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Proferred review

Clinical applications of antimicrobial host proteins lactoperoxidase, lysozyme and lactoferrin in xerostomia: efficacy and safety

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Innate human salivary defence proteins, lysozyme, lactoferrin and peroxidase, are known to exert a wide antimicrobial activity against a number of bacterial, viral and fungal pathogens *in vitro*. Therefore, these proteins, alone or in combinations, have been incorporated as preservatives in foods and pharmaceuticals as well as in oral health care products to restore salivas' own antimicrobial capacity in patients with dry mouth. These antimicrobials used in oral health care products, such as dentifrices, mouth-rinses, moisturizing gels and chewing gums, have been purified from bovine colostrum. In this review I critically evaluate the clinical efficacy and safety of this kind of preventive approach against various oral diseases and symptoms.
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Introduction

Adequate flow of saliva is a prerequisite for good oral health. Saliva not only makes oral functions possible but it also protects oral tissues from noxious agents derived from microorganisms, food or drugs. Because the human mouth is a major route of entry for foreign materials into the body, saliva with its protective capacity forms an important part of a first line of defence against exogenous, often harmful agents. A proper flow of saliva is vital not only for oral health but, obviously, also to the general health of humans (Herrera, Lyons and Johnson, 1988; Rhodus and Brown, 1990).

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Hyposalivation often leads to many oral problems: xerostomia with all its symptoms, rapid dental decay, increased susceptibility to fungal infections, mucosal infections, etc. All these impair the patients' well-being, particularly because dental and medical professionals still often fail to make proper diagnosis in this interdisciplinary area (Nederfors, 2000). In spite of their known clinical significance the prevalence of hyposalivation and xerostomia is largely unknown. Xerostomia is usually experienced when the salivary flow rate is reduced by about 50% in test subjects with normal salivary flow (Dawes, 1987). However, normal flow is an individual feature and on a population level subjective sensation of oral dryness is only poorly associated with hyposalivation, as quantitated by objective measurements (Hay *et al*, 1998). However, it seems that both hyposalivation and xerostomia increase by age, are more prevalent among women than men, and are often associated with frequent use of drugs (Närhi, 1994; Nederfors, 2000). Depending on how the questions of oral dryness are formulated, the prevalence figures for xerostomia vary in adults and elderly usually between 10 and 40% (Nederfors, 2000).

Saliva protects oral tissues in many ways. A constant flow of saliva eliminates microorganisms from the oral cavity but saliva also contains many innate or acquired defence systems (reviewed, e.g. by Brandtzaeg, 1989; Lagerlöf and Oliveby, 1994; Tenovuo, 1998; Lenander-Lumikari and Loimaranta, 2000). A number of studies have tried to find a clinically significant association between selected salivary agents and caries prevalence or incidence but the results are controversial. This may be explained by several reasons, e.g. various salivary agents interact with each other in a complicated way (Lenander-Lumikari and Loimaranta, 2000). Also inadequate immune defence (usually IgA deficiency) can be compensated by an enhanced non-immunoglobulin activity (Arnold *et al*, 1979; Kirstilä *et al*, 1994b) or by compensatory IgM response (Norhagen Engström *et al*, 1989). These examples of concerted action of salivary

antimicrobials assure host-derived protection as long as an appropriate amount of saliva is secreted.

Enhancement of the antimicrobial capacity in saliva

In connection with hyposalivation, any increase in the flow rate inevitably results in an increase in the output of saliva-borne factors and therefore all methods to increase the flow rate are encouraged. This article, however, reviews attempts to enhance or restore saliva's own antimicrobial capacity by commercially available oral health care products. The antimicrobial host proteins most widely used in these products are lysozyme, lactoferrin and lactoperoxidase. To make lactoperoxidase (LP) enzyme antimicrobial, its substrates, thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2), are also included in the products. Hydrogen peroxide is usually generated *in situ* by a glucose–glucose oxidase system.

A large amount of scientific literature exists of how these non-immunoglobulin components work separately and together (for reviews, see Tenovuo, 1998; Lenander-Lumikari and Loimaranta, 2000). The reported antimicrobial mechanisms are listed in Table 1. An interesting point is that all these three antimicrobial agents act in an additive way and even synergistic interactions have been described (Tenovuo *et al*, 1982a; Lassiter *et al*, 1987; Soukka, Lumikari and Tenovuo, 1991a,b; Lenander-Lumikari, Månson-Rahemtulla and Rahemtulla, 1992). Therefore, it seems sensible to combine them also in oral health care products.

