2008-9

Inhibition of Adhesion of Streptococcus Mutans to Hydroxylapatite by Commercial Dairy Powders and individual Milk Proteins

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Recommended Citation

Halpin, R. et al. (2008) Inhibition of adhesion of Streptococcus mutans to hydroxylapatite by commercial dairy powders and individual milk proteins. *European Food Research and Technology*. Sept. 2008, Vol. 227, Issue 5, p.1499-1506. doi:10.1007/s00217-008-0872-4

Eur Food Res Technol (2008) 227:1499–1506
DOI 10.1007/s00217-008-0872-4

ORIGINAL PAPER

Inhibition of adhesion of *Streptococcus mutans* to hydroxylapatite by commercial dairy powders and individual milk proteins

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Received: 12 July 2007 / Revised: 27 March 2008 / Accepted: 7 April 2008 / Published online: 26 April 2008

Abstract The aim of this study was to investigate the inhibitory effect of various dairy powders and milk constituents on the adhesion of a clinical isolate of *Streptococcus mutans* to hydroxylapatite (HA), an analogue of tooth enamel. Adhesion of a microorganism to a cell surface such as epithelial cells or tooth enamel is considered to be the first step in pathogenesis. Inhibiting this process may have therapeutic effects in vivo. The adherence assays were performed by incubating *S. mutans* with HA in the presence of each test material for 45 min, followed by centrifugal separation of the HA. Unbound bacteria were then quantified using a fluorescent dye. Sweet and Acid WPC80, buttermilk powder and cream powder were found to very effectively inhibit adherence of *S. mutans* to phosphate-buffered saline coated HA (PBS-HA). Sodium caseinate and the casein fractions α-,β- and κ-casein were also found to show high levels of anti-adhesive activity. A selection of test materials were assessed using saliva-coated HA (S-HA), and similar trends were observed. The results suggest commercial dairy powders, and certain milk proteins, can inhibit adhesion of *S. mutans* to HA and may have potential to control dental caries.

Keywords Dairy powders · Adhesion · *S. mutans* · Fluorescence

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Introduction

Streptococcus mutans is consistently present in dental plaque biofilms and is considered to be the primary organism involved in the formation of dental caries [1]. Dental caries is regarded as one of the most common bacterial infections in humans [2]. Control of dental plaque currently relies on good oral hygiene such as brushing and flossing, the effects of which are increased when used in conjunction with antimicrobials [3]. Bacterial adhesion is regarded as the first step in pathogenesis, thus, a logical approach to prophylaxis of dental caries centers upon the inhibition of adhesion of *S. mutans* to the tooth surface [4]. Hydroxylapatite (HA) can be used as a suitable in vitro model of adherence to tooth surfaces. The HA model [5] is currently the most common in vitro system in use for the study of adhesion of oral bacteria such as *S. mutans* [6, 7]. Phosphate-buffered saline coated HA (PBS-HA) was used in the majority of our adherence assays, however, some of our adhesion experiments were carried out in the presence of saliva for comparison purposes. Employing PBS-HA was the preferred approach for optimizing our adherence assay since it eliminated problems of variability and instability that are associated with use of saliva [8].

A vital aspect of the study of bacterial adhesion and attachment to surfaces is the requirement of accurate quantification of the microbial population attaching to a particular surface [9]. A fluorescence-based 96 well plate assay is one such approach that has been successfully employed by researchers such as Gaines et al. [7]. Many other researchers use radiolabelled bacteria for the quantification of inhibition of adhesion of cariogenic bacteria to HA. However, radiolabelling of bacteria or other reagents requires special laboratory space and techniques, and can be hazardous [9]. A major benefit of fluorescence-based

methods is their speed in comparison to visual counting [9]. The fluorescence-based method used in this study is high-throughput and reliable, and was specifically designed to measure the percentage inhibition of adhesion of *S. mutans* to HA without the use of radioactivity.

Total milk protein, casein and whey proteins have been shown to possess anticariogenic properties, primarily due to the fact that they have a positive effect on the demineralization of enamel [10]. Recent research has shown that commercial whey protein concentrate can prevent both growth and adhesion of *S. mutans*, particularly following enzyme treatment [11]. The objective of the present study was firstly to determine the effect of a variety of commercial dairy powders on the adhesion of *S. mutans* to HA, and also to examine the effects of a selection of individual milk protein constituents on this adhesion process.

