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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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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 ≥31.25μg 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.

Keywords: *Streptococcus sobrinus*, *Streptococcus salivarius*, dairy powders, inhibition of adherence, fluorescence, growth inhibition.

Abbreviations: PBS-HA; Phosphate-buffered saline-coated hydroxylapatite, S-HA; Saliva-coated hydroxylapatite, SWPC80; Sweet whey protein concentrate 80, AWPC80; Acid whey protein concentrate 80, SWPC35; Sweet whey protein concentrate 35, WPI; Whey protein isolate, WP; Whey powder, DW; Demineralised whey, BMP; Buttermilk powder, CP; Cream powder, EA; Egg albumin, PPL; Porcine pancreatic lipase.

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

capable of reducing bacterial adherence (Ofek *et al*., 2003). It is not unreasonable to speculate that the equivalent components of bovine milk and milk-derived products, such as whey, may also possess adherence inhibitory properties.

Addition of rennin or acid to milk causes the casein proteins to coagulate, while the remaining liquid phase is referred to as whey (Zadow, 1994). The main constituents of whey include protein, lactose, vitamins, minerals and traces of milkfat (Anonymous, 2003). Whey proteins are recognised as having both nutritional and functional properties (Smithers, 2008), but some biologically active peptides harboured within these proteins are latent until they are liberated by the action of hydrolytic enzymes (Sinha *et al*., 2007). Peptides exhibiting antimicrobial properties have been isolated from whey proteins such as β-lactoglobulin, α-lactalbumin and lactoferrin following proteolysis (Lopez-Exposito and Recio, 2006).

The milkfat component of whey may also possess antimicrobial activity. Bovine milkfat contains a broad range of fatty acids varying in chain length and degree of saturation (Jensen and Newburg, 1995). In the 1970's, researchers reported that the antimicrobial action observed for milkfat was dependent on the release of free fatty acids and monoglycerides by the hydrolytic action of lipases (Sun *et al.*, 2002). Generally, Gram positive microorganisms (such as streptococci) are lipid sensitive whereas Gram negatives are not (Kabara *et al*., 1972), but some exceptions to this trend exist (Sprong, 2002).

Considering these points, it is evident that both the protein and milkfat constituents of whey may have the potential to inhibit cariogenic bacteria, particularly following enzyme treatment. Further to this, it has been reported that some bioactive peptides derived from dairy proteins can possess multi-functional properties (Haque

and Chand, 2008). Thus, in addition to antibacterial peptides, hydrolysis of whey proteins may lead to production of peptides possessing anti-adhesion activity.

Research carried out in this laboratory (Halpin *et al.*, 2008) has shown that a range of untreated dairy powders reduced adherence of the cariogenic bacterium *S. mutans* to hydroxylapatite, a calcium-phosphate analogue of human tooth enamel (Gibbons *et al*., 1976, Clark and Gibbons, 1977). Further to this, more recent research carried out by this group has shown that dairy powders pre- and post-hydrolysis can inhibit adhesion of *S. mutans* to HA, and that enzyme treated SWPC80 inhibits growth of this microorganism (Halpin *et al.*, 2011). The aims of the present study were firstly to assess the effects of various untreated and enzyme-treated dairy products on the adherence of *S. sobrinus* and *S. salivarius* to hydroxylapatite. Adherence was examined in the presence and absence of saliva. In addition, the effect of enzyme-treated sweet whey protein concentrate on the growth of these cariogenic streptococci was examined.

2. Materials and Methods

2.1 Bacterial Isolates and Growth Conditions

S. sobrinus (DSM 20742) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *S. salivarius* (2184 D41287), a clinical isolate, was kindly donated by Professor Martin Cormican, Microbiology Department, National University of Ireland, Galway.

Both strains were maintained on Protect™ Bacterial Preserve beads (Technical Service Consultants Ltd, Lancashire, UK) at -80˚C. A single bead from the frozen stock culture was used to inoculate a Columbia blood agar plate (CBA: Oxoid, 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_20) 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

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

PBS to remove excess saliva. The S-HA pellets were subsequently resuspended in sterile PBS for use in the adherence assay.

