INVESTIGATION OF THE ACUTE DIGESTIVE SYMPTOMS CAUSED BY MILKS WITH DIFFERENT BETA-CASEIN PROTEIN VARIANTS IN DAIRY INTOLERANT PERSONS

by

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To my beloved family and friends for their kindness, patience, and endless support

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ABSTRACT

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Title: Investigation of the Acute Digestive Symptoms Caused by Milks with Different Beta-casein Protein Variants in Dairy Intolerant Persons
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Cows' milk generally contains two types of β-casein, A1 and A2 types. A2 beta-casein is recognized as the original beta-casein variant because it was present before a proline to histidine point mutation occurrence in the polypeptide chain at 67th position. A1 and A2 are processed differently by digestive enzymes, and once milk or milk products are consumed, a seven-amino acid bioactive opioid peptide, beta-casomorphin-7, is released as a result of incomplete digestion of A1-beta-casein. This is a single-dose, randomized and double-blinded study. Participants received four different treatments (Regular milk, A2 milk, Jersey cow milk, and lactose free milk) in a randomized order. The lactose free milk acted as a negative control. This study aimed to evaluate tolerance to milks containing different levels of A2 β-casein (Jersey and A2 milks) as compared to commercial A1 (regularmilk containing both A1 and A2 β-casein) and lactose-free milk controls in lactose digesters and maldigesters. Seven subjects completed this double-blinded, randomized, crossover trial. Lactose malabsorption (LM) was determined by breath hydrogen test and milk intolerance were assessed by validated questionnaires. Treatments were fed as a single dose with a 6-day washout period to minimize any residual effects. Each subject was fed milk containing 0.5 g lactose per kg body weight. The pilot data from the seven subjects does strongly suggest greater hydrogen production from commercial A1 milk as compared to lactose-free, A2 and Jersey milks. Regular milk containing high A1 β-casein produced significantly higher hydrogen compared to lactose-free milk from 2 hours until 5hours. This suggests biologically

relevant differences in lactose digestion among these milks. In addition, Jersey milk produced significantly higher hydrogen compared to lactose-free milk similar to regular milk between 2 and 6 hours while A2 milk was acting similar to lactose-free milk and did not result in increased hydrogen throughout the same time intervals. Taken together, these results suggest that the amount of A2 β-casein in Jersey milk was not adequate to attenuate the increased hydrogen concentration while pure A2 milk was effective. In this pilot clinical trial, abdominal pain, bloating, flatulence, diarrhea, fecal urgency and total GI symptoms were reported as measures of digestive discomfort. Although the mean values of total GI symptom scores were numerically lower on the lactose free, pure A2 and Jersey group compared to regular milk group, none were statistically different. With seven subjects reported in this pilot data, and a calculated sample size requirement of 26, we can interpret trends that ultimately could result in significant differences as additional subjects complete this protocol.

INTRODUCTION

Lactose maldigestion and Lactose intolerance

Lactose Malabsorption is different from Clinical Intolerance and Milk Allergy

Lactose malabsorption or maldigestion (LM) is thought to affect the majority of adult human populations around the world especially people from Asian or African descents (Swallow, 2003) and it is estimated that approximately 65 percent of the human population has a reduced ability to digest lactose after infancy(Matthews et al., 2005). However, only less than 5% are clinically diagnosed as Lactose Intolerant (LI) (Itan et al., 2010) (Suchy et al., 2010).

It is crucial to separate clinical lactose intolerance from hypolactasia, a low level of lactase, which commonly results in malabsorption or maldigestion. Main causes of loss of lactase is the inherited loss after weaning, secondary intestinal damage or hormonal imbalance (Usai-Satta et al., 2012). However, individuals with lactose malabsorption mostly do not develop gastrointestinal symptoms after they ingest milk products. Only people who have digestive symptoms like abdominal pain, flatulence, bloating or diarrhea are regarded and described as lactose intolerant (Vernia et al., 2004). Lactose can be also responsible from systemic symptoms including, tiredness and headaches.

It is also important to realize that a milk allergy is not the same as clinical lactose intolerance. Allergy is an immune response to food particles and is characterized by symptoms resembling lactose intolerance. (Walsh et al., 2016) However, in the case of allergy, even the smallest amount of milk causes a reaction which seems similar to lactose intolerance. Whereas in the case of

intolerance, mostly small amounts are likely be consumed without having significant problems (Heine et al., 2017).

Under normal gut conditions, (lactase persistence), the enzyme lactase-phlorizin breaks down lactose into glucose (Glu) and galactose (Gal) in the brush border of the jejunum and is absorbed into the bloodstream. Since this reaction takes place in the jejunum where the concentrations of bacteria is very low, fermentation of lactose is quite limited (Lomer et al., 2008).

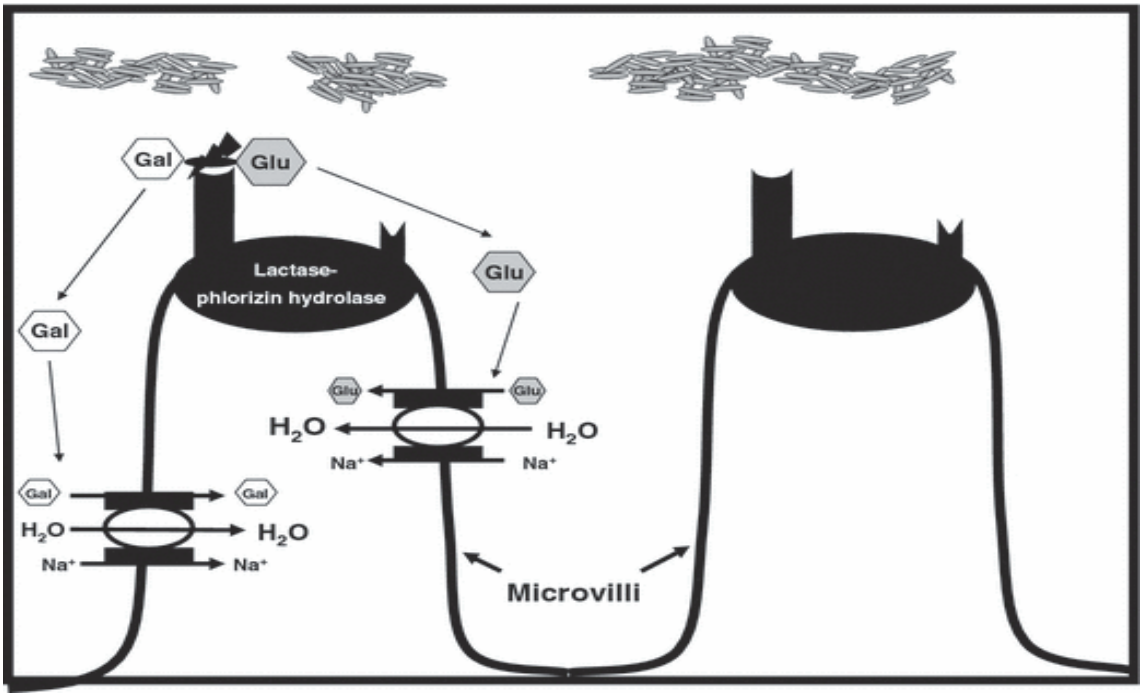

Figure 1 Digestion of lactose into glucose (Glu) and galactose (Gal) in the brush border of the jejunum under normal gut conditions