Materials and methods

Source and preparation of dairy powders

Whey protein isolate (WPI), whey powder (WP), demineralised whey (DW), sweet whey protein concentrate 80 (WPC, 80% protein), acid WPC 80 and sweet WPC 35 powders were supplied by Carbery Milk Products (Ballineen, Cork, Ireland). Buttermilk powder (BMP), cream powder (CP) and sodium caseinate were supplied by Kerry Group plc (Tralee, Co. Kerry, Ireland). α-lactalbumin, β-lactoglobulin (A and B), BSA, lactoferrin, α-casein, β-casein, κ-casein and albumin from chicken egg white (grade V) were obtained from Sigma (Poole, Dorset, UK).

Analysis of dairy powders

Compositional analysis was performed for each dairy powder using standard methods. Ash content was determined according to the international dairy federation (IDF) standard method [12]. Fat content was determined according to the method of Röse-Gottlieb [13], protein content was determined by the Kjeldahl method [14] and the moisture content was determined by the IDF reference method [15].

Bacterial isolate 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. The identity of the bacterial isolate was confirmed by 16S RNA analysis (Accugenix, Delaware, USA), and maintained on Protect™ Bacterial Preserve beads (Technical Service Consultants Ltd., Lancashire, UK) at −80 °C. For each assay, 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 of *S. mutans* was used to inoculate 20 mL of brain heart infusion (BHI) broth (BHI Broth; LabM, Lancashire, UK) and grown under aerobic conditions at 37 °C for 18 h. The culture was then subjected to centrifugation at 3,220 × *g* (Eppendorf 5810R, Cambridge, UK) for 10 min and the pellet was resuspended in sterile phosphate-buffered saline (PBS; Oxoid, Hampshire, England). Following a second centrifugation step, the bacterial pellet was resuspended in PBS, and the optical density (OD) at 630 nm was measured using a Multiskan Ascent spectrophotometer (Thermo Electron Corporation, Finland), and subsequently adjusted to 0.2 by appropriate dilution with sterile PBS. Viable colony counts were performed to verify the number of colony forming units per millilitre (CFU mL^{-1}), typically 1×10^8 CFU mL^{-1}.

Preparation of hydroxylapatite

Hydroxylapatite (HA) beads were supplied by Merck (Darmstadt, Germany). Size profile analysis of this HA was carried out using a Malvern Mastersizer (Malvern Instruments Ltd., Worcestershire, UK), and the average diameter (D [3, 4]) of the HA beads was found to be approximately 10 μm. PBS-HA was prepared immediately before use in the adherence assays, and did not undergo any pre-treatment steps. S-HA was prepared similar to the protocol set out by Gibbons and Etherden [16]. Briefly, parafilm-stilumated 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} solution of hydroxylapatite. Aliquots of saliva-coated hydroxylapatite (S-HA) were dispensed into the wells of a 96-well V-bottom plate (Sarstedt, Newton, North Carolina, 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.

Preparation of fluorescent dye

Syto® 13 dye was supplied (Molecular Probes, Oregon, USA) as a 5 mM solution in dimethylsulphoxide (DMSO). Preliminary studies established 5 μM, prepared in sterile PBS, to be a suitable concentration of fluorescent dye for our study (data not shown). A standard curve of relative

fluorescent units (RFU) vs. colony forming units per milliliter (CFU mL^{-1}) was constructed (Fig. 1), and the relationship between RFU and CFU mL^{-1} could be represented as 1 RFU $\approx$ 1 $\times$ 10^4 CFU mL^{-1}.

