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## The Effect of Untreated and Enzyme-Treated Commercial Dairy Powders on the Growth and Adhesion of *Streptococcus mutans*

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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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14 **Keywords:** *Streptococcus mutans*, dental caries, growth, adhesion, whey.

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26 **Abstract**

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

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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  $\beta$ -lactoglobulin  
78 (50%),  $\alpha$ -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<sub>2</sub>O) 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 10 $\mu$ m. Phosphate-buffered saline coated HA (PBS-HA, PBS: Oxoid,

174 Hampshire, England) was prepared by suspension of  $7.5\text{mg mL}^{-1}$  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  $60^{\circ}\text{C}$  for 30 min to  
180 inactivate degenerative enzymes, and subsequently centrifuged at  $12,000 \times g$  for 15  
181 min. The pellet was discarded and the supernatant (i.e. clarified whole saliva) was  
182 used to prepare a  $7.5\text{mg mL}^{-1}$  dispersion of HA. Aliquots ( $150\mu\text{L}$ ) 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  $30^{\circ}\text{C}$  for 1 h with gentle  
185 agitation ( $4.5 \times g$ ). Following this, the microtitre plate was centrifuged at  $805 \times g$  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  $5\text{mmol L}^{-1}$   
191 solution in dimethylsulphoxide (DMSO). This concentration was adjusted to  $5\mu\text{mol L}^{-1}$   
192 <sup>1</sup> 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  $\text{CFU mL}^{-1}$  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  $3220 \times g$  (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<sub>630nm</sub> 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<sup>-1</sup>). 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 × 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<sup>-1</sup> 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:

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

## 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<sup>-1</sup>) 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<sup>-1</sup> to 1mg mL<sup>-1</sup>. 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<sub>570nm</sub>), and readings were converted to mg mL<sup>-1</sup> protein using a standard curve of  
244 absorbance versus protein concentration (R<sup>2</sup>= 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 ( $\text{CFU mL}^{-1}$ ) 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 ( $100\mu\text{L}$ ) of test material were  
266 added to the wells of the plate, followed by  $100\mu\text{L}$  of the diluted culture; the final  
267 concentrations of test material were  $0.6\text{mg mL}^{-1}$ ,  $1.25\text{mg mL}^{-1}$ ,  $2.5\text{mg mL}^{-1}$  and  $5\text{mg}$   
268  $\text{mL}^{-1}$ . Bacterial growth in the absence of test material (i.e. control growth) was also  
269 determined. The plate was then incubated at  $37^\circ\text{C}$  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<sub>4</sub>) and hexanoic (C<sub>6</sub>) 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<sup>-1</sup>. The column  
287 temperature was 140°C and the injection block was set at 300 °C. The volatile SCFAs  
288 (C<sub>4</sub> and C<sub>6</sub>) present in the aqueous dairy powder hydrolysate (20 g L<sup>-1</sup>) samples were  
289 measured by SPME headspace analysis using iso-butanoic (iso-C<sub>4</sub>) and 4-methyl-  
290 pentanoic acids (4-Me-C<sub>5</sub>) 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<sup>-1</sup>) 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<sub>4</sub> or C<sub>6</sub>)

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

305 The response factors for the two analytes were established by carrying out SPME  
306 headspace analysis of an aqueous standard (0.2g L<sup>-1</sup> each of C<sub>4</sub>, iso-C<sub>4</sub>, C<sub>6</sub> and 4-Me-  
307 C<sub>5</sub> acids) under the same conditions as described above for the liquid hydrolysate  
308 samples.

$$309 \quad \text{Response factor (C}_4 \text{ or C}_6\text{)} = \frac{\text{peak area C}_4}{\text{peak area iso-C}_4} \text{ or } \frac{\text{peak area C}_6}{\text{peak area 4-Me-C}_5} \quad (3)$$

310 The response factor was calculated as 1 for both C<sub>4</sub> and C<sub>6</sub>. GC retention times of the  
311 SCFAs were 2.2, 2.8, 5.6 and 6.6 min for iso-C<sub>4</sub>, C<sub>4</sub>, 4-Me-C<sub>5</sub> and C<sub>6</sub>, 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 125 $\mu\text{g mL}^{-1}$ , 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  
340 125 $\mu\text{g mL}^{-1}$ , 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  $\geq 40\%$  in the PBS-HA assays. ET CP was the most potent inhibitor of *S. mutans*



344 adhesion to PBS-HA at  $31.25\mu\text{g mL}^{-1}$ , and ET CP and ET SWPC80 were found to be  
345 equally as effective ( $P>0.05$ ) inhibitors at  $62.5\mu\text{g mL}^{-1}$  and  $125\mu\text{g mL}^{-1}$ .

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  $1000\mu\text{g mL}^{-1}$  (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  $\geq 31.25\mu\text{g mL}^{-1}$ , UT SWPC80, AWPC80 and BMP significantly  
355 ( $P\leq 0.05$ ) reduced adherence of *S. mutans* to S-HA relative to the protein control (egg  
356 albumin, EA). At  $62.5\mu\text{g mL}^{-1}$  and  $125\mu\text{g mL}^{-1}$ , 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  $31.25\mu\text{g mL}^{-1}$ ,  
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  $125\mu\text{g mL}^{-1}$ , 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  $\beta$ -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  $\alpha$ -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<sup>-1</sup>) (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<sub>630</sub> during the logarithmic growth phase suggests that the maximum  
404 concentration of ET SWPC80 (5mg mL<sup>-1</sup>) 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<sub>4</sub> and C<sub>6</sub>  
409 appeared to 'level off' after c. 60 min, and were present at levels of  $52.28 \pm 6.68\mu\text{g}$   
410 mL<sup>-1</sup> and  $18.66 \pm 1.49\mu\text{g mL}^{-1}$ , 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 Wuyckhuysse, 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 ( $125\mu\text{g mL}^{-1}$ ) 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  $\kappa$ -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<sup>-1</sup> (○), 2.5mg mL<sup>-1</sup> (□), 1.25mg mL<sup>-1</sup> (Δ), 0.6mg mL<sup>-1</sup> (●) and control growth  
674 in the absence of inhibitor (■).

