FC values and associated intolerance measures. These intolerance and

Table 4. Faecal calprotectin outlier cases and associated gastrointestinal measures

Characteristic	*Three cases with high FC on A1 but not A2*[a]			*Five cases with high FC on both A1 and A2*[a]					*Median for all cases (n = 36)*
Faecal calprotectin									
A1 diet	427	102	130	103	81	51	131	61	14
A2 diet	32	38	30	367	171	50	129	53	14
Bloating									
A1 diet	1.5	0.0	0.3	0.0	1.0	0.6	0.0	0.6	0.0
A2 diet	0.0	0.0	0.1	0.0	1.0	0.6	0.0	0.0	0.0
Abdominal pain									
A1 diet	1.4	0.0	0.0	0.0	0.8	0.4	0.4	0.0	0.0
A2 diet	0.0	0.0	0.0	0.0	1.1	0.8	0.0	0.0	0.0
Flatus									
A1 diet	2.0	0.2	1.3	1.8	1.5	1.2	1.1	1.9	0.5
A2 diet	2.0	0.0	1.4	1.4	1.5	1.0	1.2	1.3	0.6
Voiding difficulty									
A1 diet	1.0	0.2	1.1	0.1	0.8	1.0	0.0	0.0	0.0
A2 diet	0.0	0.0	0.0	1.3	0.8	1.2	0.0	0.0	0.0
Stool consistency									
A1 diet	5.2	3.7	3.1	3.0	3.5	4.2	3.6	3.7	4.0
A2 diet	4.0	1.4	3.8	3.3	3.1	3.8	3.5	3.8	3.8
Bowel frequency									
A1 diet	2.1	0.9	0.6	0.9	0.9	1.1	2.4	2.0	1.3
A2 diet	1.5	0.7	0.6	1.0	1.3	1.2	2.1	2.0	1.3

Abbreviation: FC, faecal calprotectin. [a]Of the three cases with high FC on A1 but not A2, two cases had the A2 diet first. All other cases had the A1 diet first.

Table 5. Correlations (Pearson's *r*) between faecal calprotectin and measures of intolerance on each of the A1 and A2 beta-casein diets

Characteristic	*FC on A1 diet (n = 36)*	*P-value*	*FC on A2 diet (n = 36)*	*P-value*
Bloating	0.36	0.030	− 0.02	0.930
Abdominal pain	0.46	0.005	0.03	0.880
Flatus	0.39	0.020	0.32	0.060
Voiding difficulty	0.35	0.040	0.56	0.001
Composite index of four subjective intolerance measures[a]	0.50	0.002	0.32	0.060
Stool consistency	0.14	0.420	− 0.11	0.510
Bowel frequency	0.01	0.970	− 0.11	0.510

Abbreviation: FC, faecal calprotectin. [a]The composite index comprises the sum of bloating, abdominal pain, flatus and voiding difficulty.

abnormally high FC results require confirmation with a larger study of participants with perceived intolerance to ordinary A1 beta-casein-containing milk.

CONFLICT OF INTEREST

Dr Sonja Kukuljan is a salaried employee of A2 Dairy Products Australia. Professor Keith Woodford consults to A2 Corporation as an independent scientific adviser. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed to the research design (project conception, development of overall research plan and study oversight). SH and SP conducted the research (hands-on conduct of the experiments and data collection). KW analysed the data and performed statistical analyses. SK, KW, SH and SP wrote the paper. All authors had primary responsibility for the final content.

