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Untreated and Enzyme-Modified Bovine Whey Products Reduce Association of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and *Cronobacter malonaticus* (formerly *Enterobacter sakazakii*) to CaCo-2 Cells

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Abstract

Aims: Adhesion of a microorganism to a cell surface is often considered to be the first step in pathogenesis. Inhibiting this process may have therapeutic effects *in vivo.* This study investigates the inhibitory effects of various bovine whey products on the association of *Salm. typhimurium*, *E. coli* O157:H7 and *C. malonaticus* (formerly *Enterobacter sakazakii*) to the human CaCo-2 cell line. Invasion of CaCo-2 cells by *Salm. typhimurium* and *C. malonaticus* was also examined.

Methods and Results: Infection assays were performed by incubating pathogenic bacteria with CaCo-2 cells in the presence of untreated (UT) or enzyme-modified (EM) whey products. Associated microorganisms were directly quantified by plate counts. Invasion of CaCo-2 cells by *Salm. typhimurium* and *C. malonaticus* in the presence / absence of test materials was also quantified using gentamicin protection assays. At a concentration of 40mg ml^{-1}, some UT whey products reduced association and invasion, but this effect was enhanced following hydrolysis with porcine pancreatic lipase.

Conclusions: Both UT and EM Sweet whey protein concentrates (WPCs) were found to be particularly effective inhibitors of association and invasion. All EM whey products significantly ($P<0.05$) inhibited invasion of *C. malonaticus* into epithelial cells, causing a 2-log reduction in the quantity of these microorganisms internalised.

Significance and Impact of the Study: The present study suggests that whey products can inhibit association to and invasion of CaCo-2 cells by selected microorganisms, and may be useful in the treatment and/or prevention of foodborne infections.

Introduction

Food consumed by humans is rarely sterile. Microorganisms present in food can lead to spoilage and/ or foodborne illness, with the latter causing millions of cases of infection and in some cases even death every year (Meng and Doyle, 1998). *Salmonella typhimurium* and *Escherichia coli* O157:H7 (enterohaemorrhagic *E. coli*, EHEC) have been recognised as pathogens for many years, but it is only in the last 30 years that these bacteria have been considered to be predominantly foodborne (Meng and Doyle, 1998). Salmonellosis is a zoonotic infection, with infected animals being a major source of illness (Bezirtzoglou *et al*., 2000), and infection in humans is often due to consumption of undercooked poultry, eggs or egg-containing foods (Rodrigue *et al*., 1990). *Salm. typhimurium* is considered to be one of the most common causes of salmonellosis worldwide (WHO, 2005). *E. coli* is an inhabitant of the gut (both humans and animals), but some strains are pathogenic (Bezirtzoglou *et al*., 2000). *E. coli* O157:H7 is a clinically important food pathogen which can cause haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Gu *et al*., 2008). EHEC has an extremely low infectious dose, and it is estimated that as few as 100 cells is adequate to cause infection (Kaper *et al*., 2004). *Enterobacter sakazakii* was listed as a new species in 1980, but a taxonomic reclassification of this microorganism has been proposed, as *E. sakazakii* has been found to consist of five species within a new genus now referred to as 'Cronobacter' (Iversen *et al*., 2007). *Cronobacter malonaticus* is one such subspecies. This bacterium is described as an emerging opportunistic pathogen, which can cause local necrotising enterocolitis, systemic bacteremia and meningitis (Kim and Loessner, 2008). *Cronobacter* spp. has been isolated from milk powder, cheese, sausage meat, vegetables, bread, herbs and spices (Mullane *et al.*, 2007, Gurtler *et al*., 2005 and Kandhai *et al*., 2004), but powdered infant formula

(PIF) is considered to be a major vehicle of transmission, with neonates being most at risk of infection (Kim and Loessner, 2008).

Once ingested, microorganisms present in contaminated foods can adhere to the host's cell surfaces. Adhesion of a microbe to a cell surface is considered to be the first step of pathogenesis (Finlay and Falkow, 1997). Lectins on the surface of bacteria adhere to specific receptors on epithelial cells of the intestinal tract (Nakajima *et al*., 2005). Some pathogenic microorganisms are capable of entering and surviving within epithelial cells following initial adherence, a process known as invasion (Finlay and Falkow, 1997). Cultured eukaryotic cell lines are a common method employed in the study of bacterial adherence and invasion as they provide researchers with reproducible and less complicated infection models (Tang *et al*., 1993). One of the most extensively used is the CaCo-2 cell line, which was isolated from human colon carcinoma (Fogh *et al*., 1977). These cells, under standard culture conditions, differentiate to produce monolayers expressing characteristics of mature enterocytes (Pinto *et al*., 1983).

Blocking the initial adherence of foodborne pathogens to intestinal epithelial cells may be a suitable approach to preventing occurrence of infections (Nakajima *et al*., 2005). Food components capable of inhibiting initial adherence are promising agents of intervention. Several carbohydrate components of food have exhibited a positive effect against intestinal infection (Nakajima *et al*., 2005), such as sialylated oligosaccharides from bovine or human milks (Sugitu-Konishi *et al*., 2002). Recently, whey and dairy products have been shown to reduce the adherence of the dental-caries causing bacterium *Streptococcus mutans* to hydroxylapatite, an analogue of tooth enamel (Halpin *et al.*, 2008). Whey was once considered to be a waste-product of the cheese-making process, but in recent years has had its status upgraded

to co-product and is described as a ‘functional food’ (Marshall, 2004). Many studies have shown enzymatic hydrolysis of whey proteins produces a plethora of peptides, exhibiting a wide array of bioactive properties (Meisel, 1998).

