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Determination of the Effect of Dairy Powders on Adherence of Streptococcus sobrinus and Streptococcus salivarius to Hydroxylapatite and Growth of these Bacteria

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1 **Determination of the Effect of Dairy Powders on Adherence of *Streptococcus***

2 ***sobrinus* and *Streptococcus salivarius* to Hydroxylapatite and Growth of these**

3 **Bacteria**

4

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Abstract

Dental caries is a highly prevalent disease caused by colonisation of tooth surfaces by cariogenic bacteria, such as *Streptococcus sobrinus* and *S. salivarius*. Reducing initial adherence of such bacteria to teeth may delay onset of caries. Many foods, such as milk, can inhibit microbial adherence. In this investigation, the effect of untreated (UT) and enzyme-treated (ET) dairy powders on adherence of *S. sobrinus* and *S. salivarius* to hydroxylapatite (HA), an analogue of tooth enamel, was examined. UT acid whey protein concentrate (WPC) 80 inhibited streptococcal adherence to phosphate-buffered saline-coated HA (PBS-HA) and saliva-coated HA (S-HA) by >80% at $\geq 31.25 \mu g$ mL^{-1}. UT sweet WPC80, buttermilk powder and cream powder also significantly reduced adherence ($P<0.05$). Enzyme-treatment of all dairy powders reduced their anti-adhesion activity. However, ET sweet WPC80 significantly inhibited growth of these streptococci ($P<0.05$) at ≥ 0.6mg mL^{-1}. Therefore, dairy powders may reduce progression of dental caries by their anti-adhesion and /or antibacterial activity.

1. Introduction

Dental caries is a bacterial disease characterised by a localised progressive, molecular disintegration of the tooth (Marcotte and Lavoie, 1998). Tooth decay and periodontal disease are among the most common bacterial infections in humans (Loesche, 1986), affecting both children and adults (Aas *et al*., 2005). The main etiological agents of human dental caries are the mutans streptococci, such as *Streptococcus sobrinus* (Loimaranta *et al*., 1997), a strongly acidogenic bacterium (Nascimento *et al*., 2004). Though it is not a member of the mutans streptococci, *Streptococcus salivarius* is also associated with formation of dental caries (Becker *et al*., 2002). *S. salivarius* is one of the earliest colonisers of the oral cavity following birth (Carlsson *et al*., 1970), and has long been recognised as a 'potent acid producer' (Shiere *et al*., 1951). In addition to causing dental caries, microorganisms inhabiting the oral cavity can be introduced into the bloodstream, leading to occurrence of 'focal oral infections', including bacteremia, endocarditis and meningitis (Gendron *et al*., 2000, Reif *et al*., 2009).

Adherence to oral mucosa and tooth surfaces is a vital step for bacterial colonisation of the oral cavity, as adherence provides resistance to salivary flow (Marcotte and Lavoie, 1998). In the 1970's Liljemark and co-workers proposed that the initial colonisation of the tooth surface was of utmost importance when attempting to prevent or control formation of dental plaque (Liljemark *et al*., 1978). In recent years, many foods and beverages such as water-soluble protein-fraction (WSPF) of hen egg yolk (Gaines *et al*., 2003), cranberry constituents (Yamanaka *et al*., 2004), barley coffee (Papetti *et al*., 2007) and herbal extracts (Limsong *et al*., 2004, Chen *et al*., 2005) have been found to reduce adherence of caries-causing bacteria to tooth surfaces. Human milk represents a classic example of how dietary constituents are

3

75 capable of reducing bacterial adherence (Ofek *et al.*, 2003). It is not unreasonable to

76 speculate that the equivalent components of bovine milk and milk-derived products,

77 such as whey, may also possess adherence inhibitory properties.

78   Addition of rennin or acid to milk causes the casein proteins to coagulate,

79 while the remaining liquid phase is referred to as whey (Zadow, 1994). The main

80 constituents of whey include protein, lactose, vitamins, minerals and traces of milkfat

81 (Anonymous, 2003). Whey proteins are recognised as having both nutritional and

82 functional properties (Smithers, 2008), but some biologically active peptides

83 harboured within these proteins are latent until they are liberated by the action of

84 hydrolytic enzymes (Sinha *et al.*, 2007). Peptides exhibiting antimicrobial properties

85 have been isolated from whey proteins such as β-lactoglobulin, α-lactalbumin and

86 lactoferrin following proteolysis (Lopez-Exposito and Recio, 2006).

87   The milkfat component of whey may also possess antimicrobial activity.

88 Bovine milkfat contains a broad range of fatty acids varying in chain length and

89 degree of saturation (Jensen and Newburg, 1995). In the 1970's, researchers reported

90 that the antimicrobial action observed for milkfat was dependent on the release of free

91 fatty acids and monoglycerides by the hydrolytic action of lipases (Sun *et al.*, 2002).

92 Generally, Gram positive microorganisms (such as streptococci) are lipid sensitive

93 whereas Gram negatives are not (Kabara *et al.*, 1972), but some exceptions to this

94 trend exist (Sprong, 2002).

95   Considering these points, it is evident that both the protein and milkfat

96 constituents of whey may have the potential to inhibit cariogenic bacteria, particularly

97 following enzyme treatment. Further to this, it has been reported that some bioactive

98 peptides derived from dairy proteins can possess multi-functional properties (Haque

4

99 and Chand, 2008). Thus, in addition to antibacterial peptides, hydrolysis of whey

100 proteins may lead to production of peptides possessing anti-adhesion activity.

101   Research carried out in this laboratory (Halpin *et al.*, 2008) has shown that a

102 range of untreated dairy powders reduced adherence of the cariogenic bacterium *S.*

103 *mutans* to hydroxylapatite, a calcium-phosphate analogue of human tooth enamel

104 (Gibbons *et al.*, 1976, Clark and Gibbons, 1977). Further to this, more recent research

105 carried out by this group has shown that dairy powders pre- and post-hydrolysis can

106 inhibit adhesion of *S. mutans* to HA, and that enzyme treated SWPC80 inhibits

107 growth of this microorganism (Halpin *et al.*, 2011). The aims of the present study

108 were firstly to assess the effects of various untreated and enzyme-treated dairy

109 products on the adherence of *S. sobrinus* and *S. salivarius* to hydroxylapatite.

110 Adherence was examined in the presence and absence of saliva. In addition, the effect

111 of enzyme-treated sweet whey protein concentrate on the growth of these cariogenic

112 streptococci was examined.