Diagnosing Lactose Malabsorption (LM)

Lactose malabsorption or maldigestion (LM) can be determined by having study participants ingest a pre-determined amount of lactose and measuring increased levels of metabolic gases like hydrogen and methane which is generated as a result of the fermentation process of undigested lactose in the colon by probiotic bacteria and analyzed with a gas chromatography technique

(Perets et al., 2014). This method is commonly preferred since it is considered as reliable, provides accurate estimation and convenient for both subjects and researchers (Hamilton, 1992) (Furnari et al., 2013). Other diagnostic methods include measuring the lactase enzyme function through intestinal biopsy samples (Mattar et al., 2013). Even though this technique provides a very accurate estimation it is not preferred since it is regarded as too invasive for a relatively mild condition (Di Rienzo et al., 2013). There is also a genetic test available for the common polymorphism that is connected to lactase non-persistence. There is a close correlation between lactase persistence and two single nucleotide polymorphisms (SNP), C/T13910 and G/A22018 obtained from the lactase gene, (Hogenauer et al., 2005) CC/GG being associated with lactase non-persistence and lactose intolerance while homozygous thymine associated with lactose tolerant (Szilagyi et al., 2007). However, the genetics of lactose intolerance is not well documented. Even though there is a clear relationship between SNPs and lactose intolerance, the mechanism of non-persistence is not well established (Ponte et al., 2016). For instance, contrary to the expectations, even though homozygous TT are associated with lactose tolerance, several TT/AA families with lactose intolerance have been reported which suggests that this genetic difference may not explain hypolactasia/lactase persistence completely (Di Stefano et al., 2009). Thus, genetic tests are not commonly preferred and used for diagnosis.

At this point, it is important to remember that diagnosis of lactose malabsorption does not necessarily mean that subjects will have GI symptoms (Misselwitz, 2014). There are many factors affecting whether lactose maldigesters develop GI symptoms; some include the amount of lactose consumed, the residual intestinal lactase activity, whether it is consumed with food, or the ability of the probiotic bacteria to ferment lactose, and individual sensitivity to the products of lactose

fermentation (Levitt et al., 2013). Traditional management often concentrated on eliminating lactose by simply avoiding dairy products or by choosing milk products which do not contain any lactose or contain added lactase enzyme (Hertzler et al., 1996).

How does dairy avoidance affect public health?

Consumption of dairy products has been associated with an overall reduced risk of cardio metabolic diseases and some cancers (Thorning et al., 2016). In addition, many adults and children avoiding milk and other dairy products are also missing the major source of essential nutrients including Ca and Vit D (Rizzoli, 2014). Deficient intakes of calcium and vitamin D are risk factors for decreased bone mineral density. This may increase the risk of fracture throughout the life cycle, especially in postmenopausal women (Kalkwarf et al., 2003). In fact, in a recent comprehensive review, it has been revealed that adult women younger than 50 years old who avoid milk and milk products meet only 44 % of daily calcium and 57 % of potassium and magnesium recommendations (Weaver, 2009). Very low intake of vitamin D can also lead to the development of rickets, especially in children of African descent and other highly pigmented individuals.

It is not easy to meet calcium recommendations by consuming non-dairy options which is available in the form of and dark green vegetables like kale or beans and legumes, (Rozenberg et al., 2016) By analyzing the NHANES data, researcher showed that calcium requirements cannot be met while meeting other key nutrient requirements in a diet does not contain any dairy products within considering the overall current dietary pattern in United States (Gao et al., 2006). As 1 cup of milk yields 100 mg of bioavailable calcium, in order to be achieved same amount of absorbed calcium, one needs to consume 4.5 servings of broccoli, 16 servings of spinach, or 10 servings of dried beans which is neither palatable nor practical (Fulgoni et al., 2011).

Di Stefano et al divided 103 healthy subjects into 2 groups with respect to their ability to digest lactose based on the hydrogen breath samples. They further differentiated these groups as either lactose intolerant or tolerant based on self-reported symptoms and assessed their daily calcium intake by using a 3-day dietary diary method. It has been revealed that lactose intolerant group had significantly lower calcium intake (685 ± 88 mg) as opposed to tolerant group (925 ± 101 mg) ($P < 0.001$). (Di Stefano et al., 2002)

In another study conducted on more than 290 adolescents who identified themselves as milk intolerant were assessed to ingest 212 mg lower calcium per day compared to milk intolerant group.(Matlik et al., 2007) In addition, researchers further investigated the relationship between perceived milk intolerance (PMI) and bone mineral density (BMC) and the results revealed that group with PMI has significantly lower spine and hip BMC than control group ($P = 0.016$). Collectively, self -reported milk intolerance may be regarded as a risk factor for low BMC and can increase the risk of fracture especially in elderly women population. (Lee et al., 2018)

In brief, lactose intolerance dependent milk avoidance may decrease calcium intake because of the individual's avoidance of lactose-containing foods (dairy products) which are a main source of calcium. Results suggest that lactose intolerance and calcium intake are inversely related. Additionally, bone mineral density and bone mass was also inversely associated with lactose intolerance. Till today, it has been thought that that low level of lactase explain the symptoms for clinical lactose intolerance. Still, the relationship is not clear and there is a gray area that is yet to be elucidated.

Is it lactose intolerance?

Majority of subjects who identify themselves as lactose intolerant come up with such claim without completing the required clinical examination. (Brussow H., 2013) In addition, this learned behavior can be easily transferred to younger generations mostly by their parents by elimination of dairy products from daily diet and hence result in fearful individuals towards any milk product with the potential symptom development.

However, after reviewing the literature and searching for the recent evidence, experts who are composed of scientist and physicians with different specialty areas came up with a joint statement in 2010 NIH Consensus Development Conference on Lactose Intolerance & Health. The key message was clear: “For a proportion of adults who report intolerance symptoms following milk intake, lactose intolerance is not the cause, as cases of perceived lactose intolerance are more common than its prevalence in adults” and “Evidence demonstrates that many individuals who self-report lactose intolerance show no evidence of lactose malabsorption. Thus the cause of their GI symptoms is unlikely to be related to lactose.” (Suchy et al., 2010).

In a study conducted by Johnson et al, 95 people with an age range of 12-40 were identified as lactose maldigesters following a hydrogen breath exam and randomized to consume milk having either 25 g or 0 g of lactose. 33% of the subjects gave inconsistent responses and the investigators concluded that the causal relationship between consumption of lactose and GI symptoms cannot be inferred from this research.(Johnson et al., 1993) This is aligned with what have been revealed in a study in which the results showed that overall symptom scores was not different by LM status after ingestion of milk products.(Suarez et al., 1998)

In order to assess whether small doses of lactose result in symptoms, researchers conducted a randomized, crossover, double-blind trial in a lactose maldigester and digester group. After eliminating lactose from their diet for 3 days and after an overnight fast, subjects were randomized into receiving milks containing 0 g, 0.5 g 1.5 g and 7.0 g lactose and their GI symptoms were measured for 12 hrs. (Vesa et al., 1996) Results revealed that most of the maldigesters developed GI symptoms following the ingestion of milk regardless of their lactose content and changing the amount of lactose did not result in any difference in the severity of GI symptoms compared to milk without lactose. More interestingly, subjects developed the highest symptoms with the lactose-free milk. Thus, these findings suggested that the gastrointestinal symptoms in most lactose maldigesters are not induced by lactose and other elements in milk different than lactose may be responsible for the development clinical symptoms. (Vesa et al., 1996) It should be also noted that in previous studies while the subjects were informed not to consume dairy products during the investigation period, there were no control with respect to the fiber or other carbohydrates content of the diet which directly influences the transit time and can result in increased symptoms.(Shaukat et al., 2010) In this study, however, the diet was strictly controlled with respect to its fiber content, hence the symptoms induced by the lactose-free milk were surprising.

