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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 *Streptococcus 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. Untreated (UT) acid whey protein concentrate (AWPC) 80 inhibited streptococcal adherence to phosphate-buffered saline-coated HA (PBS-HA) and saliva-coated HA (S-HA) by >80% at $\geqslant 31.25\ \mu g\ mL^{-1}$. UT sweet WPC80, buttermilk powder and cream powder also significantly reduced adherence ($P < 0.05$). Enzyme-treatment of all dairy powders reduced their anti-adhesion activity. However, ET sweet WPC80 significantly inhibited growth of these streptococci ($P < 0.05$) at $\geqslant 0.6\ mg\ mL^{-1}$. Therefore, dairy powders may reduce progression of dental caries by their anti-adhesion and/or antibacterial activity.

1. Introduction

Dental caries is a bacterial disease characterised by a localised progressive, molecular disintegration of the tooth (Marcotte & Lavoie, 1998). Tooth decay and periodontal disease are among the most common bacterial infections in humans (Loesche, 1986), affecting both children and adults (Aas, Paster, Stokes, Olsen, & Dewhirst, 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, Lemos, Abranches, Goncalves, & Burne, 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, Grahnen, Jonsson, & Wikner, 1970), and has long been recognised as a 'potent acid producer' (Shiere, Georgi, & Ireland, 1951). In addition to causing dental caries,

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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.

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microorganisms inhabiting the oral cavity can be introduced into the bloodstream, leading to occurrence of 'focal oral infections', including bacteremia, endocarditis and meningitis (Gendron, Grenier, & Maheu-Robert, 2000; Reif, Roller, Rawling, & Granato, 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 & Lavoie, 1998). Liljemark, Schauer, and Bloomquist (1978) proposed that the initial colonisation of the tooth surface was of utmost importance when attempting to prevent or control formation of dental plaque. In recent years, many foods and beverages such as water-soluble protein-fraction (WSPF) of hen egg yolk (Gaines, James, Folan, Baird, & O'Farrelly, 2003), cranberry constituents (Yamanaka, Kimizuka, Kato, & Okuda, 2004), barley coffee (Papetti et al., 2007) and herbal extracts (Chen et al., 2005; Limsong, Benjavongkulchai, & Kuvatanasuchati, 2004) 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, Hasty, & Sharon, 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, Radha, Prakash, & Kaul, 2007). Peptides exhibiting antimicrobial properties have been isolated from whey proteins such as β-lactoglobulin, α-lactalbumin and lactoferrin following proteolysis (Lopez-Exposito & 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 & Newburg, 1995). In the 1970s, 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, O'Connor, & Roberton, 2002). Generally, Gram positive microorganisms (such as streptococci) are lipid sensitive, whereas Gram negatives are not (Kabara, Swieckowski, Conley, & Truant, 1972), but some exceptions to this trend exist (Sprong, Hulstein, & van der Meer, 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 & 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 (Clark & Gibbons, 1977; Gibbons, Moreno, & Spinell, 1976). 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, Brady, O'Riordan, & O'Sullivan, in press). 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 the blood agar plate was subsequently used to inoculate 20 mL of brain heart infusion (BHI) broth (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 (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). Buttermilk powder (BMP) and cream powder (CP) were supplied by Kerry Group plc (Tralee, Co. Kerry, Ireland). Albumin from chicken egg white (grade V) was supplied by Sigma (Poole, Dorset, UK).

Compositional analysis was performed on each dairy product using standard methods. Ash content was analysed according to Malkomesius and 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, 1993b) and the moisture content was determined by the IDF reference method (IDF, 1993a, 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. Hydrolysates were prepared in a Fermac 200 fermentor (Electrolab Ltd., Tewkesbury, UK) as follows: a c. 2% (w/v) solution of substrate was prepared by dissolving 20 g of dairy powder in 900 mL of sterile distilled water and heating at 37 °C with stirring for 30 min. Lipase solution (1 g of PPL in 100 mL of sterile H_2O) 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. *Preparation of hydroxylapatite*

Hydroxylapatite (HA) beads were supplied by Merck (Darmstadt, Germany). Both buffer-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 10 μm. Phosphate-buffered saline coated HA (PBS-HA, PBS: Oxoid, Hampshire, England) was prepared by suspension of 7.5 mg mL^{-1} HA in PBS immediately before use in the adherence assays.

