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1 **The Effect of Untreated and Enzyme-Treated Commercial Dairy Powders on the**

2 **Growth and Adhesion of *Streptococcus mutans***

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Abstract

Dental caries is a common bacterial infection, but the progression of this disease can be delayed by preventing initial attachment of cariogenic bacteria such as *Streptococcus mutans* to tooth surfaces. This study firstly compares the effect of untreated (UT) and enzyme-treated (ET) dairy powders on the adherence of *S. mutans* to hydroxylapatite (HA), an analogue of tooth enamel. A fluorescence-based method was used to quantify adherence of *S. mutans* to HA both in the presence (S-HA) and absence (PBS-HA) of saliva. Secondly, binding of proteins present in the test materials to HA was quantified using bicinchonic acid assays and SDS-PAGE. In addition, the effect of UT and ET dairy powders on growth of *S. mutans* was examined using an optical-density based assay. UT acid whey protein concentrate (WPC) 80, sweet WPC80, buttermilk powder (BMP) and cream powder (CP) significantly ($P<0.05$) inhibited adhesion of *S. mutans* at ≥31.25μg mL^{-1} in the presence and absence of saliva. ET dairy powders were less effective inhibitors of adhesion, but ET sweet WPC80 significantly ($P<0.05$) inhibited growth of *S. mutans* at ≥0.6mg mL^{-1}. Therefore, due to their adherence- and growth-inhibitory properties, dairy powders may be beneficial in the treatment of dental caries.

51 **1. Introduction**

52 Dental caries affects both children and adults, and is regarded as one of the
53 most common bacterial infections in humans (Aas, Paster, Stokes, Olsen, & Dewhirst,
54 2005). Individuals are susceptible to this disease throughout their lifetime (Sewitz,
55 Ismail, & Bitts, 2007). *Streptococcus mutans* is considered to be the primary
56 etiological agent involved in formation of dental caries (Loesche, 1986). Once
57 adhered to the buccal surfaces, acid(s) formed by oral bacteria due to fermentation of
58 sugars accumulate in plaque on the teeth, and in turn contribute to tooth decay
59 (Loesche, 1986). Adhesion of a pathogenic microorganism to a host tissue is
60 considered to be a vital step in colonisation and subsequent infection (Finlay and
61 Falkow, 1997). Over 25 years ago, Beachey (1981) proposed the design of therapies
62 to prevent initial adherence of a pathogen to surface receptors, thus blocking the
63 prerequisite step of infection. Therefore, a logical approach to preventing initiation of
64 the dental caries process centres upon inhibiting the adherence of cariogenic bacteria
65 such as *S. mutans* to the tooth surface (Tarsi, Muzzarelli, Guzman, & Pruzzo, 1997).
66 Many effective anti-adhesion agents have been identified in foods and beverages
67 (Ofek, Hasty, & Sharon, 2003), such as herbal extracts (Limsong, Benjavongkulchai,
68 & Kuvatanasuchati, 2004), cranberry juice (Yamanaka, Kimizuka, Kato, & Okuda,
69 2004), and water-soluble protein fraction (WSPF) from hen egg yolk (Gaines, James,
70 Folan, Baird, & O'Farrelly, 2003). In addition, some constituents of human milk are
71 known to be capable of binding to pathogenic microbes and inhibiting their adherence
72 to host surfaces (Ofek *et al.*, 2003). It is possible that the equivalent components of
73 bovine milk (and products thereof) could have similar anti-adhesion effects.

74 Research has shown that bovine milk components, including whey
75 components, possess biological activity (Brody, 2000). Whey protein has attracted

76 considerable interest as it has become evident that many of its constituents exhibit
77 bioactive properties (Marshall, 2004). Whey protein is made up of β-lactoglobulin
78 (50%), α-lactalbumin (20-25%), bovine serum albumin (10-15%), immunoglobulins
79 (10-15%), lactoferrin (0.35 to 2%) and lactoperoxidase (0.25-0.5%) (Madureira,
80 Pereira, Gomes, Pintado, & Malcata, 2007). Also, sweet whey contains
81 glycomacropeptide (GMP) at concentrations of up to 15% (Madureira *et al*., 2007).
82 Peptides derived from these precursor proteins are known to have antibacterial
83 properties (Madureira *et al*., 2007), with enzymatic digestion being the most common
84 method used to produce such peptides (Kurhonen, 2009). In addition, it has been
85 reported that peptides possessing antibacterial activity can also exhibit other
86 biological activities relating to protection of the host (Lopez-Exposito & Recio, 2006).
87 Peptide-based therapeutic agents from natural substrates (such as dairy products) that
88 can be added to food, toothpaste and mouthrinses are increasingly in demand as an
89 approach to delay progression of caries (Hayes, Ross, Fitzgerald, Hill, & Stanton,
90 2006).

91 A non-protein constituent of whey which may contribute to its bioactive
92 properties is milkfat. This component may also have potential to inhibit dental caries,
93 as it contains triglycerides and lipids that can exhibit antimicrobial effects either
94 directly or following enzymatic digestion (Sprong, Hulstein, & van der Meer, 2002).
95 The spectrum of saturated fatty acids in milkfat can vary in chain-length from C_4 to
96 C_{18}, while also containing the unsaturated fatty acids $C_{18:1}$ and $C_{18:2}$ (Sprong, Hulstein,
97 & van der Meer, 2001). It has been reported that the medium-chain fatty acids C_8 -
98 C_{12} can have a bacteriostatic effect on dental plaque bacteria (Schuster, 1980).

99 Research recently carried out in this laboratory has shown a range of
100 commercial dairy powders (including whey products such as whey protein

101 concentrates and whey protein isolates, along with buttermilk powder and cream
102 powder) are capable of inhibiting adherence of *S. mutans* to hydroxylapatite (HA)
103 (Halpin *et al*., 2008). HA is a calcium-phosphate analogue of teeth commonly used as
104 an *in vitro* model (Gibbons, Moreno, & Spinell, 1976, Clark & Gibbons, 1977, Gaines
105 *et al*., 2003) of adherence of oral bacteria to tooth surfaces. Also, it has been claimed
106 that a commercial whey product (Carbelac 80) can inhibit both growth and adherence
107 of *S. mutans* following treatment with porcine pancreatic lipase (PPL) (Brady &
108 Folan, 2003). Further to this, a separate study by Halpin, Brady, O'Riordan, &
109 O'Sullivan (2009) showed a range of commercial whey products reduced association
110 of enteric pathogens to CaCo-2 cells, and that the anti-adherence effect was enhanced
111 following PPL-treatment of the whey products.