113 **2. Materials and Methods**

114 **2.1 Bacterial Isolates and Growth Conditions**

115 *S. sobrinus* (DSM 20742) was obtained from the German Collection of

116 Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *S. salivarius*

117 (2184 D41287), a clinical isolate, was kindly donated by Professor Martin Cormican,

118 Microbiology Department, National University of Ireland, Galway.

119 Both strains were maintained on Protect™ Bacterial Preserve beads (Technical

120 Service Consultants Ltd, Lancashire, UK) at -80˚C. A single bead from the frozen

121 stock culture was used to inoculate a Columbia blood agar plate (CBA: Oxoid,

122 Hampshire, England) and grown aerobically at 37˚C for 48 h. A single colony from

123   the blood agar plate was subsequently used to inoculate 20mL of brain heart infusion

124   (BHI) broth (BHI Broth: LabM, Lancashire, UK) and grown under aerobic conditions

125   without shaking at 37˚C for 18 h.

126   **2.2 Source and Characterisation of Dairy Powders**

127   Sweet whey protein concentrate (SWPC80), acid WPC 80 (AWPC80), sweet WPC 35

128   (SWPC35), whey protein isolate (WPI), whey powder (WP) and demineralised whey

129   (DW) powders were supplied by Carbery Milk Products (Ballineen, Cork, Ireland).

130   Buttermilk powder (BMP) and cream powder (CP) were supplied by Kerry Group plc

131   (Tralee, Co. Kerry, Ireland). Albumin from chicken egg white (grade V) was supplied

132   by Sigma (Poole, Dorset, UK).

133   Compositional analysis was performed on each dairy product using standard methods.

134   Ash content was analysed according to Malkomesius & Nehring (1951). Fat content

135   was determined according to the method of Röse-Gottlieb (International Dairy

136   Federation, IDF, 1987), protein content was determined by the Kjeldahl method (IDF,

137   1993) and the moisture content was determined by the IDF reference method (IDF,

138   1993).

139   **2.3 Hydrolysate Preparation Conditions**

140   Crude porcine pancreatic lipase (PPL, 100-400 units/ mg protein) (Sigma, Poole,

141   Dorset, England) was used throughout the study. Hydrolysates were prepared in a

142   Fermac 200 fermentor (Electrolab Ltd, Tewkesbury, UK) as follows: a c. 2% (w/v)

143   solution of substrate was prepared by dissolving 20g of dairy powder in 900mL of

144   sterile distilled water and heating at 37˚ C with stirring for 30 min. Lipase solution (1g

145   of PPL in 100mL of sterile H_2O) was added to the substrate solution to give a final

146   incubation volume of 1 L. The substrates were then incubated for 18 h at 37˚C with

6

147   stirring. The resulting hydrolysates were heated at 60˚C for 10 min in order to

148   denature the enzyme(s). Each hydrolysate was then placed on ice and allowed to cool

149   to less than 10˚C (approx. 45 min), before being frozen using liquid nitrogen and

150   subsequently lyophilised (Moduloyo, Edwards High Vacuum, Manor Royal, Crawley,

151   Sussex, UK).

152   **2.4 Adhesion Assay**

153   **2.4.1 Preparation of Hydroxylapatite**

154   Hydroxylapatite (HA) beads were supplied by Merck (Darmstadt, Germany). Both

155   buffer-coated and saliva-coated HA were used throughout the study Particle size

156   analysis using a Malvern Mastersizer (Malvern Instruments Ltd., Worcestershire, UK)

157   showed the average diameter (D [4,3]) of the HA beads to be approximately 10μm.

158   Phosphate-buffered saline coated HA (PBS-HA, PBS: Oxoid, Hampshire, England)

159   was prepared by suspension of 7.5mg mL^{-1} HA in PBS immediately before use in the

160   adherence assays.

161   Saliva-coated-HA (S-HA) was prepared similarly to the protocol set out by Gibbons

162   and Etherden (1982) as follows: parafilm-stilumated whole saliva was collected in an

163   ice-chilled tube from two healthy donors (1 male, 1 female) at least 1 h after eating,

164   drinking or brushing of teeth. The saliva was heated at 60ºC for 30 min to inactivate

165   degenerative enzymes, and subsequently centrifuged at 12,000 × g for 15 min. The

166   pellet was discarded and the supernatant (i.e. clarified whole saliva) was used to

167   prepare a 7.5mg mL^{-1} dispersion of HA. Aliquots (150μL) of this dispersion were

168   dispensed into the wells of a 96-well V-bottomed plate (Sarstedt, Newton, North

169   Carolina, USA), and incubated at 30ºC for 1 h with gentle agitation (4.5 × g).

170   Following this, the microtitre plate was centrifuged at 805 × g for 2 min, the

171   supernatants discarded and the S-HA pellets washed twice with sterile pre-warmed

7

172 PBS to remove excess saliva. The S-HA pellets were subsequently resuspended in

173 sterile PBS for use in the adherence assay.

2.4.2 Preparation of Syto® 13 dye

175 Syto® 13 dye (Molecular Probes, Oregon, USA) was supplied as a 5mM solution in

176 dimethylsulphoxide (DMSO). This concentration was adjusted to 5μM by appropriate

177 dilution in sterile PBS, and was used only on the day of preparation. Standard curves

178 were constructed to show the relationship between relative fluorescent units (RFU)

179 and colony forming units per millilitre (CFU mL^{-1}) for *S. sobrinus* and *S. salivarius*,

180 which had correlation coefficient values (R^2) of 0.993 (Figure 1(a)) and 0.989 (Figure

181 1(b)), respectively.

2.4.3 Assay Protocol

183 Overnight cultures of *S. sobrinus* and *S. salivarius* were subjected to centrifugation at

184 $3220 \times g$ (Eppendorf 5810R, Cambridge, UK) for 10 min and each of the pellets were

185 washed once in sterile PBS. Following a second centrifugation step, the bacterial

186 pellets were re-suspended in PBS, and the OD_{630nm} of the suspensions measured using

187 a Multiskan Ascent spectrophotometer, and adjusted to 0.2 by appropriate dilution

188 with sterile PBS.

189 The adherence assays were carried out as previously described (Halpin *et al*., 2008,

190 Halpin *et al.*, 2011), using sterile 96-well polystyrene microtitre half-area plates

191 (Nunc, Roskilde, Denmark). Dairy powders were prepared to the required

192 concentration by dispersing the dried powder in PBS. Briefly, 50μL of test material

193 solution at various concentrations was added to the wells, followed by 50μL of PBS-

194 HA or S-HA (7.5 mg mL^{-1}). Bacterial suspension (50μL) was added to the wells, so

195 that the final volume of each well was 150μL. Control wells (no bacteria and/ or no

196 HA) were included in each assay. The plate was incubated at room temperature for 45

8

197   min, and manually inverted at 5 min intervals to prevent settling of the HA

198   suspension. The plate was subsequently centrifuged at $201 \times g$ to sediment the HA

199   and any adhering bacteria, leaving the non-adhering bacteria in suspension. These

200   non-adhering bacteria were labelled with 10μL of 5μmol L^{-1} Syto® fluorescent dye.