Saliva-coated-HA (S-HA) was prepared similar to the protocol set out by Gibbons and 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 60 °C 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 7.5 mg mL^{-1} dispersion of HA. Aliquots (150 μL) of this dispersion were dispensed into the wells of a 96-well V-bottomed plate (Sarstedt, Newton, NC, USA), and incubated at 30 °C 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.

2.4.2. *Preparation of Syto 13 dye*

Syto 13 dye (Molecular Probes, Eugene, OR, USA) was supplied as a 5 mM solution in dimethylsulphoxide (DMSO). This concentration was adjusted to 5 μM 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 (Fig. 1(a)) and 0.989 (Fig. 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

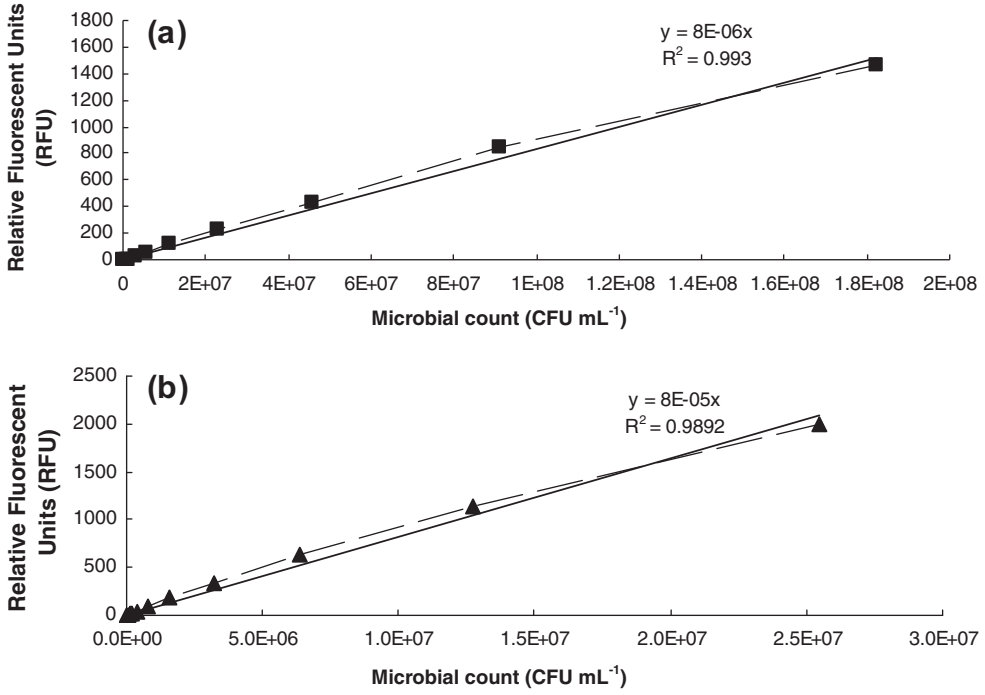

Fig. 1 – Standard curves of relative fluorescent units (RFU) vs colony forming units per millilitre (CFU mL^{-1}) for (a) *S. sobrinus* and (b) *S. salivarius*.

