# Differential effects of sheep and cow skim milk before and after fermentation on gastrointestinal transit of solids in a rat model.

Short title: Milk species alters gastrointestinal transit

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**Abstract**

Fermentation of milk is considered to improve ease of digestion. The protein composition of sheep milk differs from cow milk. We hypothesized that sheep milk would produce bioactive properties with different effects on gastrointestinal (GI) motility compared with cow milk and that this would also differ following fermentation. We compared the effect of sheep and cow milk drinks, pre and post fermentation, fed to rats over two weeks, on the rate of GI transit of beads over 12-hours using X-ray imaging. Stomach emptying in animals fed sheep yoghurt was more complete than that for cow yoghurt. GI transit was increased for sheep milk treated animals than for cow milk, and colonic transit was increased, with a similar pattern observed for the yoghurts. The increased colonic transit for sheep milk compared with cow milk reveals prominent species differences, regardless of whether or not the milk was fermented.

## 1. Introduction

Milk and dairy products are considered healthy protein sources associated with maintaining muscle, bone and digestive health. Gastrointestinal (GI) dysmotility can be a symptom of functional GI disorders such as Irritable Bowel Syndrome resulting in faster or slower GI transit (Mayer, Labus, Tillisch, Cole, & Baldi, 2015). Because dairy proteins can alter GI transit, they have potential as functional foods. Dairy protein may also help to reduce the risk of metabolic disorders such as Type 2 diabetes and obesity (Bendtsen, Lorenzen, Bendsen, Rasmussen, & Astrup, 2013; McGregor & Poppitt, 2013) as well as cardiovascular disease (Marcone, Belton, & Fitzgerald, 2017). The composition and processing of dairy protein has an impact on digestion and absorption (Barbé, Ménard, et al., 2014; Barbé et al., 2013; Claeys et al., 2014), therefore manipulation of dairy protein composition through combinations of specific protein components in milk or fermented milk may provide a way to maximize benefits for specific health outcomes.

Milk is used to produce a variety of dairy products including fermented milk products such as yoghurt or drinking yoghurt. Fermentation of milk is thought to improve cardiovascular function via angiotensin-converting-enzyme (ACE) inhibitors, used to treat high blood pressure (Hideaki et al., 1990; Kohmura et al., 1989; Pihlanto-Leppälä, Rokka, & Korhonen, 1998). Effects of fermented milk on digestion are largely attributed to a combined effect of the culture bacteria together with the bioactive peptides released during the fermentation process (Beermann & Hartung, 2013; McKinley, 2005), which occurs due to the activity of lactic acid bacteria (Chaves-López et al., 2014; Hafeez et al., 2014; Hayes, Ross, Fitzgerald, & Stanton, 2007). In addition, milk proteins are digested at various points in the human GI tract to give rise to an array of bioactive peptides that can elicit a variety of physiologic effects in humans (Silva & Malcata, 2005). The rate of digestion and transit, however, could depend on the format

of dairy products (e.g. milk vs. yoghurt) and types of dairy proteins (e.g. caseins vs. whey proteins) because processing alters protein structure and aggregation, thus leading to different peptides being released (Boutrou et al., 2013; Chabance et al., 1998).

Although sheep milk production worldwide is small compared with cow milk, it is a fast emerging dairying industry (Broadhurst, 2016). The health benefits and nutritional value of sheep milk are far from being fully understood. Not only is the protein content higher in sheep milk than cow milk but the proteins differ in their composition resulting in different physiochemical properties (Park, Juárez, Ramos, & Haenlein, 2007). This difference may affect how proteins behave during processing and their biological actions once ingested.

The main proteins in cow and sheep milk are casein and whey proteins from which most bioactive peptides are derived (Nielsen, Beverly, Qu, & Dallas, 2017). Sheep milk is considered more easily digested than cow milk and of lower allergenicity, but the precise reasons for these putative differences are unknown. Sheep milk has a different casein protein composition from cow milk, being low in α-casein and high in β-casein (Park et al., 2007). This compositional change could lead to differences in micelle size and structure and soluble caseins, which could make it more easily digested providing greater potential for improving GI comfort and transit.

How fermentation of dairy protein affects transit of contents from the stomach to the colon during digestion has not been thoroughly investigated. Previous research has focussed on the probiotic effect of fermentation altering the microbiome (Veiga et al., 2014) which may, in turn, affect GI transit rather than the possibility of direct effects of the peptides themselves. Fermented infant formulas are examples of fermented milk drinks that do not contain

significant amounts of viable bacteria yet can improve digestive symptoms (Szajewska, Skórka, & Pieścik-Lech, 2015). These observations might be indicative of direct peptide action.

Understanding the biological effects of cow and sheep milk pre and post fermentation may suggest possible long-term approaches to self-management of mild dysmotility, for example through dietary intervention.

The aim of this study was to investigate differences in milk from different species, and the effects of fermentation, on food function and physiology. In it, we compared the effect of the milk and yoghurt drinks from cow and sheep (standardised to 3 % protein) on peptide profile and correlated this with GI transit in a rat model. Due to the sequence differences between sheep and cow milk proteins, we hypothesized that sheep milk would produce different bioactive properties from cow milk following fermentation with the same bacterial cultures, resulting in different GI transit rates. We freeze-dried the yoghurt to reduce the influence of the culture and studied the peptides resulting from fermentation. The technique used to track GI transit has been used in previous rodent studies and approximates that in humans for semi-solid contents (Dalziel, Fraser, et al., 2017; Dalziel, Young, et al., 2016). Understanding how milk peptide composition affects GI transit at specific GI locations will help determine the health attributes they may impart as functional foods.