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

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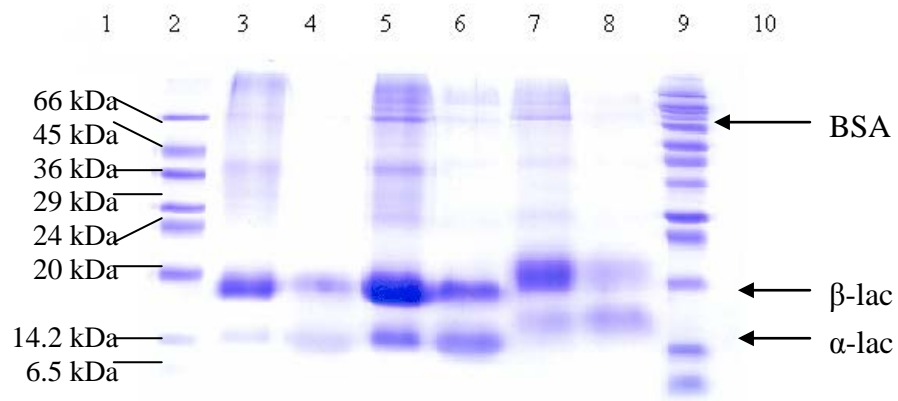
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689 **Figure 1:**



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691 **Footnotes:** Bands representing bovine serum albumin (BSA),  $\beta$ -lactoglobulin and  $\alpha$ -  
692 lactalbumin correspond to the markers labelled at 66 kDa, 18 kDa and 14k Da, respectively.

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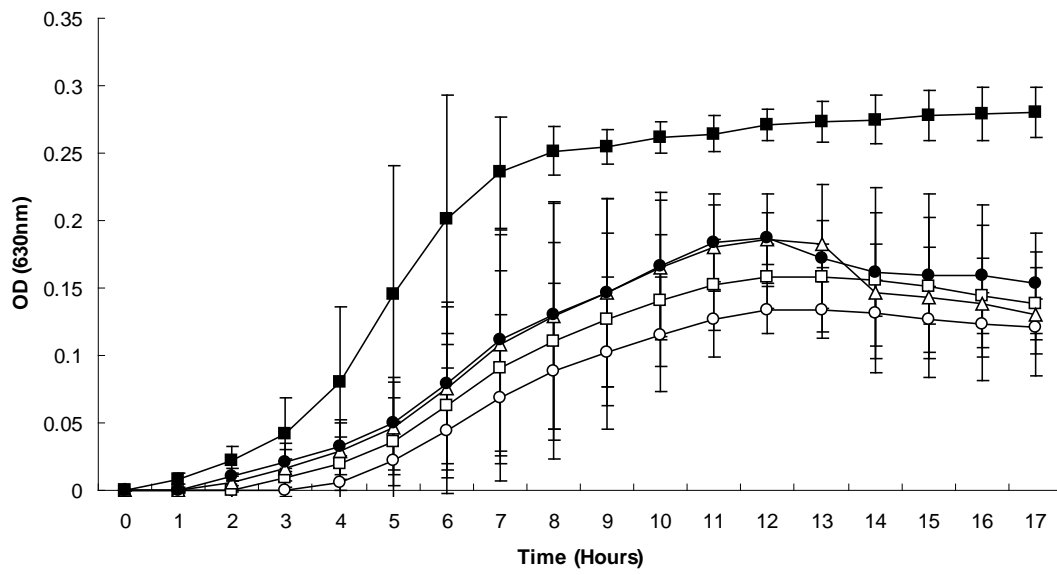
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708 **Figure 2:**

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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
<b>Protein</b>	75.5	78.2	34.3	86.6	12.5	13	30.2	16.4
<b>Fat</b>	8	7.7	3.4	0.1	1	1.8	10.8	49.1
<b>Moisture</b>	7.5	6.3	5.4	5.8	3.1	3.5	3.9	2.1
<b>Ash</b>	3	5.9	6.2	2.6	9.5	0.8	6.9	4.5
<b>Lactose</b>	6	1.9	50.7	4.9	73.9	80.9	48.2	27.9
<b>Total</b>	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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**Table 2: Proportion of *S. mutans* (%) not adhering to PBS-HA in the presence of dairy powders at various concentrations.**

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

740 Footnotes:

741 Data presented represent the means ( $\pm$  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.

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

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

751 Footnotes:

752 Data presented represent the means ( $\pm$  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.

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758 **Table 4:** Initial concentrations of various test materials and quantity of protein in the test material interacting with hydroxylapatite (n=1).  
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<b>µg protein per mg HA</b>						
<b>Quantity of Test Material (µg mL<sup>-1</sup>)</b>	<b>SWPC80</b>	<b>AWPC80</b>	<b>SWPC35</b>	<b>WPI</b>	<b>BMP</b>	<b>CP</b>
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