201   For more information regarding the development and validation of the assay described

202   here, the reader should refer to Halpin *et al*., 2008.

203   **2.5 Quantification of Bacterial Adherence**

204   Aliquots (100μL) of supernatant from the adherence assay containing the non-

205   adhering bacteria were transferred from each well of the half-area plate to the

206   corresponding wells of a black microtitre plate (Costar, Corning Inc., Corning, USA).

207   This plate was allowed to stand at room temperature for 5 min in the dark before

208   reading the fluorescence using a Fluoroskan Ascent plate reader (Thermo Electron

209   Corporation, Finland). The excitation wavelength was 485 nm and the emission

210   intensity was monitored at 538 nm. Three measurements were taken at 5 min

211   intervals, and the average fluorescence calculated. The fluorescence due to the

212   number of bacteria present in the supernatant was determined as a direct readout from

213   the fluorimeter as relative fluorescent units (RFU). The background fluorescence due

214   to non-bacterial components of the assay (i.e. dairy powder and HA) were subtracted.

215   The percentage inhibition of adhesion was calculated as follows:

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

217 **2.6 Growth Assays**

218 Growth assays were carried out in sterile 96-well plates (Nunc, Roskilde, Denmark).

219 Overnight cultures of *S. sobrinus* and *S. salivarius* were prepared in BHI broth as

220 described earlier (section 2.1).

221 A working culture containing c. 10^8 colony forming units per millilitre (CFU mL^{-1})

222 was prepared by adding 1mL of overnight culture to 9mL of sterile BHI broth. Test

223 materials were prepared by dispersing dried dairy powders or hydrolysates in BHI

224 broth to the desired concentration. Aliquots (100μL) of test material were added to the

225 wells of the plate, followed by 100μL of the diluted culture; the final concentrations

226 of test material were 0.6mg mL^{-1}, 1.25mg mL^{-1}, 2.5mg mL^{-1} and 5mg mL^{-1}. Bacterial

227 growth in the absence of test material (i.e. control growth) was also determined. The

228 plate was then incubated at 37˚C for 18 h in a Multiskan Ascent plate reader (Thermo

229 Electron Corporation, Finland). Immediately prior to incubation the plate was shaken

230 for 1 min in order to disperse the suspensions. The optical density (OD) readings at

231 630nm for each well were subsequently recorded at 1 h intervals, with the plate being

232 shaken for 30 s immediately prior to measurement. The initial OD_{630nm} reading,

233 recorded at time 0, of each well was subtracted from all other readings for the

234 corresponding wells over the 18 h incubation time (i.e. to subtract the background

235 OD_{630nm} values). Growth inhibition (%) of *S. sobrinus* and *S. salivarius* due to the

236 presence of dairy powder was calculated using OD_{630nm} values at mid-stationary phase

237 according to the following equation:

238
$$\left[\frac{(OD\ Control\ Growth) - (OD\ Growth\ in\ Presence\ of\ Dairy\ Powder)}{(OD\ Control\ Growth)}\right] \times 100 \quad \textbf{(2)}$$

239 **2.7 Statistical Analysis**

240 All growth / adherence assays were performed at least three times (n=3). Results were

241 expressed as the mean ± standard deviation (S.D.). Differences between

242 concentrations within treatments were determined using least significant difference

243 (LSD) test, while differences between treatments were determined using Duncan's

244 test. Both analyses were performed using SAS Version 9.1.3. Data were considered

245 significantly different if $P<0.05$.

246 **3. Results**

247 Compositional analysis of protein, fat, moisture, ash and lactose content of each dairy

248 powder was determined, and is summarised in Table 1. These were typical of their

249 product types.

250 **3.1 Adherence Assays**

251 Standard curves were constructed to show the relationship between relative

252 fluorescent units (RFU) and colony forming units per millilitre (CFU mL^{-1}) for *S.*

253 *sobrinus* and *S. salivarius*, and are shown in Figure 1 (a) and (b), respectively.

254 **3.1.1 *S. sobrinus***

255 <u>(i) Adherence to Phosphate-Buffered Saline-Coated Hydroxylapatite (PBS-HA)</u>

256 Typically, c. 28% of any given culture of *S. sobrinus* used throughout this study did

257 not adhere to PBS-HA in the absence of test material ('control' in Table 2).

258 Of the UT dairy powders, AWPC80 was the most effective inhibitor of *S. sobrinus*

259 adherence to PBS-HA at 31.25μg mL^{-1} and 62.5μg mL^{-1} ($P<0.05$). At 62.5μg mL^{-1},

260 UT SWPC80, UT BMP and UT CP showed a significant concentration dependent

261 increase ($P<0.05$), and at the maximum concentration examined (125μg mL^{-1}) UT

262 AWPC80, UT SWPC80, UT BMP and UT CP were found to be equally effective

11

263 (*P*<0.05).   Of the untreated dairy powders, WPI, WP and DW were the poorest

264 inhibitors of *S. sobrinus* adherence to PBS-HA at all concentrations.

265 Following enzyme-treatment, the anti-adhesion activity of all powders was reduced.

266 At 31.25μg mL^{-1}, all ET dairy powders were only equally as effective as the protein

267 control, egg albumin (*P*>0.05). ET BMP was significantly (*P*<0.05) the most effective

268 inhibitor at 62.5μg mL^{-1} and 125μg mL^{-1}. ET SWPC35, WPI, WP and DW had no

269 inhibitory effect on adherence of *S. sobrinus* to PBS-HA at any concentration, relative

270 to the control (*P*>0.05). The loss in anti-adhesion activity due to enzyme-treatment

271 was most noticeable at the highest concentration (125μg mL^{-1}), with all powders

272 (except WP) being significantly (*P*<0.05) less effective when compared to its

273 equivalent untreated form.

274 (ii) Adherence to Saliva-Coated Hydroxylapatite (S-HA)

275 For the adherence assays carried out using *S. sobrinus*, c. 46% of microorganisms in

276 any given culture did not adhere to S-HA under our assay conditions ('control' in

277 Table 3). This value was markedly higher than the control level observed for PBS-

278 HA.

279 The egg albumin protein control inhibited adherence of *S. sobrinus* to S-HA to a

280 greater extent than UT SWPC35, UT WP and UT DW at 31.25μg mL^{-1} (*P*<0.05), with

281 UT SWPC35 actually significantly (*P*<0.05) promoting adherence. This was also

282 evident for UT WP and UT DW at 62.5μg mL^{-1}. At 125μg mL^{-1}, UT SWPC80, UT

283 AWPC80, UT WPI and UT CP appeared to be the most effective inhibitors of

284 adherence of *S. sobrinus* to S-HA and exhibited similar levels of activity, yet these

285 values were not significantly different from those observed for egg albumin (*P*>0.05).