Products with antimicrobial proteins

It is relatively simple to add lysozyme and lactoferrin into oral health care products, such as toothpastes and mouth-rinses. These antimicrobial proteins can be purified in a cost-effective way from bovine colostrum and commercial products comprising such bovine lysozyme and lactoferrin preparations include at least Biotene®, Oralbalance®, BioXtra® and Zendium Saliva®. The latter two contain 'whey extract' or 'colostrum' and therefore also bovine immunoglobulins, which, however, are not reported by the manufacturer to be specific against human oral pathogens. It is possible that these products contain also colostrum-containing growth factors, such as IGF-1, TGF-β1 and TGF-β2, which are known to be present in many colostral products (Wei, Loimaranta, Tenovuo *et al*, 2002). Whether these have any effects on mucosal tissues is not known.

Human lactoferrin, or its antimicrobial 25-residue peptide fragment lactoferricin B, has recently been cloned by fermentation of *Aspergillus niger* (Headon, 2000). In future, it is likely that bovine milk derived lactoferrin will be replaced by recombinant human lactoferrin, which is reported to be active and safe for human clinical use (Headon, 2000). The possible difference in the efficacy of iron-free versus iron-saturated lactoferrin in oral health care products is not yet known.

The mimicking of salivary peroxidase systems is somewhat more complicated than lysozyme or lactoferrin. Both salivary peroxidase and myeloperoxidase, the two peroxidase enzymes in human mixed saliva, have been purified but only for research purposes. Large-scale purification from human saliva (salivary peroxidase) or from human polymorphonuclear leukocytes (myeloperoxidase) is difficult and far too expensive for commercial purposes. Therefore, for commercial purposes bovine lactoperoxidase (purified from milk or colostrum) has been used because this enzyme is structurally and catalytically very close to human salivary peroxidase (Månsson-Rahemtulla *et al*, 1988). Thiocyanate (SCN^-) is added as potassium thiocyanate but H_2O_2 is generated *in situ* in the mouth by a glucose–glucose oxidase system added to these oral hygiene products. The lactoperoxidase system is incorporated into commercial products like Biotene®, BioXtra®, Zendium Saliva® and Oralbalance®. Many of these comprise not only a toothpaste, but also a non-alcoholic mouth-rinse, a xylitol-flavoured chewing gum, moisturizing gels and/or denture adhesives for patients with dry mouth problems. The lactoperoxidase system retains its antibacterial activity for 1–2 years (Bosch, Van Doorne and De Vries, 2000) but data of the possible reduction in lysozyme or lactoferrin functions are not available. All lactoperoxidase system containing products are non-foaming because detergents inactivate the enzymes (Hoogendoorn, 1985).

Table 1 Antimicrobial mechanisms reported for lysozyme, lactoferrin and peroxidase enzyme systems. A more detailed description of these mechanisms with references is found in Tenovuo (1989)

Lysozyme
Muramidase activity (lysis of peptidoglycan layer)
Cationic-dependent activation of bacterial autolysins
Aggregation of bacteria
Inhibition of bacterial adhesion to tooth surfaces
Inhibition of bacterial glucose uptake and acid production
De-chaining of streptococci
Lactoferrin
Deprivation of iron from bacteria (bacteriostatic action)
Bactericidal action by direct contact of iron-free form of lactoferrin (apo-LF)
Inhibition of bacterial adhesion to tooth surfaces
Aggregation of bacteria
Activation of phagocytic cells
Salivary peroxidase systems
Inactivation of bacterial glycolytic enzymes
Inhibition of bacterial glucose uptake and acid production
Inhibition of bacterial transport of amino acids
Damage of the bacterial cell wall
Killing of bacteria (with I^- as a substrate instead of SCN^-)
Inhibition of bacterial adhesion to saliva-coated hydroxyapatite

Do these products work *in vivo*?