112 As whey contains both protein and fat, enzymatic digestion can potentially
113 liberate an array of peptides and fatty acids, respectively. In the present study, a varied
114 range of whey and dairy products which were previously found to effectively reduce
115 adherence of *S. mutans* to phosphate-buffered saline-coated HA (PBS-HA) (Halpin *et*
116 *al*., 2008) were subjected to enzyme-treatment in order to determine if this would
117 increase their anti-adhesion activity. Therefore, the main objective of the present
118 study was to examine the effect of enzyme-treatment on the anti-adhesion efficacy of
119 these dairy powders, using both PBS-HA and saliva-coated HA (S-HA). Association
120 of the proteinaceous component of a selection of dairy powders to HA beads was also
121 investigated. In addition, the effect of both untreated and enzyme-treated dairy
122 powders on the growth of *S. mutans* was examined.

123 2. Materials and Methods

124 2.1 Bacterial Isolates and Growth Conditions

125 A clinical isolate of *S. mutans* (LAN-SVHERC-1997sm1) was obtained from the
126 Microbiology Department, St. Vincent's University Hospital, Dublin, Ireland.
127 Bacteria were maintained on Protect™ Bacterial Preserve beads (Technical Service
128 Consultants Ltd, Lancashire, UK) at -80˚C. A single bead from the frozen stock
129 culture was used to inoculate a Columbia blood agar plate (CBA: Oxoid, Hampshire,
130 England) and grown aerobically at 37˚C for 48 h. A single colony from the blood agar
131 plate was subsequently used to inoculate 20mL of brain heart infusion (BHI) broth
132 (LabM, Lancashire, UK) and grown under aerobic conditions without shaking at 37˚C
133 for 18 h.

134 **2.2 Source and Characterisation of Dairy Powders**

135 Sweet whey protein concentrate 80 (SWPC80), acid WPC 80 (AWPC80), sweet WPC
136 35 (SWPC35), whey protein isolate (WPI), whey powder (WP) and demineralised
137 whey (DW) powders were supplied by Carbery Milk Products (Ballineen, Cork,
138 Ireland). The principal differences in composition between SWPC80 and SWPC35
139 should be noted. Although both of these whey products are derived from sweet whey,
140 SWPC80 contains 80% protein (i.e. 80 grams of protein per 100g of product) and only
141 6% lactose (i.e. 6 grams of lactose per 100g of product). However, SWPC35 contains
142 almost 35% protein and approximately 51% lactose. In addition, SWPC35 contains
143 only half of the amount of fat that is present in SWPC80 (refer to Table 1).

144 Buttermilk powder (BMP) and cream powder (CP) were supplied by Kerry Group plc
145 (Tralee, Co. Kerry, Ireland). Albumin from chicken egg white (grade V) and lactose
146 were supplied by Sigma (Poole, Dorset, UK).

147 Compositional analysis was performed on each dairy product using standard methods.
148 Ash content was analysed according to Malkomesius & Nehring (1951). Fat content
149 was determined according to the method of Röse-Gottlieb (International Dairy

150 Federation, IDF, 1987), protein content was determined by the Kjeldahl method (IDF,
151 1993a) and the moisture content was determined by the IDF reference method (IDF,
152 1993b).

153 **2.3 Hydrolysate Preparation Conditions**

154 Crude porcine pancreatic lipase (PPL, 100-400 units/ mg protein) (Sigma, Poole,
155 Dorset, England) was used throughout the study. Preliminary experiments
156 demonstrated the presence of both peptidase and lipase activities in this enzyme
157 preparation (data not shown). Hydrolysates were prepared in a Fermac 200 fermentor
158 (Electrolab Ltd, Tewkesbury, UK) as follows: a c. 2% (g/100mL) solution of substrate
159 was prepared by dissolving 20g of dairy powder in 900mL of sterile distilled water
160 and heating at 37˚ C with stirring for 30 min. Lipase solution (1g of PPL in 100mL of
161 sterile H_20) was added to the substrate solution to give a final incubation volume of 1
162 L. The substrates were then incubated for 18 h at 37˚C with stirring. The resulting
163 hydrolysates were heated at 60˚C for 10 min in order to denature the enzyme(s). Each
164 hydrolysate was then placed on ice and allowed to cool to less than 10˚C (approx. 45
165 min), before being frozen using liquid nitrogen and subsequently lyophilised
166 (Moduloyo, Edwards High Vacuum, Manor Royal, Crawley, Sussex, UK).

167 **2.4 Adhesion Assay**

168 **2.4.1 (a) Preparation of Hydroxylapatite**

169 Hydroxylapatite (HA) beads were supplied by Merck (Darmstadt, Germany). Both
170 phosphate buffered saline-coated and saliva-coated HA were used throughout the
171 study. Particle size analysis using a Malvern Mastersizer (Malvern Instruments Ltd.,
172 Worcestershire, UK) showed the average diameter (*D* [4,3]) of the HA beads to be
173 approximately 10µm. Phosphate-buffered saline coated HA (PBS-HA, PBS: Oxoid,

174 Hampshire, England) was prepared by suspension of 7.5mg mL^{-1} HA in PBS
175 immediately before use in the adherence assays.
176 Saliva-coated-HA (S-HA) was prepared by a modification of the protocol set out by
177 Gibbons & Etherden (1982) as follows: parafilm-stimulated whole saliva was
178 collected in an ice-chilled tube from two healthy donors (1 male, 1 female) at least 1 h
179 after eating, drinking or brushing of teeth. The saliva was heated at 60ºC for 30 min to
180 inactivate degenerative enzymes, and subsequently centrifuged at 12,000 × g for 15
181 min. The pellet was discarded and the supernatant (i.e. clarified whole saliva) was
182 used to prepare a 7.5mg mL^{-1} dispersion of HA. Aliquots (150µL) of saliva-coated
183 hydroxylapatite (S-HA) were dispensed into the wells of a 96-well V-bottomed plate
184 (Sarstedt, Newton, North Carolina, USA), and incubated at 30ºC for 1 h with gentle
185 agitation (4.5 × g). Following this, the microtitre plate was centrifuged at 805 × g for
186 2 min, the supernatants discarded and the S-HA pellets washed twice with sterile pre-
187 warmed PBS to remove excess saliva. The S-HA pellets were subsequently
188 resuspended in sterile PBS for use in the adherence assay.

189 **2.4.1 (b) Preparation of Syto® 13 dye**

190 Syto® 13 dye (Molecular Probes, Oregon, USA) was supplied as a 5mmol L^{-1}
191 solution in dimethylsulphoxide (DMSO). This concentration was adjusted to 5µmol L^{-}
192 ^{1} by appropriate dilution in sterile PBS, and was used only on the day of preparation.
193 A standard curve of relative fluorescent units (RFU) versus CFU mL^{-1} was
194 constructed for *S. mutans* (R^2= 0.9942).