286 For the enzyme-treated dairy powders, at maximum concentration (125μg mL^{-1}), only

287 ET AWPC80 was significantly more effective than egg albumin (*P*<0.05). Also, at

288   this concentration ET WPI, ET DW and ET CP did not reduce adherence of *S.*

289   *sobrinus* to S-HA relative to the control ($P>0.05$). However, at 125μg mL^{-1} ET

290   AWPC80, ET SWPC80 and ET BMP significantly inhibited adherence of *S. sobrinus*

291   to S-HA, causing the non-binding population of bacteria to increase to $\geq 80\%$.

292   **3.1.2 *S. salivarius***

293   <u>(i) Adherence to PBS-HA</u>

294   Approximately 41% of any given culture of *S. salivarius* used throughout this study

295   did not adhere to PBS-HA in the absence of test material ('control' in Table 4).

296   With the exception of DW, at 31.25μg mL^{-1} all of the UT test materials (including egg

297   albumin) significantly ($P<0.05$) reduced adherence of *S. salivarius* to PBS-HA

298   relative to the control. At 31.25μg mL^{-1}, UT AWPC80, UT WP and UT BMP

299   exhibited similar levels of inhibition of *S. salivarius* adhesion to PBS-HA (resulting in

300   a non-binding population of 85-90%) and were significantly ($P<0.05$) more potent

301   than the other untreated test materials. UT AWPC80, UT WPI, UT BMP and UT CP

302   were equally as effective at 62.5μg mL^{-1} and 125 μg mL^{-1} ($P>0.05$). However, UT

303   SWPC80 showed an equivalent level of anti-adhesion activity at 125μg mL^{-1}. Also at

304   this concentration (125μg mL^{-1}), all UT powders were more effective than the protein

305   control, egg albumin ($P<0.05$).

306   Subjecting the dairy powders to enzyme treatment reduced their ability to inhibit

307   adherence of *S. salivarius* to PBS-HA. No significant difference was found between

308   any ET test materials ($P>0.05$); furthermore, no ET dairy powder was more effective

309   than the protein control, egg albumin ($P>0.05$).

310   <u>(ii) Adherence to S-HA</u>

13

311 Due to the large non-binding population of *S. salivarius* to S-HA (c. 66%) it was

312 difficult to establish the efficacy of test materials in reducing adherence of this

313 microorganism to S-HA (Table 5).

314 At 31.25μg mL^{-1}, only UT SWPC80 and UT AWPC80 were found to be more potent

315 inhibitors of *S. salivarius* adhesion to S-HA than egg albumin ($P<0.05$). However, at

316 62.5μg mL^{-1} and 125μg mL^{-1}, all test materials (including egg albumin) showed equal

317 levels of efficacy ($P>0.05$).

318 Following enzyme-treatment, many of the hydrolysed dairy powders significantly

319 ($P<0.05$) inhibited adherence of *S. salivarius* to S-HA relative to the control, but only

320 ET WPI was found to be more effective than egg albumin ($P<0.05$). At 31.25μg mL^{-}

321 ^{1}, ET CP was the least effective inhibitor of *S. salivarius* adherence to S-HA ($P<0.05$).

322 No ET test material was more effective than egg albumin ($P>0.05$) at 62.5μg mL^{-1}

323 and 125μg mL^{-1}. At the maximum concentration examined (125μg mL^{-1}), only ET

324 SWPC35, ET WPI, ET WP and ET DW significantly ($P<0.05$) reduced adherence of

325 *S. salivarius* to S-HA relative to the control ($P<0.05$).

326 **3.2 Growth Assays**

327 ET SWPC80 was found to significantly ($P<0.05$) inhibit growth of *S. sobrinus* and *S.*

328 *salivarius* at all concentrations examined (Figure 2). Previous work in this laboratory

329 demonstrated that ET SWPC80 significantly inhibited growth of the highly cariogenic

330 microorganism *S. mutans* (Halpin *et al*., 2011), with no other enzyme-treated whey

331 product exhibiting an antibacterial effect against this microorganism (O'Connor *et al*.,

332 2006). Therefore, in the present study only ET SWPC80 was assessed for its

333 antibacterial activity against *S. sobrinus* and *S. salivarius*. The percentage growth

334 inhibition was calculated using formula (1) described earlier (section 2.6). A time

335   point for each *Streptococcus* was chosen, depending on the time taken for the

336   particular microorganism to reach mid-stationary phase. For *S. sobrinus* and *S.*

337   *salivarius* 10 hours and 9 hours incubation were chosen, respectively. Growth was on

338   average inhibited by 85.6% ± 5.9 for *S. sobrinus* at all concentrations. ET SWPC80

339   was less effective at inhibiting growth of *S. salivarius* when compared to inhibition

340   levels observed for *S. sobrinus*. However, growth was nevertheless inhibited by an

341   average of 50.6% ± 4.9 at all concentrations. Growth inhibition was significant at all

342   concentrations for both streptococci relative to control growth ($P<0.05$).

343   **4. Discussion**

344   The present study has shown that dairy powders can inhibit adherence of *S.*

345   *sobrinus* and *S. salivarius* to HA. The dairy powders were used firstly in their

346   untreated forms, and their anti-adhesion activity was again evaluated following

347   incubation with porcine pancreatic lipase (PPL). Both S-HA and PBS-HA models

348   were employed, to reflect the tooth surface in the presence and absence of saliva,

349   respectively. The S-HA model represents 'normal' conditions in the mouth, while the

350   PBS model system reflects conditions where saliva production is impaired ('dry

351   mouth' or xerostomia). In cases of xerostomia, an individual can experience severe

352   instances of dental caries. The occurrence of dry mouth is a well recognised clinical

353   problem in adults and children, and essentially occurs when the resting salivary flow

354   rate is less than that of fluid loss from the mouth (Walsh, 2008). This condition can be

355   due to use of certain medications (such as those prescribed for hypertension),

356   radiation treatment of the head and neck, or can be incurred by patients with aplasia of

357   the salivary glands (Sjogren's syndrome) (Loesche, 1986, Johansson, 2002). In the

358   present study, UT SWPC80, UT AWPC80, UT BMP and UT CP were the most

359   effective inhibitors of adhesion of both *S. sobrinus* and *S. salivarius* to HA in the

15

360 absence of saliva, and thus may be useful ingredients in the formulation of a dairy-

361 based saliva substitute. In addition, such dairy powders capable of inhibiting

362 adherence of streptococci to oral surfaces may help reduce the occurrence of focal

363 oral infections, as introduction of viridans streptococci resident in the oral cavity into

364 the bloodstream can lead to infections such as bacteremia (Gendron *et al*., 2000). This

365 occurrence is particularly problematic for patients experiencing neutropenia (Prabhu

366 *et al*., 2004).

