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Production from dairy cows of semi-industrial quantities of milk-protein concentrate (MPC) containing efficacious anti-*Candida albicans* IgA antibodies

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Bovine milk antibodies directed against human pathogenic organisms have potential as prophylactic or therapeutic treatments of disorders affecting mucosal surfaces. The cow, however, does not naturally secrete high levels of IgA in milk, the predominant immunoglobulin of the mucosal immune system. We have patented an immunisation protocol that results in increased production of IgA in ruminant milk and in this study established that our protocol can be used on a scale sufficient to produce semi-industrial quantities of milk for processing. Cows were immunised with a common pathogenic yeast, *Candida albicans* and responded with high levels of antigen-specific IgA antibodies in their milk. The spray-dried milk-protein concentrate (85% protein) powder was shown to reduce adherence of *Cand. albicans* cells in *in vitro* adherence assays, demonstrating an ability to retain efficacy through the processing. These results suggest that this milk product may be of therapeutic value if the reduction in *Cand. albicans* adhesion observed *in vitro* translates to reduced colonisation *in vivo.*

Keywords: IgA, bovine milk immunoglobulin, mucosal defence, therapeutic milk antibodies.

Antibodies in ruminant milk and colostrum have potential applications as prophylactic or therapeutic agents for the treatment of disorders affecting mucosal surfaces in humans. Antibodies harvested from bovine milk have previously been shown to provide passive protection including: protection of hospitalised children against rotavirus infection using anti-rotavirus bovine colostrum (Davidson et al. 1989); use of an immune whey protein concentrate to prevent a relapse of *Clostridium difficile*-associated diarrhoea (van Dissel et al. 2005).

Protection from, or therapy of, mucosal infections with oral dosage of exogenous antibodies requires that the antibodies are active locally at the mucosal surface. The immunoglobulin associated with mucosal surfaces is secretory IgA (SIgA) (Butler et al. 1972; Mestecky et al. 1987). Compared with other immunoglobulins, SIgA is considered to be more resistant to proteolysis (Underdown & Dorrington, 1974; Stone et al. 1979; Brown et al. 1970). However, in contrast to humans, SIgA is a minor immunoglobulin in ruminant mammary secretions, with IgG_1 being the predominant isotype (Butler et al. 1972). We have developed and patented an immunisation protocol which increases levels of SIgA in ruminant milk (Hodgkinson & Hodgkinson, 2003). Therapeutic products made from IgA-enhanced milk may potentially be more efficacious than those containing only IgG.

Candida albicans is a very common commensal of mucosal surfaces and can be found in the mouths of 20–40% of healthy people (Odds, 1988). In immunocompromised people, *Cand. albicans* is an opportunistic pathogen causing a range of superficial and systemic infections. AIDS patients, for example, often suffer from severe oral candidosis (Reichart, 2003). *Cand. albicans* can exist in different morphologies, the most common a budding yeast or a filamentous hyphae. For most fungal pathogens, yeast cells are the invasive morphology (Gow et al. 2002), however, when *Cand. albicans* is associated with disease, the formation of hyphae is believed to contribute to pathogenicity (reviewed by Kumamoto & Vinces, 2005). In order to cause an infection, the yeast must first adhere to host surfaces and then proliferate. During

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acquisition, colonisation and carriage as a commensal, the yeast morphology of *Cand. albicans* predominates (Cannon & Chaffin, 1999). Thus, the ability to prevent adherence of *Cand. albicans* yeast cells, and hence colonization, would present the possibility of precluding candidosis in susceptible individuals.

The objective of this trial was to produce semi-industrial quantities of a milk-protein concentrate (MPC) powder from cows immunised with yeast cells of *Cand. albicans* using the patented immunisation protocol, and to test the *in vitro* efficacy of this product in models of *Cand. albicans* adherence.