195 **2.4.2 Assay Protocol**

196 An overnight culture of *S. mutans* was centrifuged at 3220 × g (Eppendorf 5810R,
197 Cambridge, UK) for 10 min and the pellet resuspended in sterile PBS. Following a
198 second centrifugation step, the bacterial pellet was resuspended in PBS, and the

199 OD_{630nm} of the suspension measured using a Multiskan Ascent spectrophotometer
200 ((Thermo Electron Corporation, Vantaa, Finland), and adjusted to 0.2 by appropriate
201 dilution with sterile PBS.
202 The adherence assays were carried out as previously described (Halpin *et al*., 2008),
203 using sterile 96-well polystyrene microtitre half-area plates (Nunc, Roskilde,
204 Denmark). Dairy powders were prepared to the required concentration by dispersing
205 the dried powder in PBS. Briefly, 50µL of test material solution at various
206 concentrations was added to the wells, followed by 50µL of PBS-HA or S-HA (7.5
207 mg mL^{-1}). Bacterial suspension (50µL) was added to the wells, so that the final
208 volume of each well was 150µL. Control wells (no bacteria and/ or no HA) were
209 included in each assay. The plate was incubated at room temperature for 45 min, and
210 manually inverted at 5 min intervals to prevent settling of the HA suspension. The
211 plate was subsequently centrifuged at 201 $\times$ g to sediment the HA and any adhering
212 bacteria, leaving the non-adhering bacteria in suspension. These non-adhering bacteria
213 were labelled with 10µL of 5µmol L^{-1} Syto® fluorescent dye. For more information
214 regarding the development and validation of the assay described here, the reader
215 should refer to Halpin *et al*., 2008.

216 **2.5 Quantification of Bacterial Adherence**

217 Aliquots (100µL) of supernatant from the adherence assay (Section 2.4.2) containing
218 the non-adhering bacteria were transferred from each well of the half-area plate to the
219 corresponding wells of a black microtitre plate (Costar, Corning Inc., Corning, USA).
220 This plate was allowed to stand at room temperature for 5 min in the dark before
221 reading the fluorescence using a Fluoroskan Ascent plate reader (Thermo Electron
222 Corporation, Vantaa, Finland). The excitation wavelength was 485 nm and the
223 emission intensity was monitored at 538 nm. Three measurements were taken at 5 min

224 intervals, and the average fluorescence calculated. The fluorescence due to the total
225 number of bacteria present in the supernatant was determined as a direct readout from
226 the fluorimeter as relative fluorescent units (RFU). The background fluorescence due
227 to non-bacterial components of the assay (i.e. dairy powder and HA) were subtracted.
228 The percentage inhibition of adhesion was calculated as follows:

229 $$\frac{(Fluorescence\ due\ to\ unbound\ bacteria)}{(Fluorescence\ due\ tototal\ input\ bacteria)} \times 100 \qquad \textbf{(1)}$$

230 **2.6 Protein Adherence Assay**

231 The ability of the proteinaceous component of the various dairy powders to adhere to
232 the HA beads was assessed as follows; a dispersion of HA (20 mg mL^{-1}) was mixed
233 with an equal volume of test material at various concentrations, so that the final
234 concentration of test material ranged from 0.0625mg mL^{-1} to 1mg mL^{-1}. The mixture
235 was gently inverted at 5 min intervals for a period of 45 min, before being centrifuged
236 (201 × g, 10 min). Supernatants were subsequently decanted into plastic tubes and
237 shaken vigorously before determination of protein content by the bicinchonic acid
238 (BCA) method (Smith *et al.*, 1986), for which all reagents were supplied by Sigma
239 (Poole, Dorset, UK). Aliquots (25µL) of supernatant were added to wells of a 96-well
240 plate, followed by 200µL of BCA reagent, and the plate was subsequently incubated
241 for 30 min at 37˚C. A plate reader (Spectra Max; Molecular Devices Corp., U.K.) was
242 used to measure sample absorbance values. Absorbance was measured at 570nm
243 (Abs_{570nm}), and readings were converted to mg mL^{-1} protein using a standard curve of
244 absorbance versus protein concentration (R^2= 0.9983), which was prepared using
245 bovine serum albumin (BSA; Sigma, Poole, Dorset, UK).

246 **2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

247 To investigate the selectivity of any HA/ protein interactions, the protein profiles of
248 the resulting supernatants from the protein adherence assay were compared to that of
249 the starting material by SDS-PAGE (Laemmli, 1970) using the Bio-Rad protein mini-
250 gel system (Bio-Rad Laboratories, Richmond, California). Briefly, this was achieved
251 by incubating untreated WPCs with HA beads or alone (as described in Section 2.6).
252 Sedimentation recovered the HA beads along with any bound protein, leaving
253 unbound protein in the supernatant which was quantified using SDS-PAGE. All
254 reagents were purchased from Sigma (Poole, Dorset, UK). Resolving gel and stacking
255 gel were prepared to 15g/100mL and 4g/100mL acrylamide, respectively. In order to
256 visualise protein bands, gels were stained using coomassie blue dye. Protein bands
257 were quantified using densitometry software (Alphaview Version 1.3.0.7, Innovatech
258 Corporation).

259 **2.8 Growth Assays**

260 Growth assays were carried out in sterile 96-well plates (Nunc, Roskilde, Denmark).
261 Overnight cultures of *S. mutans* were prepared in BHI broth as described earlier
262 (Section 2.1). A working culture containing c. 10^8 colony forming units per millilitre
263 (CFU mL^{-1}) was prepared by adding 1mL of overnight culture to 9mL of sterile BHI
264 broth. Test materials were prepared by dispersing dried dairy powders or hydrolysates
265 in BHI broth to the desired concentration. Aliquots (100µL) of test material were
266 added to the wells of the plate, followed by 100µL of the diluted culture; the final
267 concentrations of test material were 0.6mg mL^{-1}, 1.25mg mL^{-1}, 2.5mg mL^{-1} and 5mg
268 mL^{-1}. Bacterial growth in the absence of test material (i.e. control growth) was also
269 determined. The plate was then incubated at 37°C for 18 h in a Multiskan Ascent plate
270 reader (Thermo Electron Corporation, Vantaa, Finland). Immediately prior to
271 incubation the plate was shaken for 1 min in order to disperse the suspensions. The

272 optical density (OD) readings at 630nm for each well were subsequently recorded at 1
273 h intervals, with the plate being shaken for 30 s immediately prior to measurement.
274 The initial OD reading, recorded at time 0, of each well was subtracted from all other
275 readings for the corresponding wells over the 18 h incubation time (i.e. to subtract the
276 background OD values).

277 **2.9 Solid Phase Micro Extraction / Gas chromatography (SPME/GC) headspace**
278 **analysis of short chain fatty acids (SCFAs)**

279 SPME/GC analysis was performed in order to confirm the presence of the free fatty
280 acids butanoic (C_4) and hexanoic (C_6) in SWPC80 post-hydrolysis with PPL. This
281 was carried out according to the method of Noronha, Cronin, O'Riordan, &
282 O'Sullivan (2008). Briefly, GC analysis was performed on an ATI Unicam Model
283 6100 gas chromatograph fitted with a flame ionisation detector (FID) and interfaced
284 to a Spectra-Physics SP4290 computing integrator. The column used was a 15 m
285 FFAP (Quadrex Inc.) fused silica column (0.53 mm i.d., film thickness, 1μM).
286 Hydrogen was used as a carrier gas at a flow rate of 8mL min^{-1}. The column
287 temperature was 140˚C and the injection block was set at 300 ˚C. The volatile SCFAs
288 (C_4 and C_6) present in the aqueous dairy powder hydrolysate (20 g L^{-1}) samples were
289 measured by SPME headspace analysis using iso-butanoic (iso-C_4) and 4-methyl-
290 pentanoic acids (4-Me-C_5) as internal standards (IS), respectively, at various time-
291 points after addition of the PPL. The SPME fibres (Carboxen/ PDMS, 75μM
292 thickness) were obtained from Supelco (Supelco-Aldrich, Dublin, Ireland) and were
293 conditioned under a flow of nitrogen (10mL min^{-1}) at 300˚C for 2.5h prior to use.