## 2. Materials and methods

### *2.1 Yoghurt drinks*

Cow skim milk powder (SMP 001 (111115)) was kindly provided by NZ Food Innovation (Waikato) Ltd, Hamilton, New Zealand, and sheep skim milk powder (031215 Cypher number KY03) was kindly provided by Blue River Dairy, Invercargill, New Zealand.

The fermentation of cow and sheep milk was carried out using a standard laboratory preparation procedure for set yoghurt production using thermophilic cultures that were freeze-dried then rehydrated to a drinking yoghurt . Cow skim milk powder (38 % protein, <0.1 % fat, 45 % lactose) (2.1 kg/15 L water) and sheep skim milk powder (52 % protein, 1 % fat, 37 % lactose) (1.575 kg/15 L water) were rehydrated to liquid milk over 2 h using a stick blender. They were then heated to 85 °C slowly over 2 h and held at this temperature for 30 min with constant stirring. The milk was then cooled to 43 °C (over 60 min) and a commercial starter culture containing a 1:1 ratio of *Streptococcus thermophilus* and *Lactobacillus debrueckii* subsp. *bulgaricus* (CHR Hansen YF-L811 – Yo Flex®) was added to the milk at a concentration of 0.26 U/L. The inoculated milk was incubated at 43 °C for 5-6 h until the pH dropped to 4.5. The yoghurt was then frozen at -20 °C in shallow trays (in 3-4 L batches). To improve the freeze-drying process and to also reduce bacterial viability, the yoghurt was annealed by partially thawing to -5 °C and then re-freezing to -20 °C before freeze-drying.

Four dairy drinks (3 % protein) were studied for cow and sheep milk, pre and post fermentation. The milk and yoghurt drinks were prepared by reconstituting the milk or yoghurt powder (at 3 % protein) with water and blended for 30 s in a Waring blender. Drinks were made up daily and provided as two feeds with half kept at 4 °C prior to use.

The viscosity of the drinks (20 mL sample) was measured using a Paar Physica controlled-stress rheometer (Model MCR 301, PHYSICA Mebtechnik GmbH, Stuttgart, Germany) equipped with a cup and bob geometry (the inner diameter of the cup was 28.9 mm and the diameter of the bob was 26.6 mm) giving a gap of 1.15 mm. Samples were allowed to rest for 5 min before applying a shear rate sweep between 0.1 and 100 $s^{-1}$. Measurements were performed in triplicate at a constant temperature of 20 °C.

*2.3 Bacterial quantification*

The viable strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii ss bulgaricus* were assessed in freeze-dried powder prior to the animal study. All dairy samples were reconstituted in sterile Milli-Q water at 3 % protein by blending for 30 sec, serially diluted in phosphate-buffered saline and grown on the appropriate medium. This system also sterile filters the water to ensure no microbial contamination and the milk provides the mineral content for the animal.

*Streptococcus thermophilus* was grown on Mitis-Salivarius Agar with 5 % $CO_2$ at 37 °C for 24-48 h, and *L. delbrueckii ss bulgaricus* was grown on MRS (Fort Richard Laboratories Ltd, Auckland, NZ) pH 5.2 Agar and incubated in an anaerobic jar with Anaerobic GasPak at 45 °C for 72 h. Following fermentation and annealing the yoghurt drinks contained no *Lactobacillus bulgaricus* from the starter culture, while a *Streptococcus thermophilus* count of only $4.5 \times 10^6$ CFU/mL remained for cow yoghurt and $1.5 \times 10^4$ CFU/mL for sheep yoghurt. The reconstituted milk samples were negative for both strains.

*2.3 Animal care*

This study was conducted following ethical approval (AE13501) by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand) in accordance with the Animal Welfare Act, 1999 (NZ). Male Sprague-Dawley rats, 400 g, 12 weeks old, were bred at the AgResearch Small Animal Breeding Unit (Hamilton, New Zealand). The animals were housed individually at a constant temperature of 21 °C and maintained under a 12/12 hour light/dark cycle. At 10 weeks of age, they were fed a solid diet of AIN-93M OpenStandard Rodent Diet (Research Diets, Inc. New Brunswick, NJ, USA) in which the protein source was egg white. This was supplemented with dairy drinks: cow milk, cow yoghurt, sheep milk or sheep yoghurt, provided *ad libitum* for two weeks. To be able to assess the effect of dairy drinks on GI transit the animals were fed a dairy-free nutritionally balanced diet in which egg white

was the protein source. The animals were monitored three times weekly for weight, food intake, and General Health Score (1-5; NZ Animal Health Care Standard). At the end of the study, the rats were euthanized using carbon dioxide inhalation overdose followed by cervical dislocation.

*2.4 GI transit procedures and measurements*

The methods used have been described previously (Dalziel, Fraser, et al., 2017; Dalziel, Young, et al., 2016; Dalziel, Young, McKenzie, Haggarty, & Roy, 2017). Each rat received six solid stainless steel beads, d=1.4 mm (Bal-tec, Los Angeles, CA, USA) via oral gavage in 2 mL of 15 % barium sulfate (E-Z-HD 98 % w/w, Cat. No. 764, E-Z-EM Canada Inc., kindly provided by Palmerston North Hospital, New Zealand). Isoflurane anesthesia was induced in a chamber and persisted for 5 min during which gavage was performed upon recovery of the swallow reflex.