Adherence assay

Adherence assays using PBS-HA and S-HA were carried out in a similar manner. A preliminary study was conducted to establish the time required for maximum binding of bacteria to the HA (data not shown), and 45 min was the time allocated for adherence to occur. All dairy powders and milk and whey components were prepared to a stock solution concentration of 6 mg mL^{-1} by dissolution of dried powders in sterile PBS. A sterile polystyrene 96-well half-area microtitre plate (Nunc, Roskilde, Denmark) was set up as follows: 50 µL of dairy powder or milk component suspension (prepared in sterile PBS) at various concentrations was added to all wells. 50 µL of PBS-HA or S-HA (7.5 mg mL^{-1}) was added to the wells, followed by 50 µL of the bacterial suspension, so that the final volume of all wells was 150 µL. Control wells (no bacteria and/or HA) were included in each assay. The plate was incubated for 45 min at room temperature, and manually inverted at 5 min intervals to prevent settling of the HA suspension. The plate was subsequently centrifuged at 201 $\times$ *g* (Eppendorf 5810R, Cambridge, UK) to sediment the HA and adhering bacteria, leaving the non-adhering bacteria in suspension. These non-adhering bacteria were labeled with 10 µL of 5 µM Syto 13® fluorescent dye.

Quantification of inhibition of bacterial adhesion to HA

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

$$(\text{Fluorescence due to unbound bacteria}/\text{Fluorescence due to total input bacteria}) \times 100$$

Note: Because the adherence assay described here measures the unbound population (i.e. the non-adhering microorganisms), experiments were performed to verify that the sum of the RFU values of all populations (i.e. bound and unbound bacteria) was equal to the RFU value of the control wells containing the total input bacteria (data not shown).

Statistical analysis of data

All experiments were repeated at least three times. Results were expressed as the mean ± standard deviation (SD). Differences between treatments were determined using the general linear models (GLM) function of SAS Version 9.1.3. Data were considered significantly different if $P < 0.05$.

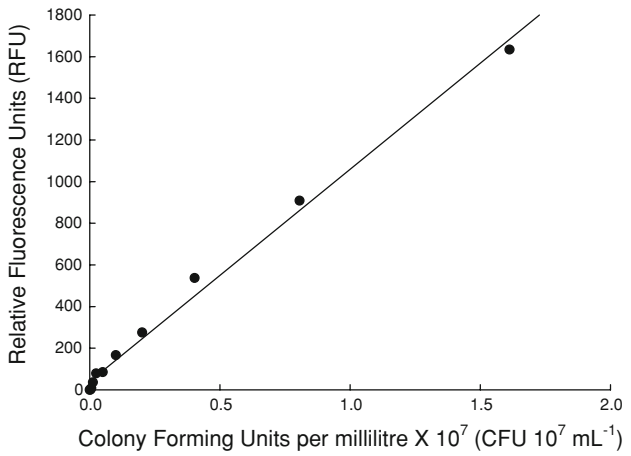

Fig. 1 Standard curve of relative fluorescent units (RFU) Vs colony forming units per milliliter (CFU mL^{-1}) for *S. mutans*

Results and discussion

Composition of dairy powders

Table 1 shows the composition of dairy powders used in the present study. These were typical of their product types.

Assessment of anti-adhesive effect of whey and dairy products

In every assay there was a small proportion ($\sim$10–20%) of bacteria that did not bind to PBS-HA in the absence of any potential inhibitor, and were considered to constitute a 'non-binding' population. Preliminary studies showed that increasing the ratio of PBS-HA to bacterial cells did not eliminate this proportion of microorganisms (data not shown). In all cases, inhibition of adhesion increased as

Table 1 Compositional analysis of dairy powders used in this study (%)

	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

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

concentration of dairy powder was increased (Fig. 2). Egg albumin was included in this study as a negative control protein, as it is not a component of milk or whey protein. This protein exhibited no inhibition of adhesion across the range of concentrations shown in Figs. 2 and 3.

WPI and DW were only found to exhibit inhibition of adhesion at $\geq$125 μg mL^{-1}, while WP showed no inhibition greater than its control adhesion value (0 μg mL^{-1}) across the range of concentrations examined ($P \leq 0.05$) (Fig. 2a). CP and BMP were found to be very effective inhibitors of *S. mutans* adhesion to PBS-HA (Fig. 2b), with both dairy powders observed to be significantly more effective than egg albumin at 17 and 33 μg mL^{-1}, respectively ($P \leq 0.05$). At 125 μg mL^{-1} both dairy powders exhibited >80% inhibition of adhesion.