367   The level of 'control' adhesion for both *S. sobrinus* and *S. salivarius* varied

368 greatly between PBS-HA and S-HA model systems. In the presence of saliva, UT

369 SWPC80, UT AWPC80, UT WPI and UT CP were the most effective inhibitors of *S.*

370 *sobrinus* adhesion to S-HA. However, all UT dairy powders (with the exception of

371 SWPC35 and WPI) significantly reduced adherence of *S. salivarius* to S-HA

372 ($P<0.05$). The findings of the present study are difficult to explain, as different levels

373 of anti-adhesion activity were observed for each of the of dairy powders against *S.*

374 *sobrinus* and *S. salivarius*, and the level of inhibition also varied depending on

375 whether PBS-HA or S-HA models were used. A possible reason for the varied levels

376 of efficacy exhibited by the dairy powders against *S. sobrinus* and *S. salivarius* may

377 be due to the different adherence mechanisms of these strains. *S. sobrinus* (a member

378 of the mutans streptococci) possesses a surface adhesin (SpaA) (Tokuda *et al*., 1990)

379 and genes capable of producing glucosyltransferases (Gilmore *et al*., 1990), whereas

380 strains of *S. salivarius* (which is not a member of the mutans streptococci) contain

381 proteinaceous components associated with a fibrillar layer outside the cell wall,

382 referred to as the 'fuzzy coat'. This fuzzy coat is believed to mediate attachment of *S.*

383 *salivarius* to host surfaces (Weerkamp *et al*., 1986). Thus, it is not surprising that the

16

384   dairy powders (and enzyme-treated versions thereof) do not interact with the different

385   surface proteins of these two streptococci in a similar manner.

386   In general, enzyme-treatment with PPL reduced the anti-adhesion efficacy of

387   the dairy powders in both PBS-HA and S-HA assays, but the degree of reduction was

388   less apparent for the latter. A possible reason for this may be interactions occurring

389   between constituents of the hydrolysates and components of saliva e.g. salivary

390   proteins or peptides. However, this is merely speculative and further research would

391   be required if the exact cause were to be determined. Of the enzyme-treated dairy

392   powders, ET SWPC80, ET AWPC80 and ET BMP were found to be the most

393   effective inhibitors of *S. sobrinus* adherence to S-HA. The majority of ET powders

394   appeared to reduce adherence of *S. salivarius* to S-HA, but this may have been due to

395   a non-specific protein effect, as egg albumin was also observed to reduce *S. salivarius*

396   adherence to S-HA, by about the same amount.

397   While the way in which the dairy powders used in this study are inhibiting

398   adherence of streptococci to HA has not yet been elucidated, protein adsorption

399   experiments performed previously by this research group indicated that proteins

400   present in the dairy powders were associating with the HA beads (Halpin *et al*., 2011).

401   This is likely to be contributing to the reduction in streptococcal adherence, as the

402   highest level of protein association was observed for UT AWPC80, which was also

403   the most effective inhibitor of streptococcal adherence to PBS-HA. However, it is

404   acknowledged in the context of such complex natural products that this may not be

405   the sole factor involved in inhibiting the adherence of streptococci to HA. In addition,

406   it should be noted that the less effective inhibitors were those which were lowest in

407   fat.

408   Another aspect of the present study was to determine the effect of ET

409   SWPC80 on the growth of *S. sobrinus* and *S. salivarius*. This hydrolysate inhibited

410   growth of these cariogenic bacteria by up to 85% at concentrations as low as 0.6mg

411   mL^{-1} (*P*<0.05). The crude PPL used in the present study is known to contain both

412   proteases and lipases (Birner-Grunberger *et al*., 2003), and it may be that enzyme

413   treatment of the dairy powders used in the present study releases both peptides and

414   free fatty acids that are inactive within the untreated material. Thus, the component(s)

415   of ET SWPC80 contributing to the observed antibacterial activity against *S. sobrinus*

416   and *S. salivarius* may on one hand be antibacterial peptides derived from whey

417   proteins such as β-lactoglobulin, α-lactalbumin or lactoferrin, as these proteins are

418   known to harbour antibacterial peptides that can be released by proteolysis (Lopez-

419   Exposito and Recio, 2006). Alternatively, the antibacterial activity could be due to

420   peptides cleaved from the glycomacropeptide (GMP), which is present in sweet whey

421   products due to the action of chymosin on κ-casein. A study by Malkoski *et al*. (2001)

422   showed that kappacin, a non-glycosylated, phosphorylated form of κ-casein, exhibited

423   significant antibacterial activity against oral pathogens. In addition to the peptide

424   hypothesis, it is possible that free fatty acids present in SWPC80 following enzyme-

425   treatment may have contributed to the antibacterial activity of this hydrolysate.

426   Previous work in this laboratory confirmed the presence of butyric (C$_4$) and caproic

427   (C$_6$) acids in SWPC80 after digestion with PPL (Halpin *et al*., 2011), and it is possible

428   that other fatty acids were present after hydrolysis. However, the exact mechanism of

429   action for the antibacterial activity of ET SWPC80 remains to be elucidated.

430   Nonetheless, the action of PPL on SWPC80 produced an effective antibacterial agent

431   possessing potent antimicrobial activity against caries-causing streptococci.

5. Conclusion

This study has demonstrated that UT dairy powders, in particular sweet and acid WPC80 are effective inhibitors of streptococcal adhesion to buffer-coated and saliva-coated HA. Thus, dairy powders, which are readily available and relatively inexpensive materials, may be suitable dental caries-protective agents for both normal mouth conditions and individuals suffering from xerostomia. The anti-adhesion properties of these dairy powders against streptococci may also potentially reduce occurrence of more serious infections such as bacteremia as a consequence. In addition, it is evident from this study that ET SWPC80 is an effective antimicrobial agent active against *S. sobrinus* and *S. salivarius*. However, future work is necessary in order to establish which specific components of the different products are responsible for the observed inhibition, and also to examine whether the~~extend the~~ observations of the present study to the oral cavity; thereby ~~and~~ establishing the efficacy of dairy products as therapeutic products *in vivo*.