As early as 1983 Månsson-Rahemtulla *et al* showed that it was indeed possible to design a mouth-rinse which *in vivo* elevates the hypothiocyanite levels to bacteriostatic concentrations. The volume of the rinse, its pH and the amount of H_2O_2 generated were found to the

critical determinants, but at pH 5.5–6.5 high HOSCN/$OSCN^-$ concentrations, as high as >200 μM, could be generated *in vivo* (Månsson-Rahemtulla *et al*, 1983). The average whole saliva concentrations of HOSCN/$OSCN^-$ range from 30 to 70 μM (mean 44 μM) in resting whole saliva with much lower concentrations (mean 5 μM) in paraffin-stimulated whole salivas (Tenovuo, Pruitt and Thomas, 1982b). One of the first products, Biotene® toothpaste, was later shown to increase human whole saliva hypothiocyanite levels up to a mean of 200 μM concentration within 20 min after brushing (Lenander-Lumikari, Mikola and Tenovuo, 1993). No such effect was found with a non-lactoperoxidase/non-H_2O_2 generating toothpaste. Daily use of Biotene® toothpaste for 1 month also resulted in a slight increase in the resting levels of salivary hypothiocyanite (Lenander-Lumikari *et al*, 1993).

Although the number of studies actually giving the *in vivo* concentrations of hypothiocyanite is limited, it seems that it is possible to significantly elevate the salivary levels of hypothiocyanite, the antimicrobial agent in saliva. However, no data are available of any increased concentrations of salivary or plaque lysozyme and lactoferrin in connection with the use of these products.

One of the advantages of peroxidase-containing products in patients with dry mouth is that administered lactoperoxidase-enzyme rapidly consumes intraorally generated H_2O_2 (by aerobic bacteria) whose accumulation in the mouth could be detrimental to oral mucosal cells (Hänström, Johansson and Carlsson, 1983; Tenovuo and Larjava, 1984).

Antimicrobial effects

There are a number of studies which describe the antimicrobial mechanisms of lysozyme, lactoferrin and HOSCN/$OSCN^-$ (see review by Tenovuo, 1989 and Table 1). Most of these studies are done *in vitro* using *Streptococcus mutans* as a target bacterium; also, the effects against periodontal pathogens have been published. In most conditions which imitate the *in vivo* situation in oral fluids, the effects are bacteriostatic (Pruitt and Reiter, 1985) (Table 1), but the spectrum against various microbial pathogens is large (Table 2). The observations with the oral health care products with host proteins parallel the above findings *in vitro*. In laboratory conditions Biotene® was superior to Zendium® to inhibit the growth of *S. mutans*, *S. sobrinus* and *Lactobacillus casei* and the Biotene® effect was comparable with that of chlorhexidine (1%)–sodium fluoride (0.2%) gel. No antimicrobial effect was observed with Biotene® in which the LP-system was inactivated (Tenovuo, Lumikari and Soukka, 1991).

In vivo studies using a commercial Zendium® toothpaste have yielded conflicting results in relation to prevention of oral diseases. Human studies have indicated slight positive effects regarding plaque accumulation and prevention of gingivitis and dental caries (Koch, Edlund and Hoogendoorn, 1973; Koch and Strand, 1979; Rotgans and Hoogendoorn, 1979; Schoenfeld *et al*, 1983; Midda and Cooksey, 1986). However, Afseth and Rölla (1983) found no reduction in plaque formation or acidogenicity in subjects using Zendium® and Moran and Addy (1984) did not detect any inhibitory effect by Zendium® against *S. mutans*. Quite surprisingly, salivary or plaque levels of hypothiocyanite were not reported in any of these studies.

Another toothpaste, Biotene®, containing all the peroxidase system components (LP, KSCN, H_2O_2-generating system) and lysozyme and lactoferrin has been launched in Europe some 10 years ago after being available in the USA. As stated above, this toothpaste effectively generates hypothiocyanite *in vivo* (Lenander-Lumikari *et al*, 1993) but no Biotene®-induced antibacterial effects against total streptococci, mutans streptococci, lactobacilli or total anaerobic flora were detected (Lenander-Lumikari *et al*, 1993; Kirstilä *et al*, 1994a), neither in whole saliva nor in dental plaque. Also plaque pH, acidogenicity or lactic acid production were unaffected by Biotene® (Kirstilä *et al*, 1994a) in a double-blind crossover study with 20 volunteers with normal salivary flow rate.

