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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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39 **Abstract**

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

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

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

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

62

63

64 **Introduction**

65 Food consumed by humans is rarely sterile. Microorganisms present in
66 food can lead to spoilage and/ or foodborne illness, with the latter causing millions of
67 cases of infection and in some cases even death every year (Meng and Doyle, 1998).
68 *Salmonella typhimurium* and *Escherichia coli* O157:H7 (enterohaemorrhagic *E. coli*,
69 EHEC) have been recognised as pathogens for many years, but it is only in the last 30
70 years that these bacteria have been considered to be predominantly foodborne (Meng
71 and Doyle, 1998). Salmonellosis is a zoonotic infection, with infected animals being a
72 major source of illness (Bezirtzoglou *et al*., 2000), and infection in humans is often
73 due to consumption of undercooked poultry, eggs or egg-containing foods (Rodrigue
74 *et al*., 1990). *Salm. typhimurium* is considered to be one of the most common causes
75 of salmonellosis worldwide (WHO, 2005). *E. coli* is an inhabitant of the gut (both
76 humans and animals), but some strains are pathogenic (Bezirtzoglou *et al*., 2000). *E.*
77 *coli* O157:H7 is a clinically important food pathogen which can cause haemorrhagic
78 colitis and haemolytic uremic syndrome (HUS) (Gu *et al*., 2008). EHEC has an
79 extremely low infectious dose, and it is estimated that as few as 100 cells is adequate
80 to cause infection (Kaper *et al*., 2004). *Enterobacter sakazakii* was listed as a new
81 species in 1980, but a taxonomic reclassification of this microorganism has been
82 proposed, as *E. sakazakii* has been found to consist of five species within a new genus
83 now referred to as 'Cronobacter' (Iversen *et al*., 2007). *Cronobacter malonaticus* is
84 one such subspecies. This bacterium is described as an emerging opportunistic
85 pathogen, which can cause local necrotising enterocolitis, systemic bacteremia and
86 meningitis (Kim and Loessner, 2008). *Cronobacter* spp. has been isolated from milk
87 powder, cheese, sausage meat, vegetables, bread, herbs and spices (Mullane *et al.*,
88 2007, Gurtler *et al*., 2005 and Kandhai *et al*., 2004), but powdered infant formula

89 (PIF) is considered to be a major vehicle of transmission, with neonates being most at
90 risk of infection (Kim and Loessner, 2008).
91 Once ingested, microorganisms present in contaminated foods can adhere
92 to the host's cell surfaces. Adhesion of a microbe to a cell surface is considered to be
93 the first step of pathogenesis (Finlay and Falkow, 1997). Lectins on the surface of
94 bacteria adhere to specific receptors on epithelial cells of the intestinal tract
95 (Nakajima *et al*., 2005). Some pathogenic microorganisms are capable of entering and
96 surviving within epithelial cells following initial adherence, a process known as
97 invasion (Finlay and Falkow, 1997). Cultured eukaryotic cell lines are a common
98 method employed in the study of bacterial adherence and invasion as they provide
99 researchers with reproducible and less complicated infection models (Tang *et al*.,
100 1993). One of the most extensively used is the CaCo-2 cell line, which was isolated
101 from human colon carcinoma (Fogh *et al*., 1977). These cells, under standard culture
102 conditions, differentiate to produce monolayers expressing characteristics of mature
103 enterocytes (Pinto *et al*., 1983).
104 Blocking the initial adherence of foodborne pathogens to intestinal
105 epithelial cells may be a suitable approach to preventing occurrence of infections
106 (Nakajima *et al*., 2005). Food components capable of inhibiting initial adherence are
107 promising agents of intervention. Several carbohydrate components of food have
108 exhibited a positive effect against intestinal infection (Nakajima *et al*., 2005), such as
109 sialylated oligosaccharides from bovine or human milks (Sugitu-Konishi *et al*., 2002).
110 Recently, whey and dairy products have been shown to reduce the adherence of the
111 dental-caries causing bacterium *Streptococcus mutans* to hydroxylapatite, an analogue
112 of tooth enamel (Halpin *et al.*, 2008). Whey was once considered to be a waste-
113 product of the cheese-making process, but in recent years has had its status upgraded