Materials and Methods

Yeast strains and vaccine preparation

For vaccine preparation, *Cand. albicans* strain ATCC10231 (American Type Culture Collection, Manassas, VA, US) was grown in Malt Extract broth at 37 °C and then killed by incubation at 70 °C for 2 h. Microscopy of the inoculum confirmed cells were of yeast morphology. Heat-killed cells were recovered by centrifugation, lyophilised then resuspended in sterile saline (50 g dry weight/l), sonicated and protein concentration estimated using the Lowry method (BioRad, Auckland, NZ). The vaccine contained an emulsion of cell suspension (0·5 g protein/l) in one part sterile saline solution (0·9%, Baxter Healthcare, NSW, Australia) to three parts Incomplete Freund's Adjuvant (Sigma Chemical Co., St Louis, MO, USA).

For adherence assays, *Cand. albicans* ATCC10231 was used, except in some experiments (adherence to saliva-coated hydroxyapatite beads and immobilised saliva proteins), the laboratory strain ATCC10261 and a clinical isolate strain designated I1 (strain hp11an; Schmid et al. 1995) were used. Cells were grown on yeast extract peptone dextrose agar plates, containing per litre: 10 g yeast extract, 20 g Bacto® peptone (Becton Dickinson, Sparks, MD, USA), 20 g glucose, 20 g agar (Germantown Co., Manakau City, NZ), at 30 °C for 24–48 h, or in liquid Glucose Salts Biotin medium (Holmes & Shepherd, 1988) with aeration at 30 °C. For adherence assays using [^{35}S]methionine-labelled yeast cells, radiolabelled cells were prepared as described previously (Holmes et al. 2002). The specific radioactivity of cells labelled with [^{35}S]methionine, calculated by measuring the radioactivity of known numbers of cells, was similar for each experiment, with a mean value of 32 ± 18 cells/cpm.

Immunisation

Fifty pregnant dairy cows of mixed age and breed (Friesian and Jersey) were immunised by intra-peritoneal injections (4 ml) into the peritoneal cavity on the left-hand side of the animal and intra-muscular injections (2 ml/side) into the anterior neck muscle at 8, 4 and 1 week prior to expected calving dates. Intra-mammary immunisations were administered via the teat orifice (2 ml/quarter) at 4 and 2 weeks prior to expected calving dates. After the first intra-mammary immunisation, antibiotics (Clavulox®L.C, GlaxoSmithKline NZ Ltd., Auckland, NZ) were also administered via the teat. Nine non-immunised animals acted as controls. All animal treatments were approved by the Ruakura Animal Ethics Committee.

Sample collection and milk processing

Individual milk samples were collected for antibody analysis from all animals on the first day following calving and 28 days later. Bulk milk for the manufacture of MPC product was collected over a 6-week period. Days-in-milk for individual cows at the beginning of the milk collection varied from 5–77 d, with an average of 16–54 d, due to different calving dates. Cows were milked twice daily and milk stored in a refrigerated vat. Every 2–3 d, milk was collected from the vats, skimmed at 50 °C, pasteurised (72·5–74·5 °C/15 s), packaged in 25 kg amounts and frozen. In total, 13 000 l skimmed milk were frozen. Samples of raw, skimmed and pasteurised milk were retained from each processed batch for analysis.

For processing, milk was thawed with the addition of approximately equal volumes of demineralised water and re-pasteurised at 63–66 °C for 30 min. A MPC product containing 85% protein (immune-MPC) was prepared by a proprietary process using a semi-industrial scale ultra-filtration and spray-drying plant. Retentate from the ultra-filtration process (300 l) was also freeze-dried to compare treatment effects. Samples of ultra-filtration retentate and permeate, and dried MPC powders were stored for analysis. A standard MPC product containing 85% protein (designated non-immune-MPC), was processed from milk collected from non-immunised cows.

Antibody analysis

Milk samples were centrifuged (1650 ***g***, 10 min, 4 °C), and the fat layer removed. Skimmed samples were diluted (20% v/v) with phosphate buffered saline (PBS; 10 mM-NaH_2PO_4/Na_2HPO_4, 150 mM-NaCl, pH 7·4) and re-centrifuged (11 600 ***g***, 1 h, 4 °C). Supernatants were retained for analysis. Samples from MPC processing were treated as above for skimmed samples. MPC powder samples were reconstituted in PBS (10% w/v) and mixed end-over-end at room temperature (RT) for at least 30 min, and then treated as above for skimmed samples.

