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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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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$31.25μg 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$0.6mg 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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1. Introduction

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

Research has shown that bovine milk components, including whey 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_2O) 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 $\times$ g for 15

181   min. The pellet was discarded and the supernatant (i.e. clarified whole saliva) was

182   used to prepare a 7.5mg 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 $\times$ 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 5mmol L^{-1}

191   solution in dimethylsulphoxide (DMSO). This concentration was adjusted to 5µmol L^{-1}

192   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 $\times$ 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_{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 $$\frac{(Fluorescence\ due\ to\ unbound\ bacteria)}{(Fluorescence\ due\ to\ total\ input\ bacteria)} \times 100 \qquad \textbf{(1)}$$

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

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

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 $125\mu g\ mL^{-1}$, 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 $125\mu g\ mL^{-1}$, 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 31.25µg 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 $\geq$ 31.25µg mL^{-1}, UT SWPC80, AWPC80 and BMP significantly

355 ($P\leq0.05$) 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.

558 **References**

559 Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I. & Dewhirst, F.E. (2005) Defining the

560 normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology, 43,* 5721-

561 5723.

562 Aimutis, W.R. (2004) Bioactive properties of milk proteins with particular focus on

563 anticariogenesis. *Journal of Nutrition, 134,* 989S-995S.

564 Beachey, E.H. (1981) Bacterial adherence: adhesin-receptor interactions mediating

565 the attachment of bacteria to mucosal surface. *Journal of Infectious Disease, 143,*

566 325-345.

567   Birner-Grunberger, R., Scholze, H., Faber, K. & Hermetter, A. (2003) Identification

568   of various lipolytic enzymes in crude porcine pancreatic lipase preparations using

569   covalent fluorescent inhibitors. *Biotechnology and Bioengineering*, *85,* 147-154.

570   Brady, D. & Folan, M. (2003) The use of milk serum apoproteins in the prophylaxis

571   of bacterial and or viral infections. Patent Number US2005042299.

572   Brody, E.P. (2000) Biological activities of bovine glycomacropeptide. *British Journal*

573   *of Nutrition, 84,* S39-S46.

574   Clark, W.B. & Gibbons, R.J. (1977) Influence of salivary components and

575   extracellular polysaccharide synthesis from sucrose on the attachment of

576   *Streptococcus mutans* 6715 to hydroxylapatite surfaces. *Infection and Immunity, 18,*

577   514-523.

578   Dashper, S.G., Liu, S.W. & Reynolds, E.C. (2007) Antimicrobial peptides and their

579   potential as oral therapeutic agents. *International Journal of Peptide Research and*

580   *Therapeutics, 13,* 505-516.

581   Finlay, B.B. & Falkow, S. (1997) Common themes in microbial pathogenicity

582   revisited. *Microbiology and Molecular Reviews, 61,* 136-169.

583   Gaines, S., James, T.C., Folan, M., Baird, A.W. & O'Farrelly, C. (2003) A novel

584   spectrofluorometric microassay for *Streptococcus mutans* adherence to

585   hydroxylapatite. *Journal of Microbiological Methods, 54,* 315-23.

586   Gibbons, R.J., Moreno, E.C. & Spinell, D.M. (1976) Model delineating the effects of

587   a salivary pellicle on the adsorption of *Streptococcus miteor* onto hydroxylapatite.

588   *Infection and Immunity, 14,* 1109-112.

589   Gibbons, R. & Etherden, I. (1982) Enzymatic modification of bacterial receptors on

590   saliva-treated hydroxyapatite surfaces *Infection and Immunity, 36,* 52-58.

591 Halpin, R.M., O'Connor, M.M., McMahon, A., Boughton, C., O'Riordan, E.D.,

592 O'Sullivan, M. & Brady, D.B. (2008) Inhibition of adhesion of *Streptococcus mutans*

593 to hydroxylapatite by commercial dairy powders and individual milk proteins.

594 *European Food Research and Technology, 227,* 1499-1506.

595 Halpin, R.M., Brady, D.B., O'Riordan, E.D. & O'Sullivan, M. (2009) Untreated and

596 Enzyme-Modified Bovine Whey Products Reduce Association of *Salmonella*

597 *typhimurium, Escherichia coli* O157:H7 and *Cronobacter malonaticus* (formerly

598 *Enterobacter sakazakii*) to CaCo-2 Cells *Journal of Applied Microbiology, DOI*

599 10.1111/j.1365-2672.2009.04436.x

600 Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C. & Stanton, C. (2006) Casein-derived

601 antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Applied and*

602 *Environmental Microbiology, 72,* 2260-2264.