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, Brady, O'Riordan, & O'Sullivan, in press; 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, 50 μL aliquots of test material solution at various concentrations were added to the wells, followed by 50 μL of PBS-HA or S-HA (7.5 mg mL^{-1}). Bacterial suspension (50 μL) was added to the wells, so that the final volume of each well was 150 μL. 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 $201 \times g$ to sediment the HA and any adhering bacteria, leaving the non-adhering bacteria in suspension. These non-adhering bacteria were labelled with 10 μL of 5 μmol 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 (100 μL) 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, MA, 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 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:

$$\text{Adhesion inhibition (\%)} = \frac{(\text{Fluorescence due to unbound bacteria})}{(\text{Fluorescence due to total input bacteria})} \times 100 \quad (1)$$

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 1 mL of overnight culture to 9 mL of sterile BHI broth. Test materials were prepared by dispersing dried dairy powders or hydrolysates in BHI broth to the desired concentration. Aliquots (100 μL) of test material were added to the wells of the plate, followed by 100 μL of the diluted culture; the final concentrations of test material were 0.6, 1.25, 2.5 and 5 mg 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 630 nm 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:

$$\text{Growth inhibition (\%)} = \frac{[(\text{OD Control Growth}) - (\text{OD Growth in Presence of Dairy Powder})]}{(\text{OD Control Growth})} \times 100 \quad (2)$$

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

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; DW, demineralised whey; BMP, buttermilk powder; CP, cream powder.

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[A]	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[B]		38.9 ± 11.6$^{a,f(x)}$	37.1 ± 7.6$^{c,d(x)}$	33.8 ± 5.8$^{e(w,x)}$			

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'.
[A] $n = 52$.
[B] Egg albumin is included for the sake of comparison only as a protein control.
¥ Significant difference ($P < 0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.

(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 Fig. 1(a) and (b), respectively.

3.1.1. *S. sobrinus*

3.1.1.1. 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 31.25 and 62.5 μg mL^{-1} ($P < 0.05$). At 62.5 μg mL^{-1}, UT SWPC80, UT BMP and UT CP showed a significant concentration dependent increase ($P < 0.05$), and at the maximum concentration examined (125 μg mL^{-1}) UT AWPC80, UT SWPC80, UT BMP and UT CP were found to be equally effective ($P < 0.05$). Of the untreated dairy powders, WPI, WP and DW were the poorest inhibitors of *S. sobrinus* adherence to PBS-HA at all concentrations.

Following enzyme-treatment, the anti-adhesion activity of all powders was reduced. At 31.25 μg mL^{-1}, all ET dairy powders were only equally as effective as the protein control, egg albumin ($P > 0.05$). ET BMP was significantly ($P < 0.05$) the most effective inhibitor at 62.5 and 125 μg mL^{-1}. ET SWPC35, WPI, WP and DW had no inhibitory effect on adherence of *S. sobrinus* to PBS-HA at any concentration, relative to the control ($P > 0.05$). The loss in anti-adhesion activity due to enzyme-treatment was most noticeable at the highest concentration (125 μg mL^{-1}), with all powders (except WP) being significantly ($P < 0.05$) less effective when compared to its equivalent untreated form.

3.1.1.2. Adherence to saliva-coated hydroxylapatite (S-HA). For the adherence assays carried out using *S. sobrinus*, c. 46% of microorganisms in any given culture did not adhere to S-HA under our assay conditions ('control' in Table 3). This value was markedly higher than the control level observed for PBS-HA.

The egg albumin protein control inhibited adherence of *S. sobrinus* to S-HA to a greater extent than UT SWPC35, UT WP and UT DW at 31.25 μg mL^{-1} ($P < 0.05$), with UT SWPC35 actually significantly ($P < 0.05$) promoting adherence. This was also evident for UT WP and UT DW at 62.5 μg mL^{-1}. At 125 μg mL^{-1}, UT SWPC80, UT AWPC80, UT WPI and UT CP appeared to be the most effective inhibitors of adherence of *S. sobrinus* to S-HA and exhibited similar levels of activity, yet these values were not significantly different from those observed for egg albumin ($P > 0.05$).