However, one of the leading ideas in designing these products has been the purpose to (partially) restore saliva's own antimicrobial capacity among patients who suffer from dry mouth. This idea seems sound to add physiological salivary antimicrobial agents into the mouth that lacks saliva-mediated protection. Therefore, studies with patients with hyposalivation (and xerostomia) are of greater interest than studies with those who have normal salivation and, consequently, normal outputs of saliva-borne antimicrobials. Kirstilä *et al* (1996) observed during a 4-week clinical trial that out of 20 patients with Sjögrens' syndrome, 16 experienced a significant relief of their dry mouth symptoms (xerostomia) although no increase in salivary flow rate or its hypothiocyanite levels were found. No major changes

Table 2 Selected oral or orally transmitted pathogens which are susceptible to peroxidase-mediated inhibition or to inhibition by lactoferrin or lysozyme

Gram-positive bacteria
Mutans streptococci
Lactobacilli
Gram-negative bacteria
Actinobacillus actinomycetemcomitans
Porphyromonas gingivalis
Fusobacterium nucleatum
Listeria monocytogens
Salmonella typhimurium
Escherichia coli
Viruses
Human immunodeficiency virus (HIV)
Herpes simplex type 1
Respiratory syncytial virus (RSV)
Yeasts
Candida albicans

Many of the observations are only from *in vitro* experiments and depend on the concentration of the inhibitory agent. See reviews by Pruitt and Reiter (1985), Tenovuo (1998) and Lenander-Lumikari and Loimaranta (2000).

occurred in salivary microflora either. Other studies with patients having, e.g. radiation-induced hyposalivation and xerostomia have shown Biotene® to improve gingival health via reduced formation of supragingival plaque compared with a control toothpaste (Banoczy *et al*, 1994; van Steenberghe *et al*, 1994; Toljanic *et al*, 1996). Some studies have combined Biotene® toothpaste with Oralbalance® gel with good results in subjective relief of radiation-induced xerostomic symptoms (Epstein *et al*, 1999; Warde, Kroll, O'Sullivan *et al*, 2000) but the lack of appropriate controls makes it impossible to rule out the placebo effect.

To facilitate the efficacy and compatibility of these products in a dry mouth, toothpastes are not foaming but lack the mucosa-irritating effect of sodium lauryl sulphate (Herlofson and Barkvoll, 1994). In fact, the early assumption that the antimicrobial proteins in these toothpastes may have inhibited the appearance of aphthous ulcers (Hoogendoorn and Piessens, 1987) may be explained by the absence of ulcer-provoking sodium lauryl sulphate in all these antimicrobial toothpastes (Brokstad-Herlofson and Barkvoll, 1996). This is, however, not the whole truth because in carefully controlled double-blind crossover studies the Zendium® mouth-rinse has been found to reduce the incidence of aphthous ulcers over 50% when compared with sodium lauryl sulphate-free placebo rinse (Fridh and Koch, 1999). The mechanism of inhibition is unclear, as the etiology of aphthous ulcers is not known.

The mouth-rinses with host proteins are non-alcoholic, to be less irritable particularly to dry oral mucosal surfaces. Banoczy *et al* (1994) reported enhanced keratinization and decreased number of inflammatory cells in both buccal and mucosal smears of xerostomic patients who had used a Biotene/Oralbalance® combination for 1 month. The only brief report of the benefits of Biotene® mouth-rinse is by Mulligan, Navazesh and Slots (1992) who found regular use of this mouth-rinse to notably decrease the total salivary microbial counts *in vivo*. However, Biotene® mouth-rinse has been observed to cause demineralization of a presoftened and also of a sound tooth enamel *in vitro* (Kielbassa, Shohadai and Schulte-Mönting, 2000). This is obviously because of the relatively low pH (5.15), high acid content and non-fluoride content of this mouth-rinse. Therefore, as it is now available, it can hardly be recommended to dentate patients with problems of dry mouth.

To conclude, study designs and small number of subjects in various clinical reports do not allow any direct comparisons with other type of clinical regimens. However, a rough estimate is that in human trials the reduction in plaque accumulation and gingivitis is 10–20%, but as high as 50–90% in the occurrence of aphthous ulcers (Hoogendoorn, 1985; Banoczy *et al*, 1994; Kirstilä *et al*, 1996).