In the case of the WPCs (Fig. 2c), AWPC80, SWPC80 and SWPC35 were more effective than their control adhesion values at 17, 33 and 66 μg mL^{-1}, respectively ($P \leq 0.05$). At 125 μg mL^{-1}, no significant difference was observed between SWPC80 and SWPC35, but both of these dairy powders were less effective than AWPC80 ($P \leq 0.05$). It is interesting to note that both Sweet and Acid WPC80 contain a high level of protein ($\sim$80%), and approximately 8% fat (protein: fat = 10:1), yet exhibit different levels of anti-adhesion activity. Glycomacropeptide (GMP), which is present in Sweet WPCs, is known to inhibit adherence of cariogenic bacteria to S-HA in the oral cavity [17–19], and can be incorporated into gum and toothpastes as a preventative measure against plaque and caries [20]. This constitutes an unusual finding in the present study where PBS-HA was used, as AWPC80 exhibited higher levels of anti-adhesion activity when compared to SWPC80 and SWPC35, yet does not contain GMP, perhaps suggesting there is a more potent active ingredient present in AWPC80 responsible for inhibiting adhesion of *S. mutans* to PBS-HA.

It appears that of the commercial dairy powders examined, AWPC80, CP and BMP were the most effective inhibitors of adhesion of *S. mutans* to PBS-HA. The exact mechanism of how these dairy powders used in our study can cause such inhibition is unclear and remains to be elucidated. In order to speculate as to which component(s) of dairy powders results in optimum anti-adhesion activity, it is worthwhile to compare the compositional analyses of each of these dairy products (Table 1). Cream powder contains $\sim$16% protein and $\sim$50% fat (protein: fat = 1:3), and buttermilk powder contains $\sim$30% protein and $\sim$10% fat (protein: fat = 3:1), yet both of these dairy powders were observed to be as effective as AWPC80 (protein: fat = 10:1) at 125 μg mL^{-1}. However, WPI contains an even higher level of protein than the WPC80s, CP and BMP ($\sim$87%) and negligible level of fat, yet resulted in less anti-adhesive activity. These findings suggest that the inhibitory effect of a dairy powder may be dependent on a balance between protein and fat content of that particular powder (Table 1). Also, the lactose content varies between powders, but we have found that this carbohydrate has no effect on the adhesion of *S. mutans* to PBS-HA, even at 1,000 μg mL^{-1} (data not shown), indicating that lactose is not contributing to the inhibitory effect.

Assessment of anti-adhesive effect of milk components

In an attempt to identify the active component(s) of the dairy powders examined, a selection of isolated milk components were assessed for their inhibitory properties. Of the range of purified milk proteins examined, neither α-lactalbumin, β-lactoglobulin (A or B) nor BSA showed any inhibitory activity higher than their control adhesion value across the range of concentrations shown in Fig. 3a. However, the caseins, lactoferrin and sodium caseinate were found to be extremely potent inhibitors of *S. mutans* adhesion to PBS-HA (Fig. 3b). At the lowest concentration examined (17 μg mL^{-1}), sodium caseinate was found to be significantly more effective than all other components tested ($P \leq 0.05$), but α-, β- and κ- casein were observed to be almost as effective at higher concentrations (>85% inhibition of adhesion). All milk proteins shown in Fig. 3b, with the exception of lactoferrin, were observed to be significant inhibitors of adhesion at the lowest concentration examined