*2.4.1 X-ray imaging*

GI transit was tracked at three time points by X-ray imaging under brief isoflurane anesthesia to monitor: exit from the stomach (4 h), small intestine transit (9 h) and large intestine transit (12 h). The metallic beads were visualised by X-ray, and the relatively opaque barium sulfate outlined the GI tract, enabling identification of bead location. Ventral and right lateral views were taken using a portable X-ray unit (Porta 100HF 2.0kW High Frequency, Job Corporation, Yokohama, Japan). This included a camera and digital cassette (Canon 55G DR sensor panel) in conjunction with a laptop computer (Lenovo ThinkPad W530) and image viewing software (Lenovo ThinkPad W530). Image files (DICOM) were visualised using MicroDicom DICOM Viewer v8.7 (Simeon Antonov Stoykov, Sofia, Bulgaria).

*2.4.2 Stomach emptying*

Comparative measures of stomach emptying were obtained by determining the proportion of beads that had exited the stomach at 4, 9, and 12 h. Five animals across three feeding groups (5/48) were excluded from analysis because no meaningful transit measurements were possible due to stomach emptying being substantially delayed, as previously reported to occur in approximately 10 % of animals using this method (Dalziel et al., 2016).

*2.4.3 GI transit score*

The rating scale (Table 1) used to classify GI bead location comprised six beads, each given a numeric score depending on its location within the GI tract (range 0=stomach to 6=expelled from GI tract). The total transit score was the sum of the individual bead scores (maximum = 35 if all expelled).

*2.4.4 Colonic transit*

The movement of beads between 9 h (when the majority were in the caecum or distal small intestine) and 12 h (when a proportion had moved to the colon or rectum) was observed to assess possible differences between feeding groups in colonic transit. The number of beads per rat that had moved from the small intestine/caecum to the colon/rectum over 3 h was determined and compared between strains.

*2.5 Peptide analysis*

Skim milk powders and freeze-dried yoghurt powders were reconstituted in water to 10 % solids (w/v). Peptides were extracted using a modified chloroform/methanol procedure (Wessel & Flügge, 1984) and enriched and desalted by solid-phase extraction on C18 Sep-Pak cartridges (1cc, 50 mg) obtained from Waters (Milford, MA, USA). Peptide extracts were

analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a ProntoSIL C18 AQ (100 µm x 150 mm, 3 µm, 200 Å) column (NanoLCMS Solutions, Rancho Cordova, CA, USA), using a nanoAdvance UHPLC (Bruker-Daltonics, Bremen, Germany) coupled to a maXis Impact HD ultra-high-resolution quadrupole time-of-flight mass spectrometer (Bruker-Daltonics). Bioinformatic analysis was carried out using PEAKS Studio 8 software (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). 'No-enzyme' searches were performed against *Bos taurus* and *Ovis aries* SwissProt protein databases with the peptide spectrum match threshold set to a false discovery rate of 1 %. Identified peptides were matched to peptides with known bioactive properties.

### *2.6 Statistical analysis*

All analyses were carried out using GenStat version 18 (VSN International Limited, Hemel Hempstead, UK) and Minitab 17 Statistical software (Minitab Inc., State College, PA, USA). Results are expressed as the mean ± SEM. Stomach emptying and GI transit score data were analysed using a linear mixed model (REML) with treatment group as the factor to compare differences between treatment group and Fisher's least significant differences used for the post-hoc test. GI transit score data were square-root transformed, and both datasets met the assumptions of normality and homogeneity. Large intestine transit data were analysed using ANOVA with treatment group as the factor, and square-root transformed to meet the assumptions of normality and homogeneity.

## 3. Results and discussion

### *3.1 Cow and sheep milk composition*

The consistency of the milk following fermentation required an initial assessment to ensure free flow from drink bottles. The composition was adjusted to 3 % protein (w/v). The fat,

lactose, total solids contents, and viscosity of the drinks are shown in Table 2. The viscosity of the yoghurt drinks was approximately 6.2–7.4 mPa.s, slightly higher than their respective reconstituted milk drinks. This level of viscosity enables free flow.

### *3.2 Physiological effects*

#### *3.2.1 Food Intake*

Over the 14 days of the experiments, the rats had a normal solid food intake of 27 g per day and gained 16.5 % body weight. Dairy drink daily intake was not different among the treatment groups (87 ± 4 ml for cow milk, 104 ± 4 mL for cow yoghurt drink, 95 ± 9 mL for sheep milk, and 98 ± 6 mL for sheep yoghurt drink; $p<0.07$).

#### *3.2.2 Stomach emptying*

Representative examples of ventral and right lateral X-ray image views show the location of six metallic beads over time in the groups for cow milk and sheep milk (Fig. 1) and cow yoghurt drink and sheep yoghurt drink (Fig. 2) at post gavage times of (A) 4 h, (B) 9 h, and (C) 12 h. The mean percentages of beads that had exited the stomach per animal at each time point are shown for each feeding group in Fig. 3. The bead movement from the stomach was similar for cow milk and sheep milk at 4 h. Following fermentation, however, 23 % more beads had exited the stomach per animal for sheep yoghurt drink than for cow yoghurt drink (Fig. 2A, Fig. 3). A long delay in stomach emptying was evident for cow milk at 9 h which was slowed compared with cow yoghurt drink (Fig. 3). The comparatively faster stomach emptying for animals fed with the cow yoghurt drink suggests easier expulsion from the stomach in the presence of fermented milk.