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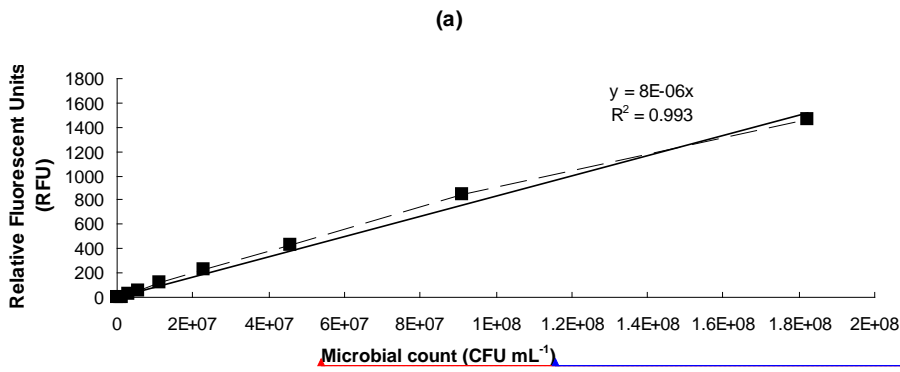582

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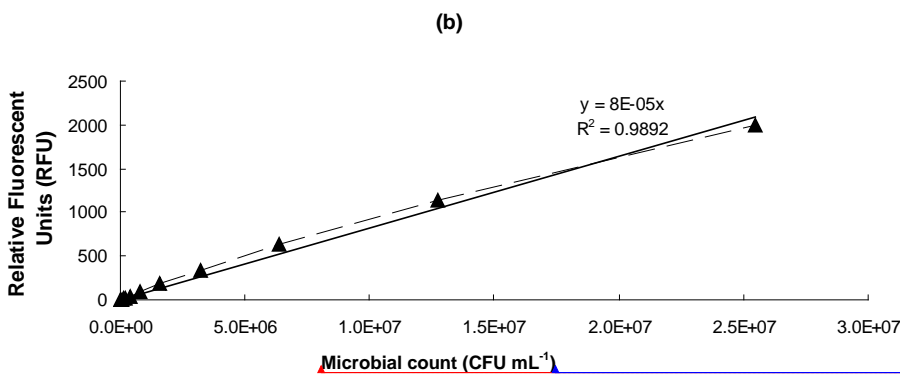588

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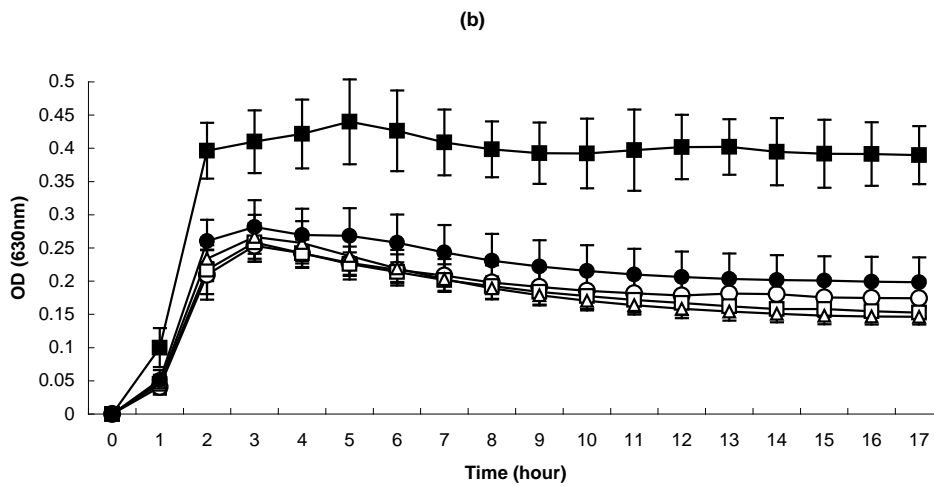

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Figure 1: Standard curves of relative fluorescent units (RFU) Vs colony forming
units per millilitre (CFU mL^{-1}) for (a) *S. sobrinus* and (b) *S. salivarius*.

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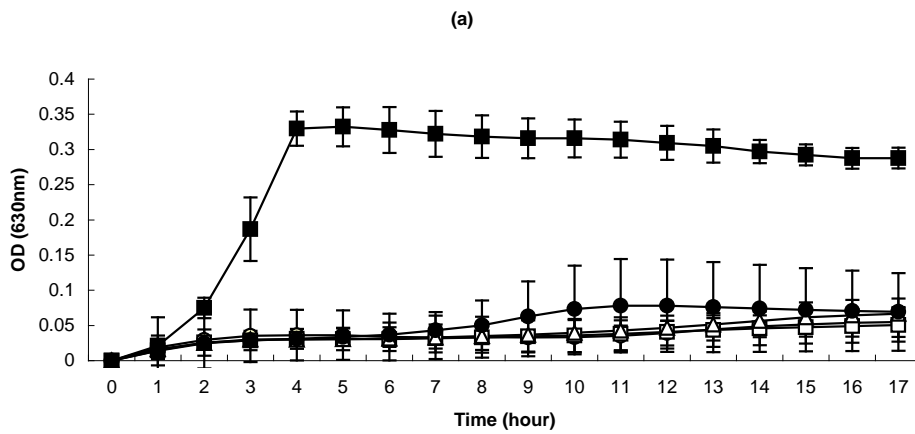
Figure 2: Effects of enzyme-treated Sweet WPC80 on the growth of (a) *S. sobrinus* and (b) *S. salivarius*, at 5mg mL^{-1} (○), 2.5mg mL^{-1} (□), 1.25mg mL^{-1} (Δ), 0.6mg mL^{-1} (●) and control growth in the absence of inhibitor (■). (Data= mean ± standard deviation, n=4).

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625 <u>**List of Tables:**</u>

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627 **Table 1:** Compositional analysis of dairy powders used in this study (%).

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

628 Abbreviations: SWPC80= Sweet Whey Protein Concentrate 80, AWPC80= Acid
629 WPC80, SWPC35= Sweet Whey Protein Concentrate 35, WPI= Whey Protein Isolate,
630 WP= Whey Powder and DW= Demineralised whey, BMP= Buttermilk Powder, CP=
631 Cream Powder.