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

3.1.2.1. Adherence to PBS-HA. Approximately 41% of any given culture of *S. salivarius* used throughout this study did

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[A]	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 ± 12.1$^{e(w)}$	61.9 ± 14.7$^{b,d(x)}$	69.4 ± 10.4$^{b,c,d(x)}$	62.1 ± 18.7$^{a,b(x)}$	78.9 ± 13.1$^{a,b,c(x)}$	80.1 ± 16.7$^{a,b,c(x)}$
CP		57.1 ± 6.6$^{d,e(w)}$)	62.6 ± 3.7$^{b,d(x)}$	71.1 ± 9.2$^{a,b,c,d(x)}$	62.2 ± 10.8$^{a,b(x)}$	58.1 ± 20.5$^{c,d,e(w,x)}$	55.8 ± 22.2$^{d,e(w,x)}$
Egg albumin[B]		51.2 ± 5.5$^{e(w,x)}$	65.5 ± 12.1$^{b,d(x,y)}$	76.1 ± 7.4$^{a,b,c(y)}$			

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'.
[A] $n = 53$.
[B] Egg albumin is included for the sake of comparison only as a protein control.
¥ Significant difference ($P < 0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.

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[A]	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[B]		60.6 ± 10.1$^{a,b(x)}$	56.7 ± 16.2$^{c(x,y)}$	41.6 ± 1.8$^{e(w,y)}$			

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'.
[A] $n = 59$.
[B] Egg albumin is included for the sake of comparison only as a protein control.
¥ Significant difference ($P < 0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.

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

With the exception of DW, at 31.25 μg 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 31.25 μg 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 62.5 and 125 μg mL^{-1} ($P > 0.05$). However, UT SWPC80 showed an equivalent level of anti-adhesion activity at 125 μg mL^{-1}. Also at this concentration (125 μg 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 ($P > 0.05$).

3.1.2.2. Adherence to S-HA. Due to the large non-binding population of *S. salivarius* to S-HA (c. 66%) it was difficult to establish the efficacy of test materials in reducing adherence of this microorganism to S-HA (Table 5).

At 31.25 μg mL^{-1}, only UT SWPC80 and UT AWPC80 were found to be more potent inhibitors of *S. salivarius* adhesion to S-HA than egg albumin ($P < 0.05$). However, at 62.5 and 125 μg mL^{-1}, all test materials (including egg albumin) showed equal levels of efficacy ($P > 0.05$).

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[A]	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[B]		66.2 ± 12.5$^{b(w)}$	75.8 ± 9.2$^{a(w)}$	76.9 ± 8.2$^{a(w)}$			

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'.
[A] $n = 57$.
[B] Egg albumin is included for the sake of comparison only as a protein control.
¥ Significant difference ($P < 0.05$) between the untreated dairy powder and enzyme-treated form thereof at that particular concentration.

Following enzyme-treatment, many of the hydrolysed dairy powders significantly ($P < 0.05$) inhibited adherence of *S. salivarius* to S-HA relative to the control, but only ET WPI was found to be more effective than egg albumin ($P < 0.05$). At 31.25 μg mL^{-1}, ET CP was the least effective inhibitor of *S. salivarius* adherence to S-HA ($P < 0.05$). No ET test material was more effective than egg albumin ($P > 0.05$) at 62.5 and 125 μg mL^{-1}. At the maximum concentration examined (125 μg mL^{-1}), only ET SWPC35, ET WPI, ET WP and ET DW significantly ($P < 0.05$) reduced adherence of *S. salivarius* to S-HA relative to the control ($P < 0.05$).