2.4.2 Preparation of Syto® 13 dye

Syto® 13 dye (Molecular Probes, Oregon, USA) was supplied as a 5mM solution in dimethylsulphoxide (DMSO). This concentration was adjusted to 5µM by appropriate dilution in sterile PBS, and was used only on the day of preparation. Standard curves were constructed to show the relationship between relative fluorescent units (RFU) and colony forming units per millilitre (CFU mL^{-1}) for *S. sobrinus* and *S. salivarius*, which had correlation coefficient values (R^2) of 0.993 (Figure 1(a)) and 0.989 (Figure 1(b)), respectively.

2.4.3 Assay Protocol

Overnight cultures of *S. sobrinus* and *S. salivarius* were subjected to centrifugation at 3220 × g (Eppendorf 5810R, Cambridge, UK) for 10 min and each of the pellets were washed once in sterile PBS. Following a second centrifugation step, the bacterial pellets were re-suspended in PBS, and the OD_{630nm} of the suspensions measured using a Multiskan Ascent spectrophotometer, and adjusted to 0.2 by appropriate dilution with sterile PBS.

The adherence assays were carried out as previously described (Halpin *et al*., 2008, Halpin *et al.*, 2011), using sterile 96-well polystyrene microtitre half-area plates (Nunc, Roskilde, Denmark). Dairy powders were prepared to the required concentration by dispersing the dried powder in PBS. Briefly, 50µL of test material solution at various concentrations was added to the wells, followed by 50µL of PBS-HA or S-HA (7.5 mg mL^{-1}). Bacterial suspension (50µL) was added to the wells, so that the final volume of each well was 150µL. Control wells (no bacteria and/ or no HA) were included in each assay. The plate was incubated at room temperature for 45

min, and manually inverted at 5 min intervals to prevent settling of the HA suspension. The plate was subsequently centrifuged at 201 × g to sediment the HA and any adhering bacteria, leaving the non-adhering bacteria in suspension. These non-adhering bacteria were labelled with 10µL of 5µmol L^{-1} Syto® fluorescent dye. For more information regarding the development and validation of the assay described here, the reader should refer to Halpin *et al*., 2008.

2.5 Quantification of Bacterial Adherence

Aliquots (100µL) of supernatant from the adherence assay containing the non-adhering bacteria were transferred from each well of the half-area plate to the corresponding wells of a black microtitre plate (Costar, Corning Inc., Corning, USA). This plate was allowed to stand at room temperature for 5 min in the dark before reading the fluorescence using a Fluoroskan Ascent plate reader (Thermo Electron Corporation, Finland). The excitation wavelength was 485 nm and the emission intensity was monitored at 538 nm. Three measurements were taken at 5 min intervals, and the average fluorescence calculated. The fluorescence due to the number of bacteria present in the supernatant was determined as a direct readout from the fluorimeter as relative fluorescent units (RFU). The background fluorescence due to non-bacterial components of the assay (i.e. dairy powder and HA) were subtracted. The percentage inhibition of adhesion was calculated as follows:

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

2.6 Growth Assays

Growth assays were carried out in sterile 96-well plates (Nunc, Roskilde, Denmark). Overnight cultures of *S. sobrinus* and *S. salivarius* were prepared in BHI broth as described earlier (section 2.1).

A working culture containing c. 10^8 colony forming units per millilitre (CFU mL^{-1}) was prepared by adding 1mL of overnight culture to 9mL of sterile BHI broth. Test materials were prepared by dispersing dried dairy powders or hydrolysates in BHI broth to the desired concentration. Aliquots (100μL) of test material were added to the wells of the plate, followed by 100μL of the diluted culture; the final concentrations of test material were 0.6mg mL^{-1}, 1.25mg mL^{-1}, 2.5mg mL^{-1} and 5mg mL^{-1}. Bacterial growth in the absence of test material (i.e. control growth) was also determined. The plate was then incubated at 37˚C for 18 h in a Multiskan Ascent plate reader (Thermo Electron Corporation, Finland). Immediately prior to incubation the plate was shaken for 1 min in order to disperse the suspensions. The optical density (OD) readings at 630nm for each well were subsequently recorded at 1 h intervals, with the plate being shaken for 30 s immediately prior to measurement. The initial OD_{630nm} reading, recorded at time 0, of each well was subtracted from all other readings for the corresponding wells over the 18 h incubation time (i.e. to subtract the background OD_{630nm} values). Growth inhibition (%) of *S. sobrinus* and *S. salivarius* due to the presence of dairy powder was calculated using OD_{630nm} values at mid-stationary phase according to the following equation:

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

2.7 Statistical Analysis

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

3. Results

Compositional analysis of protein, fat, moisture, ash and lactose content of each dairy powder was determined, and is summarised in Table 1. These were typical of their product types.