REFERENCES

1 Davies DT, Law AJR. The content and composition of creamery milks in south-west Scotland. *J Dairy Res* 1980; **47**: 80–90.
2 Walstra P, Jenness R. Proteins. In: *Dairy Chemistry and Physics*. John Wiley & Sons: New York, 1984, pp 98–122.
3 Ng-Kwai-Hang KF, Grosclaude F. Genetic polymorphism of milk proteins. In: Fox PF, McSweeney PLH (eds). *Advanced Dairy Chemistry: Volume 1: Proteins, Parts A & B*. Kluwer Academic/Plenum Publishers: New York, 2002, pp 739–816.
4 Scientific Report of EFSA prepared by a DATEX Working Group on the potential health impact of β-casomorphins and related peptides. *EFSA Sci Rep* 2009; **231**: 1–107.
5 Boutrou R, Gaudichon C, Dupont D, Jardin J, Airinei G, Marsset-Baglieri A *et al.* Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *Am J Clin Nutr* 2013; **97**: 1314–1323.
6 De Noni I, Cattaneo S. Occurrence of beta-casomorphins 5 and 7 in commercial dairy products and in their digests following in vitro simulated gastro-intestinal digestion. *Food Chem* 2010; **119**: 560–566.
7 De Noni I. Release of b-casomorphins 5 and 7 during simulated gastro-intestinal digestion of bovine b-casein variants and milk-based infant formulas. *Food Chem* 2008; **110**: 897–903.
8 Jinsmaa Y, Yoshikawa M. Enzymatic release of neocasomorphin and beta-casomorphin from bovine beta-casein. *Peptides* 1999; **20**: 957–962.
9 Cielinkska A, Kostyra EB, Kostyra H, Olenski K, Fiedorowicz E, Kaminski SA. Milk from cows of different beta-casein genotypes as a source of beta-casomorphin-7. *Int J Food Sci Nutr* 2012; **63**: 426–20.
10 Schmelzer CE, Schops R, Reynell L, Ulbrich-Hofmann R, Neubert RH, Raith K. Peptic digestion of beta-casein. Time course and fate of possible bioactive peptides. *J Chromatogr A* 2007; **1166**: 108–115.
11 Brantl V, Teschemacher H, Blasig J, Henschen A, Lottspeich F. Opioid activities of beta-casomorphins. *Life Sci* 1981; **28**: 1903–1909.
12 Pleuvry BJ. Opioid receptors and their ligands: natural and unnatural. *Br J Anaesth* 1991; **66**: 370–380.
13 Haq MR, Kapila R, Sharma R, Saliganti V, Kapila S. Comparative evaluation of cow β-casein variants (A1/A2) consumption on Th2-mediated inflammatory response in mouse gut. *Eur J Nutr* 2013; **53**: 1039–1049.
14 Barnett MPG, McNabb WC, Roy NC, Woodford K, Clarke AJ. Dietary A1 β-casein affects gastrointestinal transit time, dipeptidyl peptidase-4 activity, and inflammatory status relative to A2 β-casein in Wistar rats. *Int J Food Sci Nutr* 2014; e-pub ahead of print 20 March 2014; doi:10.3109/09637486.2014.898260.
15 Kreil G, Umbach M, Brantl V, Teschemacher H. Studies on the enzymatic degradation of beta-casomorphins. *Life Sci* 1983; **33** (Suppl 1), 137–140.
16 Drucker DJ. The biology of incretin hormones. *Cell Metab* 2006; **3**: 153–165.
17 Holst JJ, Gromada J. Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *Am J Physiol Endocrinol Metab* 2004; **287**: E199–E206.
18 Imeryuz N, Yegen BC, Bozkurt A, Coskun T, Villanueva-Penacarrillo ML, Ulusoy NB. Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. *Am J Physiol* 1997; **273**: G920–G927.
19 Schirra J, Sturm K, Leicht P, Arnold R, Goke B, Katschinski M. Exendin(9–39)amide is an antagonist of glucagon-like peptide-1(7–36)amide in humans. *J Clin Invest* 1998; **101**: 1421–1430.
20 Schirra J, Nicolaus M, Roggel R, Katschinski M, Storr M, Woerle HJ *et al.* Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans. *Gut* 2006; **55**: 243–251.
21 Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC *et al.* Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2007; **2**: 119–129.
22 Elitsur Y, Luk GD. Beta-casomorphin (BCM) and human colonic lamina propria lymphocyte proliferation. *Clin Exp Immunol* 1991; **85**: 493–497.
23 Kayser H, Meisel H. Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *FEBS Lett* 1996; **383**: 18–20.
24 Zoghbi S, Trompette A, Claustre J, El Homsi M, Garzon J, Jourdan G *et al.* beta-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a mu-opioid pathway. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1105–G1113.
25 Trompette A, Claustre J, Caillon F, Jourdan G, Chayvialle JA, Plaisancie P. Milk bioactive peptides and beta-casomorphins induce mucus release in rat jejunum. *J Nutr* 2003; **133**: 3499–3503.
26 Svedberg J, de Haas J, Leimenstoll G, Paul F, Teschemacher H. Demonstration of beta-casomorphin immunoreactive materials in in vitro digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. *Peptides* 1985; **6**: 825–830.
27 Kost NV, Sokolov OY, Kurasova OB, Dmitriev AD, Tarakanova JN, Gabaeva MV *et al.* Beta-casomorphins-7 in infants on different type of feeding and different levels of psychomotor development. *Peptides* 2009; **30**: 1854–1860.
28 Wasilewska J, Sienkiewicz-Szlapka E, Kuzbida E, Jarmolowska B, Kaczmarski M, Kostyra E. The exogenous opioid peptides and DPPIV serum activity in infants with apnoea expressed as apparent life threatening events (ALTE). *Neuropeptides* 2011; **45**: 189–195.
29 Booth M. Assessment of physical activity: an international perspective. *Res Q Exerc Sport* 2000; **71**: S114–S120.
30 Gearry R, Barclay M, Florkowski C, George P, Walmsley T. Faecal calprotectin: the case for a novel non-invasive way of assessing intestinal inflammation. *N Z Med J* 2005; **118**: U1444.
31 Däbritz J, Musci J, Foell D. Diagnostic utility of faecal biomarkers in patients with irritable bowel syndrome. *World J Gastroenterol* 2014; **20**: 363–375.
32 O'Donnell LJ, Virjee J, Heaton KW. Detection of pseudodiarrhoea by simple clinical assessment of intestinal transit rate. *BMJ* 1990; **300**: 439–440.
33 Degen LP, Phillips SF. How well does stool form reflect colonic transit? *Gut* 1996; **39**: 109–113.
34 Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol* 1997; **32**: 920–924.
35 Davies GJ, Crowder M, Reid B, Dickerson JW. Bowel function measurements of individuals with different eating patterns. *Gut* 1986; **27**: 164–169.
36 Mourad FH, Barada KA, Bou Rached NA, Khoury CI, Saade NE, Nassar CF. Inhibitory effect of experimental colitis on fluid absorption in rat jejunum: role of the enteric nervous system, VIP, and nitric oxide. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G262–G268.

37 Sundaram U, Wisel S, Coon S. Neutral Na-amino acid cotransport is differentially regulated by glucocorticoids in the normal and chronically inflamed rabbit small intestine. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G467–G474.
38 Jussila J, Launiala K, Gorbatow O. Lactase deficiency and a lactose-free diet in patients with 'unspecific abdominal complaints'. *Acta Med Scand* 1969; **186**: 217–222.
39 Carroccio A, Montalto G, Cavera G, Notarbatolo A. Lactose intolerance and self-reported milk intolerance: relationship with lactose maldigestion and nutrient intake. Lactase Deficiency Study Group. *J Am Coll Nutr* 1998; **17**: 631–636.
40 Johnson AO, Semenya JG, Buchowski MS, Enwonwu CO, Scrimshaw NS. Correlation of lactose maldigestion, lactose intolerance, and milk intolerance. *Am J Clin Nutr* 1993; **57**: 399–401.