In summary, a NIH statement identifies and acknowledges a gap in our current knowledge. After reviewing the available literature, it can be clearly stated that lactose maldigestion is not the same as clinical intolerance but the question still remains regarding the responsible factors in developing GI symptoms (Swallow, 2003).

Together, the results from the previous studies were not consistent and do not explain and suggest a clear and causal relationship between lactose consumption and gastrointestinal symptoms due to variations in study designs, not objective symptom evaluations and different dose of lactose administrations. Other natural components in milk may be responsible for stimulating some of the remaining gastrointestinal intolerance responses and indeed in the recent years, preclinical and some clinical studies concentrated on the effect of certain bioactive peptides released from A1 Beta-Casein and there is recent evidence to support that A1 beta-casein protein from cows' milk may have a role in developing inflammation and causing GI disturbances in animal and some people. (Jianqin et al., 2016)

Is there a Role for dairy protein in causing inflammation and developing GI Symptoms?

A1 and A2 Beta-casein milk protein variants

The milk proteins are divided into two main types; namely, caseins and whey proteins. The casein proteins including α, β, and κ make up approximately 80% of total milk protein, while the remaining 20% consists of whey proteins, mainly β-LG and α-LA (Kunz et al., 1990). Overall, casein proteins contains a high number of proline residues and no disulfide bridges. As a result, they have limited α-helix and β-sheet secondary structure (Adamson et al., 1995). The biochemistry of casein molecules is relatively hydrophobic, making them poorly soluble in water (Lucey, 2002). Caseins are found in milk as a suspension of particles, called casein micelles and are different from surfactant micelles as the interior of a casein micelle is highly hydrated. In addition, the caseins proteins contained in the micelles are held together by calcium cations and hydrophobic interactions (Dalgleish, 1998).

Alpha casein is the most prevalent version of casein molecule present in bovine milk.(Adams et al., 2008) and it has been reported to exhibit antioxidant and radical scavenging properties due to its biochemical structure (Adamson et al., 1997). There is much more interest in β-Casein and its fragments for research purposes since they have been implicated in a number of biological functions (Brantl et al., 1979). β-Caseins are also a source of casomorphin peptides which exhibit opioid activity binding to opioid receptors (Dalgleish, 1998; Henschen et al., 1979).

Beta-casein proteins constitutes approximately 30% of the total protein of cows' milk and can be seen as one of two major genetic sub variants; known as A1 and A2 variants (Kruif, 2003). According to anthropologists, emergence of A1 variant goes back to approximately 2 thousand years ago in certain European breeds like Holstein which Western countries mostly rely on (Kwai-Hang, 2002). A2 beta-casein is recognized as the original beta-casein variant because it was present before a proline to histidine point mutation occurrence in the polypeptide chain at 67^{th} position. (Daniel G Bradley, 1998) As of today, most Asian and African bos indicus and taurus cattle breeds produce only the A2 beta-casein containing milk even though some Asian or African cattle produce some A1 variant as a result of cross-breeding with other cows (D. G. Bradley et al., 1996) (MacHugh et al., 1997). In addition to bovine sources, goat and human breast milk also contains only A2 variants (Daniel G Bradley, 1998) (Grigson, 1991).

β-casomorphin-7 (BCM-7) is a Digestion Product of A1 β-Casein

There are several compounds in food proteins which are known as bioactive peptides (BAP) and one particular peptide sequence in β-casein has received more research attention than other BAP. (Rutherfurd-Markwick et al., 2005) This class of peptides was isolated from β-casein hydrolysates and is referred to as b-casomorphins (Gobbetti et al., 2002).

Due to the way that beta-casein interacts with enzymes found in the digestive system, A1 and A2 are processed differently by digestive enzymes, and once milk or milk products are consumed, a seven-amino acid bioactive opioid peptide, beta-casomorphin-7, (BCM-7) is released as a result of incomplete digestion of A1-beta-casein. (Kwai-Hang, 2002) A1 beta casein contains a histidine residue at position 67, which allows cleavage of the preceding seven amino acid residues to yield the peptide BCM-7. (I. D. Noni, 2008) In contrast, under normal intestinal conditions, BCM-7 is not released as a result of digestion of A2 beta-casein mainly due to the presence of proline amino acid at the polypeptide chain blocking the release. (Ul Haq et al., 2015)

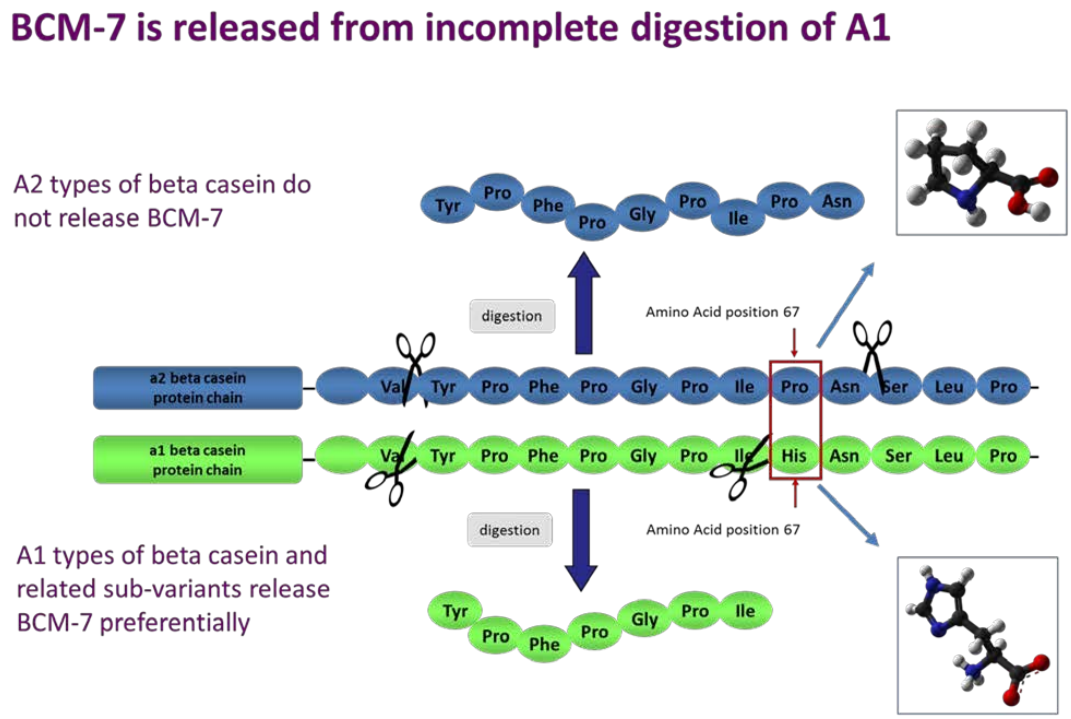

Figure 2 β-casomorphin-7 (BCM-7) is a Digestion Product of A1 β-casein

It has been recently revealed that BCM-7 is also released from other dairy products like yoghurt and cheese (I. D. Noni, 2008) but compared to milk, this release is much less since probiotic

bacteria present in the yoghurt products breaks down BCM-7 into smaller non-bioactive fragments (Nguyen et al., 2014).