The objective of this study was to examine the influence of a variety of whey products on the interaction of *Salm. typhimurium*, *E. coli* O157:H7 and *Cronobacter malonaticus* with CaCo-2 cells. The effect of pre-treating the whey products with PPL on any such influence was also examined.

Materials and Methods

Source and Analysis of Dairy Powders

Sweet whey protein concentrate (WPC, 80% protein), acid WPC 80 (AWPC80), sweet WPC 35 (SWPC35), whey protein isolate (WPI), whey powder (WP) and demineralised whey (DW) powders were supplied by Carbery Milk Products (Ballineen, Cork, Ireland). Albumin from chicken egg white (grade V) was supplied by Sigma (Poole, Dorset, UK).

Compositional analysis was performed on each whey product using standard methods. Ash content was determined according to Malkomesius & Nehring (1951). Fat content was determined according to the method of Röse-Gottlieb (International Dairy Federation (IDF) 1987), protein content was determined by the Kjeldahl method (IDF, 1993) and the moisture content was determined by oven drying (IDF, 1993).

Hydrolysis conditions

Crude porcine pancreatic lipase (PPL, Sigma, Poole, Dorset, England)) containing 100-400 units/ mg protein was used throughout the study. Hydrolysates were prepared in a Fermac 200 fermentor (Electrolab Ltd, Tewkesbury, UK) as follows: a c. 2% (w/v) solution of substrate was prepared by dissolving 20g of whey product in 900ml of sterile distilled water and heating to 37˚ C with stirring for 30mins. Lipase solution

(1g of PPL in 100ml of sterile H_20) was added to the substrate solution to give a final incubation volume of 1 L. The hydrolysates were then incubated for 2 h at 37˚C with stirring. Following this, hydrolysates were heated at 60˚C for 10 min in order to denature the enzyme(s). Each hydrolysate was placed on ice and allowed to cool to below 10˚C (approx. 45 min), before being frozen using liquid nitrogen and subsequently lyophilised (Moduloyo, Edwards High Vacuum, Manor Royal, Crawley, Sussex, UK).

Bacteria and Growth Conditions

Salm. typhimurium (ATCC 14028) and *E. coli* O157:H7 (ATCC 43888) were obtained from the American Type Culture Collection (Rockville, MD, USA), and *C. malonaticus* (DSM 18702) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Primary cultures were grown overnight in 10ml of Luria-Bertani (LB) broth (Sigma, Poole, Dorset, UK) at 37°C. A 1% inoculum was prepared by adding 400µl of overnight culture to 39.6ml of fresh pre-warmed LB broth before re-incubating at 37°C. *Salm. typhimurium* and *E. coli* O157:H7 were grown to mid-log phase. *C. malonaticus* was grown to late-log phase, as it has been recently reported that adherence of *E. sakazakii* is at it's maximum at this stage of growth, after at least 4 h of culturing (Mange *et al.*, 2006). Bacteria were then collected by centrifugation at 3220 × g (Eppendorf 5810R, Cambridge, UK) for 10 min and were resuspended in supplement-free Dubleco's Modified Eagle's Medium (DMEM, Gibco), so that the final concentration of bacteria was approx. 10^8 cells per millilitre.

CaCo-2 Cell Culture

CaCo-2 cells were obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). At late confluency these cells express both structural and functional

characteristics of enterocytes present in the small intestine (Hendricks *et al.*, 1996). Cells were routinely cultured in DMEM supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% non-essential amino acids 100X, 1% penicillin/streptomycin solution and 1% fungizone containing 250μg/ml amphotericin B. All supplements were supplied by Gibco. The cells were initially grown in T75cm^2 flasks (Sarstedt, Nümbrecht, Germany) and upon confluency (approx. 1.5 $\times 10^6$ cells/ml, 7-10 days) were passaged using 0.25% trypsin (Gibco). For infection assays, monolayers were cultured in 24-well tissue culture plates (Sarstedt, Nümbrecht, Germany). CaCo-2 cells were seeded at a density of 5×10^5 cells/well, and growth medium was changed every other day. These cells are known to be fully differentiated after being cultured for 19 days (Koninkx, 1995). Maintenance of cells and subsequent experiments were carried out at 37°C in a 5% CO_2-95% air atmosphere (Binder Apt Line C150, Tuttlingen, Germany), between passage number 37 and 55, with c. 10^6 CaCo-2 cells per well.

Viabilty of Bacterial and Epithelial Cells

The trypan blue (Sigma, Poole, Dorset, UK) dye exclusion test was used to determine if test materials affected viability of CaCo-2 cells. Test materials were prepared in supplement-free DMEM (SFM) and added to wells containing monolayers, followed by incubation for 1 hour at 37°C and 5% CO_2. The monolayers were then washed twice with SFM, before being trypsinised and added to 0.4% trypan blue (1:1). An inverted light microscope (Ceti, Belgium) was used to examine cells to determine viability. Viable epithelial cells exclude trypan blue while dead cells allow entry, and appear blue when viewed under the microscope. The percentage viability was calculated as follows:

100-(Number of dead cells/ Total Number of cells × 100)

Viability of bacteria was determined by direct contact studies, where each microorganism was incubated with test materials under typical assay conditions and subsequently enumerated by spread plates following appropriate dilution. As whey products are not sterile, selective agars were used to quantify the number of pathogenic bacteria (i.e. to eliminate any 'background count' due to microorganisms such as lactobacilli). Brilliant green, McConkey and Chromogenic *Enterobacter sakazakii* agars (DFI formulation) (Oxoid, Hampshire, UK) were used to selectively cultivate *Salm. typhimurium, E. coli* O157:H7 and *Cronobacter malonaticus*, respectively.