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

μg mL^{-1}	Control*	Untreated			Enzyme-treated		
		31.25	**62.5**	**125**	**31.25**	**62.5**	**125**
	28 ±7.1$^{(w)}$						
SWPC80		33.7±3.6$^{a,b,c\ (w)}$	63 ±3.1$^{a\ (x)}$ ¥	91 ±3.2$^{a,b\ (y)}$ ¥	31 ±5.6$^{a\ (w,x)}$	32.2 ±5.9$^{a,b\ (w,x)}$	40.3 ±7.3$^{a\ (x)}$
AWPC80		90.7 ±6.1$^{d\ (x)}$ ¥	99.6 ±0.8$^{b\ (x)}$ ¥	100 ±4.7$^{a\ (x)}$ ¥	45.4^{a}	44.7^{b,c}	39.8^{a,b}
SWPC35		34.6 ±6.2$^{a,b\ (w)}$	44.5 ±9$^{c\ (x)}$	66.8 ±6.8$^{c\ (y)}$ ¥	35.1 ±18.9$^{a\ (w)}$	27.7 ±4.9$^{b\ (w)}$	34.5 ±9.8$^{a,b,c\ (w)}$
WPI		23.9 ± 4.2$^{c\ (w)}$	31.4 ±5.6$^{d\ (w)}$	43 ±6.7$^{d,e\ (x)}$ ¥	34.6 ±11.9$^{a\ (w)}$	30.1 ±7.2$^{b\ (w)}$	25.2 ± 2.8$^{b,c\ (w)}$
WP		26.9 ±4.1$^{b,c\ (w)}$	32 ±5$^{d\ (w,x)}$	37.5 ±4.7$^{d,e\ (x)}$	24^{a}	23.8^{b}	23.5^{c}
DW		30.4 ±3.2$^{a,b,c\ (w)}$	32.4 ±4.7$^{d\ (w)}$	44.7 ±9$^{d\ (x)}$ ¥	29.3 ±0.9$^{a\ (w)}$	24.5 ±2$^{b\ (w)}$	26.4 ±3.9$^{a,b,c\ (w)}$
BMP		73.1 ±4.4$^{e\ (x)}$ ¥	85.1 ±5$^{e\ (y)}$ ¥	98.4 ±3.2$^{a,b\ (z)}$ ¥	45.9 ±4.8$^{a\ (x)}$	56 ±3.6$^{c\ (x,y)}$	65.4 ±10.3$^{d\ (y,z)}$
CP		47.3 ±6.3$^{f\ (x)}$ ¥	67.4 ±7$^{a\ (y)}$ ¥	90.1 ±8.6$^{b\ (z)}$ ¥	31.3 ±3.3$^{a\ (w,x)}$	34 ±7.1$^{a,b\ (w,x)}$	39 ±4.4$^{a,b\ (x,y)}$
Egg Albumin†		38.9 ±11.6$^{a,f\ (x)}$	37.1 ±7.6$^{c,d\ (x)}$	33.8 ±5.8$^{e\ (w,x)}$			

Footnotes: PBS-HA= phosphate-buffered saline-coated hydroxylapatite.
Data presented represent the means (± SD) of 3 replicates. Within each column, means bearing different superscripts (a,b,c etc.) are significantly ($P<0.05$) different. Data within each row bearing different superscripts (x,y,z) show significant ($P<0.05$) differences between concentrations within (i) untreated and (ii) enzyme-treated dairy powders, with control adherence bearing the superscript 'w'.
¥ denotes significant difference ($P<0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.
*n=52, †= egg albumin is included for the sake of comparison only as a protein control.

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

μg mL^{-1}	Control*	Untreated 31.25	Untreated 62.5	Untreated 125	Enzyme-Treated 31.25	Enzyme-Treated 62.5	Enzyme-Treated 125
	45.8 ±10.8$^{(w)}$						
SWPC80		72.1 ±8.7$^{a\ (x)}$	87 ±9.7$^{a\ (x)}$	87 ±10.2$^{a,b\ (x)}$	82.9 ±12$^{a\ (x)}$	89.3 ±8.2$^{a\ (x)}$	96.8 ±5.6$^{a,b\ (x)}$
AWPC80		83.4 ±1.2$^{b\ (x)\ ¥}$	88.2 ±2.3$^{a\ (x)}$	89.1 ±9.7$^{a\ (x)}$	60.3 ±9.1$^{a,b,c\ (x)}$	81.4 ±7.4$^{a,b\ (y)}$	100$^{a\ (z)}$
SWPC35		38 ±6$^{c\ (w)}$	47.7 ±7.1$^{b,c\ (w,x)\ ¥}$	62.3 ±8.3$^{c,d\ (x)}$	57.4 ±23.7$^{b,c\ (w,x)}$	68.2 ±8.3$^{b,c,d\ (x)}$	76 ±15$^{b,c,d\ (x)}$
WPI		64.3 ±3.1$^{a,d\ (x)\ ¥}$	78.7 ±4.9$^{a,d\ (x,y)\ ¥}$	89.6 ±4.8$^{a\ (y)\ ¥}$	47.3 ±5.8$^{b,c\ (w)}$	54.6 ±6.6$^{d,e\ (w)}$	58.5 ±14$^{c,d,e\ (w)}$
WP		27.4 ±4.3$^{c\ (x)\ ¥}$	41 ±13.3$^{c\ (w,x,y)}$	53.3 ±16.8$^{d,e\ (w,y)}$	55.4 ±10.8$^{b,c\ (w,x)}$	63.4 ±10.6$^{b,c,d\ (x,y)}$	76.1 ±2.8$^{b,c,d\ (y)}$
DW		36.7 ±4.2$^{c\ (w)}$	41.8 ±9.7$^{c\ (w)}$	44.3 ±9.3$^{e\ (w)}$	37.5 ±10.3$^{c\ (w)}$	39.6 ±11$^{e\ (w)}$	48.4 ±11$^{e\ (w)}$
BMP		52.1 ±12.1$^{e\ (w)}$	61.9 ±14.7$^{b,d\ (x)}$	69.4 ±10.4$^{b,c,d\ (x)}$	62.1 ±18.7$^{a,b\ (x)}$	78.9 ±13.1$^{a,b,c\ (x)}$	80.1 ±16.7$^{a,b,c\ (x)}$
CP		57.1 ±6.6$^{d,e\ (w)}$)	62.6 ±3.7$^{b,d\ (x)}$	71.1 ±9.2$^{a,b,c,d\ (x)}$	62.2 ±10.8$^{a,b\ (x)}$	58.1 ±20.5$^{c,d,e\ (w,x)}$	55.8 ±22.2$^{d,e\ (w,x)}$
Egg Albumin†		51.2 ±5.5$^{e\ (w,x)}$	65.5 ±12.1$^{b,d\ (x,y)}$	76.1 ±7.4$^{a,b,c\ (y)}$			

Footnotes: S-HA= saliva-coated hydroxylapatite.
Data presented represent the means (± SD) of 3 replicates. Within each column, means bearing different superscripts (a,b,c etc.) are significantly ($P<0.05$) different. Data within each row bearing different superscripts (x,y,z) show significant ($P<0.05$) differences between concentrations within (i) untreated and (ii) enzyme-treated dairy powders, with control adherence bearing the superscript 'w'.
¥ denotes significant difference ($P<0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.
*n=53, †= egg albumin is included for the sake of comparison only as a protein control.

Table 4: Proportion of *S. salivarius* (%) not adhering to PBS-HA in the presence of dairy powders at various concentrations.