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

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

121 **Materials and Methods**

122 **Source and Analysis of Dairy Powders**

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

128 Compositional analysis was performed on each whey product using standard methods.
129 Ash content was determined according to Malkomesius & Nehring (1951). Fat
130 content was determined according to the method of Röse-Gottlieb (International Dairy
131 Federation (IDF) 1987), protein content was determined by the Kjeldahl method (IDF,
132 1993) and the moisture content was determined by oven drying (IDF, 1993).

133 **Hydrolysis conditions**

134 Crude porcine pancreatic lipase (PPL, Sigma, Poole, Dorset, England)) containing
135 100-400 units/ mg protein was used throughout the study. Hydrolysates were prepared
136 in a Fermac 200 fermentor (Electrolab Ltd, Tewkesbury, UK) as follows: a c. 2%
137 (w/v) solution of substrate was prepared by dissolving 20g of whey product in 900ml
138 of sterile distilled water and heating to 37˚ C with stirring for 30mins. Lipase solution

139 (1g of PPL in 100ml of sterile $H_20$) was added to the substrate solution to give a final
140 incubation volume of 1 L. The hydrolysates were then incubated for 2 h at 37˚C with
141 stirring. Following this, hydrolysates were heated at 60˚C for 10 min in order to
142 denature the enzyme(s). Each hydrolysate was placed on ice and allowed to cool to
143 below 10˚C (approx. 45 min), before being frozen using liquid nitrogen and
144 subsequently lyophilised (Moduloyo, Edwards High Vacuum, Manor Royal, Crawley,
145 Sussex, UK).

146 **Bacteria and Growth Conditions**

147 *Salm. typhimurium* (ATCC 14028) and *E. coli* O157:H7 (ATCC 43888) were
148 obtained from the American Type Culture Collection (Rockville, MD, USA), and *C.*
149 *malonaticus* (DSM 18702) was obtained from the German Collection of
150 Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Primary
151 cultures were grown overnight in 10ml of Luria-Bertani (LB) broth (Sigma, Poole,
152 Dorset, UK) at 37°C. A 1% inoculum was prepared by adding 400μl of overnight
153 culture to 39.6ml of fresh pre-warmed LB broth before re-incubating at 37°C. *Salm.*
154 *typhimurium* and *E. coli* O157:H7 were grown to mid-log phase. *C. malonaticus* was
155 grown to late-log phase, as it has been recently reported that adherence of *E. sakazakii*
156 is at it's maximum at this stage of growth, after at least 4 h of culturing (Mange *et al.*,
157 2006). Bacteria were then collected by centrifugation at 3220 × g (Eppendorf 5810R,
158 Cambridge, UK) for 10 min and were resuspended in supplement-free Dubleco's
159 Modified Eagle's Medium (DMEM, Gibco), so that the final concentration of bacteria
160 was approx. $10^8$ cells per millilitre.

161 **CaCo-2 Cell Culture**

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

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

178 **Viabilty of Bacterial and Epithelial Cells**

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

188 100-(Number of dead cells/ Total Number of cells × 100)

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

198 **Infection Assays**

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

203 **(i) Quantification of Association**

204 Aliquots (500μl) of test material dispersed in SFM (80mg $ml^{-1}$) or 500μl of SFM (to
205 act as a negative control) were added to each well, followed by 500μl of pathogens
206 suspended in SFM (c. $10^8$ CFU $ml^{-1}$). The final concentration of test material was
207 40mg $ml^{-1}$. Monolayers were challenged in triplicate wells at a multiplicity of
208 infection (MOI) of 100:1 (bacteria: epithelial cells). The plates were then incubated at
209 37°C and 5% $CO_2$ for 1 h. Following this, the monolayers were washed twice with
210 SFM in order to remove non-adhered and loosely adhered bacteria**.** Cells were
211 overlayed with SFM and further incubated for 30min at 37°C and 5% $CO_2$.
212 Monolayers containing associated (i.e. adhered and invaded) bacteria were lysed (in
213 order to liberate the microorganisms) with a 1ml volume of 1% triton-X-100 (Sigma,