In 2013, Boutrou et al provided time line and amount of BCM- 7 generation following the consumption of milk protein. They characterized the kinetics of bioactive peptides derived from human jejunum with mass spectrometry method following the 15N-labeled casein consumption. After casein ingestion BCM-7 peptides which have potential opioid activity, were released in amounts that were compatible with a biological action which peaks at around 30 min and present for four hours following intake of milk.(Boutrou et al., 2013) In addition, BCM-7 has also been found in urine samples of children. (Sokolov et al., 2014) Naturally, the presence of BCM-7 does not say much about its biologic functions and potential physiologic role and effects of BCM-7 still needs to be clarified.

What does β-casomorphin-7 (BCM-7) potentially moderate?

BCM7 increases mucin production in rat and human gut cells

Mucus Gel covers the mucosal surface and it is considered as one of the most potent factor regarding the defense of the organisms and mucins of the mucin (MUC) genes are mainly responsible for physiological proprieties of Gastrointestinal mucus. (Burger-van Paassen et al., 2012)In a recent research study, DHE cells: a mucin-producing rat colon cells were incubated with or without B-CM-7 for 30 min to 24 h. The release of mucinlike glycoprotein under the influence of B-CM-7 was raised after 2 h and reached to a maximum level after 8 h of stimulation different from the control. To establish whether B-CM-7 can also modulate mucins in humans, they extended their study to a HT29 MTX cells: human colon goblet-like cell line. The addition of B-

CM-7 into the incubation medium of HT29 MTX cells significantly elicited an increase in the level of mucin mRNA expression. (Zoghbi et al., 2006)

Hence, based on these results, investigators concluded that since intestinal mucin helps to protect the intestinal wall, it is possible that the mucin response to BCM-7 might be a protective mechanism against inflammatory effects of BCM-7. (Zoghbi et al., 2006)

A1 variant activates the innate immune system

Evidence regarding the inflammatory effects of BCM-7 within gastrointestinal system is well documented.(I. d. Noni et al., 2010) In a recent investigation, 3 variants of bovine casein peptides and their immunological responses were studied in a mouse model. In addition to the expression of inflammatory biomarkes MCP-1 and IL-4, level of total immunoglobulin antibodies and expression of toll-like receptors were also measured. In this particular investigation, mice were fed with either a control diet or a diet including pure A1, pure A2 and mixture of A1 and A2 protein. Results revealed that consumption of b-casein containing A1 variant significantly raised the expression of TLR-4 compared to control and pure A2 group. (Ul Haq et al., 2014) Activation of the transmembrane protein TLR4 induces intracellular signaling cascade reactions by triggering NF-κB and other cytokine generation which collectively result in activation of innate immune response.

Furthermore, ingestion of A1 like variants significantly increased the levels of MPO, IL-4, MCP-1, antibodies IgE, IgG, IgG1 and the number of total leukocytes compared to pure A2 group in intestine. Specifically, the increase in the levels IL-4 which is known to be a Th2 cytokine further

agrees with previous results and points towards an intestinal inflammation mediated by Th2 pathway with the ingestion of beta casein containing A1 variant. (Ul Haq et al., 2014) These results were consistent with the findings of Rungkat-Zakaria et al who revealed earlier that consumption of 100 mg casein per day elevated histamine release significantly in a mouse model. (Rungkat-Zakaria F, 1992)

Dietary A1 β-casein affects gastrointestinal transit time and inflammatory markers

In another study, researchers fed male rats with skim milk-based diets including β-casein of A1 or A2 variant and animals were orally administered with an inert tracer titanium dioxide [TiO_2] to be able to measure the transit time. In addition, they further divided animals into 2 additional groups and one group received a mu opioid receptor blocker, naloxone dissolved in a saline solution. After collecting the urine samples, it has been shown that feeding rats with A1 milk resulted in significantly delayed gastrointestinal transit time compared with A2 variant. More interestingly, this delay is eliminated by injection of the opioid blocker naloxone, which suggests that the GI transit delay with A1 feeding is an opioid-mediated effect (Barnett et al., 2014).

Collectively these results suggest that BCM-7 derived from A1 β-casein is responsible and can modulate mucin production, activate innate immune system by changing the level of inflammatory markers and slower transit time in animal models which has negative effects on lactose digestion and potential to increase GI symptoms.

Does A1 β-casein account for the non-lactase intolerances associated with milk consumption in humans?

Unfortunately, there is not many RCTs investigating the effect of A1 β-casein on GI functions in humans. In a recent study conducted in Chinese adults, after washout period with rice milk for 2 weeks, 45 participants were randomized into 2 sequence, In the first sequence, subjects started consuming conventional bovine milk containing both A1 and A2 β-casein for 2 weeks followed by 2 weeks wash-out period and switched to milk containing only A2 β-casein for the 2 weeks. In sequence 2, this order was reversed (Jianqin et al., 2016). They also divided subjects as being lactose tolerant and intolerant based on the GI symptom score before starting the study. Visual analogue scales (VAS) is a well validated and verified psychometric measuring instrument designed to document the characteristics of disease-related symptom severity in individual patients (Wu et al., 2014).

Results revealed that ingestion of conventional milk containing A1 β-casein significantly increased the severity of gastrointestinal symptoms in both sequences. This effect was observed in both lactose intolerant and lactose tolerant groups. In addition to GI symptoms, A1 β-casein consumption also elevated the levels of inflammatory markers such as IL-4, IgG, IgE and IgG1 compared to milk containing only A2 variant (Jianqin et al., 2016).

To date, it is clear that data from animal and cell culture models, and to a very limited extent human studies, reveal that the A1–derived bioactive peptide BCM-7 has pro-inflammatory effects (Fiedorowicz et al., 2011). However, it is not clear whether the GI symptoms are resulting from directly inflammatory effects of BCM-7 or indirect effects of slower transit modifying different biochemical reactions. The current gastrointestinal evidence is strongly linked to BCM-7 and μ-

opioid signaling pathway, the exact mechanism responsible for GI disturbances in most dairy avoiders remain unknown. Available clinical studies have also certain limitations including limited sample size, lack of consideration of individual body size differences while determining lactose dose. There is certainly a need for more and well-designed clinical studies investigating the effects of A1 variant in a population group with various ages, ethnicities, and different genetic haplotypes.

METHODS

The study was conducted in Accordance with the Declaration of Helsinki and was approved by IRB (Protocol #: 1710019781:). All subjects provided written informed consent prior to inclusion in the study.

Study Objectives

Primary Aim

The primary study objective was to evaluate tolerance to milks containing different levels of A2 β-casein (Jersey and A2 milks) as compared to commercial A1 milk and lactose-free milk controls.

Secondary Aim

To determine if lactose digestion is similar between pure A2, high A2 (Jersey) and high A1 (commercial) β-casein milks as measured by breath hydrogen.