Transit scores summarised from 6 solid beads to compare GI transit tracked over 12 h for the animals (n = 10-12 animals per group) fed with cow milk, cow yoghurt drink, sheep milk, and sheep yoghurt drink are shown in Fig. 4. The location of the beads relative to the stomach by 4 h (Fig. 4) indicated that more beads had transited into the small intestine for sheep yoghurt drink than for cow yoghurt drink. The bead transit score of ~1 for cow milk, cow yoghurt and sheep milk drinks at 4 h means that few beads had left the stomach, whereas the score of 3 for sheep yoghurt drink means that more beads had transited to the proximal intestine. This suggests although species-dependent effects of milk fermentation on stomach emptying are more prominent, there are also differences in stomach emptying between fermented and non-fermented cow and sheep milk. The immediate stomach emptying effect was most prominent for the sheep yoghurt drink compared with the cow yoghurt drink and was not detected between the unfermented drinks, implicating fermentation products in the sheep yoghurt drink in a gastric promotility effect, or conversely the cow yoghurt drink in slowing motility. We note that stomach emptying was slower in this study across all four feeding groups compared with previous studies using this method and rat strain (Dalziel, Fraser, et al., 2017; Dalziel, Young, et al., 2016), which may be attributed to egg white being the protein source in the solid feed rather than soy or casein as in previous work.

*3.2.3 GI transit*

Bead transit to the small intestine transit (9 h) was not different among the animals fed with different dairy drinks. However, bead transit to the large intestine (12 h) was greater for sheep milk compared with cow milk (Fig. 1B&C, Fig. 4). Thus by 12 h, most beads were in the caecum (score of 18) for the animals fed with cow milk or yoghurt drink whereas at least half were in the colon for the animals that consumed sheep milk or yoghurt drink.

To better resolve differences in large intestine transit we took the caecum as a marker point at 9 h and measured how many beads had transited from the small intestine/caecum region into the colon/rectum or exited by 12 h (Fig. 5). For the animals fed with sheep milk, more beads had moved from into the colon over 9-12 h compared with the animals fed with cow milk. Similarly, more beads had moved into the colon over 9-12 h for the animals consuming sheep yoghurt drink compared with cow yoghurt drink. Our findings indicate a strong species effect demonstrating that sheep milk increased colonic transit of solid contents relative to cow milk in rats and that this effect also occurred for the corresponding fermented milks. Because the protein content was matched, and the milks were low fat, it is the peptides released from the proteins we consider to be largely responsible for the GI transit differences detected.

Delayed stomach emptying resulting in food remaining in the stomach for a longer time is referred to as gastroparesis ("partial paralysis") in humans. This would be for longer than 4 hours for a rat. Irritable bowel syndrome (IBS) is an example of a non-pathological GI state that can include other parts of the digestive tract in addition to the colon. Delayed stomach emptying of solids and constipation occur in a large proportion of study participants with IBS (Caballero-Plasencia, Valenzuela-Barranco, Herrerías-Gutiérrez, & Esteban-Carretero, 1999). The sheep yoghurt drink may be a useful supplement for those who suffer from functional GI conditions such as gastroparesis and constipation.

*3.3 Peptide profile differences*

Because the protein content was matched are low fat and similar in carbohydrate, it is the protein that is most likely to confer any biological difference in effect. The peptides potentially released from the proteins during digestion were therefore considered to be the most likely source of bioactives to contribute to the GI transit differences detected (Kamau et al., 2010).

Peptide analysis revealed that although there were 29 % fewer peptides detected in sheep milk than cow milk, the proportional increase in peptides in the yoghurt drinks was similar at 37 % for cow yoghurt and 36 % for sheep yoghurt (Nielsen et al., 2017). These numbers were probably underestimated because a limitation of the technique used is that small peptides less than five amino acid would not be detected. Differences in the number of peptides present in the milk and yoghurt drinks are depicted in a Venn diagram (Fig. 6). The cow and sheep peptide sequences were aligned with numbering for the parent cow proteins (Supplementary data information). The peptides known to withstand GI enzymatic digestion *in vivo* or *in vitro*, or to reach the bloodstream, and with known biological activities, are summarised in Table 3. Peptide bioactives detected included those with antihypertensive, antioxidant, mucin production, immune modulators, antibacterial, $GABA_A$, bradykinin, opioid and other neuropeptide modulators.

To determine how differences in the peptide composition of the dairy drinks might contribute to the changes detected in stomach emptying and GI transit, these were correlated with the physiological effects of: 1) the enhanced stomach emptying effect with sheep yoghurt drink compared with cow yoghurt drink; 2) the more complete (9 h) stomach emptying with cow yoghurt drink compared with cow milk; and 3) the enhanced colonic transit conferred by sheep milk compared with cow milk (and also for sheep yoghurt drink compared with cow yoghurt drink).

Differences between cow and sheep milk that might account for the species difference effect on colonic transit might include small peptides as these are not expected to be degraded further by fermentation or hydrolysis by digestive enzymes.

*1) Enhanced stomach emptying effect with sheep yoghurt drink compared with cow yoghurt drink*

We found a peptide precursor for the bioactive β-casomorphin was present in cow yoghurt and encrypted in a larger peptide in cow milk, but was not present in sheep milk or yoghurt. This is because the sheep milk β-casein sequence in this region (YPFTGPI) is different to that of cow milk (Table 3). In addition, only proline was observed at position 67 ($P^{67}$) in sheep milk or yoghurt β-casein, whereas both $P^{67}$ (A2 variant) and $H^{67}$ (histidine, A1 variant) were found in cow milk (Table 3). Therefore, it is expected that β-casomorphin would be released from cow milk drinks during GI digestion (Svedberg, de Haas, Leimenstoll, Paul, & Teschemacher, 1985). Because this peptide is a known mu opioid agonist (Allescher, Storr, Piller, Brantl, & Schusdziarra, 2000; Dalziel et al., 2014; Daniel, Vohwinkel, & Rehner, 1990) its presence would be expected to contribute to relatively slower stomach colon motility for the cow yoghurt drink compared with the sheep yoghurt drink.