Infection Assays

Prior to infection assays, CaCo-2 cells were washed twice in sterile phosphate-buffered saline (PBS, Sigma, Poole, Dorset, UK) to remove traces of antibiotic, and equilibrated in SFM at 37°C and 5% CO_2 for at least 2 h. (i) Association and (ii) invasion of pathogenic bacteria were examined as follows:

(i) Quantification of Association

Aliquots (500µl) of test material dispersed in SFM (80mg ml^{-1}) or 500µl of SFM (to act as a negative control) were added to each well, followed by 500µl of pathogens suspended in SFM (c. 10^8 CFU ml^{-1}). The final concentration of test material was 40mg ml^{-1}. Monolayers were challenged in triplicate wells at a multiplicity of infection (MOI) of 100:1 (bacteria: epithelial cells). The plates were then incubated at 37°C and 5% CO_2 for 1 h. Following this, the monolayers were washed twice with SFM in order to remove non-adhered and loosely adhered bacteria**.** Cells were overlayed with SFM and further incubated for 30min at 37˚C and 5% CO_2. Monolayers containing associated (i.e. adhered and invaded) bacteria were lysed (in order to liberate the microorganisms) with a 1ml volume of 1% triton-X-100 (Sigma,

Poole, Dorset, UK) prepared in sterile PBS, for 5min at room temperature. This 1ml volume was serially diluted in PBS and spread plates were prepared using selective agars.

(ii) Quantification of Invasion

CaCo-2 cells were treated as previously described for the association assay. After the non-adherent bacteria had been removed by washing with SFM, CaCo-2 cells were treated with gentamicin (Gibco) in order to quantify invasion. Gentamicin (an antibiotic) does not diffuse into CaCo-2 cells, so any externally adhered bacteria are rapidly killed but the viability of any invaded bacteria is not affected. Briefly, gentamicin was prepared to a concentration of 50μg ml^{-1} in SFM and added to wells, and monolayers were again incubated for 30min at 37°C and 5% CO_2, before being washed twice with PBS to remove excess antibiotic. Epithelial cells were then lysed in order to liberate invaded pathogens and spread plates prepared as described earlier. The quantity of associated/ invaded pathogenic bacteria in SFM was assigned to 100%. Thus, percentage association/ invasion was expressed relative to the control (in the absence of test material) as follows: (Number of bacteria associated or invaded in presence of test material/ Number of bacteria associated or invaded in presence of DMEM alone) × 100

Mechanism of Inhibition Assays

In an attempt to determine if test material interacted with either epithelial cells or bacteria or both, CaCo-2 cells and bacteria were separately pre-incubated with test material for 1 h at 37˚C and 5% CO_2 prior to performing infection assays as described earlier.

Statistical Analysis

Experiments were carried out using three bacterial cultures (n=3) for each treatment. Results were expressed as the mean ± standard deviation (S.D.). Differences between inhibitory effects of each treatment were determined using the general linear models (GLM) function of SAS Version 9.1.3. Data were considered significantly different if $P<0.05$.

Results

The compositional analysis of each test material was determined, along with the pH value of each whey product in its untreated and enzyme-modified form (Table 1). Test materials were dispersed in DMEM at a concentration of 40mg ml^{-1} prior to measuring pH, and the pH values of DMEM alone and egg albumin were 7.1 and 7.08, respectively. Enzyme-treatment was found to lower the pH of all whey products. Growth curves for each microorganism are shown in Figure 1. Test materials were not found to reduce viability of either CaCo-2 cells or bacteria at a concentration of 40mg ml^{-1} (data not shown).

(i) Salm. typhimurium

In the absence of test material, *Salm. typhimurium* associated to and invaded CaCo-2 cells at levels of 10^7 and 10^6 CFU ml^{-1}/well, respectively, and this is represented by the DMEM bars in Figure 2(a) and (b) as 100% association/ invasion. Of the untreated materials tested, all but AWPC80, DW and the protein control, egg albumin, significantly reduced association of *Salm. typhimurium* to CaCo-2 cells ($P<0.05$). UT SWPC80 was significantly more effective than the other materials, reducing association by c. 60% ($P<0.05$). With one exception (WP), pre-treatment of these whey products with PPL increased their ability to subsequently reduce *Salm. typhimurium* association, although the reduction in association brought about by this treatment was not always significant (Figure 2(a)).

In the invasion assays, of the untreated products examined, only SWPC80 and SWPC35 and to a lesser extent WPI reduced invasion significantly (P<0.05). UT AWPC80 appeared to enhance invasion of *Salm. typhimurium* into CaCo-2 cells (Figure 2(b)). All EM whey products were significant inhibitors of invasion (P<0.05), with EM-SWPC35 showing the greatest reduction (c. 75%). EM-SWPC80, EM-AWPC80, EM-WPI and EM-WP exhibited similar levels of activity, reducing invasion of this microorganism into CaCo-2 cells by 40-50%. EM-DW was the least effective of the hydrolysates, but still reduced invasion by 26%.