Investigational Agent

Participants were asked to consume four different commercially available milks in random order. The samples were fed for breakfast separated by at least 6 days, after overnight fasts. The commercial milk treatments include; high A1 β-casein milk, high A2 β-casein milk, Jersey cattle milk (which contains a mixture of A1 and A2 β-casein), and lactose free milk as a control. Milk samples contain 2% fat to control for transit. Milk is considered a GRAS (Generally Regarded As Safe) substance, due to its common use in the United States food supply prior to 1958.

Intervention Regimen

Treatments were fed in a randomized order at least 6 days apart to minimize any residual effects. If the subject did not make it to the scheduled appointment, they could reschedule as far as 30 days after the original meeting. If conflicts continued to arise, the subjects were needed to be withdrawn.

Dose Rationale

Each subject was fed milk containing 0.5 g lactose per kg body weight. This dose of lactose is in the physiological range of typical dietary consumption and has historically resulted in some elevation of symptoms. Each subject served as their own control by completing all four treatments. Controlling the amount of lactose consumed allowed us to observe if the variant of protein casein in milk influences the ability for maldigesters to digest and tolerate lactose.

Study Design

Overview or Design Summary

This is a single-dose, randomized and double-blinded study. Participants received four different treatments (Regular milk, A2 milk, Jersey cow milk, and lactose free milk) in a randomized order. The lactose free milk acted as a negative control. The Study Coordinators were blinded to the treatments until the end of the study.

Inclusion Criteria

- Ability/desire to provide informed consent
- 18 to 65 years of age inclusive at screening
- Current or recent history of intolerance to and avoidance of milk of at least one month duration (by self-reported symptoms)

- Agrees to refrain from all other treatments and products used for lactose intolerance (e.g., Lactaid® Dietary Supplements) during study involvement
- Willing to return for all study visits and complete all study related procedures, including fasting before and during the HBT test
- Able to understand and provide written informed consent in English
- Baseline Lactose Challenge Symptom Score

(4 symptom categories with severity measured on from 0 to 5) as defined by one of the following:
At least one score of “moderately severe” or “severe” on a single symptom during the 6 hour HBT test;
A score of “moderate” or greater for a single symptom on at least two (2) time points during the 6 hour HBT test; or
At least one “moderate” score or greater on each of two symptoms during the 6 hour HBT test

- Baseline lactose challenge HBT of at least 20 parts per million greater than baseline at least 2 time points during the 6 hour HBT

Exclusion Criteria

- Milk allergy
- Currently pregnant
- Currently lactating
- Cigarette smoking or other use of tobacco or nicotine containing products within 3 months of screening

- Diagnosed with any of the following disorders known to be associated with abnormal gastrointestinal motility such as; Gastroparesis, amyloidosis, neuromuscular diseases (including Parkinson's disease), collagen vascular diseases, alcoholism, uremia, malnutrition, or untreated hypothyroidism
- History of surgery that alters the normal function of the gastrointestinal tract including, but not limited to: gastrointestinal bypass surgery, bariatric surgery, gastric banding, vagotomy, fundoplication, pyloroplasty [Note: history of uncomplicated abdominal surgeries such as removal of an appendix more than 12 months prior to screening will not be excluded]
- Past or present : Organ transplant, chronic pancreatitis, pancreatic insufficiency, symptomatic biliary disease, Celiac disease, chronic constipation, diverticulosis, inflammatory bowel disease (IBD), ulcerative colitis (UC), Crohn's disease (CD), small intestine bacterial overgrowth syndrome (SIBO), gastroparesis, gastro-esophageal reflux disease (GERD), Irritable Bowel Syndrome (IBS) or any other medical condition with symptoms that could confound collection of adverse events.
- Active ulcers, or history of severe ulcers
- Diabetes mellitus (type 1 and type 2)
- Congestive Heart Failure (CHF)
- Human Immunodeficiency Virus (HIV), Hepatitis B or Hepatitis C
- BMI > 35 kg/m2

- Recent bowel preparation for endoscopic or radiologic investigation within four weeks of screening (e.g., colonoscopy prep)
- Use of concurrent therapy(ies) or other products (e.g., laxatives, stool softeners, Pepto Bismol®, Lactaid® Dietary Supplements) used for symptoms of lactose intolerance within 7 days of screening
- Chronic antacid and/or PPI use (more than twice in the last 3 months)
- Recent use of systemic antibiotics defined as use within 30 days prior to screening
- Recent high colonic enema, defined as use within 30 days prior to screening
- Any concurrent disease or symptoms which may interfere with the assessment of the cardinal symptoms of lactose intolerance (i.e., gas, diarrhea, bloating, cramps, stomach pain)
- History of ethanol abuse in the past 12 months defined as three or more alcoholic beverages per day;
- History of drug abuse within 12 months prior to screening
- Use of any investigational drug or participation in any investigational study within 30 days prior to screening
- Prior enrollment in this study
- Any other conditions/issues noted by the Study Coordinator and/or Principal Investigator that would impact participation and/or protocol compliance.

Recruitment of Subjects and Obtaining Informed Consent

Potential participants were invited to voluntarily participate through advertisements via departmental email, flyers posted on bulletin boards on campus, advertisements posted in the campus and local newspaper (The Exponent, West Lafayette, Indiana; Journal and Courier, Lafayette, Indiana; The Indianapolis Star, Indianapolis, Indiana) the Ismail newsletter (Purdue University, West Lafayette, Indiana) and various Purdue websites (Purdue internal and external websites; appropriate external websites). Abbreviated inclusion/exclusion criteria was included in the advertisements, in an attempt to reach the appropriate audience. Study staff contact information was included, so interested parties can contact study staff either via phone or email. Participants who are interested in the study were given the Informed Consent document to review. This was either be mailed or e-mailed to the potential participant, or given to them in person. The potential participant came to Stone Hall to discuss the study with either the study staff or Principal Investigator, to ensure understanding of study guidelines and requirements. If Informed Consent is granted, the participant was enrolled in the study.

Statistical Analysis

Sample size was determined based on the selection of a 20% decrease in AUC $\Delta H2$ as the minimal difference that would be clinically significant. Power calculations indicate the completion of the protocol with a crossover design by 26 subjects is adequate to demonstrate 80% statistical significance that is consistent with biological relevance using $\alpha = 0.05$ to detect a 20%.

Before parametric analyses were done, Shapiro-Wilk test was used to determine adherence to a normal distribution. Hydrogen concentrations (ppm) and total symptom scores did not require a transformation, but individual symptom scores were transformed by using log transformation. Data

were analyzed using SPSS ® version 24.0 for Windows. Descriptive Statistics has been conducted and mean and standard error values of the dataset for hydrogen concentration and sum of symptoms have been calculated.

Differences in AUC ΔH2 concentrations (primary outcomes) among milk phases were examined by repeated-measures analysis of variance (ANOVA). Repeated-measures ANOVA was also used to test for differences within each of the symptom categories (secondary outcomes) after transforming to correct for nonstationary variance. For both the H2 concentrations and symptom levels, to be able to detect differences between every single treatment, pairwise differences were examined using least significant difference (LSD). In each analysis, only those study participants with complete data were included in the statistical testing. All statistical tests were 2-tailed using $\alpha < .05$.

Breath Hydrogen Analysis

The primary study outcome was breath H2 excretion as measured by HBT. The increase in hydrogen concentration after the consumption of milk product is determined as lactose malabsorption.