Total IgA and IgG were measured by enzyme-linked immunosorbent assay (ELISA) kits (E10-121 and E10-118, Bethyl Laboratories, Montgomery, TX, USA), following the manufacturer's recommended protocol. Anti-*Cand. albicans* IgA and IgG titres were measured using non-competitive indirect ELISAs as described previously (Hodgkinson et al. 1995). Briefly, microtitre plates (Maxisorb F-96, Nalge Nunc International, DK-4000, Roskilde, Denmark) were coated with a suspension of the

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Cand. albicans strain ATCC10231 preparation used for vaccination (100 µl; 10 mg protein/l) in bicarbonate buffer (50 mM-$NaHCO_3$, pH 9·8). Test samples and reference sample were assayed at dilutions of 1 : 100 to 1 : 100 000. For the IgA and IgG ELISA, secondary antibodies were rabbit anti-bovine IgA (1 : 40 000; Bethyl Laboratories) and goat anti-bovine IgG (1 : 100 000; ICN Biomedicals, NSW, Australia), respectively. Tertiary antibodies were goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (1 : 12 000; DakoCytomation, DK-2600, Glostrup, Denmark) and donkey anti-goat horseradish peroxidise (1 : 70 000; Chemicon, Australia). Detection substrate contained 0·45 g H_2O_2/l and 0·1 g 3,3′,5,5′-tetramethylbenzidine/l in acetate buffer (100 mM-CH_3COONa, pH 5·4). Optical density (OD) was measured at 450 nm (Plate reader, ELP-35, Bio-Tek). Antibody titres for test samples were defined as the reciprocal of the dilution which produced an OD equal to 50% of the maximum OD above background of the reference sample. Results are expressed as titre units $\times 10^3$ (kTU) of antibody.

Preparation of MPC for testing in Cand. albicans *adherence assays*

Stock solutions were prepared by dissolving MPC (5 g/l) in pre-heated (40 °C) KCl buffer (2 mM-KH_2PO_4/K_2HPO_4; 5 mM-KCl; 1 mM-$CaCl_2$, pH 6·5) and incubating at 40 °C for 10 min with gentle shaking.

Saliva collection

After obtaining informed consent, saliva was collected according to a protocol approved by the University of Otago, Human Ethics Committee. Un-stimulated saliva from six donors was stored on ice and proteinase inhibitors added as described by O'Sullivan et al. (2000). A pool containing an equal volume from each donor was clarified by centrifugation (12 000 ***g***, 15 min, 4 °C).

Cand. albicans *adherence assays*

Saliva-coated hydroxyapatite bead assay – a model for adherence to the tooth surface (Cannon et al. 1995). Briefly, *Cand. albicans* ATCC10261 cells, radiolabelled with [^{35}S]methionine as described above, were treated with KCl buffer alone, or KCl buffer containing either immune-MPC product or non-immune-MPC (10, 5, 2·5, 1·25, 0·625 or 0 g/l) by incubation at RT for 1 h with end-over-end mixing. Adherence of the pre-treated *Cand. albicans* cells to saliva-coated hydroxyapatite beads was measured as described previously (Cannon et al. 1995) with bead-associated radioactivity determined by liquid scintillation. Percent inhibition of cell-adhesion by milk products was calculated relative to cells incubated with buffer alone.

Adherence to saliva proteins immobilised on nitrocellulose. This method undertaken as described previously (O'Sullivan et al. 1997), was performed twice for each of two strains of *Cand. albicans*. Briefly, salivary proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. *Cand. albicans* cells, (ATCC10261 or strain I1; 2×10^7 cells/ml) radiolabelled as described above, were pre-treated with KCl buffer containing immune-MPC product or non-immune-MPC (50, 5, 0·5 or 0 g/l), before incubation with strips of membrane, washing, and detection of *Cand. albicans* bound to protein bands by autoradiography. Band intensity was quantified using densitometry image software and mean band intensity calculated for each concentration of test milk-product. Percent inhibition of cell-adhesion by milk products was calculated relative to cells incubated with buffer alone.