(ii) *E. coli* O157:H7

Under the experimental conditions described here, approximately 10^6 CFU ml^{-1}/well of *E. coli* O157:H7 cells associated to CaCo-2 monolayers in the absence of test material (representing 100% association, Figure 3). The presence of all UT materials with the exception of DW significantly reduced association of *E. coli* O157:H7 with CaCo-2 cells, as observed previously (P<0.05). UT SWPC80 was again the most potent in this regard, reducing association by c. 60%. Interestingly, UT AWPC80 which did not reduce association of *Salm. typhimurium* inhibited association of *E. coli* O157:H7 with CaCo-2 cells.

Pre-treatment with PPL did not significantly increase the ability of the materials to reduce association (P>0.05). An exception in this regard was EM-AWPC80, which showed the greatest reduction in association (>60% inhibition) of the materials studied. Also it should be noted that egg albumin, the protein control, was an effective inhibitor of association of *E. coli* O157:H7 with CaCo-2 cells, being only significantly less effective than EM-AWPC80 (P<0.05).

Invasion of this microorganism in the presence of the test materials was not examined here, as it has been reported that *E. coli* O157:H7 does not invade into all cell lines,

but this does not necessarily mean that this bacterium is tissue culture non-invasive (Oelschlaeger *et al*., 1994).

(iii) C. malonaticus

This microorganism showed similar levels of association with and invasion of CaCo-2 cells as those observed for *Salm. typhimurium* (10^7 and 10^6, respectively). Again these values represent 100% association or invasion (Figure 4(a) and (b), respectively). UT AWPC80 increased association of *C. malonaticus* to CaCo-2 cells by approx. 20% (Figure 4(a)). No significant differences were noted between the potency of UT SWPC80, UT SWPC35, UT WPI and UT WP, but each of these whey products were found to be more effective than DMEM alone ($P<0.05$), causing a 35-40% reduction in association. For the enzyme-modified products, EM-SWPC80, EM-SWPC35 and EM-WPI were the most effective test materials, reducing association by 35-45%. EM-AWPC80, EM-WP and EM-DW had no significant effect on association of this bacterium to monolayers ($P>0.05$). Egg albumin was found to significantly increase association of *C. malonaticus* to the CaCo-2 cell line, and showed least inhibitory activity when compared to untreated and enzyme-modified whey products ($P<0.05$).

For the invasion assays, UT AWPC80 appeared to increase invasion of *C. malonaticus* into epithelial cells (Figure 4(b)). Of the untreated whey products, the most effective inhibitors of invasion were UT WP and UT WPI, which showed greater reductions than UT SWPC80 and UT SWPC35 ($P <0.05$). UT DW had no significant ($P>0.05$) effect on invasion. However, all enzyme-modified whey products greatly reduced invasion of *C. malonaticus* into CaCo-2 cells, causing a 2-log reduction when compared to DMEM alone ($10^6 \rightarrow 10^4$ CFU ml^{-1}/well). EM-WP and EM-DW were slightly less effective than other test materials, nevertheless causing a

reduction of 92 and 88%, respectively (Figure 4(b)). Egg albumin, the protein control, neither increased nor reduced invasion of this microorganism into CaCo-2 cells.

(iv) Mechanism of Action

EM-SWPC80 was the test material chosen for mechanistic experiments, as this whey product was found to be a very effective inhibitor of association of *Salm. typhimurium*, *E. coli* O157:H7 and *C. malonaticus* to CaCo-2 cells. A reduction in association was observed for these microorganisms when the bacteria and test material were added simultaneously to the test wells. However, with the exception of *C. malonaticus*, pre-treatment of the microorganisms with EM-SWPC80 generally did not reduce association, nor did pre-treatment of the epithelial cells (Table 2).

Discussion

Adherence to the host's cell surface is a vital step in pathogenesis of gastrointestinal tract (GIT) infection as it prevents microorganisms from being swept away by bulk fluid movement. Once a microbe adheres, it can readily access nutrients, while being able to deliver toxins into host tissues before eventually penetrating these tissues (Acord *et al.*, 2005). The aim of anti-adhesion therapy is essentially to reduce contact between pathogens and host tissues, by preventing or reversing adherence. Currently, some of the most efficient known inhibitors of adhesion are found in foodstuffs (Ofek *et al.*, 2003). In the present study, the effect of native and enzyme-modified whey products on association of *Salm. typhimurium*, *E. coli* O157:H7 and *C. malonaticus* to human CaCo-2 cells was examined, along with their effects on the invasion of such cells by *Salm. typhimurium* and *C. malonaticus*. Whey is currently described as a 'functional food', as it possesses a wide array of health benefits, and undenatured whey can provide high concentrations of intact proteins such as lactoferrin and immunoglobulins (Marshall, 2004). The results

described here indicate that, with the exception of AWPC80 and DW, most UT whey products were effective inhibitors of both association and invasion. However, the activity of these whey products was enhanced following hydrolysis with PPL, with EM-SWPC80 and EM-SWPC35 causing particularly high reductions.

Each whey product has varying levels of fat, protein, moisture, ash and lactose (Table 1). Sweet and acid WPCs have similar levels of protein and fat etc., yet the sweet WPCs were found to have greater anti-adhesion/ invasion activity. WPI has almost no fat, yet still exhibited significant anti-adhesive and anti-invasion activity. WP and DW contain less protein and fat than the WPCs and WPI, but have a high content of lactose. In a report by Coppa *et al*. (2006), lactose from human milk (concentration not given) was not found to inhibit association of *E.coli* (serotype O119) or *Salm. fyris* to CaCo-2 cells. In the present study, inhibition of association and invasion of pathogenic bacteria by the whey products was generally more potent than that of egg albumin, suggesting the activity is not due to a non-specific protein effect. Whey proteins are recognised as having superior biological activity to other proteins, and the activity of peptides encrypted within such proteins usually remains latent until they are subjected to proteolytic action of enzymes (Sinha *et al*., 2007). The crude PPL used in the present study is known to contain both proteases and lipases, and it may be that enzyme pre-treatment of the whey products listed here releases species such as specific peptides or free fatty acids that are latent within the untreated material. The observation that potency of these materials was not diminished after enzyme-treatment may indicate that the active components could survive passage through the gut. This would be a favourable characteristic for using whey products to prevent/ treat infection of the GIT.