Those determined eligible after the screening were invited to complete a 6-hour HBT: after an overnight fast, end-alveolar air samples containing 20 mL or more were collected. Breath hydrogen production was measured every 30 minutes until 2 hours and hourly until 6 hours. Alveolar air samples were collected via plastic syringes fitted with stopcocks and Hydrogen and Carbon dioxide concentrations were measured and analyzed within 24 hours after an oral load of regular milk using gaseous chromatography (Breath Tracker Digital Microlyzer, model SC; Quintron

Instruments). Observed hydrogen values were auto corrected for contamination of alveolar air resulting from room air by normalization of the carbon dioxide concentrations. Lactose amount was calculated as 0.5 g lactose per kg body weight. Participants whose H2 levels elevated 20 ppm or more above baseline and who experienced at least moderate or moderately severe Gastrointestinal symptom(s) of lactose intolerance during the test were included and randomized in the study which were described in detail in the inclusion criteria section above. Once enrolled, participants completed a similar 6-hour HBT after consumption of the assigned milk randomly.

Gastrointestinal Intolerance Symptoms

Participants were provided a validated questionnaire and they rated GI symptoms including abdominal pain or cramps, bloating, flatulence (gas) diarrhea and fecal urgency. A Likert scale was utilized and 0 indicated no symptoms, 1 slight symptoms, 2 mild symptoms, 3 moderate symptoms, 4 moderately severe symptoms and 5 severe symptoms. The maximum potential symptom score was 30 (rating of 5 for 6 hours) for each symptom.

RESULTS

All subjects who responded to recruiting materials were screened by phone. 201 subjects were excluded as they did not meet the inclusion criteria or did not wish to participate in the trial after learning of the requirement of five milk-feeding sessions. 30 subjects qualified for initial hydrogen screening. 18 subjects did not meet the inclusion criteria of symptom scores of moderate, moderately severe and severe during the baseline milk test. Thus, 12 subjects experienced the threshold LI symptoms and were randomized to the four different milk treatments. The randomized population had a mean age of 25 years, 8 were male, 3 of the participants were Asian, 3 were African-American, and 6 were Caucasian. Five participants withdrew from the study before completing their final randomized treatments. The remaining seven subjects who completed the protocol were; 2 females, 2 Asians, 1 African-American and 3 Caucasians.

Hydrogen Breath production over the 6 hours period following consumption of the four milks is shown in Figure 3. As expected, lactose-free milk did not generate hydrogen compared to baseline over the course of 6 hours, remaining at fasting levels. Total hydrogen production (AUC) for the lactose-free group trended to be lower than both regular milk (P= 0.07) and Jersey Cattle Milk (P= 0.08) over the 6 hour period, marginally failing to be significant at the 95% level. Area under the curve (Pairwise) analysis revealed that total hydrogen production was not different between lactose-free & A1-free (P= 0.43), A1-free & low A1 (Jersey) (P= 0.34), High A1 (Regular) & low A1 (Jersey) (P= 0.93) and High A1 (Regular) & A1-free (P= 0.29) treatments over the course of total 6 hours. However, when each time point was separately analyzed, regular milk containing high A1 β-casein produced significantly higher hydrogen compared to lactose free milk at 2 hours (P= 0.048), 3-hour (P= 0.014), 4-hour (P= 0.012) and 5-hour time points (P= 0.025). In addition,

Jersey Milk, which contains lower amount of A1 β-casein, also generated significantly more hydrogen compared to lactose free group after 4 hours (P= 0.015) and 5 hours (P= 0.012). The amount of hydrogen produced when participants were fed with A2 milk was in between lactose-free and high A1 milk from 2 hours until 6 hours but AUC analyses showed that these differences did not reach to significant levels, as measured by pairwise comparisons with a p-value of 0.23 when A2 Milk group was compared to lactose-free group and 0.21 when compared to High A1 (Regular) group. In addition, AUC from 2 to 6 hours revealed that Jersey milk and high A1 (Regular) fed group also generated significantly more hydrogen compared to lactose-free milk with (P= 0.046) and (P= 0.018), respectively. When Jersey group was compared to regular milk group, the differences were also not significant (P= 0.068) within the same time intervals.

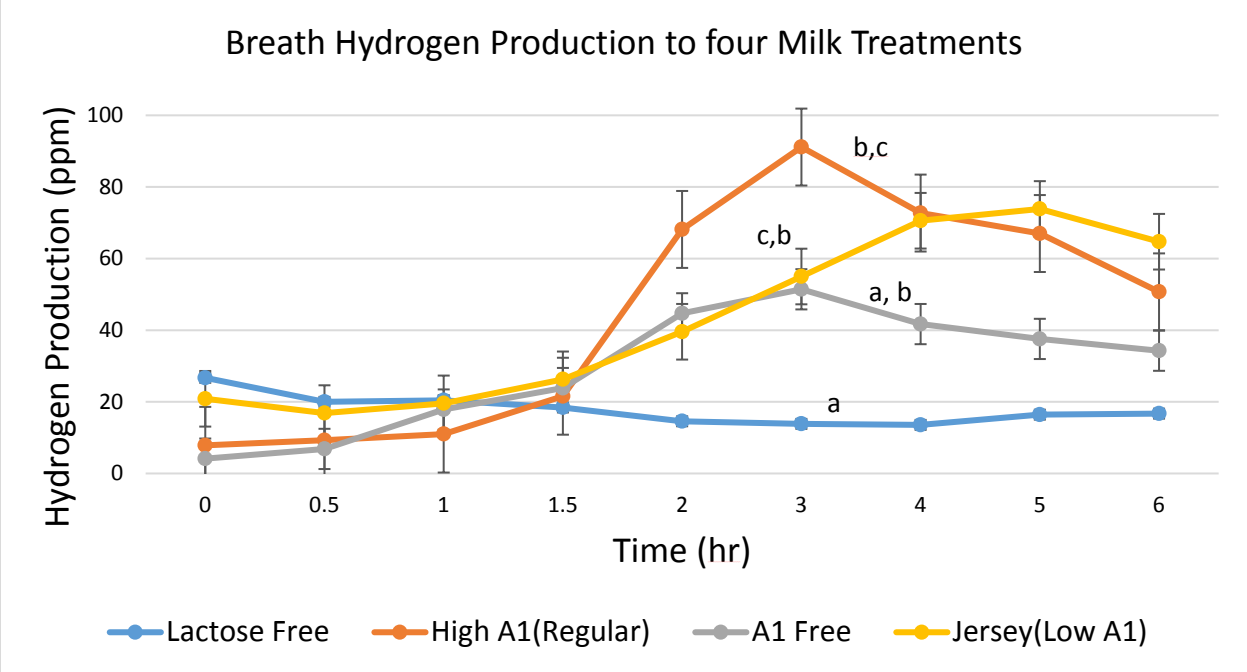

Figure 3 Breath Hydrogen Production from four Milk Treatments for 6 hrs
Data are expressed as mean ± standard error for seven subjects. ANOVA and pairwise multiple comparisons were performed on hydrogen data. Values which do not share the same letters are sig different (p≤ .05)

Over the course of 6 hours, subjects did not report significant differences in GI symptoms in response to the four milk treatments. In this pilot analysis, changing the lactose level or protein variants did not result in significant changes in the severity of individual symptoms including abdominal pain, bloating or fecal urgency as well as total GI responses. When the total symptoms summed, however, there was a very slight trend toward increased symptoms with the consumption of milk containing high A1 β-casein compared to lactose free milk (P= 0.22). This small trend aligns with Hydrogen production data. However, this difference did not reach to the conventional threshold of significance with the 7-sample size population group. Even though individual symptom scores did not differ significantly, the severity of flatulence (P= 0.15) and diarrhea (P= 0.14) were relatively closer to significant level when comparing high A1 and lactose free milks over the 6 hour period.