*2) Faster stomach emptying (9 h) with cow yoghurt drink compared with cow milk*

As far as we are aware, there has been no report to compare the stomach emptying effects of fermented and non-fermented dairy drinks. Possible reasons for the improved stomach emptying with the cow yoghurt drink could be due to it being partially 'pre-digested' by fermentation cultures. It is notable that the $\alpha_{s1}$-casein (91-100) decapeptide was only detected in cow milk; this is $GABA_A$ receptor agonist which might be expected to alter gastric motility (dela Peña et al., 2016; Krantis, Mattar, & Glasgow, 1998).

*3) Enhanced colonic transit by sheep milk and yoghurt compared with cow milk and yoghurt*

Peptides known to alter GI transit that differ between these milk species are the β-casomorphins which are present in cow milk that contain A1-type β-casein (Kamau et al., 2010). The corresponding peptide sequences differ in sheep milk. The cow milk and yoghurt drinks used in this study were a common bulk milk, therefore are expected to contain both β-casein A1 and

A2 phenotypes as we found in our analyses (Table 3). Because the A1 form of β-casein has histidine ($H^{67}$) immediately after the β-casomorphin-7 sequence (60-66) in cow milk, this site can be cleaved by proteases to release the bioactive peptide β-casomorphin-7 (Jinsmaa & Yoshikawa, 1999) when cow milk products are consumed. Some β-casomorphins activate mu opioid receptors to inhibit synchronised propagating contractions in the rat colon (Dalziel et al., 2014) and slow GI transit (Daniel et al., 1990). The inhibitory effects of opioids in the GI tract on neuronal activity reduce propulsions and delay GI transit (Jianqin et al., 2016; Sobczak, Sałaga, Storr, & Fichna, 2014).

Bovine whey hydrolysate also alters colonic motility via mu opioid receptors indicating that other peptides of whey protein origin also modulate motility (Dalziel, Anderson, et al., 2016). However, it is unlikely they would have been present at a sufficient concentration to alter motility in skim milk. Thus the probable production of the β-casomorphin-7 peptide from cow milk and yoghurt in the GI tract most likely contributed to the relatively slower colonic transit in cow milk and yoghurt drinks compared with sheep milk and yoghurt drinks. In a functional food sense the cow milk peptides would be expected to reduce colonic motility.

Larger peptides were also detected that encrypted other relevant bioactive peptides known to be released during gastric digestion and resistant to GI degradation. These, however, did not correlate directly with our GI transit results because they did not show a specific distinction between cow and sheep for either milk or yoghurts. Casoxins A & B (κ-casein 35-41 & 58-61, Table 3) were detected in the yoghurt drinks but not in the milks. The TEDEL (β-casein 41-45, Table 3) opioid agonist sequence occurred in precursor peptides for three out of four of the drinks with sheep yoghurt drink being the exception. Likewise, EMPFPK (β-casein 108-113) and YPVEP (β-casein 114-119) sequences were detected in precursor peptide sequences for all except sheep yoghurt. The EMPFPK bioactive peptide is known to be released during casein

hydrolysis and have dual actions in the nanomolar range, potentiating the effect of bradykinin to increase guinea pig ileum contractions (Perpetuo, Juliano, & Lebrun, 2003). It is possible that sheep milk contains peptides with as yet unknown biological action that contribute to the enhanced GI transit compared with cow milk.

The fermentation process itself used did not result in the release of any detectable β-casomorphin peptides for cow yoghurt. Although enzymes from the yoghurt strains used in the current study may be able to break proline bonds and potentially release smaller peptides from the β-casomorphin peptides (Donkor, Henriksson, Vasiljevic, & Shah, 2007), there is evidence to suggest that β-casomorphin peptides are found in fermented dairy products (De Noni & Cattaneo, 2010; Schieber & Brückner, 2000). The absence of the functional peptides in cow yoghurt does not preclude the release of β-casomorphin peptides from larger peptides and uncleaved whole-protein A1-type β-casein during the digestion of yoghurt and subsequent contribution to the GI transit effect observed. Likewise, we cannot rule out an influence of the intestinal microbiota in the release of opioid peptides from β-casomorphin-like peptides. Although an opiate-like bioactive peptide (e.g. β-casein 114-121) can be released from sheep milk β-casein using specific bacterial combinations for fermentation (Papadimitriou et al., 2007; Perpetuo et al., 2003), we did not detect any which is consistent with findings using similar standard yoghurt cultures (Papadimitriou et al., 2007).

The absence of opiate GI motility modulatory peptides from sheep yoghurt might contribute to this treatment having the most rapid stomach emptying. Furthermore, a recent peptidomic study identified 21 bioactive peptides sequences with opioid agonist (including β-casomorphin and exorphin) and 4 with opioid antagonist (casoxin) activity from cow caseins, but not from sheep caseins (Nielsen et al., 2017).

We note that peptides with a range of biological activities were detected including ACE-inhibitory peptides, but how these might relate to differences in colonic transit between the milk species is unclear. However, their effect at reducing blood pressure (Table 3) might assist blood flow to the GI tract. We also note that many of the casein peptides became glycosylated following fermentation. This might alter their prebiotic potential impacting on the microbiota to indirectly affect colonic motility and transit of contents.