Future aspects

It is likely that these products can be further improved, e.g. by choosing more efficient substrates for peroxidases. The oxidation products of iodide, for example, are much more potent antimicrobials against periodontal pathogens, such as *Actinobacillus actinomycetemcomitans* (Ihalin *et al*, 1998) as well as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (Ihalin *et al*, 2001). Also streptococci and *Candida albicans* are more prone to I^- oxidation than to hypothiocyanite (Courtois *et al*, 1995; Bosch *et al*, 2000; Ihalin *et al*, 2001). The major problem with iodide in connection with LP-system is that even relatively small concentrations of salivary thiocyanate abolish the bactericidal effect of the $LP/I^-/H_2O_2$ systems as SCN^- is the preferred substrate for oral peroxidases *in vivo* (van Dalen *et al*, 1997; Ihalin *et al*, 2001). An interesting new approach to overcome the blocking effect of SCN^- is to replace LP in oral hygiene products by horseradish peroxidase, which does not bind or oxidize SCN^- at neutral pH but readily oxidizes iodide (Ihalin *et al*, unpublished data). In future, the application of these antimicrobial components may be more effective if encapsulated into reactive liposomes, which have been demonstrated to be good vehicles to exploit horseradish peroxidase, lactoperoxidase, glucose oxidase and lactoferrin against oral streptococci and dental caries (Hill *et al*, 1997; Martinez-Gomis *et al*, 1999).

Also interestingly, in a double-blind placebo-controlled study on 140 subjects, positive clinical results against dental plaque and gingivitis have been obtained with a toothpaste supplemented by only SCN^- and H_2O_2, without any peroxidase enzyme (Rosin, Kocher and Kramer, 2001). This is likely because of the enhancement of lysozyme action by SCN^- (Pollock *et al*, 1981) and by the positive interaction of SCN^- with carbamide perhydrate present in these test dentifrices. This compound produces urea, which facilitates SCN^- penetration into the oral mucosa. Thiocyanate is known to enhance cell proliferation and regeneration, e.g. in wound healing (Kramer and Böhland, 1996), which might explain its value against gingival inflammation.

It also seems beneficial to combine peroxidase-mediated inhibition with specific IgG antibodies against mutans streptococci (Tenovuo *et al*, 1982a; Loimaranta, Tenovuo and Korhonen, 1998). Thus, future products may not only comprise innate host proteins but also specific antibodies against oral pathogens. It should also be stressed that fluoride ions and the peroxidase systems act in an additive manner (Lenander-Lumikari *et al*, 1997). To summarize, future products obviously comprise an array of various host-related antimicrobials at the expense of synthetic agents, such as chlorhexidine and hexetidine. This approach is also likely to be more physiological, safe and suitable for daily use as no harmful side-effects of host proteins have been described to human oral cell lines (Hänström *et al*, 1983; Tenovuo and Larjava, 1984). Although solely bovine milk derived proteins have been used so far in commercial applications, no short- (e.g. allergy) or long-term (e.g. autoimmunity) adverse effects have been observed. This may result from the immunological similarity, although not complete identity, of these proteins in cows and humans (Månsson-Rahemtulla, Rahemtulla and

Humphreys-Beher, 1990; Headon, 2000). Thus, the described enhancement or restoration of saliva's natural antimicrobial systems could be a useful and safe supplement to maintain good oral health.

Conclusions

1. Incorporation of bovine milk-derived antimicrobial proteins, particularly lysozyme, lactoferrin and lactoperoxidase (LP), into human oral health care products is a serious and commercially active attempt to enhance and restore saliva's own antimicrobial capacity.
2. There is strong evidence that LP remains active and functional in a dentifrice, producing high concentrations of the antimicrobial oxidation product hypothiocyanite ($HOSCN/OSCN^-$) *in vivo*. No such evidence of activity exists for lysozyme or lactoferrin.
3. In clinical trials these antimicrobial toothpastes, mouth-rinses or moisturizing gels relieve the symptoms of xerostomia, but whether it is because of the antimicrobial proteins is not known.
4. There is no clear-cut clinical evidence that these antimicrobial oral health care products could enhance salivas' own defence capacity if salivary flow rate is within normal limits.
5. These products may partly restore the innate immunity of saliva in patients with hyposalivation but their clinical efficacy is still poorly documented.
6. No side-effects have been reported. Because the antimicrobial components are similar, although not completely identical, to those naturally present in human salivary secretions, there seems to be no contraindication for their daily use by patients with dry mouth or by patients suffering from aphthous ulcers. The only exception is mouth-rinse whose low pH and absence of fluoride may cause tooth demineralization, particularly among patients with dry mouth.

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