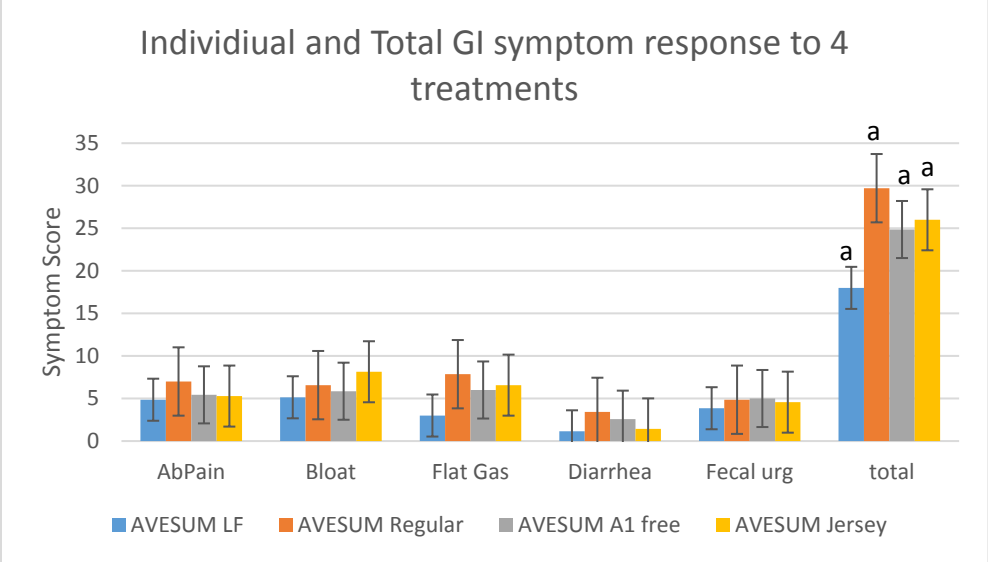

Figure 4 Summation of the symptoms reported for six hours following consumption of commercial A1 milk, lactose-free milk, A2 milk and Jersey milk in seven lactose intolerant maldigesters. Data are expressed as mean ± standard error. ANOVA and pairwise multiple comparisons were performed on symptom score. Values which do not share the same letters are sig different ($p \leq .05$)

DISCUSSION

In this study, we hypothesized that consumption of milk containing high A1 β-casein would result in an increased hydrogen production compared to A2 or Jersey milk. Further, commercial A1 milk would also be associated with greater systemic gastrointestinal symptoms resembling lactose intolerance in subjects who exhibit symptoms of intolerance when fed commercial milk. Compositional data from the food labels suggest the milk products tested in this investigation had nearly identical fat, lactose and protein content and the sole difference was the amount of β-casein variant where A1 to A2 ratio was 50:50 in regular milk, 25:75 in Jersey Milk and 0:100 in A2 Milk, respectively. With only seven subjects reported in this pilot data, and a calculated sample size requirement of 26, we can interpret trends that ultimately could result in significant differences as additional subjects complete this protocol. Thus, the discussion is written in an exploratory manner with the hypothesis that additional subjects will result in modest trends becoming significant differences.

The pilot data from the seven subjects shows greater hydrogen production from commercial A1 milk as compared to lactose-free, A2 and Jersey milk. This is somewhat remarkable given the small sample and suggests biologically relevant differences in lactose digestion among these milks. Most subjects experienced increased hydrogen production after 90 minutes regardless from the treatment type and this is biologically reasonable considering the average transit time for healthy adults. Hence, when the AUC window from 2 hours to 6 hours analyzed, the change in hydrogen production from each milk was calculated. Specifically, A2 Milk was similar to lactose-free milk with respect to its hydrogen generation, with lower production compared to regular milk, yet these

differences did not reach significance with 7 sample size. However, assuming the mean values remain the same, increasing the sample size to 26 is expected to result in significant differences between A2 Milk and Regular Milk. Jersey Milk, on the other hand, resulted in hydrogen production similar to commercial A1, milk but the difference from lactose-free milk was significant. ($p=0.046$), Overall, considering the equal amount of lactose in Regular, A2 and Jersey Milks, the differences in hydrogen production can be attributed to the their A1 β-casein content which may have a negative effect on transit time. In this pilot clinical trial, abdominal pain, bloating, flatulence, diarrhea, fecal urgency and total GI symptoms were reported as measures of digestive discomfort**.** Although the mean values of total GI symptom scores were numerically lower on the lactose free, pure A2 and Jersey group compared to regular milk group, none were statistically different. This is not surprising given the sample size. Yet, the symptom scores of A2 and Jersey Milks were located between negative controls lactose-free and positive control Regular Milk as expected. I predict that when the entire study is completed, these differences will be statistically significant, and possibly biologically relevant in terms of nutrition advice for individuals who have difficulty digesting commercial milk.

Previous studies showed that consumption of regular milk increased the GI symptoms, while milk containing only A2 β-casein significantly reduced symptoms and linked to A1 β-casein induced inflammation (He et al, 2017). He et al measured gastrointestinal symptom scores following the acute effect of different milk products. Scores were significantly lower at 1, 3 and 12 h after consumption of A2 β-casein milk relative to the regular milk in 600 Chinese adults.

Previous clinical studies also showed that consumption of milk products having both A1 and A2 β-casein significantly increased the gastrointestinal transit times, compared to the A1 β-casein free milk (Jianqin et al., 2016). The increased transit time results were consistent with preclinical trials conducted in rodent models (Barnett et al., 2014). The decreased transit time can be a factor explaining the reduction in the hydrogen generation in A2 group compared to regular milk.

Consumption of milk containing only A2 protein reduced the breath hydrogen production compared to regular milk containing both A1 and A2. These results were aligned with a recent clinical pilot study (Cameron-Smith et al, 2017). Reduced hydrogen production was statistically significant after 3 and 4 hours as the high A1 (conventional) group generated almost two times more hydrogen compared to A1-free group, which is quite remarkable given the small sample size.

The results of this pilot trial were interesting in that even if it was not powered enough to detect significant differences, it was possible to observe a relationship trend between the dose of A2 β-casein and the amount of Hydrogen produced which indicates the need of further research in this area. Inclusion of Jersey milk was done for the first time and it was quite important since it contained about 75% A2 β-casein variant which is a value between conventional milk and pure A2 Milk. The results showed that Jersey milk produced significantly higher hydrogen compared to lactose-free milk similar to regular milk between 2 and 6 hours ($p=0.046$) while A2 milk was acting similar to lactose-free milk and did not result in increased hydrogen throughout the same time intervals ($p=0.226$) These results suggest that the amount of A2 β-casein in Jersey milk was not adequate to attenuate the increased hydrogen concentration while pure A2 milk was effective. These were interesting since it implies a dose response effect of A2 β-casein with respect to lactose

maldigestion status. Considering the small sample size, these differences can reach to significant level once more subjects complete the study. Hence, a larger number of eligible subjects from different ages, gender and races are needed to either confirm or refute this hypothesis.