One potential hypothesis as to why high levels of inhibition were observed for sweet WPCs may be because of the presence of glycomacropeptide (GMP). GMP, also referred to as the 'casein macropeptide' is present in sweet (rennet) whey as a result of the action of the chymosin enzyme on κ-casein during the cheese-making process (Marshall, 2004). This peptide is known to inhibit bacterial and viral adhesion (Kawasaki *et al*., 1993, Simon, 1996), and is capable of binding to *E. coli* O157:H7 and *Salmonella enteridis* when in its sialylated form (Nakajima *et al*., 2005). In a study by Bruck *et al*. (2006), the effect of GMP on the association of enteropathogenic *E. coli* (EPEC) and *Salm. typhimurium* to CaCo-2 cells was examined, and they found that undigested GMP (0.25mg ml^{-1}) reduced association of these bacteria. They also reported that enzyme digestion with pepsin and pancreatin increased the anti-adhesive activity of GMP. It was concluded that fragments produced by digestion with both pancreatin and pepsin digestion of GMP were most potent for *Salm. typhimurium*, but peptides liberated from digestion with pepsin alone significantly reduced association levels of EPEC (P<0.05-0.001). However, isolated and purified GMP is an expensive resource. Our study has shown that native sweet WPC products, which are inexpensive and readily available, are effective inhibitors of bacterial association and invasion. Enzyme-modifying such material with PPL is also a relatively inexpensive and straightforward process, and enhances the anti-adhesive and anti-invasive activity of sweet WPCs.

In a study by de Araujo and Giugliano (2000), it was reported that whey produced from human milk (0.97mg ml^{-1}) inhibited association of diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) to HeLa cells by 9% and 16%, respectively. In a subsequent study by the same researcher in 2001, the free secretory component (105μg ml^{-1}) and lactoferrin (157μg ml^{-1}) isolated from human milk,

386 inhibited adherence of EPEC to HeLa cells by 32% and 4.5%, respectively. The
387 immunoglobulin fraction was also found to reduce adherence of these bacteria to
388 HeLa cells (de Araujo and Giugliano, 2001). da Motta Willer and co-workers (2004)
389 found that whey from human milk (2.8mg ml^{-1}) could reduce adhesion of *Shigella*
390 strains to HeLa cells by at least 40%, and invasion was reduced by more than 50%. It
391 was also reported that human lactoferrin (0.3 mg ml^{-1}) reduced invasion by 50- 65%
392 (da Motta Willer *et al*., 2004). It is possible that the equivalent components of bovine
393 milk/ whey could inhibit association and/ or invasion of pathogens to epithelial cells.
394 Non-protein components of whey include oligosaccharides and lipids,
395 including sphingolipids (Shoaf *et al*., 2006). Certain oligasaccharides are similar in
396 structure to receptor sites (recognised and adhered to by pathogens) coating epithelial
397 cells of the intestine. Thus, oligosaccharides possibly act as molecular receptor
398 decoys, competitively inhibiting microbial adherence. Conversely, instead of
399 pathogens adhering to the host cell surfaces, they would bind to the decoy
400 oligosaccharides and be displaced from the GIT (Shoaf *et al.*, 2006). Oligosaccharides
401 present in the whey products used in our study may be contributing to the anti-
402 adhesive activities of these materials. Another characteristic of the test materials used
403 in this study which should be taken into consideration are the pH values of individual
404 products when in solution. Treatment with PPL reduced the pH of all test materials,
405 due to liberation of free fatty acids and amino acids during-hydrolysis. It is possible
406 that the fimbrial protein structures of pathogenic microorganisms are influenced by
407 different pH values (close to their pI values), reducing the capability of bacteria to
408 adhere to epithelial cells (Lehto and Salminen, 1997). This may be a contributing
409 factor in the reduction in association of *Salm. typhimurium*, *E. coli* O157:H7 and *C.*
410 *malonaticus* to CaCo-2 cells in the presence of whey products, which was observed in

411 the results reported here. Preliminary results have shown that inhibition of association
412 is greatest when EM-SWPC80 and bacteria are added simultaneously to monolayers
413 (Table 2). However, whether this inhibitory effect is due to proteins, fats, pH,
414 oligosaccharides, immunoglobulins, lactobacilli etc. present in whey or a combination
415 of these is not yet clear and the exact mechanism of action of these products to
416 prevent association / invasion of pathogenic bacteria to epithelial cells remains to be
417 elucidated, but it is not unreasonable to speculate that the inhibitory effect is because
418 of the presence of GMP.

419 Although CaCo-2 cells are regarded as one of the best in vitro models of
420 mature enterocytes, such cell systems have their limitations (Giannasca *et al*., 1996),
421 such as the absence of host factors (e.g. mucus barriers, immune factors). In addition,
422 cell culture models do not possess other host cells that would normally be present in
423 vivo e.g. inflammatory cells (Finlay and Falkow, 1997). Nevertheless, the results of
424 the present study are positive from a cell culture perspective.