294 An aliquot (20 mL) of the hydrolysate was transferred to a 10 mL screw thread glass
295 vial, fitted with a magnetic stirring bar. After sealing the vial with a
296 polytetrafluoroethylene (PTFE) silicone rubber septum, the SPME needle was

297 inserted through the latter so as to position the fibre 15 mm above the surface of the
298 liquid hydrolysate. The hydrolysate was heated with stirring by placing the vial in a
299 thermostatically controlled water bath at 50˚C. After equilibration for 10 min, the
300 headspace was sampled by exposing the fibre for 10 min. The concentration of the
301 volatile fatty acids (VFAs) was determined using the following equation:
302
303 Concentration of analyte (C_4 or C_6)

304 $$= \frac{\text{peak area of analyte} \times \text{concentration IS}}{\text{peak area of IS} \times \text{response factor} \times \text{sample volume}} \qquad \textbf{(2)}$$

305 The response factors for the two analytes were established by carrying out SPME
306 headspace analysis of an aqueous standard (0.2g L^{-1} each of C_4, iso-C_4, C_6 and 4-Me-
307 C_5 acids) under the same conditions as described above for the liquid hydrolysate
308 samples.

309 $$\text{Response factor } (C_4 \text{ or } C_6) = \frac{\text{peak area } C_4}{\text{peak area iso-}C_4} \text{ or } \frac{\text{peak area } C_6}{\text{peak area 4-Me-}C_5} \qquad \textbf{(3)}$$

310 The response factor was calculated as 1 for both C_4 and C_6. GC retention times of the
311 SCFAs were 2.2, 2.8, 5.6 and 6.6 min for iso-C_4, C_4, 4-Me-C_5 and C_6, respectively.

312 **2.10 Statistical Analysis**

313 All adherence/ growth assays were performed at least three times (n=3). Results were
314 expressed as the mean ± standard deviation (S.D.). Differences between
315 concentrations within treatments were determined using the least significant
316 difference (LSD) test, while differences between treatments were determined using
317 Duncan's test. Both analyses were performed using SAS Version 9.1.3. Data were
318 considered significantly different if $P<0.05$.

319 **3. Results**

320 Compositional analysis of the dairy powders (fat, protein, moisture, ash and lactose)
321 was determined (Table 1). These were typical of their product types. Sweet and acid
322 WPC80s have similar protein and fat contents, while WPI has almost no fat. WP and
323 DW contain less protein and fat than the WPCs and WPI, but have a high content of
324 lactose. SWPC35 also contains high levels of lactose when compared to the
325 WPC80's, WPI and CP. BMP contained less protein than the WPCs and WPI, and CP
326 had the highest fat content of the test materials.

327 **3.1 Adherence Assays**

328 **(a) Adhesion to phosphate buffered saline-coated hydroxylapatite**

329 A small proportion of *S. mutans* did not bind to PBS-HA (c. 15%) under our
330 experimental conditions (shown as the 'control' value in Table 2). Of the untreated
331 dairy powders, AWPC80 appeared to be the most effective inhibitor of *S. mutans*
332 adhesion to PBS-HA at the concentrations examined (Table 2), increasing the non-
333 binding proportion of bacteria to c. 93%. However, at 125μg mL^{-1}, UT BMP and UT
334 CP were equally as effective (P>0.05). The protein control, egg albumin, did not
335 reduce adhesion at all, and resulted in similar non-binding proportions of *S. mutans* as
336 were observed in the absence of test material (i.e. control adherence).

337 In most cases, enzyme treatment was found to reduce the anti-adhesion activity of all
338 dairy powders (Table 2), in that the proportion of non-binding bacteria was markedly
339 lower for the ET dairy powders than those observed for the equivalent UT samples. At
340 125μg mL^{-1}, enzyme treatment significantly (P<0.05) reduced the anti-adhesion
341 activity of all dairy powders with the exception of WP. None of the enzyme-treated
342 dairy powders caused the non-binding proportion of *S. mutans* to increase to levels
343 ≥40% in the PBS-HA assays. ET CP was the most potent inhibitor of *S. mutans*

344 adhesion to PBS-HA at 31.25μg mL^{-1}, and ET CP and ET SWPC80 were found to be
345 equally as effective (*P*>0.05) inhibitors at 62.5μg mL^{-1} and 125μg mL^{-1}.
346 Lactose, which was present in all test materials at varying levels, was not found to
347 affect adherence of *S. mutans* to PBS-HA, even when used at concentrations up to
348 1000μg mL^{-1} (data not shown), and in fact was found to significantly increase the
349 adherence of *S. mutans* to PBS-HA (*P*<0.05).

350 **(b) Adhesion to saliva-coated hydroxylapatite**

351 In the presence of saliva, the control level of adhesion of *S. mutans* to hydroxylapatite
352 was significantly reduced when compared to that of the PBS-HA model (*P*<0.0001),
353 with c. 37% of each bacterial culture not adhering to S-HA ('control' in Table 3).
354 At concentrations ≥ 31.25μg mL^{-1}, UT SWPC80, AWPC80 and BMP significantly
355 (*P*≤0.05) reduced adherence of *S. mutans* to S-HA relative to the protein control (egg
356 albumin, EA). At 62.5μg mL^{-1} and 125μg mL^{-1}, UT SWPC80, UT AWPC80 and UT
357 WP were the most potent inhibitors of *S. mutans* adhesion to S-HA, being more
358 effective than all other UT test materials and increasing the proportion of non-binding
359 bacteria to 75-80%.
360 Consistent with our observations in the PBS-HA model system, untreated dairy
361 powders were generally more potent inhibitors of *S. mutans* adherence than the same
362 powders following enzyme-treatment, except in the case of the WPCs, which were
363 found to show similar levels of efficacy both in their untreated and enzyme-treated
364 forms. However, it is worthwhile to note that the reduction in anti-adhesion activity
365 caused by enzyme treatment of powders was not as dramatic in the case of S-HA as
366 that observed in the PBS-HA model. Following enzyme treatment, at 31.25μg mL^{-1},
367 all powders excluding ET WPI, ET WP and ET DW significantly inhibited adherence
368 of *S. mutans* to S-HA relative to the control (*P*<0.05). However, at 125μg mL^{-1}, all

369 enzyme treated dairy powders were more effective than egg albumin (P<0.05), with
370 most ET dairy powders showing similar levels of anti-adhesion.

