2011

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

Rachel Halpin
Technological University Dublin, rachel.halpin@tudublin.ie

D.B. Brady
School of Biomolecular and Biomedical Science, University College Dublin, Ireland.

E.D. O'Riordan
School of Agriculture, Food Science and Veterinary Medicine, University College Dublin

See next page for additional authors

Recommended Citation

Halpin, R. et al. (2011) The Effect of Untreated and Enzyme-Treated Commercial Dairy Powders on the Growth and Adhesion of Streptococcus mutans, *LWT, Food Science & Technology,* Volume 44, Issue 6, July 2011, Pages 1525-1532. doi:10.1016/j.lwt.2011.01.025

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

R.M. Halpin[1]*, D.B. Brady[2§], E.D. O'Riordan[1] and M. O'Sullivan[1]

[1]School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland.

[2]School of Biomolecular and Biomedical Science, University College Dublin, Ireland.

[§]Present Address: School of Science, Athlone Institute of Technology, Athlone, Ireland.

*Corresponding author: Rachel Halpin, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland.

Tel:0035317161301

E-mail address: rachel.halpin@ucd.ie

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

Abstract

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Bacterial Isolates and Growth Conditions

A clinical isolate of *S. mutans* (LAN-SVHERC-1997sm1) was obtained from the Microbiology Department, St. Vincent's University Hospital, Dublin, Ireland. Bacteria 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 the blood agar plate was subsequently used to inoculate 20mL of brain heart infusion (BHI) broth (LabM, Lancashire, UK) and grown under aerobic conditions without shaking at 37˚C for 18 h.

2.2 Source and Characterisation of Dairy Powders

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

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

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

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

2.3 Hydrolysate Preparation Conditions

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

2.4 Adhesion Assay

2.4.1 (a) Preparation of Hydroxylapatite

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

Hampshire, England) was prepared by suspension of 7.5mg mL^{-1} HA in PBS immediately before use in the adherence assays.

Saliva-coated-HA (S-HA) was prepared by a modification of the protocol set out by Gibbons & Etherden (1982) as follows: parafilm-stimulated whole saliva was collected in an ice-chilled tube from two healthy donors (1 male, 1 female) at least 1 h after eating, drinking or brushing of teeth. The saliva was heated at 60ºC for 30 min to inactivate degenerative enzymes, and subsequently centrifuged at 12,000 × g for 15 min. The pellet was discarded and the supernatant (i.e. clarified whole saliva) was used to prepare a 7.5mg mL^{-1} dispersion of HA. Aliquots (150µL) of saliva-coated hydroxylapatite (S-HA) were dispensed into the wells of a 96-well V-bottomed plate (Sarstedt, Newton, North Carolina, USA), and incubated at 30ºC for 1 h with gentle agitation (4.5 × g). Following this, the microtitre plate was centrifuged at 805 × g for 2 min, the 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.

2.4.1 (b) Preparation of Syto® 13 dye

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

2.4.2 Assay Protocol

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

OD_{630nm} of the suspension measured using a Multiskan Ascent spectrophotometer ((Thermo Electron Corporation, Vantaa, Finland), and adjusted to 0.2 by appropriate dilution with sterile PBS.

The adherence assays were carried out as previously described (Halpin *et al*., 2008), 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 (Section 2.4.2) 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, Vantaa, 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 total 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 Protein Adherence Assay

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

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

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

2.8 Growth Assays

Growth assays were carried out in sterile 96-well plates (Nunc, Roskilde, Denmark). Overnight cultures of *S. mutans* 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, Vantaa, 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 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 values).

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

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

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

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

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

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

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

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

312 **2.10 Statistical Analysis**

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

3. Results

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

3.1 Adherence Assays

(a) Adhesion to phosphate buffered saline-coated hydroxylapatite

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

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

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

Lactose, which was present in all test materials at varying levels, was not found to affect adherence of *S. mutans* to PBS-HA, even when used at concentrations up to 1000μg mL^{-1} (data not shown), and in fact was found to significantly increase the adherence of *S. mutans* to PBS-HA (P<0.05).

(b) Adhesion to saliva-coated hydroxylapatite

In the presence of saliva, the control level of adhesion of *S. mutans* to hydroxylapatite was significantly reduced when compared to that of the PBS-HA model (P<0.0001), with c. 37% of each bacterial culture not adhering to S-HA ('control' in Table 3).