Adherence of Cand. albicans *to tooth enamel.* Human teeth (incisors) used in this experiment had been extracted for clinical reasons at the University of Otago, School of Dentistry Clinic, and patients had consented to use of extracted teeth in research. Teeth were first bisected and the roots removed, then incubated for 1 h at RT with end-over-end mixing in pooled human saliva diluted (50% v/v) in KCl buffer. Teeth were washed twice with KCl buffer and non-specific protein binding then blocked with KCl buffer containing 0·1% (w/v) BSA (1 h at RT with end-over-end mixing). Concurrently, *Cand. albicans* ATCC10231 cells (2×10^7 cells/ml) were pre-treated with either non-immune-MPC (0·5 g/l) or immune-MPC (0·5 g/l) for 1 h at RT with end-over-end mixing. Individual teeth and pre-treated yeast cells (1·0 ml, 2×10^6 cells/ml in KCl) were incubated together for 1·5 h at RT with end-over-end mixing, before washing (×3 in KCl buffer) and preparation for scanning electron micrograph analysis.

Adherence of Cand. albicans *to HEp-2 human epithelial cell monolayers.* Adherence of *Cand. albicans* ATCC10231 cells to cultured human epithelial cells (HEp-2 cell line) was measured as previously described (Holmes et al. 2002). *Cand. albicans* cells, radiolabelled as described above, were pre-treated (30% v/v) with an artificial saliva buffer (1·75 mM-KH_2PO_4, 1·5 mM-$CaCl_2$, 18·3 mM-KCl, 0·35 mM-NaSCN, 15 µM-NaF, 26 mM-$NaHCO_3$, pH 6·5) containing 1 g glucose/l. Radiolabelled yeast cells (50 µl) were then added to quadruplicate microtitre plate wells containing confluent monolayers of HEp-2 cells in the presence of artificial saliva buffer (50 µl) containing either immune-MPC or non-immune-MPC (2·0, 1·0, 0·5, 0·25, 0·125 or 0 g/l). Adherence of radiolabelled cells to the HEp-2 monolayers was determined following incubation, washing and scintillation counting.

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Table 1. Titres of anti-*Cand. albicans* IgA and IgG in colostrum (day 1) and milk (day 28) collected from immunised and control cows

Days after calving	Titres of anti-*Cand. albicans* IgA (kTU)†		Titres of anti-*Cand. albicans* IgG (kTU)	
	Control cows	Immunised cows	Control cows	Immunised cows
1	0·39±0·12	26·22±7·83	0·22±0·07	16·24±2·30
28	N.D.‡	6·42±1·14	N.D.	0·41±0·08

† Titre units $\times 10^3$. Values are means of individual milk samples ± the standard error of the mean for $n=9$ control cows and for $n=49$ immunised cows
‡ Not detectable, limit of detection 0·1 kTU

Statistical analysis

Values, where appropriate, were expressed as mean ± standard error of the mean (SEM). The difference between the means of two data sets was given as the standard error of the difference (SED). A two-tailed student t-test was used to determine significance between two data sets, the test parameters dependent on the data set to be analysed.

Results

Specific antibody titres and total immunoglobulin concentrations

Titres of specific anti-*Cand. albicans* IgA and IgG were higher ($P<0·001$) in milk collected from immunised animals than in milk collected from control animals, on d 1 and 28 following calving (Table 1). For immunised animals, the average IgA antibody titre on d 28 was 24·4% of the d 1 titre whereas for IgG the d 28 titre was only 2·5% of the d 1 titre.

The average titre of anti-*Cand. albicans* IgA of the raw bulk milk collected from immunised animals was 7·4±0·5 kTU and following pasteurisation 6·9±0·1 kTU. Samples collected during the different processing steps showed no reduction in anti-*Cand. albicans* IgA titres, when adjusted for total solids (data not shown). The titre of anti-*Cand. albicans* IgA for spray-dried retentate (immune-MPC) was 10·2 kTU compared with 15·2 kTU for freeze-dried retentate, equating to a decrease in antibody activity in the immune-MPC of approximately 30%. No anti-*Cand. albicans* IgA or IgG was detected in the non-immune-MPC. The titre of anti-*Cand. albicans* IgG was 0·55 kTU in the immune-MPC. Total IgA