371 **3.2 Adherence of Whey and Dairy Powders to PBS-HA**

372 The more effective inhibitors of *S. mutans* adherence to PBS-HA were used to
373 establish if protein present in the test material was adhering to the HA beads. Table 4
374 shows the relationship between the initial protein concentrations of these dairy
375 suspensions and the amount of protein associated with HA. When sedimented from
376 solutions of increased protein content, the amount of protein associated to the HA
377 increased with increasing protein concentration in all cases, but to different extents,
378 perhaps suggesting dairy powders possessed different affinities for HA. Of the
379 materials examined, the greatest level of protein association was observed in the case
380 of AWPC80, which was also observed to be the most potent inhibitor of *S. mutans*
381 adherence to PBS-HA (Table 2).

382 **3.3 SDS-PAGE of Protein Content of WPC Supernatants Before and After**
383 **Incubation with PBS-HA**

384 The electrophoresis patterns of UT SWPC80, UT AWPC80 and UT SWPC35 before
385 and after incubation with and separation from HA are compared in Figure 1.
386 Densitometric analysis of the protein bands confirmed that the total protein content of
387 each WPC was reduced following incubation with HA. Protein contents of UT
388 SWPC80, UT AWPC80 and UT SWPC35 were reduced by 66.7%, 53.9% and 59.4%,
389 respectively. Most notably, the larger proteins (possibly the heavy and light chains of
390 the immunoglobulins and BSA) appeared to have associated with HA, as these bands
391 are not present following incubation with HA.

392 In addition, densitometric analysis indicated that the protein bands representing β-lac
393 were reduced by 51%, 41% and 63.2% for UT SWPC80, UT AWPC80 and UT

394 SWPC35, respectively. No reduction in intensity was observed for the bands
395 representing α-lac following incubation with HA, suggesting this whey protein did
396 not adhere to the HA beads.

397 **3.4 Growth Inhibition Assays**

398 None of the untreated (UT) dairy powders inhibited growth of *S. mutans* at any of the
399 concentrations examined (0.6-5mg mL^{-1}) (data not shown). Of the ET dairy powders,
400 growth inhibition of *S. mutans* was most evident for ET SWPC80 (Figure 2). Growth
401 of *S. mutans* was significantly inhibited (P<0.05) at all concentrations examined, and
402 the effect showed a slight concentration dependency Comparison of the rates of
403 increase of OD_{630} during the logarithmic growth phase suggests that the maximum
404 concentration of ET SWPC80 (5mg mL^{-1}) used reduced the rate of growth by more
405 than 2-fold over that of the control.

406 **3.5 Determination of Volatile Fatty Acids in ET SWPC80**

407 Aqueous hydrolysate samples were taken at 15 min intervals following addition of
408 lipase solution and SPME/GC performed at each time point. Levels of C_4 and C_6
409 appeared to 'level off' after c. 60 min, and were present at levels of 52.28 ± 6.68µg
410 mL^{-1} and 18.66 ± 1.49µg mL^{-1}, respectively, after 120 mins of hydrolysis time.

411 **4. Discussion**

412 The findings of a previous study by this group showed that a range of UT dairy
413 powders reduced adherence of *S. mutans* to PBS-HA (Halpin *et al*., 2008). The
414 present study examined the effect of enzyme-treatment on the anti-adhesion activity
415 of these powders, using two model systems: PBS-HA and S-HA. The S-HA model
416 represents the closest approximation to conditions in the oral cavity, while the PBS
417 model system represents a cleaner working matrix and may also serve as a model for

418 *in vivo* conditions where saliva production is impaired, e.g. in cases of 'dry mouth'.
419 Dry mouth, also referred to as xerostomia, describes a variety of conditions whereby
420 salivary flow rate is reduced, and individuals with this condition are susceptible to
421 rampant caries (Loesche, 1986). The authors do however acknowledge that dry
422 mouth patients do not have teeth free from a protein film (so called 'pellicle') and
423 bacterial biofilm, but that the proteins adhering to the tooth tissues are of origins other
424 than saliva, i.e. gingival pockets, exudate from the soft tissues, and of course, foods.
425 Under our experimental conditions, control adherence varied greatly between PBS-
426 HA and S-HA, which resulted in a different 'starting point' as such for assessing the
427 efficacy of the test materials. The more effective test materials (UT AWPC80, UT
428 SWPC80, UT BMP and UT CP) increased the proportion of *S. mutans* not adhering to
429 PBS-HA to a level similar to or greater than those observed in the presence of saliva.
430 For example, control adherence of *S. mutans* was typically 40% for S-HA, and the
431 proportion of bacteria not adhering to PBS-HA far exceeded this value in the presence
432 of the dairy powders listed above. Thus, dairy powders may be useful ingredients in
433 the development of a beverage which could potentially act as a saliva substitute. It has
434 previously been reported (Johansson, 2002) that milk and dairy-based drinks possess
435 many of the biological and physical attributes that would make them suitable saliva
436 substitutes, and the current investigation provides substantiating evidence that this
437 may be a useful application for dairy products.
438 Experiments have shown that proteins present in these dairy powders are
439 interacting with the HA beads and this may, in part at least, be contributing to the
440 reduction in adherence of *S. mutans* to PBS-HA. This observation was confirmed by
441 results from SDS-PAGE, which further suggested that some of the larger proteins in
442 the WPCs such as the immunoglobulins and BSA had associated with the HA beads.

443 Of the UT dairy powders, AWPC80 was found to be the most effective inhibitor of *S.*
444 *mutans* adhesion to HA, and exhibited the highest level of protein association with
445 HA beads. However, the level of protein associating with HA varied between test
446 materials, and a high protein content did not necessarily lead to higher levels of
447 interaction with HA beads, suggesting other factors were contributing to inhibition of
448 adherence. Furthermore, it is possible that the test materials are also interacting with
449 the bacterial cells, and this may in turn reduce the ability of the cells to adhere to
450 surfaces.

451 Although UT AWPC80, UT SWPC80, UT BMP and UT CP were found to be
452 very effective inhibitors of *S. mutans* adherence to both PBS-HA and S-HA, the active
453 component(s) of each of these test materials may not be the same. To begin with, the
454 compositions of these dairy powders are quite different, and this may have had a
455 direct influence on their level of anti-adhesion activity. The inhibitory effect may even
456 be due to multiple factors acting synergistically within a particular test material. Also,
457 these dairy powders exhibited varying levels of efficacy depending on (a) whether
458 they were used in PBS-HA or S-HA systems and (b) whether they had been subjected
459 to enzyme treatment. The latter is further complicated by the fact that the enzyme
460 used throughout this study was a crude PPL mixture, that contains both protease and
461 lipase constituents (Birner-Grunberger, Scholze, Faber, & Hermetter, 2003), which
462 could hydrolyse protein and fat components of the dairy powders, respectively.