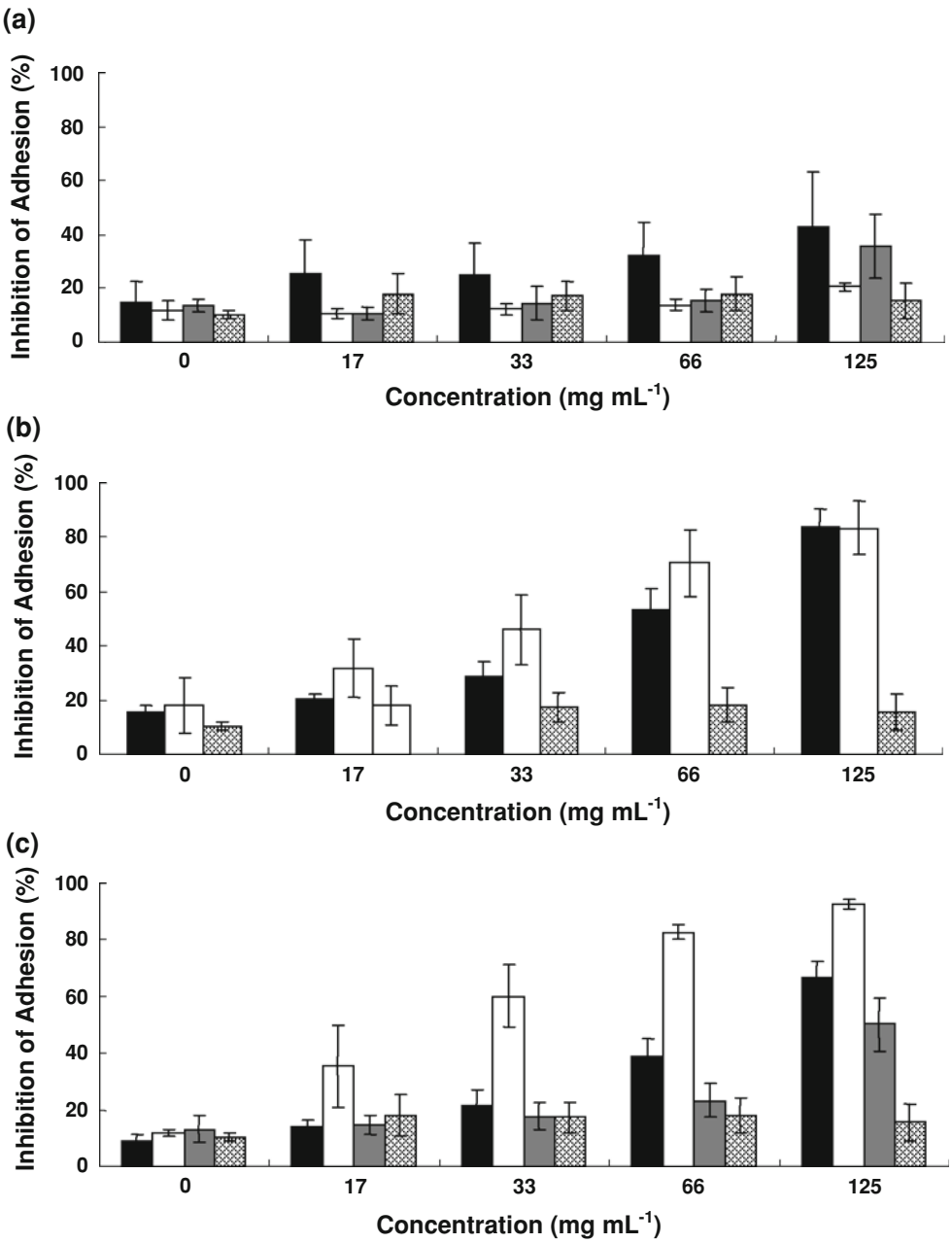

Fig. 2 Effects of dairy powders on the adhesion of *S. mutans* to PBS-HA: **a** whey protein isolate (WPI, *filled bar*), whey powder (WP, *open bar*) and demineralised whey (DW, *grey bar*), **b** buttermilk powder (BMP, *filled bar*) and cream powder (CP, *open bar*), and **c** sweet whey protein concentrate (WPC) 80 (SWPC80, *filled bar*), Acid WPC80 (AWPC80, *open bar*) and Sweet WPC35 (SWPC35, *grey bar*). Egg albumin (*dotted bar*) is included as a negative control (Data = mean ± standard deviation)

(17 μg mL^{-1}). Lactoferrin was not found to inhibit adhesion at concentrations <66 μg mL^{-1}, but exhibited potent inhibition of *S. mutans* adhesion to PBS-HA at concentrations above this.

A most interesting observation in the results presented here is that sodium caseinate, a widely available and relatively inexpensive industrial protein was the most effective inhibitor of *S. mutans* adhesion to PBS-HA, producing >80% inhibition of adhesion at the lowest concentration examined (17 μg mL^{-1}). This level of inhibition was only observed for AWPC80 at a fourfold higher concentration (66 μg mL^{-1}). Previous research has shown that sodium caseinate at 2% w/v in drinking water of rats infected with *S. mutans* caused a reduction in caries [21]. It was concluded that casein and sodium caseinate exhibited a 'topical anticariogenic effect', possibly due to incorporation of this protein into plaque, and subsequently preventing demineralization of the enamel [22]. However, the ability of sodium caseinate to inhibit the adherence of *S. mutans* to HA may be more beneficial than preventing demineralization of enamel.