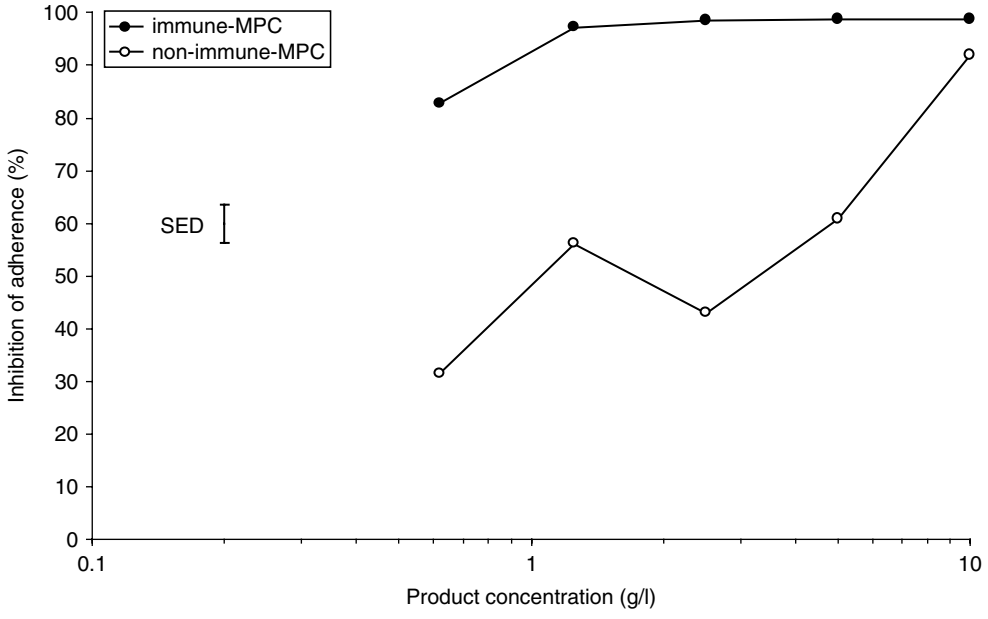

Fig. 1. Inhibition of adherence of radiolabelled *Cand. albicans* ATCC10231 cells to saliva coated hydroxyapatite beads after pre-incubation of cells with immune-MPC (●) or non-immune-MPC (○). Inhibition (%) was determined by comparison with the adherence of radiolabelled cells preincubated with buffer only to saliva-coated beads, which gave a mean value of 20±4% of input cells (1×10^5 cells). SED shows average standard error of the difference between the immune-MPC and non-immune-MPC at different concentrations.

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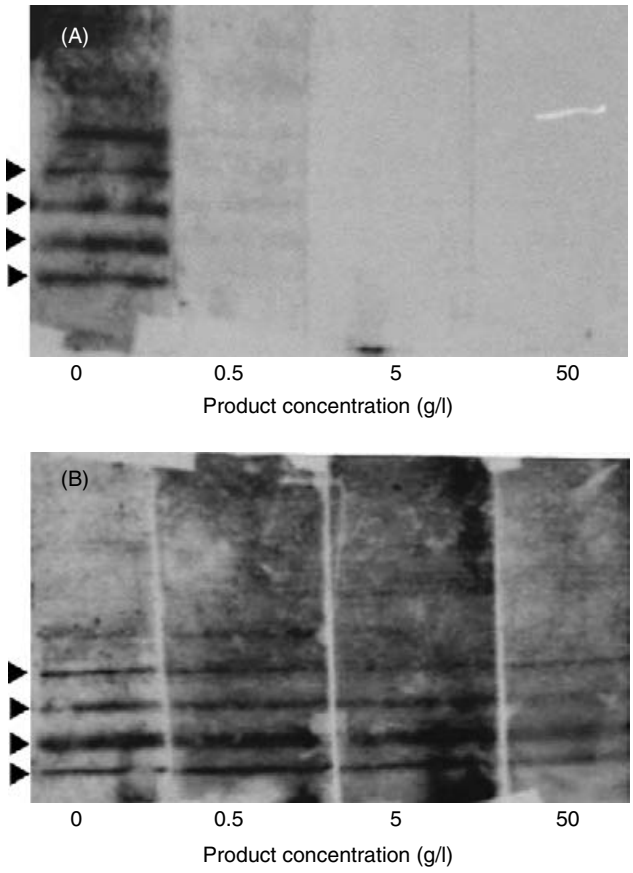