463 If protein levels of each test material are compared, WPI contains the highest
464 level of protein, yet was found to be a poor inhibitor of *S. mutans* adhesion to HA.
465 AWPC80 contains less protein than WPI, but exhibited high levels of anti-adhesion
466 activity. Interestingly, SWPC80 contains almost the same level of protein as
467 AWPC80, but was a less effective inhibitor than AWPC80. However, the lactose

468 content of SWPC80 was almost three times greater than that of AWPC80, and may
469 have been detrimental to the anti-adhesion activity of this dairy powder. Results from
470 the present study show that lactose promoted adherence of *S. mutans* to PBS-HA
471 (Table 2), and this may help explain why powders with high lactose contents (WP and
472 DW) exhibited poor anti-adhesion activity. Further to this, it could be speculated that
473 these dairy powders did indeed possess some anti-adhesion activity but this may have
474 been negated by their high lactose content. On the other hand, BMP and CP contained
475 lower levels of protein than both AWPC80 and SWPC80, and also had considerable
476 lactose levels, yet these dairy powders were potent inhibitors of *S. mutans* adherence
477 to HA. It is worthwhile to note that these dairy products (BMP and CP) contain
478 caseins, which are well known to inhibit adherence of *S. mutans* to HA (Vacca-Smith,
479 Van Wuyckhuyse, Tabak, & Bowen, 1994). It may be the case that whey protein(s) is
480 responsible for the inhibitory activity exhibited by the WPC80s, while casein fractions
481 may be contributing to the anti-adhesion activity caused by BMP and CP. Another
482 possible explanation for the variation in the anti-adherence activity of the various
483 powders may be the differences in fat content, in that in general, only the powders
484 with fat contents higher than c. 8% exhibited high levels of inhibition. Furthermore, it
485 could be speculated that anti-adhesion activity of any dairy powder is due to the
486 presence of both protein and fat, and it may be the case that fat is required in order for
487 protein(s) to effectively associate with HA and consequently reduce adherence of *S.*
488 *mutans*, as evidenced by the greater level of association of protein to HA in the case
489 of AWPC80 than was observed for WPI.

490 At the outset of this study, it was anticipated that enzyme treatment would
491 enhance the anti-adhesion efficacy of the dairy powders, as a patent by Brady & Folan
492 (2003) claimed the adhesion inhibitory properties of a lactose-free commercial whey

493 product were activated upon hydrolysis. In addition, it has been reported that certain
494 antimicrobial substances present in milk and dairy-based products (such as peptides
495 and fatty acids) only become active following enzymatic digestion (Lopez-Exposito &
496 Recio, 2006). However, in the present study the anti-adhesion efficacy of all dairy
497 powders was, in general, reduced following enzyme-treatment for both PBS-HA and
498 S-HA. For the S-HA assays, the efficacy of SWPC80, AWPC80, SWPC35 and CP
499 was slightly (but not necessarily significantly) increased by enzyme treatment at the
500 maximum concentration (125µg mL^{-1}) only. Lower levels of anti-adhesion activity
501 following enzyme treatment may have been due to hydrolysis of proteins and/ or fats,
502 as no consistent trend was observed in order to determine whether protein or fat
503 digestion caused the reduction in efficacy.

504 A previous investigation by this research group which sought to determine the
505 effect of a range of whey products on the adherence of foodborne pathogens to
506 intestinal cells found that hydrolysis generally increased the inhibitory activity of
507 these test materials (Halpin *et al*., 2009). However, the study in question employed a
508 model system in which bacterial cells were incubated with epithelial cells, unlike the
509 present study where bacteria were incubated with a mineral surface (hydroxylapatite)
510 in the presence and absence of saliva. Thus, it may be that the efficacy of test
511 materials, and hydrolysates thereof, is dependent on the surface to which
512 microorganisms are adhering to.

513 Another aspect of the present study was to examine the effect of various dairy
514 powders on the growth of *S. mutans*. Of the range of test materials, only ET SWPC80
515 caused a substantial and significant (P<0.05) reduction in the growth of *S. mutans*.
516 SPME/GC analysis confirmed the presence of the free fatty acids butyric (C_4) and
517 caproic (C_6) acids in the SWPC80 hydrolysates, and it is possible that other FAs were

518 present in the hydrolysed product, as milkfat contains a broad spectrum of FAs.
519 Studies by Sprong *et al*. (2001, 2002) have demonstrated the antibacterial activity of
520 milk-lipids. However, the inhibitory activity of ET SWPC80 could equally be due to
521 peptides produced during enzymatic digestion. Peptides liberated from GMP by the
522 action of proteolytic enzymes in PPL may also have contributed to the observed
523 antibacterial effect. A study by Malkoski, Dashper, O'Brien-Simpson, Talbo, Macris,
524 Cross, & Reynolds (2001) showed that kappacin, a peptide derived from κ-casein,
525 inhibited growth of plaque-forming bacteria, and although not established here, it is
526 possible such peptides derived from GMP are contributing to the observed
527 antimicrobial activity of ET SWPC80. Alternatively, growth inhibition of *S. mutans*
528 due to ET SWPC80 may have been the result of a synergistic effect between FAs and
529 peptides released during enzyme treatment. Small peptides exhibiting antimicrobial
530 properties have been proposed as alternatives to antibiotics (Mor, 2003). The oral
531 cavity is considered eminently suitable for the application of such peptides as it
532 provides direct access to bacterial biofilms on non-shedding surfaces (Dashper *et al*.,
533 2007). Currently, there is considerable commercial interest in the isolation and
534 characterisation of dairy-derived bioactive peptides that can be added to products such
535 as toothpastes, gels and mouth rinses (Aimutis, 2004). Our results show that an
536 enzyme-treated whey product exhibits antibacterial effects, without isolation of
537 individual peptides or FAs. Therefore, an antibacterial agent can be produced from
538 whey, which is available in large quantities and is relatively inexpensive.
539 In conclusion, the results presented here have shown that UT dairy powders
540 are effective inhibitors of *S. mutans* adherence to both PBS-HA and S-HA. In general,
541 the anti-adhesion efficacy of these dairy powders was, for the most part, reduced
542 following enzyme-treatment. However, some activity was observed following

543 enzymatic digestion, in particular for AWPC80, SWPC80, CP and BMP in both PBS-
544 HA and S-HA assays. It was also evident that ET SWPC80 is an effective
545 antimicrobial agent active against *S. mutans*. An interesting observation of the present
546 study is that although hydrolysis of a particular whey product may on the one hand
547 increase the antimicrobial activity, it may do so at the expense of the potential anti-
548 adhesion activity of the product. In other words, enzyme-treatment of dairy products
549 may enhance antimicrobial activity while suppressing their anti-adhesion efficacy.
550 Nonetheless, this present study has shown that dairy powders, which are natural
551 products and are readily available and relatively inexpensive materials, may offer
552 protection against caries in both normal and dry-mouth contexts.