µg mL^{-1}	Control*	Untreated			Enzyme-Treated		
		31.25	62.5	125	31.25	62.5	125
	40.7 ±10.6$^{(w)}$						
SWPC80		61.3 ±8.2$^{a,b\ (x)}$	75 ±8$^{a,b,c\ (x)\ ¥}$	89.5 ±2.8$^{a,b,c,d\ (y)\ ¥}$	50.9 ±9.8$^{a,b\ (w,x)}$	53.2 ±6.6$^{a\ (x)}$	56.6 ±5.4$^{a\ (x)}$
AWPC80		90.4 ±6.7$^{c\ (x)\ ¥}$	98.6 ±1.8$^{d\ (x)\ ¥}$	97.8 ±4.3$^{a\ (x)\ ¥}$	40.4 ±4.4$^{a,b\ (w)}$	40.9 ±5.7$^{a,b\ (w)}$	39.7 ±8$^{a,b\ (w)}$
SWPC35		63.2 ±9$^{a,b\ (x)}$	69.3 ±6.4$^{a,b,c\ (x)\ ¥}$	77.2 ±3.6$^{d\ (x)\ ¥}$	47.1 ±10.7$^{a,b\ (w)}$	42.2 ±8$^{a,b\ (w)}$	39.4 ±5.6$^{a,b\ (w)}$
WPI		65.4 ±16.5$^{a,b\ (x)}$	84.8 ±15.7$^{a,b,d\ (y)}$	94.2 ±5.3$^{a,b\ (y)\ ¥}$	26.2^{b}	25.6^{b}	27.2^{b}
WP		86.5 ±12.7$^{c,d\ (x)\ ¥}$	74.3 ±16.8$^{a,b,c\ (x)}$	84 ±15.7$^{b,c,d\ (x)}$	39.3 ±9.8$^{a,b\ (w)}$	51 ±24.5$^{a,b\ (w)}$	50.1 ±21.4$^{a,b\ (w)}$
DW		51.2 ±14.3$^{b\ (w)}$	67.3 ±17.7$^{b,c\ (x)}$	78.7 ±12.8$^{c,d\ (x)\ ¥}$	38.4^{a,b}	44.1^{a,b}	30.5^{b}
BMP		85.6 ±9.3$^{c,d\ (x)\ ¥}$	89.7 ±7.7$^{a,d\ (x)\ ¥}$	95.6 ±3.1$^{a,b\ (x)\ ¥}$	44.8 ±11.6$^{a,b\ (w)}$	41.6 ±8.2$^{a,b\ (w)}$	39.7 ±11$^{a,b\ (w)}$
CP		71.1 ±9.3$^{a,d\ (x)}$	83.3 ±11.6$^{a,b,d\ (x,y)\ ¥}$	90.8 ±7$^{a,b,c\ (y,z)\ ¥}$	64.2 ±19.1$^{a\ (x)}$	49.7 ±11.8$^{a,b\ (w,x,y)}$	47 ± 15.3$^{a,b\ (y)}$
Egg Albumin†		60.6 ±10.1$^{a,b\ (x)}$	56.7 ±16.2$^{c\ (x,y)}$	41.6 ±1.8$^{e\ (w,y)}$			

Footnotes: PBS-HA= phosphate-buffered saline-coated hydroxylapatite.
Data presented represent the means (± SD) of 3 replicates. Within each column, means bearing different superscripts (a,b,c etc.) are significantly ($P<0.05$) different. Data within each row bearing different superscripts (x,y,z) show significant ($P<0.05$) differences between concentrations within (i) untreated and (ii) enzyme-treated dairy powders, with control adherence bearing the superscript 'w'.
¥ denotes significant difference ($P<0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.
*n=59, †= egg albumin is included for the sake of comparison only as a protein control.

Table 5: Proportion of *S. salivarius* (%) not adhering to S-HA in the presence of dairy powders at various concentrations.

µg mL^{-1}	Control*	Untreated			Enzyme-Treated		
		31.25	**62.5**	**125**	**31.25**	**62.5**	**125**
	66.2 ±15.7$^{(w)}$						
SWPC80		95.7 ±3.4$^{a\,(x)}$	87.1 ±5.7$^{a\,(x)}$	90.9 ±6.1$^{a\,(x)\,¥}$	91.1 ±3.9$^{a,b\,(x)}$	83.8 ±6.5$^{a,b\,(w,x)}$	68.3 ± 8.3$^{a,b\,(w,x)}$
AWPC80		95.7 ±7.4$^{a\,(x)}$	93.3 ±5.8$^{a\,(x)}$	98.7 ±2.2$^{a\,(x)\,¥}$	89.7 ±13.6$^{a,b\,(x)}$	89.2 ±12.7$^{a,b\,(x)}$	60.8 ±7.7$^{a,b\,(w,y)}$
SWPC35		69.2 ± 18.1$^{b\,(w)}$	77.8 ±22.7$^{a\,(w)}$	79.7 ±18.3$^{a\,(w)}$	83.8 ±3.9$^{a,b,c\,(x)}$	91.4 ±9.9$^{a\,(x)}$	89.5 ±9.6$^{a\,(x)}$
WPI		65 ± 25$^{b\,(w)}$	70.5 ±22.8$^{a\,(w)}$	77 ±19.5$^{a\,(w)}$	93.6 ±6.5$^{a\,(x)}$	94.7 ±12.5$^{a\,(x)}$	96.4 ±5.5$^{a\,(x)}$
WP		80.7 ±10.5$^{a,b(w,x)}$	86.2 ±1.3$^{a(x)}$	87.7 ±13.8$^{a(x)}$	80.8 ±5.7$^{a,b,c\,(w,x)}$	83.4 ±8.9$^{a,b\,(w,x)}$	91.1 ±12.4$^{a\,(x,y)}$
DW		81.2 ±15.2$^{a,b\,(w,x)}$	83.8 ±14.2$^{a\,(w,x)}$	85 ±18.6$^{a\,(x)}$	84 ± 9.9$^{a,b,c\,(w,x)}$	86.7 ±7$^{a,b\,(x)}$	95.2 ±6.7$^{a\,(x)}$
BMP		87.9 ±7.9$^{a,b\,(x)}$	84.1 ±15.1$^{a\,(x)}$	91.3 ±10.2$^{a\,(x)}$	90.1 ±2.7$^{a,b\,(x)}$	70.3 ±13.2$^{a,b\,(w,x)}$	52.2 ±33.6$^{b\,(w,y)}$
CP		67 ±13.6$^{b\,(w,x)}$	72.1 ±8.4$^{a\,(w,x)}$	88.2 ±13.1$^{a\,(x)}$	62.6 ±38.6$^{c\,(w)}$	63 ±33.2$^{b\,(w)}$	69.8 ±49.7$^{a,b\,(w)}$
Egg Albumin†		66.2 ±12.5$^{b\,(w)}$	75.8 ±9.2$^{a\,(w)}$	76.9 ±8.2$^{a\,(w)}$			

Footnotes: S-HA= saliva-coated hydroxylapatite.
Data presented represent the means (± SD) of 3 replicates. Within each column, means bearing different superscripts (a,b,c etc.) are significantly ($P<0.05$) different. Data within each row bearing different superscripts (x,y,z) show significant ($P<0.05$) differences between concentrations within (i) untreated and (ii) enzyme-treated dairy powders, with control adherence bearing the superscript 'w'.
¥ denotes significant difference ($P<0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.
*n=57, †= egg albumin is included for the sake of comparison only as a protein control.