Developing a bacteriotherapy-based approach to control streptococcal infections

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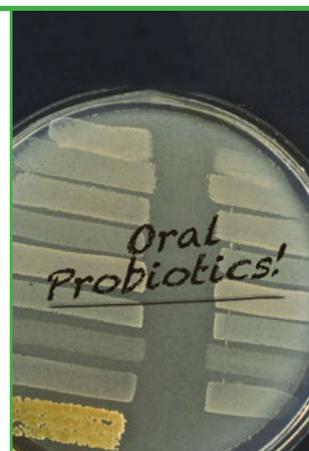
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Abstract

The author provides an account of the background to his discovery of bacteriocin production by streptococcal bacteria and the subsequent demonstration over five decades of research that the streptococcal bacteriocin-like activities are distinctive both for the abundance of their production and for the chemical heterogeneity of their structures. The initial impetus for this research was an endeavour to identify candidate bacteriocin-producing, non-disease-associated streptococci for potential development as oral probiotics capable of providing protection to young children against *Streptococcus pyogenes* infections. The practical outcome of these studies has been the development and commercial distribution of the bacteriocin-producing probiotic strains *Streptococcus salivarius* K12 (BLIS K12) and *S. salivarius* M18 (BLIS M18) for application to the control of a wide variety of bacterial infections and disequilibria of the oral microbiota, currently ranging from streptococcal pharyngitis and otitis media to tooth decay and halitosis.

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The origins of the author's involvement in streptococcal probiotic research

As far as I can recall my first significant encounter with *Streptococcus pyogenes* was as a 12-year old living in Melbourne, when a series of sore throats culminated in me developing rheumatic fever.

My most vivid memory of this episode is of all the penicillin tablets that I then had to consume daily over the following decade to help prevent any follow-up attacks of the disease. From a more positive perspective, it seemed that, unlike so many of the other youngsters worldwide who are still succumbing to rheumatic fever, for me there had been no residual heart damage. This, I was told, should be taken as a further incentive to continue my daily penicillin dosing routine, since it was known that recurrent episodes of rheumatic fever carried an increased risk of scarring of the heart valves. Nevertheless, it just seemed to me that there must surely be a better way to defend myself against streptococcal sore throats.

Following high school, I had the opportunity to study at Melbourne University and it was there, in the third year of a microbiology degree, that I was influenced by the teachings of Dr Rose Mushin concerning the potential applications of bacterial interference as a targeted and natural means

of infection prevention. Dr Mushin had recently returned from study leave visiting Professor René Dubos at The Rockefeller University and under his influence she had become a devotee of this old-world strategy for infectious disease control, the origins of which pre-date the discovery of antibiotics and indeed can be traced back to the studies of Louis Pasteur [1]. Dr Mushin managed to convince her entire microbiology class to consume milk that had been seeded with so-called 'friendly' *Escherichia coli*. These bacteria, we were told, were equipped with a bacteriocin armament that would enable them, from their proposed site of lodgement in our intestinal tracts, to kill any vulnerable salmonellae that happened to pass close by.

Dr Mushin's proposal sounded logical and feasible. Bacteriocins, she explained, were proteinaceous antibiotics produced by bacteria which had a bacteriocidal mode of action against various other relatively closely related bacteria that were potentially capable of competing with them for occupancy of the same ecological niche (i.e. they were anti-competitor molecules). As I listened to Dr Mushin it occurred to me that perhaps a similar strategy could be applied in the human oral cavity to gain some relatively specific protection against *S. pyogenes* infections.

That insight, now almost 50 years ago, provided me with an irresistible challenge – I now knew what I wanted to do. I first needed to try to familiarize myself with the idiosyncrasies of streptococcal behaviour and my conviction was that, in order to prevent *S. pyogenes* from assaulting its human host, it would be important to try to strengthen the territorial defensive capabilities of the non-virulent streptococcal component of our indigenous microbiota. It occurred to me that over the course of their lengthy co-evolution with humans, it was the indigenous oral streptococcal populations that would surely have developed the most effective and specifically targeted bacteriocin weaponry to counter competition for their space by rapidly multiplying virulent streptococci.

I next undertook PhD research at Monash University – the theme of those studies being an exploration of the relationship between *S. pyogenes* infections and the induction of the autoimmune manifestations of rheumatic fever [2]. I set about screening many hundreds of oral streptococci for their bacteriocin-producing capability (i.e. bacteriocinogenicity) and included amongst these streptococci was a series of *S. pyogenes* isolates from the Fairfield Infectious Diseases Hospital in Melbourne. On 1 September 1969, I discovered that number 22 in this series produced bacteriocin-like inhibitory activity against some other *S. pyogenes* strains when tested in a deferred antagonism assay [3]. The inhibitory agent, later given the name streptococcin A-FF22 [4], was the first of the streptococcal bacteriocins to be isolated and characterised.