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664 **Legends for Figures:**

665 **Figure 1:** SDS-PAGE showing protein bands present in centrifuged supernatants of
666 sweet whey protein concentrate (WPC) 80, acid WPC 80 and sweet WPC 35, both
667 with and without prior incubation with hydroxylapatite.

668 Lanes: 2= low range markers (range= 6,500-66,000 Da), 3= AWPC80, 4= AWPC80
669 supernatant after incubation with HA, 5= SWPC80, 6= SWPC80 supernatant after
670 incubation with HA, 7= SWPC35, 8= SWPC35 after incubation with HA and 9= wide
671 range markers (range= 6,500-205,000 Da).

672 **Figure 2:** Effects of Enzyme-Treated Sweet WPC80 on the growth of *S. mutans*, at
673 5mg mL^{-1} (○), 2.5mg mL^{-1} (□), 1.25mg mL^{-1} (Δ), 0.6mg mL^{-1} (●) and control growth
674 in the absence of inhibitor (■).

675 Data= mean ± standard deviation, n=4.

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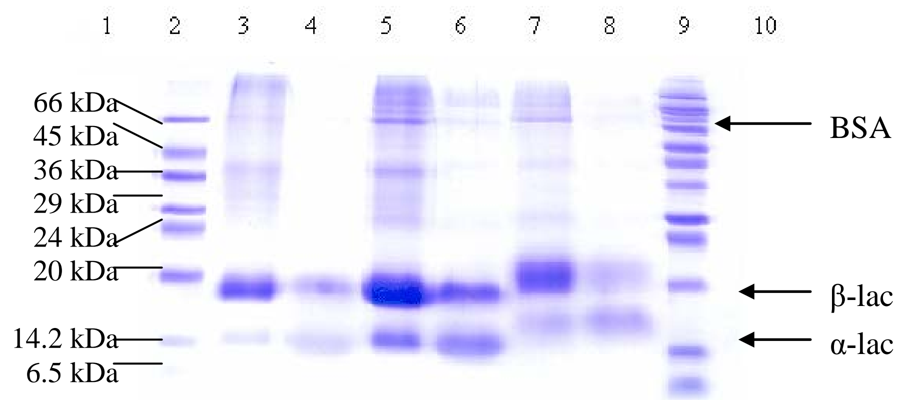
689 **Figure 1:**



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691 **Footnotes:** Bands representing bovine serum albumin (BSA), β-lactoglobulin and α-
692 lactalbumin correspond to the markers labelled at 66 kDa, 18 kDa and 14k Da, respectively.
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708 **Figure 2:**

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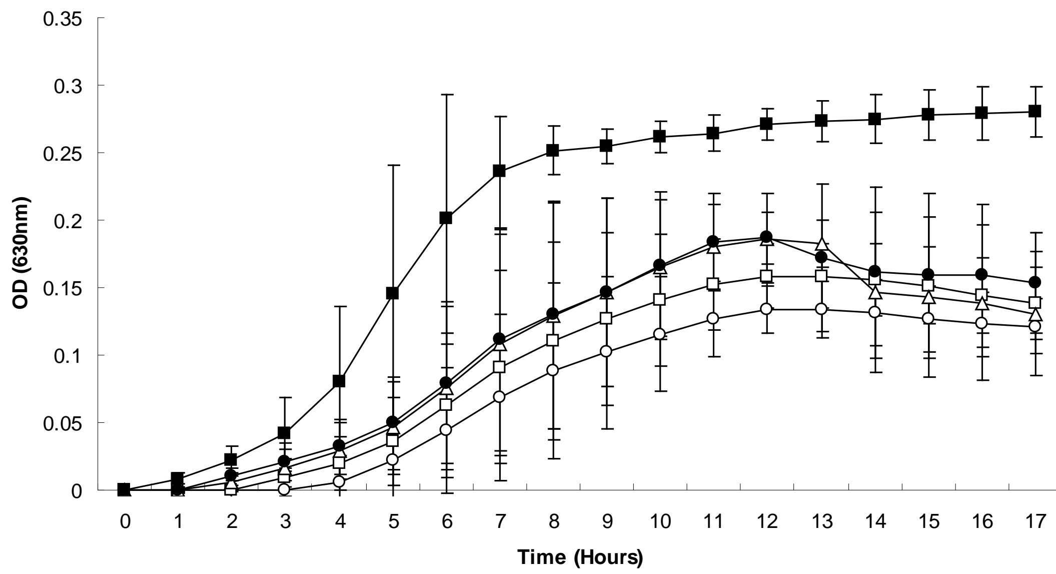

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725 **Table 1:** Compositional analysis of dairy powders used in this study (g/ 100g)

	SWPC80	AWPC80	SWPC35	WPI	WP	DW	BMP	CP
Protein	75.5	78.2	34.3	86.6	12.5	13	30.2	16.4
Fat	8	7.7	3.4	0.1	1	1.8	10.8	49.1
Moisture	7.5	6.3	5.4	5.8	3.1	3.5	3.9	2.1
Ash	3	5.9	6.2	2.6	9.5	0.8	6.9	4.5
Lactose	6	1.9	50.7	4.9	73.9	80.9	48.2	27.9
Total	100	100	100	100	100	100	100	100

726 **Abbreviations:** SWPC80= Sweet Whey Protein Concentrate 80, AWPC80= Acid
727 WPC80, SWPC35= Sweet Whey Protein Concentrate 35, WPI= Whey Protein Isolate,
728 WP= Whey Powder, DW= Demineralised Whey, BMP= Buttermilk Powder and CP=
729 Cream Powder.

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739 **Table 2: Proportion of *S. mutans* (%) not adhering to PBS-HA in the presence of dairy powders at various concentrations.**

		(i) Untreated			(ii) Enzyme-Treated		
µg mL^{-1}	**Control***	**31.25**	**62.5**	**125**	**31.25**	**62.5**	**125**
	15.1 ±4.6 [(w)]						
SWPC80		21.6 ±5.8[a,b (x)]	39.2 ±6[a (y) ¥]	66.5 ±5.9[a (z) ¥]	24.6 ±0.9[a (x)]	29.9 ±3.3[a,b (x)]	37.8 ±4.2[a (y)]
AWPC80		60.1 ±11[c (x) ¥]	82.7 ±2.6[b (y) ¥]	92.7 ±1.9[b (z) ¥]	16.5 ±3[a,b (w)]	17 ±3.4[c (w)]	18.6 ±4.4[b (w)]
SWPC35		17.8 ±4.9[a,b (w,x)]	23.3 ±6[c,d (y)]	50 ±9.4[c (z) ¥]	20.1 ±4.4[a,b (w,x)]	19.7 ±4.8[c,d (w,x,y)]	20.5 ±4.9[b (w,y)]
WPI		25.2 ±11.4[a,b (x,y)]	32.2 ±12.4[a,c (x,y) ¥]	42.9 ±20.1[c (z) ¥]	16.3 ±1.8[a,b (w)]	15.7 ±2.5[c (w)]	16.8 ±3.1[b (w)]
WP		12.2 ±2.3[b (w)]	13.7 ±2.1[d (w,x)]	20.6 ±1.7[d,e (x)]	14.6 ±2.3[b (w)]	14.4 ±3.6[c (w)]	15 ±3.6[b (w)]
DW		14.4 ±6.3[b (w)]	15.5 ±4.1[d (w)]	35.5 ±11.8[c,d (x) ¥]	18.2 ±1.4[a,b (w)]	16.3 ±3.1[c (w)]	18.7 ±5.2[b (w)]
BMP		28.2 ±5.3[a (x)]	53.2 ±7.9[e (y) ¥]	83.8 ±6.4[b (z) ¥]	22.5 ±4.2[a,b (x)]	25 ±3.2[b,d (x)]	27.9 ±4.2[c (x)]
CP		45.9 ±13.1[d (x)]	70.4 ±12.2[f (y) ¥]	83.5 ±9.8[b (z) ¥]	38.2 ±14.7[c (x)]	35.8 ±7[a (x)]	39.2 ±6.8[a (x)]
EggAlbumin†		17.4 ±5.4[a,b (w)]	18 ±6.3[d (w)]	15.7 ±6.6[e (w)]			
Lactose††		5.8 ±1.1 [(x)]	5.8 ±1 [(x)]	5.9 ±0.9[(x)]			