At concentrations ≥ 31.25μg mL^{-1}, UT SWPC80, AWPC80 and BMP significantly (P≤0.05) reduced adherence of *S. mutans* to S-HA relative to the protein control (egg albumin, EA). At 62.5μg mL^{-1} and 125μg mL^{-1}, UT SWPC80, UT AWPC80 and UT WP were the most potent inhibitors of *S. mutans* adhesion to S-HA, being more effective than all other UT test materials and increasing the proportion of non-binding bacteria to 75-80%.

Consistent with our observations in the PBS-HA model system, untreated dairy powders were generally more potent inhibitors of *S. mutans* adherence than the same powders following enzyme-treatment, except in the case of the WPCs, which were found to show similar levels of efficacy both in their untreated and enzyme-treated forms. However, it is worthwhile to note that the reduction in anti-adhesion activity caused by enzyme treatment of powders was not as dramatic in the case of S-HA as that observed in the PBS-HA model. Following enzyme treatment, at 31.25μg mL^{-1}, all powders excluding ET WPI, ET WP and ET DW significantly inhibited adherence of *S. mutans* to S-HA relative to the control (P<0.05). However, at 125μg mL^{-1}, all

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

3.2 Adherence of Whey and Dairy Powders to PBS-HA

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

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

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

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

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

3.4 Growth Inhibition Assays

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

3.5 Determination of Volatile Fatty Acids in ET SWPC80

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

4. Discussion

The findings of a previous study by this group showed that a range of UT dairy powders reduced adherence of *S. mutans* to PBS-HA (Halpin *et al*., 2008). The present study examined the effect of enzyme-treatment on the anti-adhesion activity of these powders, using two model systems: PBS-HA and S-HA. The S-HA model represents the closest approximation to conditions in the oral cavity, while the PBS 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.

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

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

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

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

At the outset of this study, it was anticipated that enzyme treatment would enhance the anti-adhesion efficacy of the dairy powders, as a patent by Brady & Folan (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

present in the hydrolysed product, as milkfat contains a broad spectrum of FAs. Studies by Sprong *et al*. (2001, 2002) have demonstrated the antibacterial activity of milk-lipids. However, the inhibitory activity of ET SWPC80 could equally be due to peptides produced during enzymatic digestion. Peptides liberated from GMP by the action of proteolytic enzymes in PPL may also have contributed to the observed antibacterial effect. A study by Malkoski, Dashper, O'Brien-Simpson, Talbo, Macris, Cross, & Reynolds (2001) showed that kappacin, a peptide derived from κ-casein, inhibited growth of plaque-forming bacteria, and although not established here, it is possible such peptides derived from GMP are contributing to the observed antimicrobial activity of ET SWPC80. Alternatively, growth inhibition of *S. mutans* due to ET SWPC80 may have been the result of a synergistic effect between FAs and peptides released during enzyme treatment. Small peptides exhibiting antimicrobial properties have been proposed as alternatives to antibiotics (Mor, 2003). The oral cavity is considered eminently suitable for the application of such peptides as it provides direct access to bacterial biofilms on non-shedding surfaces (Dashper *et al*., 2007). Currently, there is considerable commercial interest in the isolation and characterisation of dairy-derived bioactive peptides that can be added to products such as toothpastes, gels and mouth rinses (Aimutis, 2004). Our results show that an enzyme-treated whey product exhibits antibacterial effects, without isolation of individual peptides or FAs. Therefore, an antibacterial agent can be produced from whey, which is available in large quantities and is relatively inexpensive.

In conclusion, the results presented here have shown that UT dairy powders are effective inhibitors of *S. mutans* adherence to both PBS-HA and S-HA. In general, the anti-adhesion efficacy of these dairy powders was, for the most part, reduced following enzyme-treatment. However, some activity was observed following

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

Acknowledgements

This work was supported by the national development plan (NDP), with a grant from the food institution research measure (FIRM). We would like to thank Dr. Nessa Noronha, Ms. Maeve O'Connor and Mr. Tristan Rouille for their assistance with experiments.

References

Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I. & Dewhirst, F.E. (2005) Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology, 43,* 5721-5723.

Aimutis, W.R. (2004) Bioactive properties of milk proteins with particular focus on anticariogenesis. *Journal of Nutrition, 134,* 989S-995S.