Fig. 2. Auto-radiograms of radiolabelled *Cand. albicans* I1 cells bound to saliva proteins immobilised on nitrocellulose, after pre-incubation of cells with (A) immune-MPC and (B) non-immune-MPC, at concentrations of 0, 0·5, 5·0 or 50·0 g/l. The bands identified as members of the basic proline-rich proteins (bPRPs) polypeptide family are indicated by arrowheads.

concentration in the immune-MPC was 2·1 mg/g solids compared with 0·5 mg/g solids in the non-immune-MPC. Total IgG concentration was 4·3 mg/g solids in the immune-MPC compared with 1·3 mg/g solids in the non-immune MPC.

Inhibition of Cand. albicans *adherence to hydroxyapatite beads, immobilised saliva proteins, tooth enamel and HEp-2 human epithelial cell monolayers*

A comparison of the abilities of immune-MPC and non-immune MPC to inhibit the adherence of *Cand. albicans* was determined in four assays. Figure 1 shows the dose-dependent inhibitory effect of MPC on adherence of *Cand. albicans* to saliva-coated hydroxyapatite beads. The inhibition of adherence by immune-MPC was greater than that observed for non-immune-MPC ($P<0·05$) at all but one of the concentrations tested (10 g/l).

Immune-MPC also showed significantly greater ($P<0·001$) inhibition of adherence of *Cand. albicans* strains ATCC10261 and I1 to specific saliva proteins than

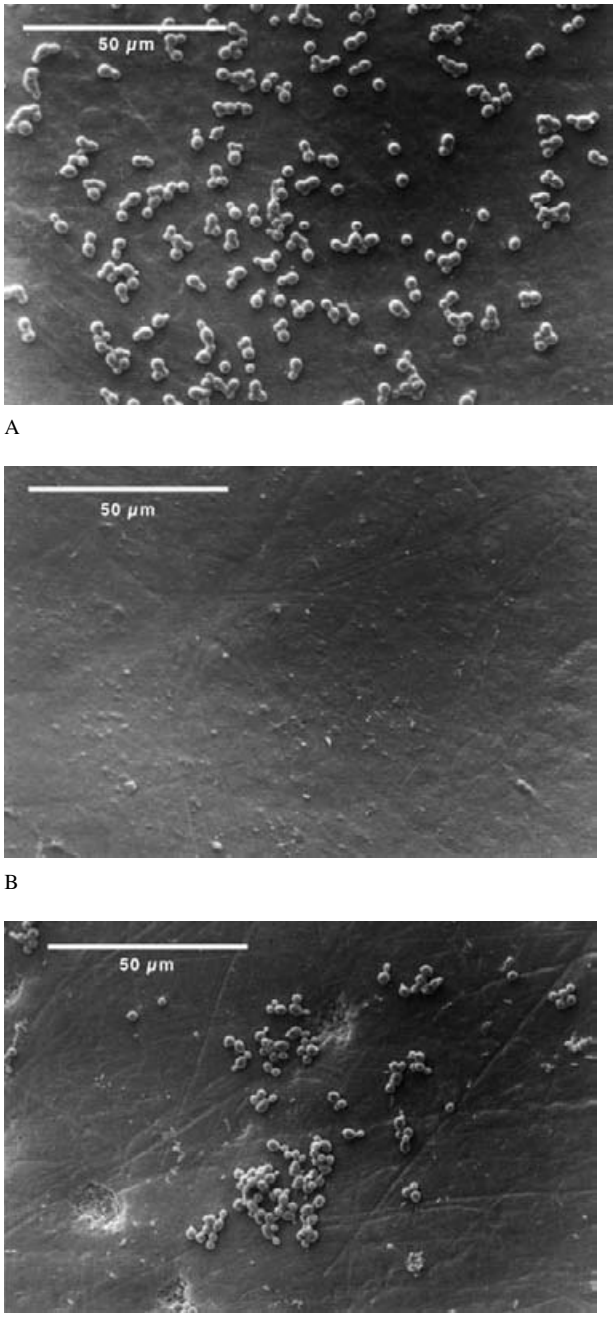

Fig. 3. Scanning electron micrograph of *Cand. albicans* cells bound to tooth surface after pre-incubation of cells: (A) with buffer; (B) immune-MPC (0·5 g/l); (C) non-immune-MPC (0·5 g/l). Bar = 50 µm.