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

217 **(ii) Quantification of Invasion**

218 CaCo-2 cells were treated as previously described for the association assay. After the
219 non-adherent bacteria had been removed by washing with SFM, CaCo-2 cells were
220 treated with gentamicin (Gibco) in order to quantify invasion. Gentamicin (an
221 antibiotic) does not diffuse into CaCo-2 cells, so any externally adhered bacteria are
222 rapidly killed but the viability of any invaded bacteria is not affected. Briefly,
223 gentamicin was prepared to a concentration of 50μg $ml^{-1}$ in SFM and added to wells,
224 and monolayers were again incubated for 30min at 37°C and 5% $CO_2$, before being
225 washed twice with PBS to remove excess antibiotic. Epithelial cells were then lysed
226 in order to liberate invaded pathogens and spread plates prepared as described earlier.
227 The quantity of associated/ invaded pathogenic bacteria in SFM was assigned to
228 100%. Thus, percentage association/ invasion was expressed relative to the control (in
229 the absence of test material) as follows: (Number of bacteria associated or invaded in
230 presence of test material/ Number of bacteria associated or invaded in presence of
231 DMEM alone) × 100

232 **Mechanism of Inhibition Assays**

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

237 **Statistical Analysis**

238 Experiments were carried out using three bacterial cultures (n=3) for each treatment.
239 Results were expressed as the mean ± standard deviation (S.D.). Differences between
240 inhibitory effects of each treatment were determined using the general linear models
241 (GLM) function of SAS Version 9.1.3. Data were considered significantly different if
242 $P<0.05$.

243 **Results**

244 The compositional analysis of each test material was determined, along with the pH
245 value of each whey product in its untreated and enzyme-modified form (Table 1). Test
246 materials were dispersed in DMEM at a concentration of 40mg $ml^{-1}$ prior to
247 measuring pH, and the pH values of DMEM alone and egg albumin were 7.1 and
248 7.08, respectively. Enzyme-treatment was found to lower the pH of all whey products.
249 Growth curves for each microorganism are shown in Figure 1. Test materials were not
250 found to reduce viability of either CaCo-2 cells or bacteria at a concentration of 40mg
251 $ml^{-1}$ (data not shown).

252 *(i) Salm. typhimurium*

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

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

271 *(ii)* *E. coli* O157:H7

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

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

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

288 but this does not necessarily mean that this bacterium is tissue culture non-invasive
289 (Oelschlaeger *et al*., 1994).
290 *(iii) C. malonaticus*
291 This microorganism showed similar levels of association with and invasion of CaCo-2
292 cells as those observed for *Salm. typhimurium* ($10^7$ and $10^6$, respectively). Again these
293 values represent 100% association or invasion (Figure 4(a) and (b), respectively). UT
294 AWPC80 increased association of *C. malonaticus* to CaCo-2 cells by approx. 20%
295 (Figure 4(a)). No significant differences were noted between the potency of UT
296 SWPC80, UT SWPC35, UT WPI and UT WP, but each of these whey products were
297 found to be more effective than DMEM alone ($P$<0.05), causing a 35-40% reduction
298 in association. For the enzyme-modified products, EM-SWPC80, EM-SWPC35 and
299 EM-WPI were the most effective test materials, reducing association by 35-45%. EM-
300 AWPC80, EM-WP and EM-DW had no significant effect on association of this
301 bacterium to monolayers ($P$>0.05). Egg albumin was found to significantly increase
302 association of *C. malonaticus* to the CaCo-2 cell line, and showed least inhibitory
303 activity when compared to untreated and enzyme-modified whey products ($P$<0.05).
304 For the invasion assays, UT AWPC80 appeared to increase invasion of *C.*
305 *malonaticus* into epithelial cells (Figure 4(b)). Of the untreated whey products, the
306 most effective inhibitors of invasion were UT WP and UT WPI, which showed
307 greater reductions than UT SWPC80 and UT SWPC35 ($P$ <0.05). UT DW had no
308 significant ($P$>0.05) effect on invasion. However, all enzyme-modified whey products
309 greatly reduced invasion of *C. malonaticus* into CaCo-2 cells, causing a 2-log
310 reduction when compared to DMEM alone ($10^6 \rightarrow 10^4$ CFU $ml^{-1}$/well). EM-WP and
311 EM-DW were slightly less effective than other test materials, nevertheless causing a