In order to assess the relevance of these in vitro adherence assays in relation to oral conditions in vivo, some experiments were carried out using saliva-coated HA. S-HA was prepared as described earlier (see 'Preparation of hydroxylapatite') and an example of poor, medium and

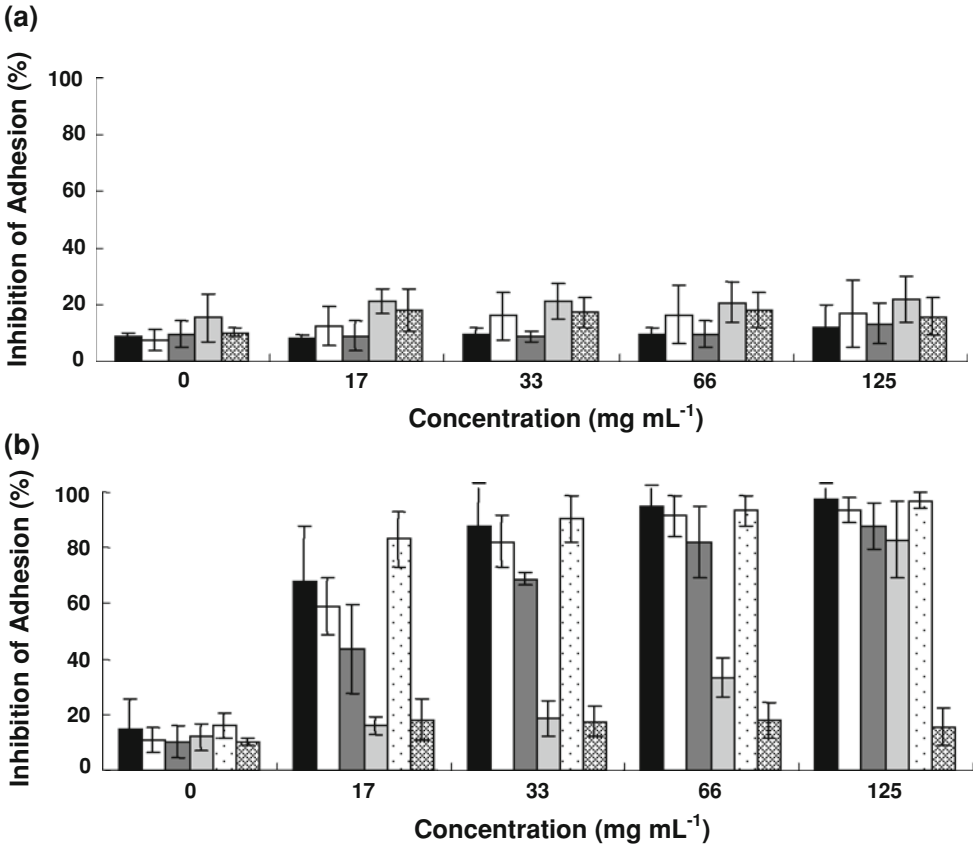

Fig. 3 Effects of milk and whey components on the adhesion of *S. mutans* to PBS-HA: **a** α-lactalbumin (■), *β*-lactoglobulin A (□), *β*-lactoglobulinB (■) and bovine serum albumin (■), and **b** α-casein (■), *β*-casein (□), *κ*-casein (■), lactoferrin (■) and sodium caseinate ( ). Egg albumin (▨) is included as a negative control (Data = mean ± standard deviation)