3.1 Adherence Assays

Standard curves were constructed to show the relationship between relative fluorescent units (RFU) and colony forming units per millilitre (CFU mL^{-1}) for *S. sobrinus* and *S. salivarius*, and are shown in Figure 1 (a) and (b), respectively.

3.1.1 *S. sobrinus*

(i) Adherence to Phosphate-Buffered Saline-Coated Hydroxylapatite (PBS-HA)

Typically, c. 28% of any given culture of *S. sobrinus* used throughout this study did not adhere to PBS-HA in the absence of test material ('control' in Table 2).

Of the UT dairy powders, AWPC80 was the most effective inhibitor of *S. sobrinus* adherence to PBS-HA at 31.25μg mL^{-1} and 62.5μg mL^{-1} ($P<0.05$). At 62.5μg mL^{-1}, UT SWPC80, UT BMP and UT CP showed a significant concentration dependent increase ($P<0.05$), and at the maximum concentration examined (125μg mL^{-1}) UT AWPC80, UT SWPC80, UT BMP and UT CP were found to be equally effective

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

this concentration ET WPI, ET DW and ET CP did not reduce adherence of *S. sobrinus* to S-HA relative to the control ($P>0.05$). However, at 125μg mL^{-1} ET AWPC80, ET SWPC80 and ET BMP significantly inhibited adherence of *S. sobrinus* to S-HA, causing the non-binding population of bacteria to increase to ≥ 80%.

3.1.2 *S. salivarius*

(i) Adherence to PBS-HA

Approximately 41% of any given culture of *S. salivarius* used throughout this study did not adhere to PBS-HA in the absence of test material ('control' in Table 4).

With the exception of DW, at 31.25μg mL^{-1} all of the UT test materials (including egg albumin) significantly ($P<0.05$) reduced adherence of *S. salivarius* to PBS-HA relative to the control. At 31.25μg mL^{-1}, UT AWPC80, UT WP and UT BMP exhibited similar levels of inhibition of *S. salivarius* adhesion to PBS-HA (resulting in a non-binding population of 85-90%) and were significantly ($P<0.05$) more potent than the other untreated test materials. UT AWPC80, UT WPI, UT BMP and UT CP were equally as effective at 62.5μg mL^{-1} and 125 μg mL^{-1} ($P>0.05$). However, UT SWPC80 showed an equivalent level of anti-adhesion activity at 125μg mL^{-1}. Also at this concentration (125μg mL^{-1}), all UT powders were more effective than the protein control, egg albumin ($P<0.05$).

Subjecting the dairy powders to enzyme treatment reduced their ability to inhibit adherence of *S. salivarius* to PBS-HA. No significant difference was found between any ET test materials ($P>0.05$); furthermore, no ET dairy powder was more effective than the protein control, egg albumin ($P>0.05$).

(ii) Adherence to S-HA

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^{-1},
321 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

point for each *Streptococcus* was chosen, depending on the time taken for the particular microorganism to reach mid-stationary phase. For *S. sobrinus* and *S. salivarius* 10 hours and 9 hours incubation were chosen, respectively. Growth was on average inhibited by 85.6% ± 5.9 for *S. sobrinus* at all concentrations. ET SWPC80 was less effective at inhibiting growth of *S. salivarius* when compared to inhibition levels observed for *S. sobrinus*. However, growth was nevertheless inhibited by an average of 50.6% ± 4.9 at all concentrations. Growth inhibition was significant at all concentrations for both streptococci relative to control growth ($P<0.05$).