312 reduction of 92 and 88%, respectively (Figure 4(b)). Egg albumin, the protein control,
313 neither increased nor reduced invasion of this microorganism into CaCo-2 cells.
314 *(iv) Mechanism of Action*
315 EM-SWPC80 was the test material chosen for mechanistic experiments, as this whey
316 product was found to be a very effective inhibitor of association of *Salm.*
317 *typhimurium, E. coli* O157:H7 and *C. malonaticus* to CaCo-2 cells. A reduction in
318 association was observed for these microorganisms when the bacteria and test
319 material were added simultaneously to the test wells. However, with the exception of
320 *C. malonaticus*, pre-treatment of the microorganisms with EM-SWPC80 generally did
321 not reduce association, nor did pre-treatment of the epithelial cells (Table 2).
322 **Discussion**
323 Adherence to the host's cell surface is a vital step in pathogenesis of
324 gastrointestinal tract (GIT) infection as it prevents microorganisms from being swept
325 away by bulk fluid movement. Once a microbe adheres, it can readily access
326 nutrients, while being able to deliver toxins into host tissues before eventually
327 penetrating these tissues (Acord *et al.*, 2005). The aim of anti-adhesion therapy is
328 essentially to reduce contact between pathogens and host tissues, by preventing or
329 reversing adherence. Currently, some of the most efficient known inhibitors of
330 adhesion are found in foodstuffs (Ofek *et al.*, 2003). In the present study, the effect of
331 native and enzyme-modified whey products on association of *Salm. typhimurium*, *E.*
332 *coli* O157:H7 and *C. malonaticus* to human CaCo-2 cells was examined, along with
333 their effects on the invasion of such cells by *Salm. typhimurium* and *C. malonaticus*.
334 Whey is currently described as a 'functional food', as it possesses a wide array of
335 health benefits, and undenatured whey can provide high concentrations of intact
336 proteins such as lactoferrin and immunoglobulins (Marshall, 2004). The results

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

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

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

381 In a study by de Araujo and Giugliano (2000), it was reported that whey
382 produced from human milk (0.97mg $ml^{-1}$) inhibited association of diffusely adherent
383 *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) to HeLa cells by 9% and 16%,
384 respectively. In a subsequent study by the same researcher in 2001, the free secretory
385 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

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

439 **Conclusion**

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

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447 Ireland for supplying the whey products used throughout this study, and also we
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449 whey products.

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585 Legends for figures:

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

588 **Figure 2:** The Effect of DMEM (■), Untreated Whey Products (□), Enzyme-
589 Modified Whey Products (■) and Egg Albumin* on the (a) Association and (b)
590 Invasion of *Salm. typhimurium* to/ into CaCo-2 cells. (Data= mean ± S.D., n=3)
591 * Egg Albumin is included in this figure for comparison only

592 **Figure 3:** The Effect of DMEM (■), Untreated Whey Products (□), Enzyme-
593 Modified Whey Products (■) and Egg Albumin* on the Association of *E. coli*
594 O157:H7 to CaCo-2 cells. (Data= mean ± S.D., n=3)
595 * Egg Albumin is included in this figure for comparison only

596 **Figure 4:** The Effect of DMEM (■), Untreated Whey Products (□), Enzyme-
597 Modified Whey Products (■) and Egg Albumin* on the (a) Association and (b)
598 Invasion of *C. malonaticus* to/ into CaCo-2 cells. (Data= mean ± S.D., n=3)
599 * Egg Albumin is included in this figure for comparison only