potent inhibitors of *S. mutans* adhesion to PBS-HA were selected and their inhibitory properties assessed. These were α-lactalbumin (poor), WPI (medium) and SWPC 80 and lactoferrin (potent). Similar trends were observed for these dairy products/components regardless of whether PBS-HA or S-HA was used, although it must be taken into consideration that a higher proportion of *S. mutans* did not bind to S-HA than for PBS-HA, thus the non-binding population in the absence of inhibitor was much greater (∼30–40%) when S-HA was employed (Table 2). Saliva is well known to reduce the adherence of *S. mutans* to HA, and this observation has been reported previously by researchers such as Liljemark et al. [23] and Clark et al. [24]. Two hypotheses exist to explain this phenomenon: (a) saliva alters the strength of the bond formed between *S. mutans* and HA and (b) saliva changes the amount of receptor or adsorption sites on HA beads, causing less bacteria to bind [24]. α-lactalbumin did not exhibit any significant level of inhibition greater than the control value across the range of concentrations examined for both types of coated HA, and was the least potent inhibitor of adhesion ($P = 0.05$). Both WPI and SWPC80 showed similar trends whether saliva was present or not, and lactoferrin was observed to be effective at inhibiting adherence of *S. mutans* to both PBS-HA and S-HA. This component of milk has previously been shown to be a potent inhibitor of adhesion of *S. mutans* (strain MT8148) to S-HA beads, and the findings of the present study are consistent with the work of Oho and co-workers [25].

It is well known that consumption of milk can be protective against occurrence of dental caries, and a study by Vacca-Smith et al. [26] reported that treatment of S-HA beads with milk caused a reduction in the adherence of *S. mutans* GS-5. In the same study, lactose was shown to have no function in the anti-adhesive properties of milk, which has also been observed in our laboratory (data not shown). Vacca-Smith and co-workers have demonstrated that adhesion of *S. mutans* to S-HA was inhibited by α-, *β*- and *κ*-casein and suggested that the caseins were interacting with both the S-HA beads and the bacterial cell surface, producing a synergistic inhibition of adhesion effect. It is possible that the same process is occurring in our adhesion assays. Other foods and beverages which are capable of inhibiting adhesion of cariogenic bacteria to S-HA include herbal extracts, cranberry juice and water-soluble protein fraction (WSPF) from hen-egg yolk, where 80, 90 and 61% inhibition of *S. mutans* adhesion to HA has been reported using 5, 250 g L^{-1} and 5 mg mL^{-1} of test substance, respectively [7, 27, 28].

Ideally, to prevent initial colonisation, an inhibitory substance against formation of dental caries should interact specifically with both oral microorganisms and the tooth

Table 2 Inhibition of adhesion values (%) of *S. mutans* to (i) Phosphate-buffered saline coated hydroxylapatite (PBS-HA) and Saliva-coated HA (S-HA) observed for α-lactalbumin, whey protein isolate (WPI), sweet whey protein concentrate 80 (SWPC80) and Lactoferrin

Conc. (μg mL^{-1})	α-lactalbumin	WPI	SWPC80	Lactoferrin
(i) PBS-HA				
0	$8.8^{a\ (x)} \pm 1.1$	$14.7^{a\ (x)} \pm 7.8$	$9.2^{a\ (w)} \pm 2.2$	$11.9^{a\ (x)} \pm 4.9$
17	$8.2^{a\ (x)} \pm 1.5$	$25.6^{b\ (x,y)} \pm 12.7$	$14.3^{a\ (w,x)} \pm 1.9$	$16.1^{a\ (x)} \pm 3.3$
33	$9.6^{a\ (x)} \pm 2.2$	$25.2^{b\ (x,y)} \pm 11.4$	$21.6^{b\ (x)} \pm 5.8$	$18.5^{a,b\ (x)} \pm 6.2$
66	$9.6^{a\ (x)} \pm 2.5$	$32.2^{b\ (x,y)} \pm 12.4$	$39.2^{b\ (y)} \pm 6$	$33.3^{b\ (y)} \pm 7.1$
125	$12^{a\ (x)} \pm 7.8$	$42.9^{b\ (y)} \pm 20.1$	$66.5^{c\ (z)} \pm 5.9$	$82.7^{c\ (z)} \pm 13.8$
(ii) S-HA				
0	$34.9^{a\ (x)} \pm 4.3$	$31.1^{a\ (x)} \pm 2.8$	$35.1^{a\ (w)} \pm 6.3$	$33.8^{a\ (x)} \pm 9.1$
17	$33^{a\ (x)} \pm 4.5$	$43^{b\ (x,y)} \pm 4.5$	$65.3^{b\ (x)} \pm 4.4$	$58.7^{b\ (y)} \pm 11.5$
33	$33.7^{a\ (x)} \pm 2.8$	$50.9^{b\ (y)} \pm 7.5$	$72.7^{b\ (x,y)} \pm 6.2$	$66.4^{b\ (y)} \pm 7.7$
66	$35.9^{a\ (x)} \pm 1.9$	$54.6^{b\ (y)} \pm 1.5$	$82.8^{b\ (z)} \pm 7.3$	$85.8^{b\ (z)} \pm 9.6$
125	$37.2^{a\ (x)} \pm 1.8$	$55.2^{b\ (y)} \pm 7.9$	$85.8^{b,c\ (z)} \pm 9.6$	$87.1^{c\ (z)} \pm 10.6$