### *4. Conclusion*

The main findings of this study are that prominent differences between species exist with respect to the effects of dairy drinks on colonic transit of solids both before and after fermentation. Because faster colonic transit for sheep milk occurred in both the unfermented and fermented drinks, this effect cannot be attributed to fermentation, but rather indicates species differences between these milks whereby sheep dairy facilitates transit of contents. Following fermentation, stomach emptying was faster for sheep yoghurt than for cow yoghurt. The peptide analysis showed that bioactive β-casomorphin precursor was found in cow milk, implying this peptide could contribute to the slower stomach emptying and GI transit for cow milk and yoghurt. Such GI modulatory actions may promote a longer sense of fullness and calm any colonic over-activity. Since the cow milk was used in this study contained both A1 and A2 types of β-casein, the finding of slower colonic transit for cow milk and yoghurt drinks (attributable to β-casomorphins), compared with sheep milk and yoghurt drinks, may not be relevant to cow milk products containing only the A2-type of β-casein, in which these peptides would be absent. Further studies, however, would be required to confirm this assumption.

### **Conflict of Interest**

The authors declare no conflict of interest.

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**Conflict of Interest Statement**

Competing Interests: the authors have no competing interests. All authors had complete access to the data that supports the publication.

**Author Contribution**

J.E.D. analysed the data, interpreted the results and wrote the paper; G.S. carried out peptide detection and qualitative analysis; S.H. analysed the peptide data against databases; C.M.M. carried out statistical analysis; J.E.D., G.S., S.H. and L.D. designed the study and critically revised the manuscript.

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Figure legends

**Figure 1.** Representative example of X-ray images showing the location of six metallic beads over time in dairy treatment groups for cow milk and sheep milk ventral (V) and right lateral (RL) view images at post-gavage: (A) 4 h, (B) 9 h, and (C) 12 h.

**Figure 2.** Representative example of X-ray images showing the location of six metallic beads over time in dairy treatment groups for cow yoghurt drink and sheep yoghurt drink ventral (V) and right lateral (RL) view images at post-gavage: (A) 4 h, (B) 9 h, and (C) 12 h.

**Figure 3.** Comparison of transit from the stomach over 12 h for cow milk, cow yoghurt drink, sheep milk, and sheep yoghurt drink treated animals (n = 10-12 animals per group). The percentage of beads that had exited the stomach per animal (mean per treatment). Asterisks indicate the significance difference between treatments (* $p < 0.05$). Data show mean ± SEM.

**Figure 4.** Comparison of gastrointestinal transit tracked over 12 hours for cow milk, cow yoghurt drink, sheep milk, and sheep yoghurt drink treated animals (n = 10-12 animals per group). (A) Transit scores for 6 solid beads. Transit scoring is detailed in Table 1. Asterisks indicate the significance difference between treatments (* $p < 0.05$). Data show mean ± SEM.

**Figure 5.** Large intestine transit. The number of beads per animal that moved from the small intestine/caecum at 9 hours to the colon/rectum at 12 hours are shown for cow milk (n=8), cow yoghurt drink (n=6), sheep milk (n=8), and sheep yoghurt drink (n=7) treated animals using back-transformed square root data (* $p < 0.05$). Data show mean ± SEM.

**Figure 6.** Venn diagram of the unique sequence combination of peptides in the four dairy drinks.

**Table 1. GIT Bead Location Scoring**

| **Location** | **Stomach** | **Proximal small intestine** | **Distal small intestine** | **Caecum** | **Colon** | **Rectum** | **Exited GI tract** |
|---|---|---|---|---|---|---|---|
| Bead score* | 0 | 1 | 2 | 3 | 4 | 5 | 6 |

* Indicates the number points allocated to a bead for its location.

**Table 2. Composition of reconstituted milk and yoghurt drinks fed to animals.**

| Dairy drink | Protein [a] (%) | Fat [b] (%) | Lactose [b] (%) | Total solid [b] (%) | Viscosity (mPa.s) |
|---|---|---|---|---|---|
| Cow milk | 3.0 | <0.01 | 3.6 | 8.0 | 1.71 |
| Sheep milk | 3.0 | <0.094 | 2.2 | 5.8 | 1.50 |
| Cow yoghurt | 3.0 | <0.01 | ND | 8.0 | 6.21 |
| Sheep yoghurt | 3.0 | <0.094 | ND | 5.8 | 7.41 |

[a] Determined using Kjeldahl method. [b] Calculated from 10 % milk solution by Milkoscan.

ND: not determined using milkoscan due to high viscosity.