425 Individuals most at risk from foodborne infection include newborns, elderly
426 people and those who are immunocompromised (Sprong *et al.*, 1999). Treatments for
427 infection due to foodborne pathogens such as those caused by *E. coli* O157:H7 is for
428 the most part supportive, as practitioners are reluctant to prescribe antibiotics due to
429 the risk of complications, such as acute renal failure in patients with HUS (Meng and
430 Doyle, 1998). Also, the emergence of multi-drug resistant strains of *Salm.*
431 *typhimurium* has led to a limited number of treatment options, in particular for
432 invasive infections (Meng and Doyle, 1998). Thus, the use of antibiotics to treat
433 foodborne illness is no longer desirable due to complications which are likely to
434 occur. These include incidences of drug resistant strains and the potential for chronic
435 toxicity (Lin *et al.*, 2007). As a result, alternative approaches to preventing infections

of the GIT have been sought. Whey products, in either their untreated or enzyme-modified form may be suitable alternatives to treat or preferably prevent illness due to foodborne pathogens.

Conclusion

Both untreated and enzyme-modified whey products are effective inhibitors of association of *Salm. typhimurium*, *E. coli* O157:H7 and *C. malonaticus* to CaCo-2 cells and may be suitable for in vivo use to prevent and/ or treat GIT infection due to foodborne pathogens.

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Legends for figures:

Figure 1: Growth curves of *Salm. typhimurium* (●), *E. coli* O157:H7 (■) and *C. malonaticus* (▲) in LB broth.

Figure 2: The Effect of DMEM (▩), Untreated Whey Products (□), Enzyme-Modified Whey Products (■) and Egg Albumin* on the (a) Association and (b) Invasion of *Salm. typhimurium* to/ into CaCo-2 cells. (Data= mean ± S.D., n=3)

* Egg Albumin is included in this figure for comparison only

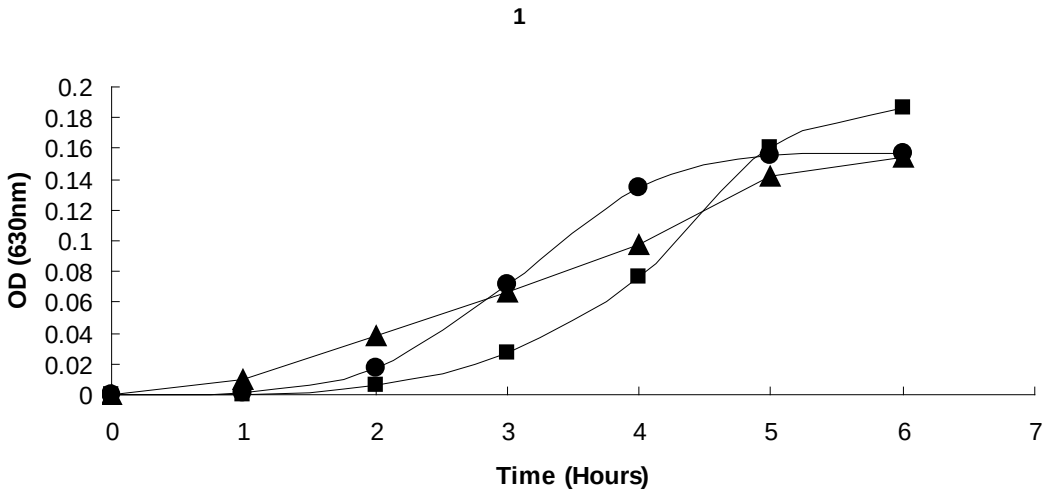
Figure 3: The Effect of DMEM (▩), Untreated Whey Products (□), Enzyme-Modified Whey Products (■) and Egg Albumin* on the Association of *E. coli* O157:H7 to CaCo-2 cells. (Data= mean ± S.D., n=3)

* Egg Albumin is included in this figure for comparison only

Figure 4: The Effect of DMEM (▩), Untreated Whey Products (□), Enzyme-Modified Whey Products (■) and Egg Albumin* on the (a) Association and (b) Invasion of *C. malonaticus* to/ into CaCo-2 cells. (Data= mean ± S.D., n=3)