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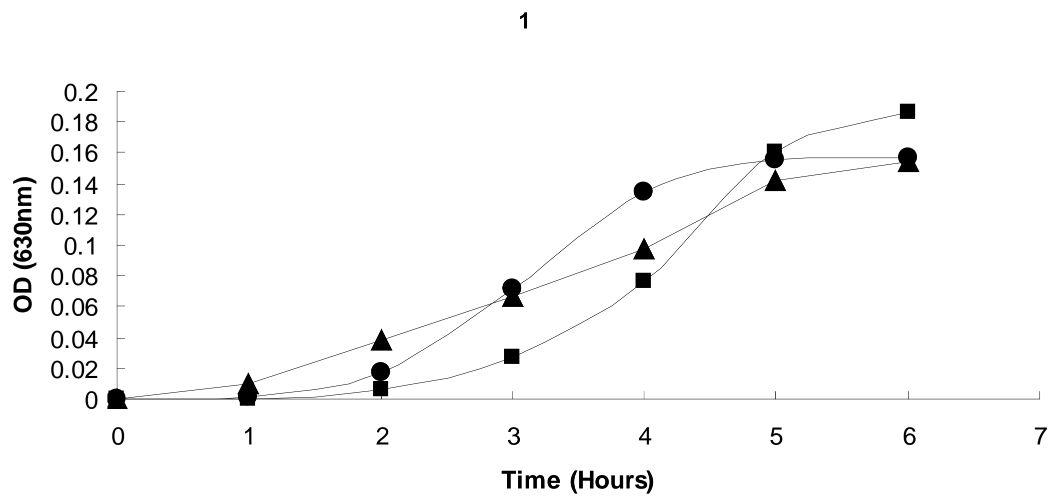
609 **Figure 1**

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627 **Figure 2:**

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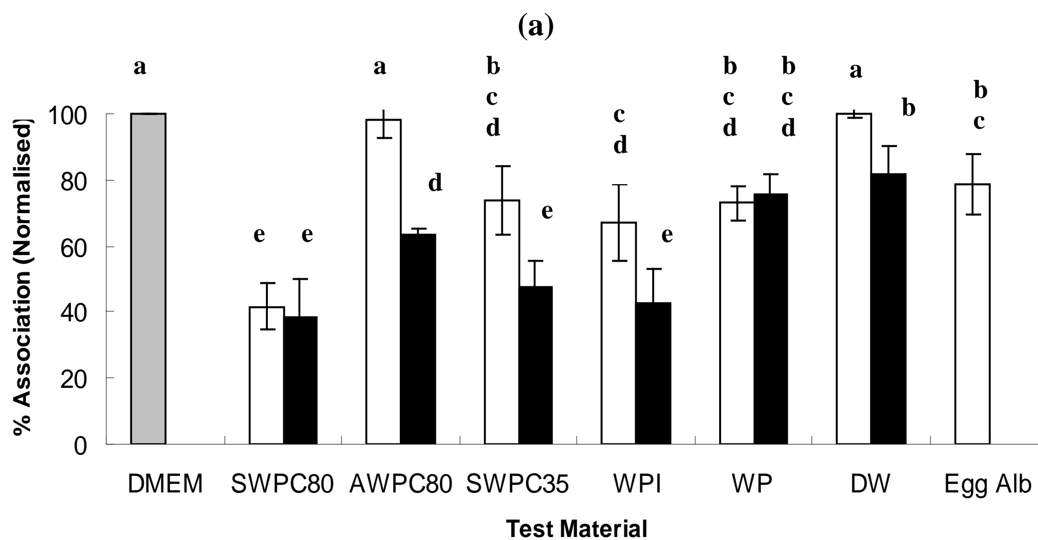
(a)