**Table 3.** Peptides detected in milk drinks that are known to be GI stable for the cow homologue.

| Position | Cow milk | Sheep milk | Cow yoghurt | Sheep yoghurt | Activity | GI stable |
|---|---|---|---|---|---|---|
| $\alpha_{s1}$-casein 1-21 | **RPKHPIKHQ**GLPQEVLNENLL | | | | Antibacterial, immunomodulator | Human plasma (Chabance et al., 1998); Mini-pig (Barbé, Le Feunteun, et al., 2014); Calf (Yvon & Pelissier, 1987) |
| $\alpha_{s1}$-casein 23-34 | **FFVAPFPEVFGK** | FVVAPFPEVFR | | | Antihypertensive (ACE inhibitor), anticancer, bitter | Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| $\alpha_{s1}$-casein 24-32 | | | **FVAPFPEVF** | | Antihypertensive (ACE inhibitor) (Ong & Shah, 2008) | Human (Boutrou et al., 2013) |
| $\alpha_{s1}$-casein 25-32 | | | **VAPFPEVF** | V**VAPFPEVF** | Antihypertensive (ACE inhibitor) | Human (Boutrou et al., 2013) |
| $\alpha_{s1}$-casein 80-90 | **HIQKEDVPSER** | | | | Antioxidant | Human (Boutrou et al., 2013); Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| $\alpha_{s1}$-casein 91-100 | **YLGYLEQLLR** | | | | Anti-stress (GABA A receptor) (dela Peña et al., 2016) | Mini-pig (Barbé, Le Feunteun, et al., 2014); |
| $\alpha_{s1}$-casein 104-119 | **YKVPQL**EIVPNSAEER | | | | Antihypertensive (ACE inhibitor) | Human (Boutrou et al., 2013) |
| $\alpha_{s1}$-casein 143-149 | | | **AYFYPEL** | | Antihypertensive (Contreras, Carrón, Montero, Ramos, & Recio, 2009); mucin production (Martínez-Maqueda, Miralles, Cruz-Huerta, & Recio, 2013) | *In vitro* digestion (Sánchez-Rivera et al., 2014) |

| Position | Cow milk | Sheep milk | Cow yoghurt | Sheep yoghurt | Activity | GI stable |
|---|---|---|---|---|---|---|
| $\alpha_{s1}$-casein 157-164 | **DAYPSGAW** | **DAYPSGAW** | **DAYPSGAW** | | Antihypertensive (ACE inhibitor) (Pihlanto-Leppälä et al., 1998) | Calf (Yvon & Pelissier, 1987); *In vitro* digestion (Sánchez-Rivera et al., 2014) |
| $\alpha_{s1}$-casein 180-193 | | | **SDIPNPIGSENSEEK** | SDIPNPIGSENSGK | Antimicrobial | Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| $\alpha_{s2}$-casein 171-180 | YQK**FALPQYL(K)** | | | | IgE interaction, persistent allergy | Peptide cutter prediction |
| $\alpha_{s2}$-casein 172-180 | | | QK**FALPQYLK** | | IgE interaction, persistent allergy | Peptide cutter prediction |
| $\alpha_{s2}$-casein 189-197 | AMKPWIQP | | **AMKPWIQPK** * | | Antihypertensive (ACE inhibitor) \ | Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| $\alpha_{s2}$-casein 198-204 | **TKVIP**YV | (TNAIPYV) | | | Antihypertensive (Maeno, Yamamoto, & Takano, 1996) | Human (Boutrou et al., 2013) |
| β-casein 6-14 | | | **LNVPGEIVE** | | Antihypertensive (ACE inhibitor) | Human (Boutrou et al., 2013) |
| β-casein 7-14 | | | **NVPGEIVE** | | Antihypertensive (ACE inhibitor) | Human (Boutrou et al., 2013) |
| β-casein 37-48 | | EQQQ**TEDEL**QDK | | | Opioid agonist (Boutrou et al., 2013) | Human (Boutrou et al., 2013) |
| β-casein 41-46 | | | **TEDEL**Q | | Opioid agonist (Boutrou et al., 2013) | Human (Boutrou et al., 2013) |
| β-casein 41-49 | **TEDEL**QDKI | | | | Opioid agonist (Boutrou et al., 2013) | Human (Boutrou et al., 2013) |
| β-casein 58-72 (57-68 β-CM precursor) Note: A1 cow milk β-casomorphins | AQTQSLV\|**YPFPGPI**HN\|SLPQNIPPLTQTPV (A1: 53-82) and | **YPFTGPI**PN\|SLPQNILP(60-76) | **PFPGPI**HNSLPQ (A1: 61-72) V\|**YPFPGPIH** (A1: 59-67) | **(Y)PFTGPI**PNSLP (61-71) And | μ opioid agonist – intestinal motility (Beermann & Hartung, 2013) Protease/peptidase | Mini-pig (Barbé et al., 2014; Meisel et al., 1986); Human (Svedberg et al., 1985); β-casomorphin 7 in human plasma (Kost et al., |

| Position | Cow milk | Sheep milk | Cow yoghurt | Sheep yoghurt | Activity | GI stable |
|---|---|---|---|---|---|---|
| range from 4-11 amino acids, e.g. β-casomorphin 7 (60-66) | AQTQSLV\|**YPFPG PI**PN\|S (A2: 53-69) | | LV\|**YPF**P**GPI**HN \|**S**LPQ (A1: 58-72) and LV**YPFPGPI**PN (A2: 58-68) **PFPGPI**PNSLPQ (A2: 61-72) V\|**YPFPGPI**PN\|S LPQ (A2: 59-72)<br><br>LV\|**YPFPGPI**PN\| SLPQ (A2: 58-72) | LV**YPFTGPI**P NSLPQNILPL (58-77) | inhibitor, antihypertensive (ACE inhibitor) | 2009); *in vitro* digestion (Jinsmaa & Yoshikawa, 1999) |
| | | | | | | |
| β-casein 73-82 | | | **NIPPLTQTPV** | | Antihypertensive (ACE inhibitor) | Human (Boutrou, Henry, & Sanchez-Rivera, 2015) |
| β-casein 98-105 | **VKEAMAPK** | VKETMVPK | * | | Neuropeptide, antioxidant (Korhonen & Pihlanto, 2007) | Mini-pig (Barbé, Le Feunteun, et al., 2014); *In vitro* digestion (Sánchez-Rivera et al., 2014) |
| β-casein 108-115 | **EMPFPK**YP | | | | **EMPFPK** potentiates bradykinin, opiate analgesia (Perpetuo, Juliano & Lebrun 2003), anti-hypertensive (Boutrou et al., 2015) | Human (Boutrou et al., 2013) |
| β-casein 108-119 | **EMPFPK**\|YPVQPF (A1) | | **EMPFPK\|YPVE P**F (A2) | | **YPVEP** (114-119) Neocasomorphin δ-opioid agonist ($IC_{50}$ = 56 μM) (Jinsmaa | Human (Boutrou et al., 2013) |