4. Discussion

The present study has shown that dairy powders can inhibit adherence of *S. sobrinus* and *S. salivarius* to HA. The dairy powders were used firstly in their untreated forms, and their anti-adhesion activity was again evaluated following incubation with porcine pancreatic lipase (PPL). Both S-HA and PBS-HA models were employed, to reflect the tooth surface in the presence and absence of saliva, respectively. The S-HA model represents 'normal' conditions in the mouth, while the PBS model system reflects conditions where saliva production is impaired ('dry mouth' or xerostomia). In cases of xerostomia, an individual can experience severe instances of dental caries. The occurrence of dry mouth is a well recognised clinical problem in adults and children, and essentially occurs when the resting salivary flow rate is less than that of fluid loss from the mouth (Walsh, 2008). This condition can be due to use of certain medications (such as those prescribed for hypertension), radiation treatment of the head and neck, or can be incurred by patients with aplasia of the salivary glands (Sjogren's syndrome) (Loesche, 1986, Johansson, 2002). In the present study, UT SWPC80, UT AWPC80, UT BMP and UT CP were the most effective inhibitors of adhesion of both *S. sobrinus* and *S. salivarius* to HA in the

absence of saliva, and thus may be useful ingredients in the formulation of a dairy-based saliva substitute. In addition, such dairy powders capable of inhibiting adherence of streptococci to oral surfaces may help reduce the occurrence of focal oral infections, as introduction of viridans streptococci resident in the oral cavity into the bloodstream can lead to infections such as bacteremia (Gendron *et al*., 2000). This occurrence is particularly problematic for patients experiencing neutropenia (Prabhu *et al*., 2004).

The level of 'control' adhesion for both *S. sobrinus* and *S. salivarius* varied greatly between PBS-HA and S-HA model systems. In the presence of saliva, UT SWPC80, UT AWPC80, UT WPI and UT CP were the most effective inhibitors of *S. sobrinus* adhesion to S-HA. However, all UT dairy powders (with the exception of SWPC35 and WPI) significantly reduced adherence of *S. salivarius* to S-HA ($P<0.05$). The findings of the present study are difficult to explain, as different levels of anti-adhesion activity were observed for each of the of dairy powders against *S. sobrinus* and *S. salivarius*, and the level of inhibition also varied depending on whether PBS-HA or S-HA models were used. A possible reason for the varied levels of efficacy exhibited by the dairy powders against *S. sobrinus* and *S. salivarius* may be due to the different adherence mechanisms of these strains. *S. sobrinus* (a member of the mutans streptococci) possesses a surface adhesin (SpaA) (Tokuda *et al*., 1990) and genes capable of producing glucosyltransferases (Gilmore *et al*., 1990), whereas strains of *S. salivarius* (which is not a member of the mutans streptococci) contain proteinaceous components associated with a fibrillar layer outside the cell wall, referred to as the 'fuzzy coat'. This fuzzy coat is believed to mediate attachment of *S. salivarius* to host surfaces (Weerkamp *et al*., 1986). Thus, it is not surprising that the

dairy powders (and enzyme-treated versions thereof) do not interact with the different surface proteins of these two streptococci in a similar manner.

In general, enzyme-treatment with PPL reduced the anti-adhesion efficacy of the dairy powders in both PBS-HA and S-HA assays, but the degree of reduction was less apparent for the latter. A possible reason for this may be interactions occurring between constituents of the hydrolysates and components of saliva e.g. salivary proteins or peptides. However, this is merely speculative and further research would be required if the exact cause were to be determined. Of the enzyme-treated dairy powders, ET SWPC80, ET AWPC80 and ET BMP were found to be the most effective inhibitors of *S. sobrinus* adherence to S-HA. The majority of ET powders appeared to reduce adherence of *S. salivarius* to S-HA, but this may have been due to a non-specific protein effect, as egg albumin was also observed to reduce *S. salivarius* adherence to S-HA, by about the same amount.

While the way in which the dairy powders used in this study are inhibiting adherence of streptococci to HA has not yet been elucidated, protein adsorption experiments performed previously by this research group indicated that proteins present in the dairy powders were associating with the HA beads (Halpin *et al*., 2011). This is likely to be contributing to the reduction in streptococcal adherence, as the highest level of protein association was observed for UT AWPC80, which was also the most effective inhibitor of streptococcal adherence to PBS-HA. However, it is acknowledged in the context of such complex natural products that this may not be the sole factor involved in inhibiting the adherence of streptococci to HA. In addition, it should be noted that the less effective inhibitors were those which were lowest in fat.