740 Footnotes:
741 Data presented represent the means (± SD) of 3 replicates. Within each column, means bearing different superscripts (a,b,c etc.) are significantly
742 (*P*<0.05) different. Data within each row bearing different superscripts (x,y,z) show significant (*P*<0.05) differences between concentrations
743 within (i) untreated and (ii) enzyme-treated dairy powders, with control adherence bearing the superscript ‘w’.
744 ¥ denotes significant difference (*P*<0.05) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.
745 *n=60, †= egg albumin is included for the sake of comparison only as a protein control. †† lactose n=2.
746 Abbreviations:
747 SWPC80= Sweet Whey Protein Concentrate 80, AWPC80= Acid WPC80, SWPC35= Sweet Whey Protein Concentrate 35, WPI= Whey Protein
748 Isolate, WP= Whey Powder, DW= Demineralised Whey, BMP= Buttermilk Powder and CP= Cream Powder.
749

750 **Table 3: Proportion of *S. mutans* (%) not adhering to S-HA in the presence of dairy powders at various concentrations.**

		(i) Untreated			(ii) Enzyme-Treated		
µg mL^{-1}	**Control***	**31.25**	**62.5**	**125**	**31.25**	**62.5**	**125**
	36.8±7.7$^{(w)}$						
SWPC80		72.7 ±6.2$^{a,b,c\ (x)}$ ¥	82.8 ±7.3$^{a\ (x)}$ ¥	76.4 ±7.8$^{a\ (x)}$	52.8 ±8.8$^{a,b\ (x)}$	60.9 ±14.9$^{a\ (x)}$	80.3 ±0.5$^{a\ (y)}$
AWPC80		74.7 ±1.7$^{a,b\ (x)}$ ¥	76.3 ±3.5$^{a,b\ (x)}$ ¥	75.2 ±7.1$^{a\ (x)}$	50.9 ±7.6$^{a,b\ (x)}$	57.7 ±11.1$^{a\ (x)}$	86.4 ±13.7$^{a\ (y)}$
SWPC35		53.1± 16.7$^{d\ (x)}$	58.7 ±12$^{c,d\ (x)}$	55 ±7.6$^{b\ (x)}$ ¥	47.9 ±11.3$^{a,b\ (x)}$	52.2 ±10.1$^{a\ (x)}$	78.3 ±8.6$^{a\ (y)}$
WPI		50.9 ±7.5$^{d\ (x)}$	54.6 ±1.5$^{c,d\ (x)}$ ¥	55.2 ±7.9$^{b\ (x)}$ ¥	37.6 ±7.1$^{b\ (w)}$	39.3 ±5.9$^{a,b\ (w)}$	42.4 ±2.1$^{c\ (w)}$
WP		61.6 ±2.7$^{b,c,d\ (x)}$ ¥	79.3 ±1.8$^{a\ (y)}$ ¥	73.5 ±9.3$^{a\ (x,y)}$ ¥	33.9 ± 3.6$^{b\ (w)}$	40.3 ± 5.8$^{a,b\ (w)}$	42.7 ±4.9$^{c\ (w)}$
DW		61.4 ±4.7$^{b,c,d\ (x)}$ ¥	65.1 ±3.1$^{b,c\ (x)}$ ¥	62.6 ±5.1$^{a,b\ (x)}$ ¥	34.8 ±5$^{b\ (w)}$	33.7 ± 10.8$^{b\ (w)}$	42.8 ±6.4$^{c\ (w)}$
BMP		71.8 ± 1.6$^{a\ (x)}$ ¥	63.2 ± 1.8$^{b,c,d\ (y,z)}$ ¥	59.2 ± 1.8$^{b\ (y,z)}$ ¥	44.6 ±2.7$^{a,b\ (x)}$	40.8 ±5$^{a,b\ (w,x)}$	42 ± 4.9$^{c\ (w,x)}$
CP		58 ±10$^{c,d\ (x)}$	63.1 ±15$^{b,c,d\ (x)}$	59.2 ±7.3$^{b\ (x)}$ ¥	56.3 ±7.8$^{a\ (x)}$	52.6 ±10.7$^{a\ (x)}$	74 ±9.9$^{a\ (y)}$
EggAlbumin†		47.6 ±9.2$^{d\ (x)}$	48.9 ±7$^{d\ (x)}$	57.3 ±13.8$^{b\ (x)}$			

751 Footnotes:
752 Data presented represent the means (± SD) of 3 replicates. Within each column, means bearing different superscripts (a,b,c etc.) are significantly
753 (P<0.05) different. Data within each row bearing different superscripts (w,x,y,z) show significant (P<0.05) differences between concentrations
754 within (i) untreated and (ii) enzyme-treated dairy powders, with control adherence bearing the superscript 'w'.
755 ¥ denotes significant difference (P<0.05) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.
756 *n=60, †= egg albumin is included for the sake of comparison only as a protein control.
757

758 **Table 4:** Initial concentrations of various test materials and quantity of protein in the test material interacting with hydroxylapatite (n=1).
759

	µg protein per mg HA					
Quantity of Test Material (µg mL^{-1})	**SWPC80**	**AWPC80**	**SWPC35**	**WPI**	**BMP**	**CP**
31.25	3	4.9	2.2	4	1	0
62.5	5	9.1	4.2	9	4	3
125	10.5	19	7.8	15	8	7
250	14.0	28.5	8.2	23	15	13
500	21.4	60.3	8	27	26	N/ D

760 Abbreviations:
761 SWPC80= Sweet Whey Protein Concentrate 80, AWPC80= Acid WPC80, SWPC35= Sweet Whey Protein Concentrate 35, WPI= Whey Protein
762 Isolate¸BMP= Buttermilk Powder and CP= Cream Powder