non-immune MPC as demonstrated by autoradiography (Fig. 2). Radiolabelled *Cand. albicans* cells were specifically bound by several PAGE-separated salivary proteins including four (17, 20, 24 and 27 kDa) that have previously been identified as basic proline-rich proteins (O'Sullivan et al. 1997). For strain ATCC10261, the mean inhibition of adherence of *Cand. albicans* cells by

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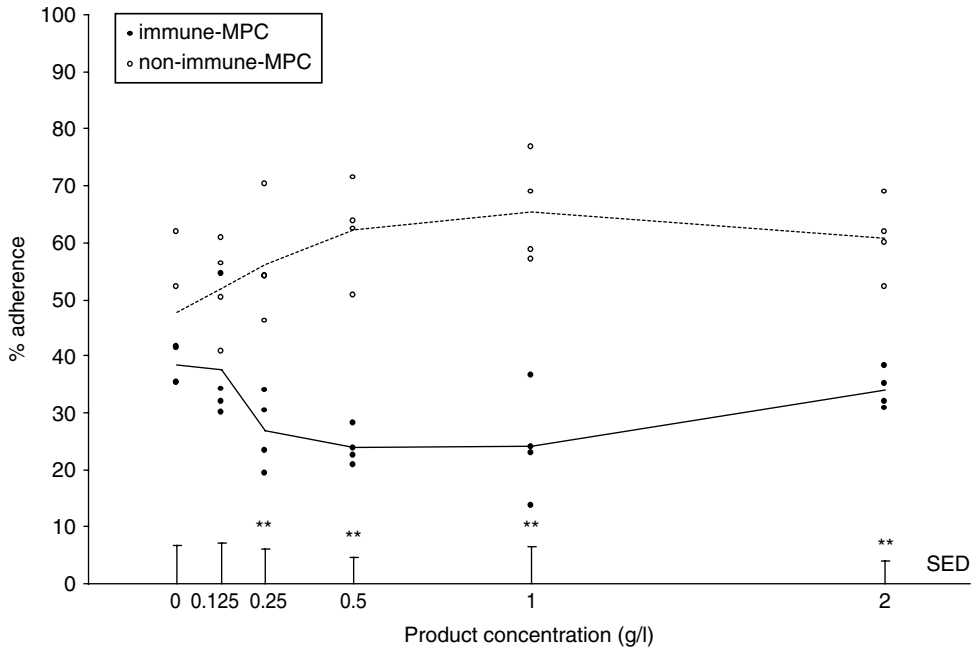

Fig. 4. Inhibition of adherence of *Cand. albicans* cells to HEp2 monolayers after pre-incubation of cells with various concentrations of immune-MPC (●) or non-immune-MPC (○). Lines show means of plotted observations. Concentrations at which there was a significant difference in inhibition of adherence between non-immune-MPC and immune-MPC are marked (** $P<0{\cdot}01$). Inhibition (%) was determined by comparison with the adherence of radiolabelled cells preincubated with buffer only to HEp2 cells, which gave a mean value of 45±4% of input cells ($5{\cdot}5\times10^4$ cells).

immune-MPC and non-immune MPC was 91·2 and 59·5% (SED 3·42), respectively. For strain I1, the mean inhibition of adherence by immune-MPC and non-immune MPC was 87·4 and 52·3% (SED 4·03), respectively.

Scanning electron micrography showed that *Cand. albicans* cells bound to saliva-coated tooth enamel when no MPC product was present (Fig. 3A). In contrast, the immune-MPC completely prevented adherence of *Cand. albicans* to the surface of the tooth (Fig. 3B). Non-immune-MPC caused clumping of *Cand. albicans* cells, however, it did not appear to prevent adherence of cells to the tooth (Fig. 3C).