Another aspect of the present study was to determine the effect of ET SWPC80 on the growth of *S. sobrinus* and *S. salivarius*. This hydrolysate inhibited growth of these cariogenic bacteria by up to 85% at concentrations as low as 0.6mg mL^{-1} ($P<0.05$). The crude PPL used in the present study is known to contain both proteases and lipases (Birner-Grunberger *et al*., 2003), and it may be that enzyme treatment of the dairy powders used in the present study releases both peptides and free fatty acids that are inactive within the untreated material. Thus, the component(s) of ET SWPC80 contributing to the observed antibacterial activity against *S. sobrinus* and *S. salivarius* may on one hand be antibacterial peptides derived from whey proteins such as β-lactoglobulin, α-lactalbumin or lactoferrin, as these proteins are known to harbour antibacterial peptides that can be released by proteolysis (Lopez-Exposito and Recio, 2006). Alternatively, the antibacterial activity could be due to peptides cleaved from the glycomacropeptide (GMP), which is present in sweet whey products due to the action of chymosin on κ-casein. A study by Malkoski *et al*. (2001) showed that kappacin, a non-glycosylated, phosphorylated form of κ-casein, exhibited significant antibacterial activity against oral pathogens. In addition to the peptide hypothesis, it is possible that free fatty acids present in SWPC80 following enzyme-treatment may have contributed to the antibacterial activity of this hydrolysate. Previous work in this laboratory confirmed the presence of butyric (C_4) and caproic (C_6) acids in SWPC80 after digestion with PPL (Halpin *et al*., 2011), and it is possible that other fatty acids were present after hydrolysis. However, the exact mechanism of action for the antibacterial activity of ET SWPC80 remains to be elucidated. Nonetheless, the action of PPL on SWPC80 produced an effective antibacterial agent 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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597 **List of Figures:**

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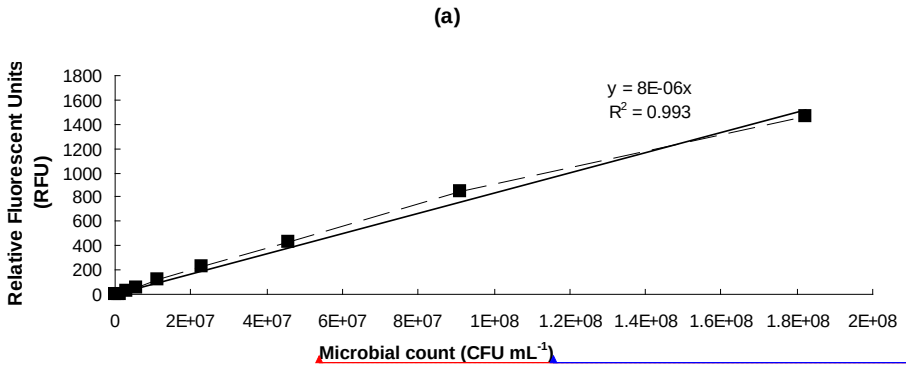

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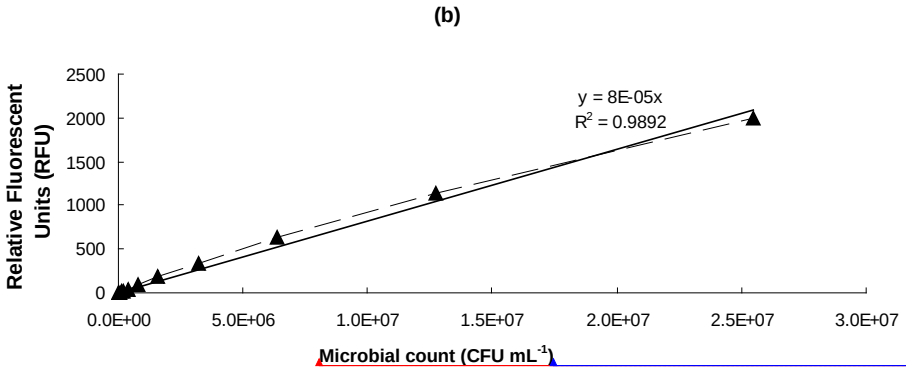

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602 **Figure 1:** Standard curves of relative fluorescent units (RFU) Vs colony forming
603 units per millilitre (CFU mL^{-1}) for (a) *S. sobrinus* and (b) *S. salivarius*.