603 International Dairy Federation (1987) Standard 9C: determination of fat content of

604 dried milk, dried whey, dried buttermilk and dried butter.

605 International Dairy Federation (1993a) Milk, determination of the nitrogen content: II.

606 Block digestion method (standard 20B) Brussels: International Dairy Federation.

607 International Dairy Federation (1993b) Dried Milk and Cream-Determination of

608 Water Content. Brussels: International Dairy Federation.

609 Johansson, I. (2002) Milk and dairy products: possible effects on dental health.

610 *Scandinavian J Nutr, 46(3)*, 119-122.

611 Kurhonen, H. (2009) Milk-derived bioactive peptides: From science to applications.

612 *Journal of Functional Foods, DOI* 10.1016/j.jff.2009.01.007.

613 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head

614 of bacteriophage T4. *Nature, 277,* 680-685.

615 Limsong, J., Benjavongkulchai, E. & Kuvatanasuchati, J. (2004) Inhibitory effect of

616 some herbal extracts on adherence of *Streptococcus mutans. Journal of*

617 *Ethnopharmacology, 92,* 281-289.

618 Loesche, W.J. (1986) Role of *Streptococcus mutans* in human dental decay.

619 *Microbiological Reviews, 50,* 353-380.

620 Lopez-Exposito, I. & Recio, I. (2006) Antibacterial activity of peptides and folding

621 variants from milk proteins. *International Dairy Journal, 16,* 1294-1305.

622 Madureira, A.R., Pereira, C.I., Gomes, A. M. P., Pintado, M.E. & Malcata, F.X.

623 (2007) Bovine whey proteins: Overview on their main biological properties. *Food*

624 *Research International, 40,* 1197-1211.

625 Malkomesius, P. E. & Nehring, K. (1951) Chemische Untersuchung von

626 Futtermitteln. In: *Handbuch der Landwirtschaftlichen Versuchs-und*

627 *Untersuchungsmethodik, (Band 3).* (Ed. Herrmann, R.). Naumann Verlag, Berlin,

628 Germany. pp 15-25.

629 Malkoski, M., Dashper, S.G., O'Brien-Simpson, N.M., Talbo, G.H., Macris, M.,

630 Cross, K.J. & Reynolds, E.C. (2001) Kappacin, a novel antibacterial peptide from

631 bovine milk. *Antimicrob Agents and Chemotherapy, 45,* 2309-2315.

632 Marshall, K. (2004) Therapeutic applications of whey protein. *Alternative Medicine*

633 *Reviews, 9,* 136-156.

634 Mor, A. (2003) Gene-encoded antimicrobial peptides. *Peptides, 24,* 1645.

635 Noronha, N., Cronin, D. A., O'Riordan, E.D. & O'Sullivan, M. (2008) Flavouring of

636 imitation cheese with enzyme-modified cheeses (EMCs): Sensory impact and

637 measurement of aroma active short chain fatty acids (SCFAs). *Food Chemistry, 106,*

638 905-913.

639   Ofek, I., Hasty, D.L. & Sharon, N. (2003) Anti-adhesion therapy of bacterial diseases:

640   prospects and problems. *FEMS Immunology and Medical Microbiology, 38,* 181-191.

641   Schuster, G.S. (1980) Anticaries and antiplaque potential of free fatty acids *in vitro*

642   and *in vivo*. *Pharmaceutical Therapeutics in Dentistry, 5,* 25-33.

643   Sewitz, R.H., Ismail, A.I. & Bitts, N.B. (2007) Dental caries. *Lancet, 369,* 51-59.

644   Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H.,

645   Provenzano,.M.D., Fuzimoto, E.K., Goeka, N.M., Olson, B.T. & Klend, D.C. (1986)

646   Measurement of protein using bicinchonic acid. *Analytical Biochemistry, 150,* 76-85.

647   Sprong, R.C., Hulstein, M.F.E. & van der Meer, R. (2001) Bactericidal activities of

648   milk lipids. *Antimicrobial Agents and Chemotherapy, 45,* 1298-1301.

649   Sprong, R.C., Hulstein, M.F.E. & van der Meer, R. (2002) Bovine milk fat

650   components inhibit food-borne pathogens. *International Dairy Journal, 12,* 209-215.