Beachey, E.H. (1981) Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. *Journal of Infectious Disease, 143,* 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.
660
661
662
663

Legends for Figures:

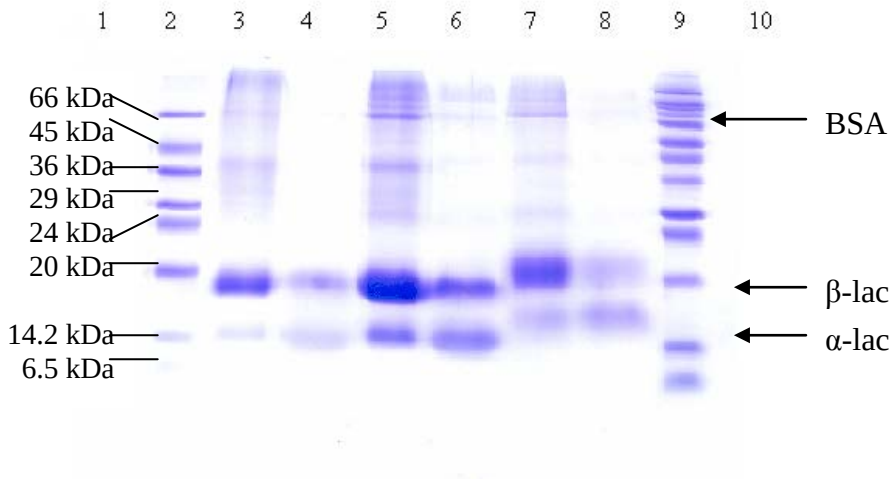
Figure 1: SDS-PAGE showing protein bands present in centrifuged supernatants of sweet whey protein concentrate (WPC) 80, acid WPC 80 and sweet WPC 35, both with and without prior incubation with hydroxylapatite.

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

Figure 2: Effects of Enzyme-Treated Sweet WPC80 on the growth of *S. mutans*, 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.

Figure 1:



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

Figure 2:

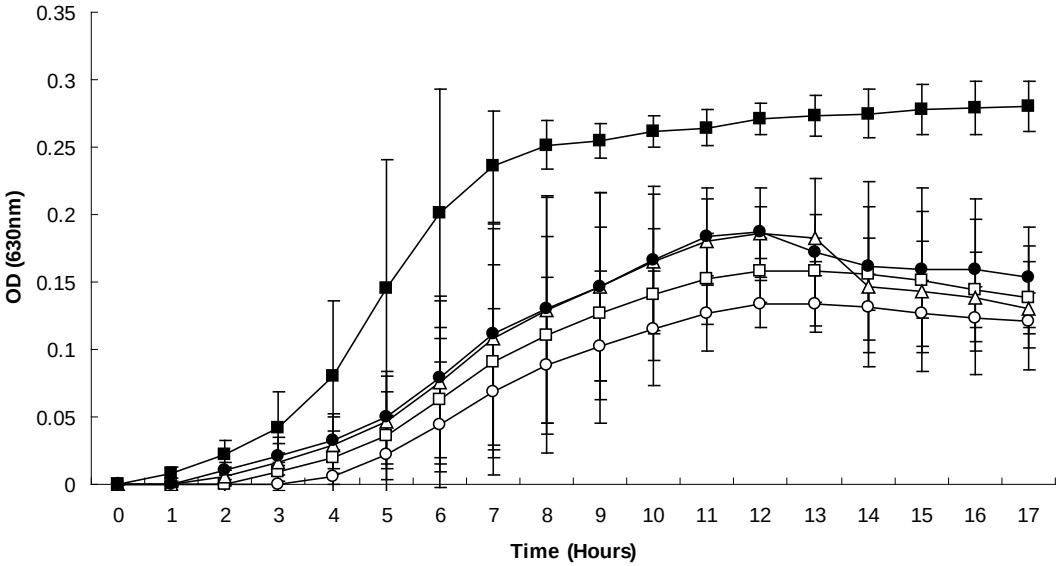

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
OD (630nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time (Hours)

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

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

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

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

Footnotes:

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=60, †= egg albumin is included for the sake of comparison only as a protein control. †† lactose n=2.

Abbreviations:

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

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

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

Footnotes:

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 (w,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=60, †= egg albumin is included for the sake of comparison only as a protein control.

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

	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

Abbreviations:

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