CONCLUSION

In this pilot clinical trial, results revealed greater hydrogen production from commercial A1 milk as compared to lactose-free, A2 and Jersey milk. Regular milk containing high A1 β-casein produced significantly higher hydrogen compared to lactose-free milk from 2 hours until 5hours. In addition, Jersey milk produced significantly higher hydrogen compared to lactose-free milk similar to regular milk between 2 and 6 hours while A2 milk was acting similar to lactose-free milk and did not result in increased hydrogen throughout the same time intervals. Taken together, these results suggest that the amount of A2 β-casein in Jersey milk was not adequate to attenuate the increased hydrogen concentration while pure A2 milk was effective.

The mean values of total GI symptom scores were numerically lower on the lactose free, pure A2 and Jersey group as compared to regular milk group, however, these were not statistically different. With seven subjects reported in this pilot data, and a calculated sample size requirement of 26, we can interpret trends that ultimately could result in significant differences as additional subjects complete this study. Therefore, these findings warrant further examination in a larger cohort or with a longer intervention time. In the future, it is being planned to use of ultrafiltration to formulate completely controlled milks varying only in A2 and A1 β-casein protein and lactose levels to confirm the importance of bioactive peptides such as BCM7 released from only A1 type. Furthermore, studies on transit time of A2 and A1 milks using MRI is being planned following the completion of this trial in order to be able to measure differences in transit time more accurately.

APPENDIX

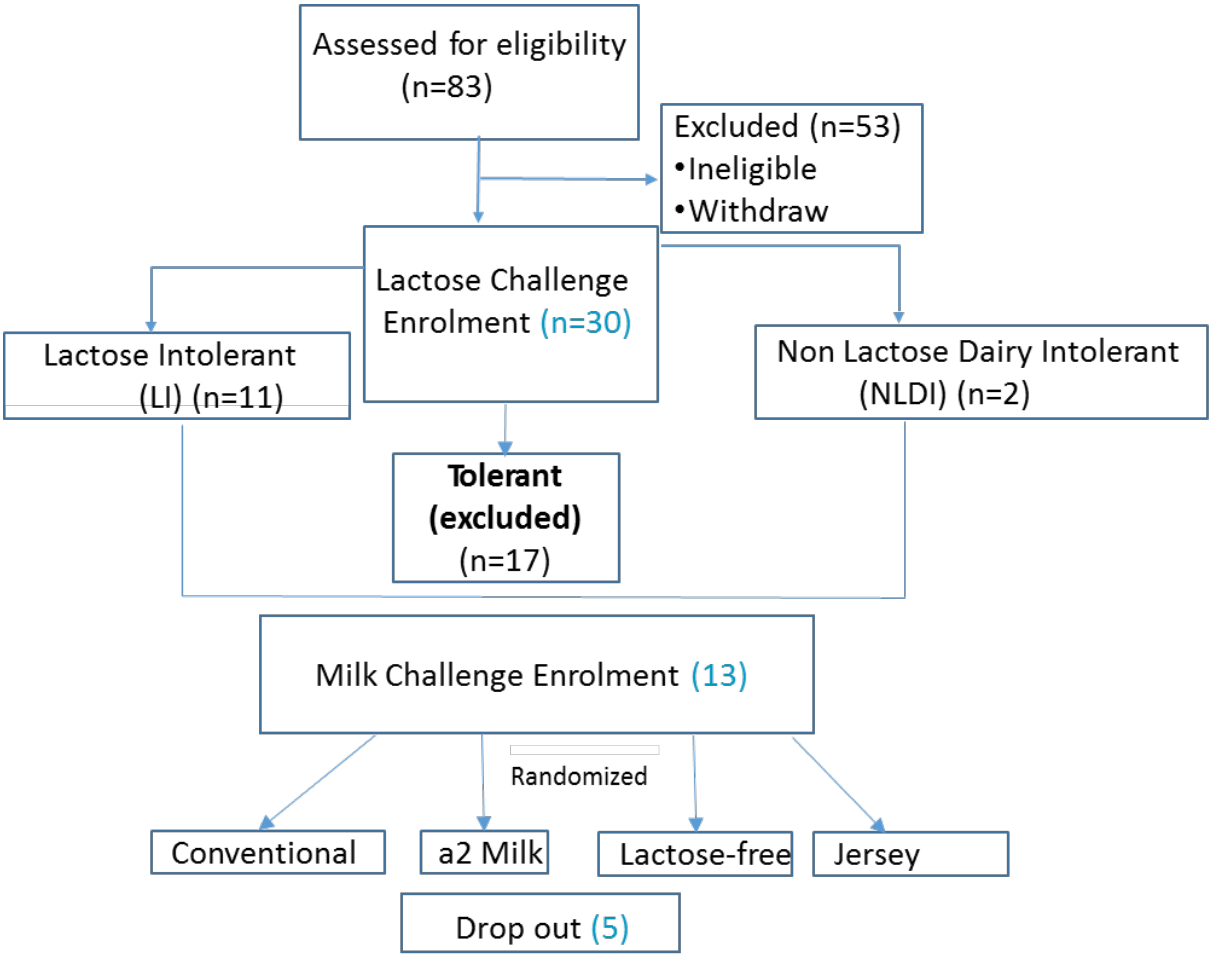

Figure 5 Flow chart of participants included for analysis

Hydrogen Breath Test/Symptom Report/Time: 1 Hour

Subject ID: ☐☐☐ **Date:** ☐☐ / ☐☐ / ☐☐
Month Day Year

Study Visit (check visit)		**Time of Sample**
☐ Screening ☐ Visit 1 ☐ Visit 2 ☐ Visit 3 ☐ Visit 4		☐☐ Hour ☐☐ Minutes ☐ AM ☐ PM

Instructions: Circle the number next to each symptom to indicate the degree to which you experienced each of these symptoms for the specified time-point. If you do not experience the symptom, select 0 (0 = none). The more discomfort you feel, the larger the number. Please rate your experience of abdominal pain/cramps, bloating, flatulence (gas), and diarrhea and any other symptoms.

	None	Slight	Mild	Moderate	Moderately Severe	Severe
Abdominal pain or cramps	0	1	2	3	4	5
Bloating	0	1	2	3	4	5
Flatulence (Gas)	0	1	2	3	4	5
Diarrhea	0	1	2	3	4	5
Fecal urgency	0	1	2	3	4	5
Other: (Please write in below and rate)						
	0	1	2	3	4	5
	0	1	2	3	4	5

Figure 6 Likert Scale Symptom Score Survey

Hydrogen Breath Test Sample Analysis

Subject ID: ☐☐☐ **Date:** ☐☐ / ☐☐ / ☐☐
Month Day Year

Study Visit (check visit)

☐ Screening ☐ Visit 1 ☐ Visit 2 ☐ Visit 3 ☐ Visit 4

Time	Hydrogen (ppm)	Methane (ppm)	CO2 (%)	Corr Factor
0				
30 min.				
1 hour				
90 minutes				
2 hours				
3 hours				
4 hours				
5 hours				
6 hours				

COMMENTS (note any issues encountered when analyzing samples):

Figure 7 Breath Hydrogen Production (ppm) Form

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