| Position | Cow milk | Sheep milk | Cow yoghurt | Sheep yoghurt | Activity | GI stable |
|---|---|---|---|---|---|---|
| | | | | | & Yoshikawa, 1999) | |
| β-casein 108-124 | **EMPFPK**\|YPVQPF TESQS (A1) **EMPFPK**\|**YPVEP**F TESQS (A2) | | | | see above | 114-124 Human (Boutrou et al., 2013); *In vitro* digestion (Sánchez-Rivera et al., 2014) |
| β-casein 108-132 | | **EMPFPK**\|**YPV EP**FTESQSLTL TDVEK | | | see above | Human (Boutrou et al., 2013) |
| Chabance cβ-casein 109-124 | | MPFPK\|**YPVE PF**TESQS | | | see above | *In vitro* digestion (Sánchez-Rivera et al., 2014) |
| β-casein 114-125 | **YPVEP**FTESQSL (A2) | | | | see above | 114-124: Human (Boutrou et al., 2013); *In vitro* digestion (Picariello et al., 2010) |
| β-casein 166-175 | | SQPKVLPVPQ K | **SQSKVLPVPQ** (A2 only) * | SQPKVLPVPQ | Antihypertensive (ACE inhibitor) (Hayes, Stanton, et al., 2007) | Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| β-casein 170-176 | VLPVPQK (A2 only) | | | | Antioxidant | Human (Boutrou et al., 2013) |
| β-casein 183-190 | | | **RDMPIQAF** | **RDMPIQAF** | Antioxidant | *In vitro* digestion (Picariello et al., 2010) |
| β-casein 191-209 | **LLYQEPVLGPVR GPFPIIV** | LLYQEPVLGP VRGPFPILV | **LLYQEPVLGP VRGPFPIIV** * | LLYQEPVLGP VRGPFPILV | Antihypertensive (ACE inhibitor) (Yamamoto, Akino, & Takano, 1994) | Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| β-casein 192-209 | **LYQEPVLGPVRG PFPIIV** | LYQEPVLGPV RGPFPILV | **LYQEPVLGPV RGPFPIIV** | LYQEPVLGPV RGPFPILV | Immunomodulatory | Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| β-casein 193-207 | **YQEPVLGPVRGP FPI** | YQEPVLGPVR GPFPI | **YQEPVLGPVR GPFPI** | YQEPVLGPVR GPFPI | Antimicrobial | Mini-pig (Barbé, Le Feunteun, et al., 2014) |
| β-casein 193-209 | **YQEPVLGPVRGP FPIIV** | YQEPVLGPVR GPFPILV | **YQEPVLGPVR GPFPIIV** * | YQEPVLGPVR GPFPILV | Immunomodulator, antihypertensive (ACE inhibitor) antibacterial | Calf (Yvon & Pelissier, 1987); mini-pig (Barbé, Le Feunteun, et al., 2014); *in* |

| Position | Cow milk | Sheep milk | Cow yoghurt | Sheep yoghurt | Activity | GI stable |
|---|---|---|---|---|---|---|
| | | | | | (Yamamoto et al., 1994) | *vitro* digestion (Picariello et al., 2010) |
| β-casein 194-209 | **QEPVLGPVRGPFPIIV** | QEPVLGPVRGPFPILV | **QEPVLGPVRGPFPIIV** * | QEPVLGPVRGPFPILV | Antihypertensive (ACE inhibitor), protease/peptidase inhibitor (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Yamamoto et al., 1994) | Mini-pig (Barbé, Le Feunteun, et al., 2014); *in vitro* digestion (Picariello et al., 2010) |
| β-casein 195-206 | **EPVLGPVRGPFP** | **EPVLGPVRGPFP** | | | Antihypertensive (ACE inhibitor) | Human (Boutrou et al., 2013) |
| κ-casein 33-43 | | | SR**YPSYGLNY**Y | | Casoxin A (35-41) | |
| κ-casein 33-48 | | | SR**YPSYGLN**YYQQKPV | SR**YPSYGLN**YYQQRPV | Casoxin A (35-41) | |
| κ-casein 55-66 | | | | LP**YPYY**AKPVA | Casoxin B (58-61) | |
| κ-casein 56-65 | | | LP**YPYY**AKPA | LP**YPYY**AKPVYPYYAKPV (58-65) | Casoxin B (58-61) | |
| κ-casein 96-106 | | | **ARHPHPHLSFM** | | Antioxidant | Mini-pig (Barbé, Le Feunteun, et al., 2014) |

Sequences in bold are those of known peptide sequences. Underlined sequences indicate amino acid differences between species or genetic variants. Sequence in brackets was not detected. Yoghurt produced using 1:1 ratio of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (CHR Hansen YF-L811 – Yo Flex®). | predicted chymotrypsin cut site; || predicted pepsin cut site

* Indicates peptides detected in products of bovine sodium caseinate fermented by *Streptococcus thermophiles* 4F44 strain (Chang et al., 2014).