3.2. *Growth assays*

ET SWPC80 was found to significantly ($P < 0.05$) inhibit growth of *S. sobrinus* and *S. salivarius* at all concentrations examined (Fig. 2). Previous work in this laboratory demonstrated that ET SWPC80 significantly inhibited growth of the highly cariogenic microorganism *S. mutans* (Halpin et al., in press), with no other enzyme-treated whey product exhibiting an antibacterial effect against this microorganism (O'Connor et al., 2006). Therefore, in the present study only ET SWPC80 was assessed for its antibacterial activity against *S. sobrinus* and *S. salivarius*. The percentage growth 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 and 9 h 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) (Johansson, 2002; Loesche, 1986). 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

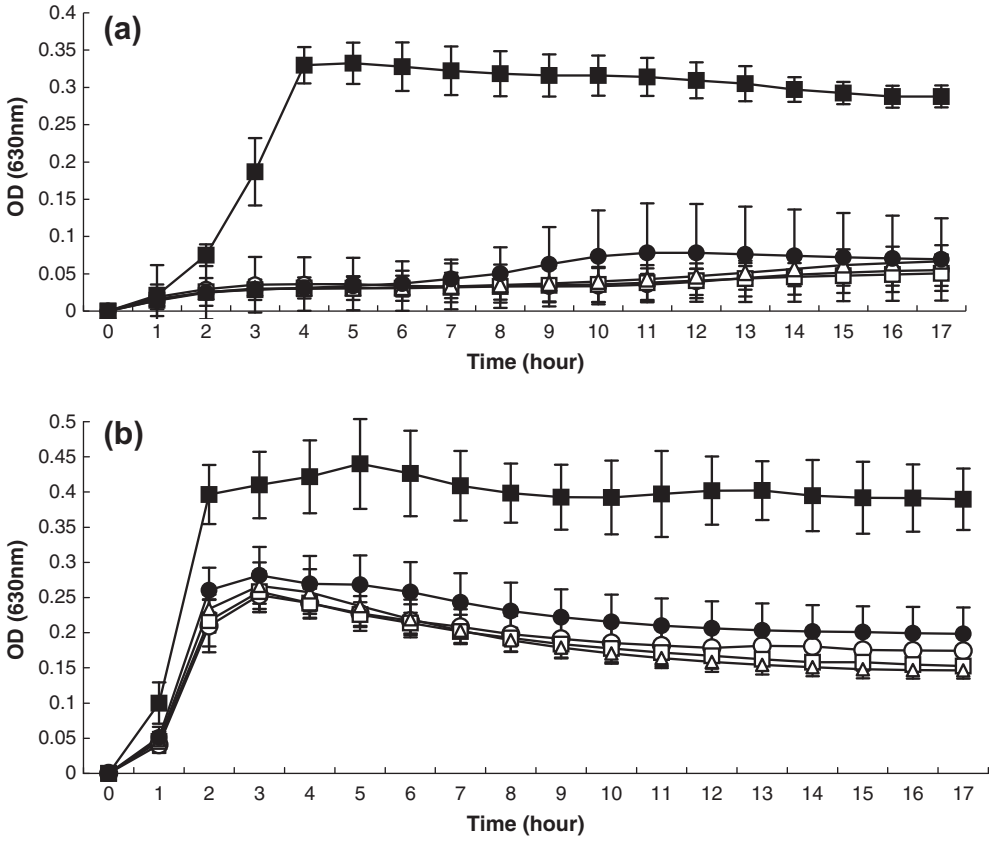

Fig. 2 – Effects of enzyme-treated sweet WPC80 on the growth of (a) *S. sobrinus* and (b) *S. salivarius*, at 5 mg mL^{-1} (○), 2.5 mg mL^{-1} (□), 1.25 mg mL^{-1} (△), 0.6 mg mL^{-1} (●) and control growth in the absence of inhibitor (■). (Data = mean ± standard deviation, *n* = 4.)

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, Russell, & Ferretti, 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, Handley, Baars, & Slot, 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., in press). 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 0.6 mg mL^{-1} ($P < 0.05$). The crude PPL used in the present study is known to contain both proteases and lipases (Birner-Grunberger, Scholze, Faber, & Hermetter, 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 contain antibacterial peptide sequences that can be released by proteolysis (Lopez-Exposito & 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., in press), 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 observations of the present study can be extended to the oral cavity; thereby establishing the efficacy of dairy products as therapeutic products *in vivo*.

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