* Egg Albumin is included in this figure for comparison only

Figure 1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0.14
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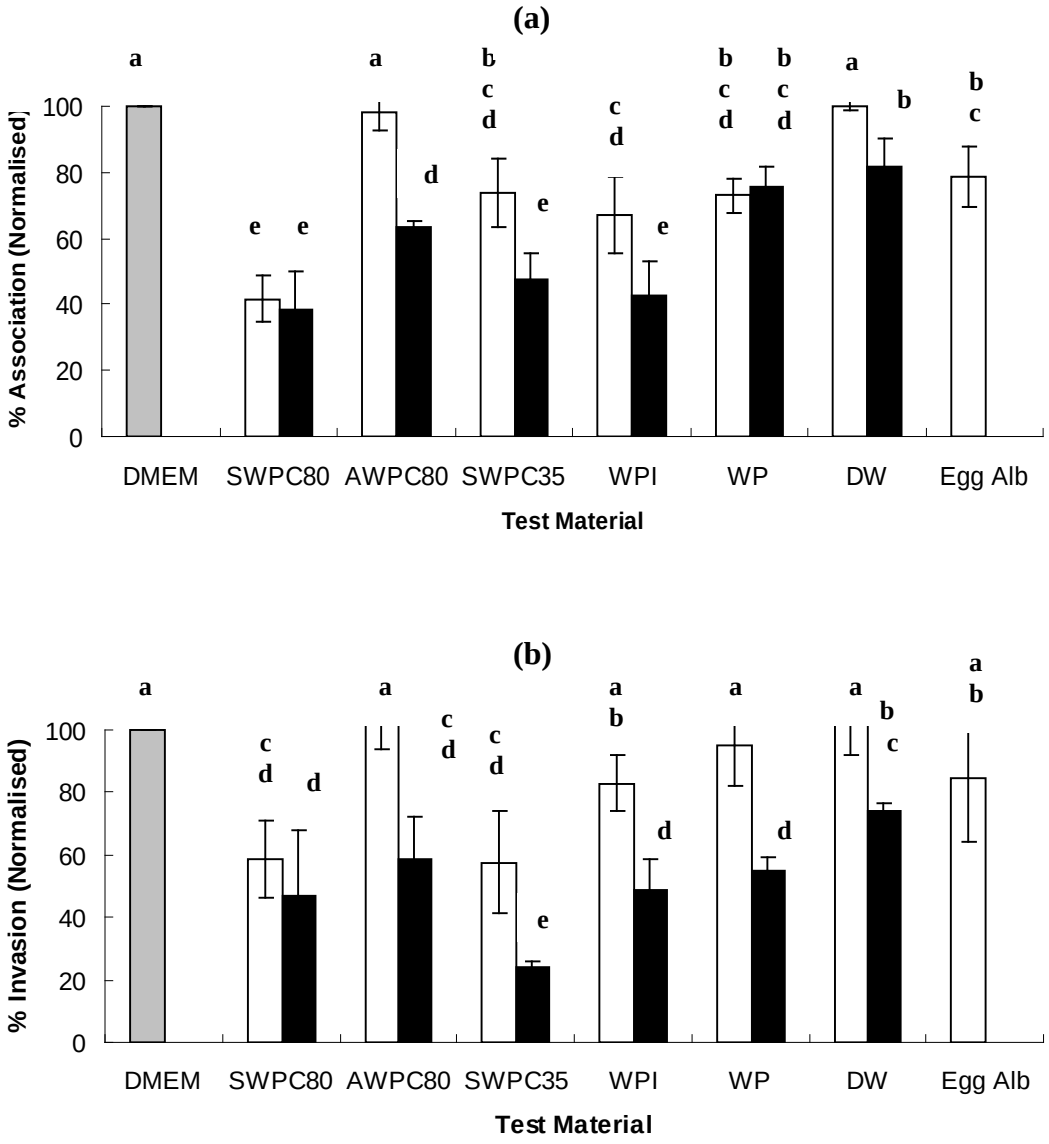
Time (Hours)

Figure 2:



Notes: All test materials were used at a concentration of 40mg mL^{-1}. Means with the same letter are not significantly different (at the 5% significance level).

Abbreviations: DMEM= Dubleco's Modified Eagle's Medium, SWPC80= Sweet Whey Protein Concentrate 80, AWPC80= Acid WPC80, SWPC35= Sweet Whey Protein Concentrate 35, WPI= Whey Protein Isolate, WP= Whey Powder, DW= Demineralised Whey, Egg Alb= Egg Albumin.

Figure 3:

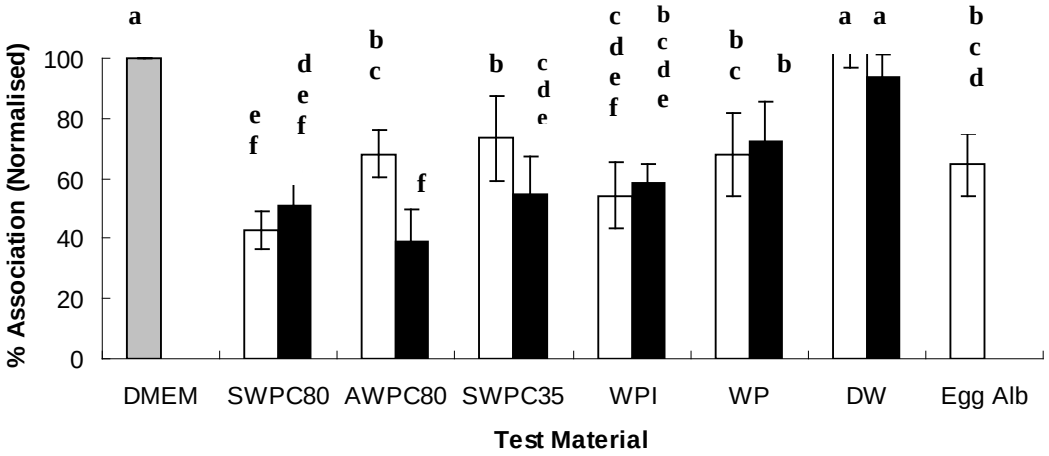


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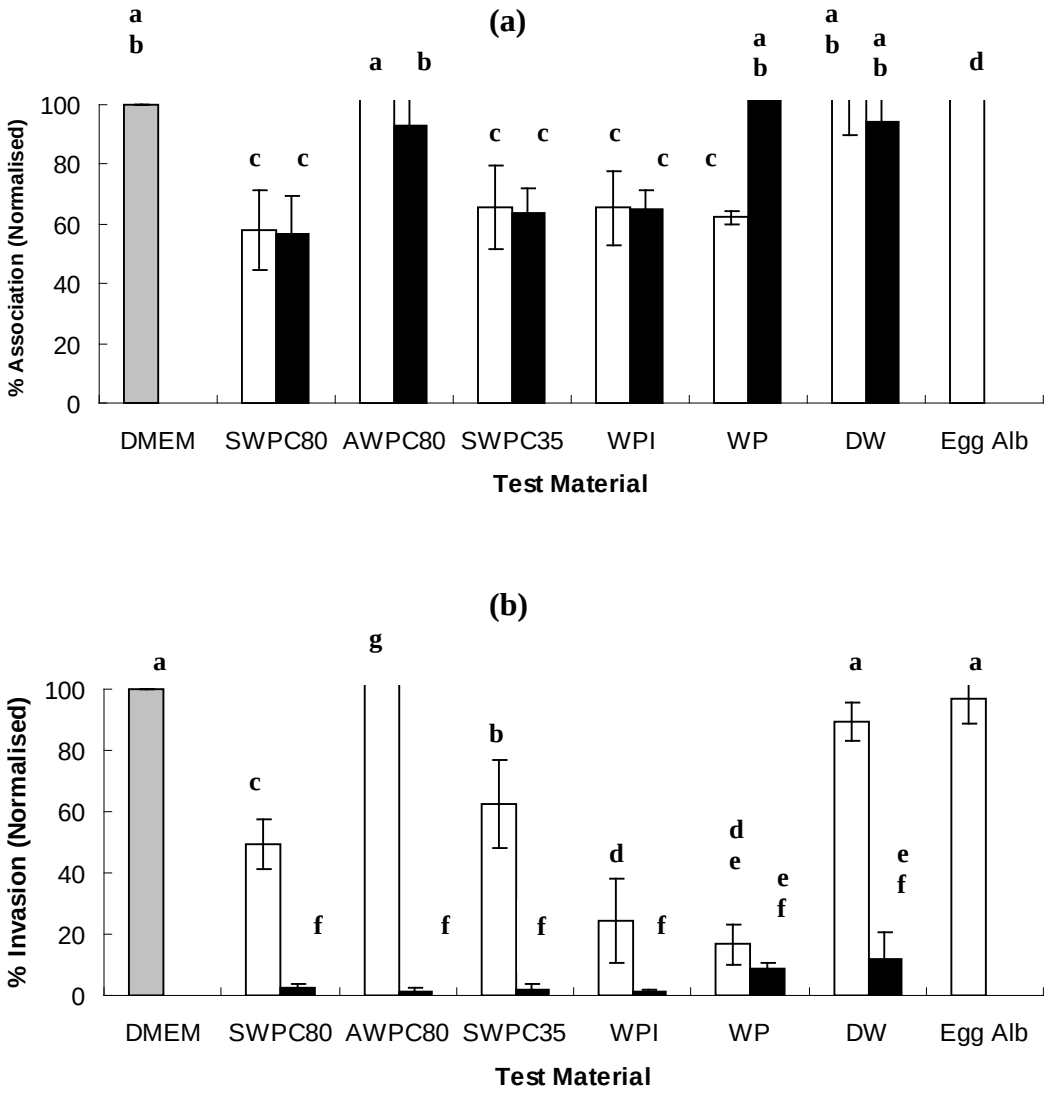
Figure 4:



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672 **Table 1:** Compositional analysis of whey products used in this study (%) and pH
673 values of test materials before and after treatment with porcine pancreatic lipase.

	SWPC80	AWPC80	SWPC35	WPI	WP	DW
Protein	75.5	78.2	34.3	86.6	12.5	13
Fat	8	7.7	3.4	0.1	1	1.8
Moisture	7.5	6.3	5.4	5.8	3.1	3.5
Ash	3	5.9	6.2	2.6	9.5	0.8
Lactose	6	1.9	50.7	4.9	73.9	80.9
pH UT	7.16	7.26	7.22	7.11	7.17	7.22
pH EM	6.48	6.56	6.58	6.56	7.01	6.97

674
675 Abbreviations: SWPC80= Sweet Whey Protein Concentrate 80, AWPC80= Acid
676 WPC80, SWPC35= Sweet Whey Protein Concentrate 35, WPI= Whey Protein Isolate,
677 WP= Whey Powder, DW= Demineralised Whey.
678 UT= untreated, EM= enzyme-modified with porcine pancreatic lipase.
679
680 **Table 2:** Levels of Association (%) of *S. typhimurium*, *E. coli* O157:H7 and *C.*
681 *malonaticus* to CaCo-2 Cells in the presence of EM- SWPC80 (40mg ml^{-1}) under
682 Varying Assay Conditions.

	S. typhimurium	***E. coli* O157:H7**	***C. malonaticus***
(a) EM-SWPC80 and bacteria added simultaneously	(i) 33.3 (ii) 44.1 (iii) 61.8	(i) 50.8 (ii) 67.5 (iii) 73.7	(i) 62.2 (ii) 43.6
(b) Bacteria pre-treated with EM-SWPC80	(i) No reduction (ii) No reduction (iii) No reduction	(i) 93.2 (ii) No reduction (iii) 71.9	(i) 58.1 (ii) 39.2
(c)CaCo-2 cells pre-treated with EM-SWPC80	(i) No reduction (ii) No reduction (iii) No reduction	(i) 88.5 (ii) No reduction (iii) No reduction	(i) No reduction (ii) No reduction

683 Abbreviations: EM-SWPC80= Enzyme-modified Sweet Whey Protein Concentrate 80
684 Association of bacteria in DMEM alone was assigned to 100%