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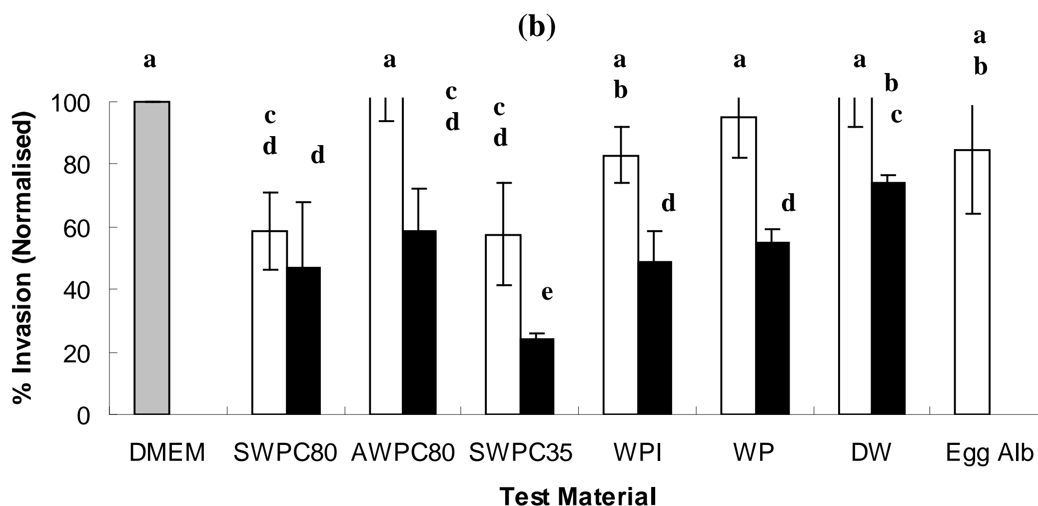
(b)

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631 Notes: All test materials were used at a concentration of 40mg $mL^{-1}$. Means with the
632 same letter are not significantly different (at the 5% significance level).
633 Abbreviations: DMEM= Dubleco's Modified Eagle's Medium, SWPC80= Sweet
634 Whey Protein Concentrate 80, AWPC80= Acid WPC80, SWPC35= Sweet Whey
635 Protein Concentrate 35, WPI= Whey Protein Isolate, WP= Whey Powder, DW=
636 Demineralised Whey, Egg Alb= Egg Albumin.

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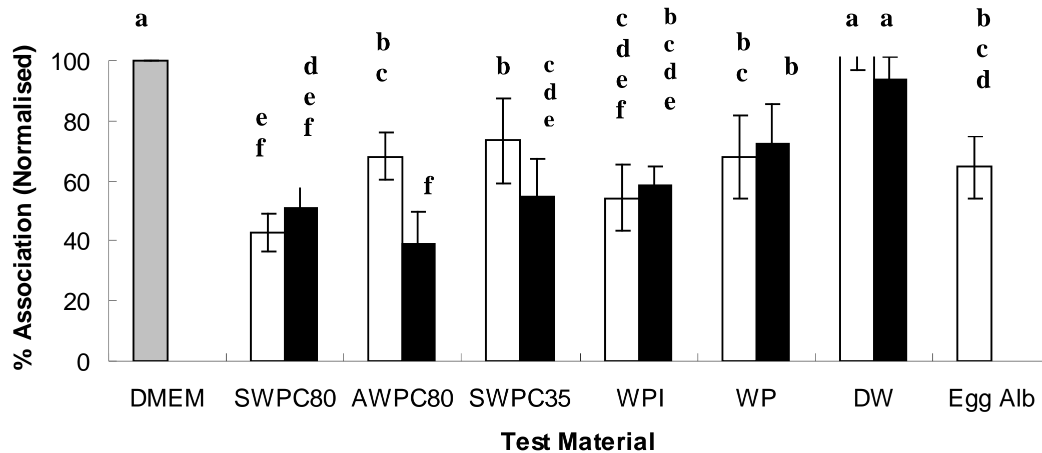
**Figure 3:**




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.