651   Tarsi, R., Muzzarelli, R.A., Guzman, C.A. & Pruzzo, C. (1997) Inhibition of

652   *Streptococcus mutans* adsorption to hydroxylapatite by low-molecular weight

653   chitosans. *Journal of Dental Research, 76,* 665-672.

654   Vacca-Smith, A.M, Van Wuyckhuyse, B.C., Tabak, L.A. & Bowen, L.H. (1994) The

655   effect of milk and casein proteins on the adherence of *Streptococcus mutans* to saliva-

656   coated hydroxylapatite. *Archives of Oral Biology, 39,* 1063-1069.

657   Yamanaka, A., Kimizuka, R., Kato, T. & Okuda, K. (2004) Inhibitory effects of

658   cranberry juice on attachment of oral streptococci and biofilm formation. *Oral*

659   *Microbiology and Immunology, 19,* 150-154.

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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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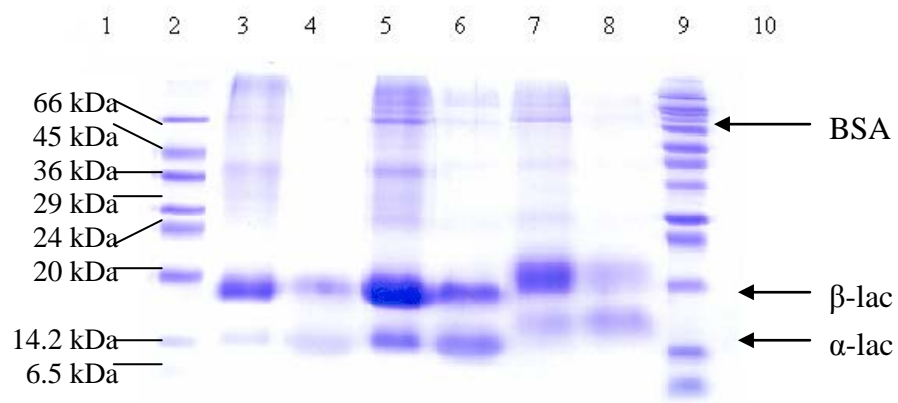677

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689 **<u>Figure 1:</u>**

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691 **<u>Footnotes:</u>** 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.
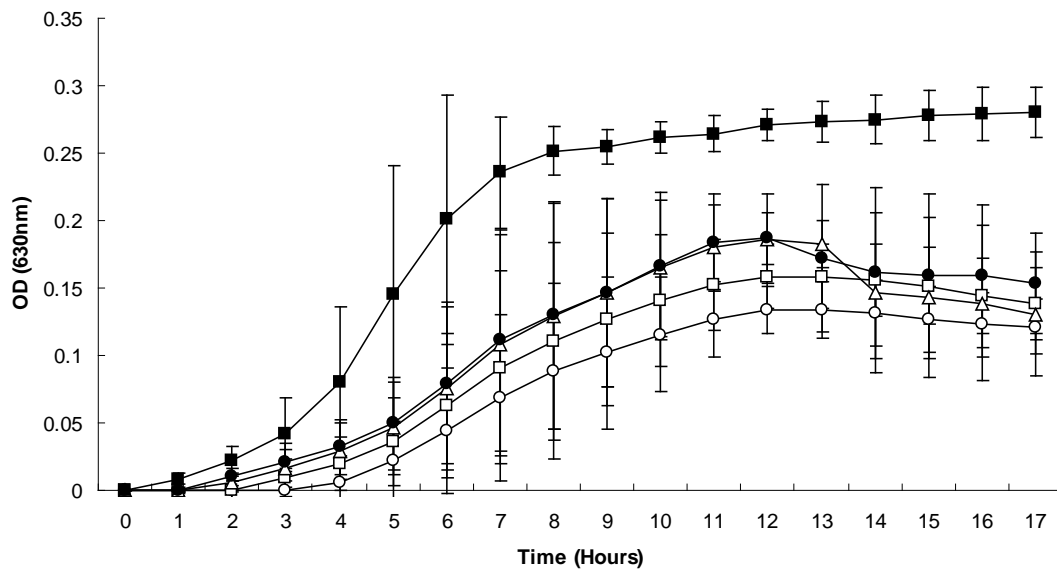693

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Figure 2:

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		
$\mu g\ mL^{-1}$	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

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

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

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.
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