Adherence *of Cand. albicans* cells to HEp-2 monolayers was significantly inhibited by immune-MPC at a concentration of 0·5 g/l ($P<0{\cdot}01$; Fig. 4). Non-immune-MPC appeared to increase the adherence to HEp-2 cells, although, this difference was not statistically significant. The difference in adherence of cells between the non-immune-MPC and immune-MPC products was significant at concentrations ranging from 0·25–2·0 g/l ($P<0{\cdot}01$).

Discussion

We have previously described the development of a proprietary immunisation protocol to increase levels of antigen-specific IgA in bovine milk (Hodgkinson & Hodgkinson, 2003). In this study, we demonstrated that our patented procedure can be used to immunise a large number of cows and the immune-milk may be processed using existing industrial processes and retain biological activity of the antibody in the end-product. There appeared to be no decrease in antibody-binding activity, as determined by ELISA, when pasteurised milk was compared with raw milk, although, there was some loss in antigen-binding activity observed when spray-dried immune-MPC product was compared with freeze-dried product. However, the spray-dried immune-MPC product retained sufficient activity to have significantly greater effect in all the assays measuring inhibition of adherence compared with non-immune-MPC. Interestingly, a higher percentage of anti-*Cand. albicans* IgA persisted in milk than anti-*Cand. albicans* IgG. In addition, IgA titres were 26·2-fold higher in immune-MPC compared with colostrum collected from non-immunised cows, while IgG titres were only 2·5-fold higher. These data suggest that the concentrations of anti-*Cand. albicans* IgA in milk from immunised cows are sustained at higher values than anti-*Cand. albicans* IgG concentrations.

Adhesion of *Cand. albicans* cells to oral surfaces is believed to be an important step in the development of oral candidosis. All oral surfaces in contact with saliva rapidly become coated with salivary components and these components have been shown to mediate *Cand. albicans* adherence to surfaces such as hydroxyapatite, a model for the tooth surface (Cannon et al. 1995) and epithelial cells (Holmes et al. 2002). There is surface-specific differential adsorption of salivary components

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that forms a conditioning film or pellicle (Carlen et al. 2003; Vitorino et al. 2004). Basic proline-rich proteins, for example, are selectively adsorbed to oral streptococcal cells (O'Sullivan et al. 1997, 2000) and hydroxyapatite beads (Cannon et al. 1995). This report used assays of *Cand. albicans* adherence to demonstrate that the immune-MPC inhibited adherence of *Cand. albicans* cells to hydroxyapatite beads, immobilised salivary proteins, and epithelial cells to a significantly greater extent than a control non-immune MPC product. Although both immune and non-immune MPC preparations showed non-specific inhibitory effects at the higher concentrations tested in the assays, in all assays there was a greater inhibitory effect by immune MPC at lower or intermediate concentrations. These findings indicate that the antibodies in the immune-MPC were active factors in adherence inhibition, and suggest the possibility that the immune-MPC may act to reduce colonisation by *Cand. albicans* of different oral surfaces. This possibility was also supported by the demonstration that colonisation of the surface of extracted teeth was prevented in the presence of immune-MPC but not with non-immune MPC. Furthermore, a previous study has reported that elevated titres of autologous sIgA specific to *Cand. albicans* in human saliva correlated with reduced adherence of *Cand. albicans* to human epithelial cells in the presence of the saliva sample (Holmes et al. 2002), supporting an hypothesis that the presence of increased sIgA may reduce adherence of *Cand. albicans*.

This is the first study to show industrial-scale production of a bovine antibody preparation that inhibits *Cand. albicans* adherence *in vitro*. The immune-MPC product inhibited adherence of *Cand. albicans* cells in all assays tested, and as well as inhibiting adherence of the vaccine strain ATCC10231, also reduced adherence of two other strains, ATCC10261 and I1. These strains were previously shown to have adherence properties representative of many *Cand. albicans* strains (Holmes et al. 1995). These results suggest that immune-MPC containing specific antibodies to *Cand. albicans* may be effective in preventing the adherence of *Cand. albicans* cells *in vivo*.

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