Values are ± standard deviation. Means with the same letters are not significantly different (at the 5% significance level). Letters within the same rows indicate significant differences between test materials for (i) PBS-HA and (ii) S-HA, while letters within the same column (in parentheses) indicate significant differences between concentrations within the individual test material

surface [21]. Preliminary experiments have indicated that the HA beads used in our laboratory are being coated with these dairy proteins, which is possibly contributing to a reduction in adherence of *S. mutans*. Adsorption of proteins to HA is quite complex since HA carries both positive and negative charges due to the presence of Ca^{2+} and PO_4^{3-}, and the affinity of the former for negatively charged groups is higher than the affinity for phosphate groups for positively charged residues [29]. However, we cannot report that the inhibition of *S. mutans* to HA is solely due to this process, as there are other possible mechanisms that could be contributing to this inhibition, such as charge-charge-repulsion/attraction, hydrophobic interactions, steric hindrance and possible interference to the surface binding proteins associated with *S. mutans*.

Our adherence assays may be useful for the initial screening of inhibitory substances capable of reducing/preventing adherence of cariogenic bacteria to PBS-HA prior to use in assays employing S-HA. Preliminary studies using various test materials can be carried out in the absence of saliva, and if favorable results are obtained, the test material in question can subsequently be included in S-HA adherence assays. Preparation of S-HA is a time-consuming process, and is susceptible to many natural variations, for example diet, hormone fluctuations (females), smoking and use of antibiotics or other drugs. Also, logistical problems are often associated with saliva collection as it is difficult if not impossible to collect all samples and perform assays on the same day. Freezing can have an undesirable influence on saliva, as adherence of *S. mutans* can vary between different samples from an individual after freezing [30]. In addition, precautions associated with the handling of bodily fluids are not required if PBS-HA is employed, and the adherence assay is more sensitive as there is little variation associated with using a buffer of which the exact composition is known. Thus, our method using PBS-HA is a more convenient model system for assessing the anti-adhesion properties of various test materials of a cariogenic microorganism to a tooth surface. In addition, the effect of an inhibitory substance on the adherence of *S. mutans* to uncoated HA can provide useful information for the development of products for individuals with xerostomia or 'dry mouth syndrome', such as cancer patients receiving radiation treatment to the head and neck, frequent users of narcotics and patients with aplasia of the salivary glands [1]. Such conditions can result in rampant caries, and a substitute for saliva may have many beneficial effects. One such substitute is milk or milk component based drinks, as these beverages possess many of the biological and physical properties associated with saliva [31], and would be easily consumed by patients with these conditions.

The novel method described in this study enabled us to carry out assays which were non-hazardous, rapid and easy to perform, the results of which were highly reproducible, with the coefficient of variation being typically <10% between the RFU values recorded for replicates. This adherence assay may also be useful in assessing the inhibitory properties of other substances to HA (both PBS-coated and saliva-coated). In conclusion, inhibition of *S. mutans* adhesion to hydroxylapatite and therefore ability to control dental caries is an application for dairy products. The results presented here further substantiate the potential of dairy based compounds to delay the progression of dental caries by impairing adherence of the cariogenic microorganism *S. mutans* to HA.

Acknowledgments This work was supported by the national development plan (NDP), with a grant from the food institutional research measure (FIRM). We would like to thank Una Scallan and Joshua Arimi (UCD) for their help with statistical analysis.

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