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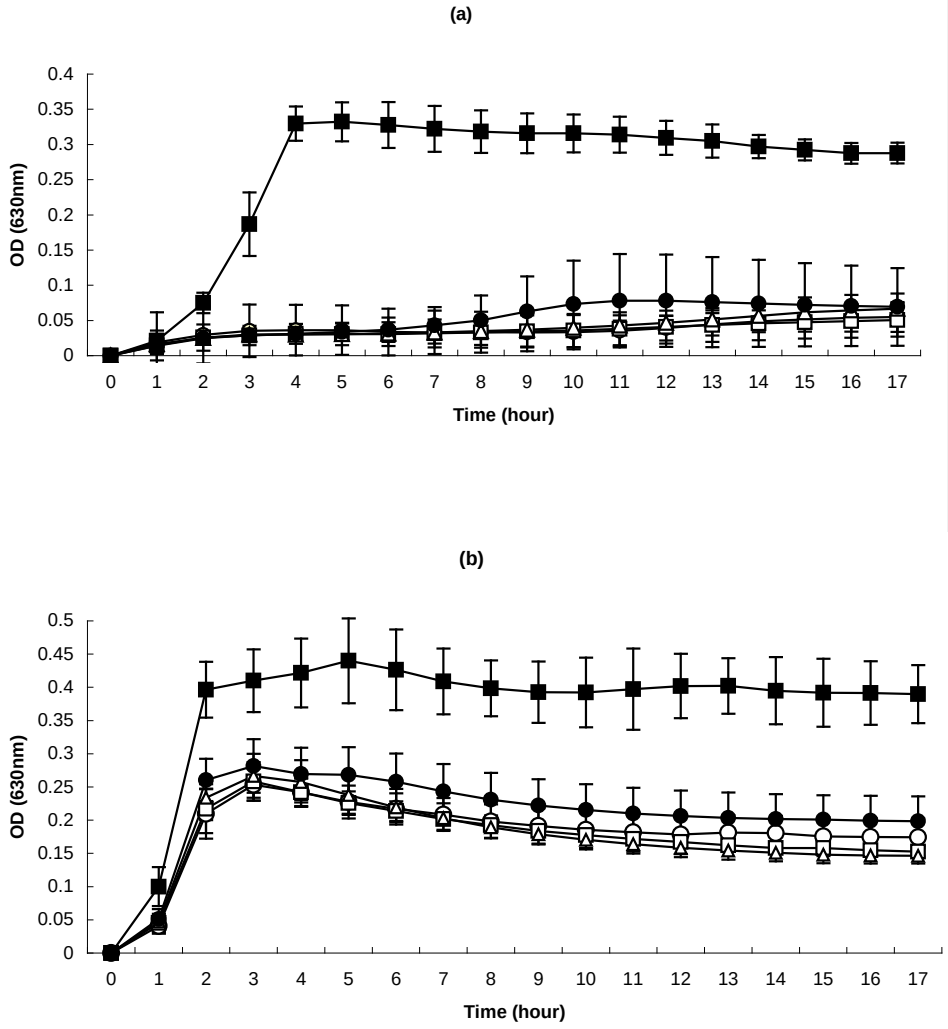

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615 **Figure 2:** Effects of enzyme-treated Sweet WPC80 on the growth of (a) *S. sobrinus*
616 and (b) *S. salivarius*, at 5mg mL^{-1} (○), 2.5mg mL^{-1} (□), 1.25mg mL^{-1} (Δ), 0.6mg mL^{-1}
617 (●) and control growth in the absence of inhibitor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

List of Tables:

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

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

		Untreated			Enzyme-treated		
µg mL^{-1}	**Control***	**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.

		Untreated			Enzyme-Treated		
µg mL^{-1}	**Control***	**31.25**	**62.5**	**125**	**31.25**	**62.5**	**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.l$^{e\ (w)}$	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.l ±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.

		Untreated			Enzyme-Treated		
µg mL^{-1}	**Control***	**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.

		Untreated			Enzyme-Treated		
µg mL^{-1}	**Control***	**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.