659 **Figure 4:**

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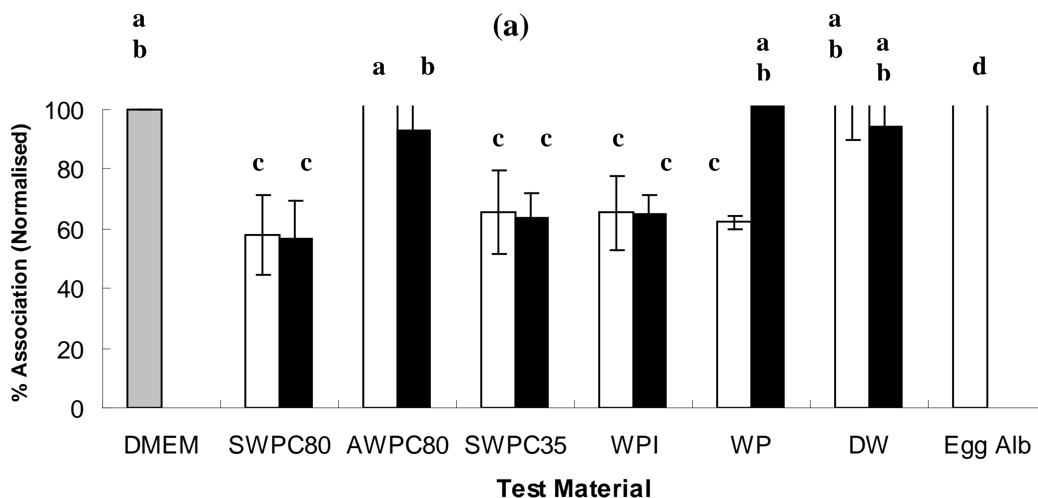


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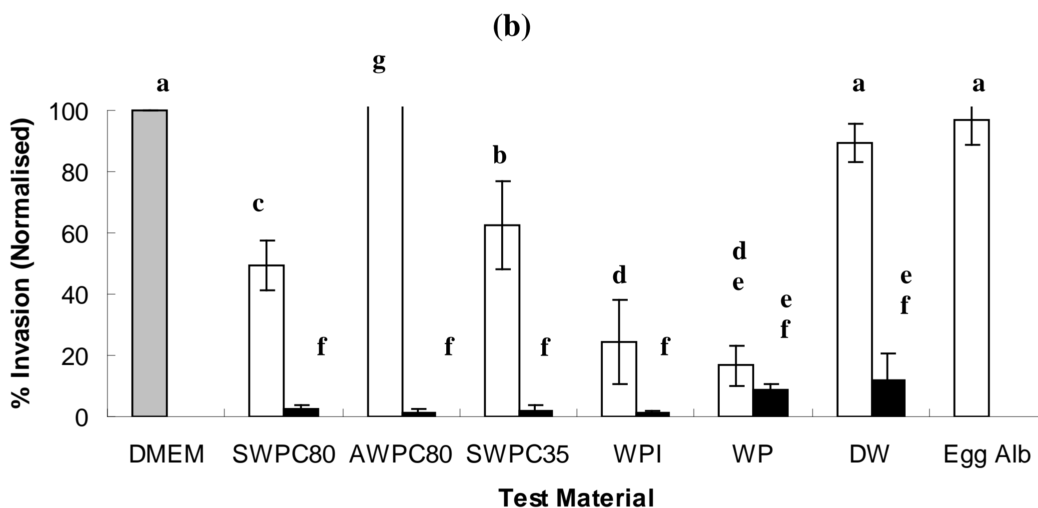


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669 Protein Concentrate 35, WPI= Whey Protein Isolate, WP= Whey Powder, DW=

670 Demineralised Whey, Egg Alb= Egg Albumin.

671

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<br>(ii) 44.1<br>(iii) 61.8 | (i) 50.8<br>(ii) 67.5<br>(iii) 73.7 | (i) 62.2<br>(ii) 43.6 |
| (b) Bacteria pre-treated with EM-SWPC80 | (i) No reduction<br>(ii) No reduction<br>(iii) No reduction | (i) 93.2<br>(ii) No reduction<br>(iii) 71.9 | (i) 58.1<br>(ii) 39.2 |
| (c)CaCo-2 cells pre-treated with EM-SWPC80 | (i) No reduction<br>(ii) No reduction<br>(iii) No reduction | (i) 88.5<br>(ii) No reduction<br>(iii) No reduction | (i) No reduction<br>(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%