Next it was time for me to undertake a post-doctoral apprenticeship, and from my reading of the current scientific literature I knew where I wanted to continue my studies. Dr Lewis Wannamaker at the University of Minnesota was an influential leader in the field of streptococcal research and indeed he had played a major role in developing the original guidelines for the use of penicillin prophylaxis as a preventative against rheumatic fever recurrences [5]. In 1972 he hosted a workshop on 'Streptococci and Streptococcal Diseases' at the University of Minnesota which was attended by many of the leading *S. pyogenes* researchers of that era. In his workshop summary Dr Wannamaker remarked that "working with the streptococcus is like a love affair, which I guess explains why so many of us find it difficult to give up" [6]. I could relate to this sentiment and wrote to him asking if I could work together with him, seeking a bacterial interference-based alternative to the use of penicillin for rheumatic fever prophylaxis. Three years of total streptococcal research immersion followed. Dr Wannamaker was generous in his support for my endeavours to find a harmless oral streptococcus capable of effectively engaging in bacteriocin-mediated warfare against *S. pyogenes*, at the same time maintaining his personal view that what *S. pyogenes* really needed was greater scientific understanding – not extermination. Before leaving Minnesota I wrote the first major review of the bacteriocins of gram-positive bacteria [7] and upon purifying streptococcin A-FF22 demonstrated that it was closely similar to nisin, the best

known and still the most widely applied of all the bacteriocins of gram-positive bacteria [8].

Rheumatic fever is a major public health concern in New Zealand, with a particularly high occurrence in the native Maori and Pacific Islander populations. In view of this, I was excited in 1975 to obtain an academic position in the Microbiology Department at the University of Otago in Dunedin. My research agenda in New Zealand became firmly focused on finding a harmless oral streptococcal antagonist of *S. pyogenes*. I developed a procedure for the bacteriocin 'fingerprinting' of streptococci based on the deferred antagonism test in which a set of nine standard indicator bacteria are evaluated for their relative sensitivity to inhibitory substances released into an agar medium during the growth of a diametric streak culture of the test bacterium [9]. It soon became clear to me that most (if not all) streptococci were probably capable of producing some sort of bacteriocin-like inhibitory activity. The pattern of inhibition of the nine standard BLIS indicator bacteria when converted to code format is referred to as the Production (P) type of the test bacterium [9,10].

The cover picture of this issue of Nutrafoods illustrates the P-type 777 inhibition of all 9 indicator bacteria given by *S. salivarius* strain K12 (note of the Editor-in-Chief).

Over the next several decades my laboratory screened many thousands of streptococcal isolates for their bacteriocin activities and subsequently documented a remarkably heterogeneous array of proteinaceous inhibitory agents, ranging from post-translationally modified nisin-like peptides of the lantibiotic family to non-modified small peptides and relatively large proteins, some muralytic in their activity and others having an unusual circular conformation [11, 12]. Streptococci, especially those of the species uberis, mutans and salivarius, appear to have been particularly inventive and acquisitive of unusual bacteriocin loci.

My second approach to identifying a naturally occurring antagonist of *S. pyogenes* came in the form of a prospective study of one hundred 5-year-old Dunedin schoolchildren. The objective was to document changes occurring in the composition of the children's oral microfloras over the next 6 years and, in particular, to record if and when each child acquired *S. pyogenes*. Despite frequent outbreaks of *S. pyogenes* pharyngitis occurring in the school classrooms, not all similarly exposed children experienced streptococcal infections. A particularly interesting observation was that many of the children who seldom acquired *S. pyogenes* had large populations of strong bacteriocin-producing *S. salivarius* on their tongues (i.e. the bacteriocin producers comprised at least 5% of the child's total *S. salivarius* population) [13].

This led to the hypothesis that the presence in the oral cavity of certain bacteriocin-producing *S. salivarius* may afford some protection against *S. pyogenes* infection. A follow-up study of 780 Dunedin school children identified two major types of bacteriocin-producing *S. salivarius*, the corresponding P-type patterns being 226 (11% of children positive) and 677 (9% positive) [14]. A further 20% of the children had *S. salivarius* of various other P-type designations, including some (approximately 1% of all tested subjects) having particularly strong (P-type 777) bacteriocin activity. The children harbouring populations of either P-type 677 or P-type 777 *S. salivarius* had a significantly reduced rate of acquisition of *S. pyogenes* during the 10-month study period [14]. Strains of P-type 677 *S. salivarius* typically produce a 2315 Da bacteriocin of the lantibiotic class named salivaricin A (SalA) [15]. SalA differs from most other lantibiotics in that its inhibitory activity against *S. pyogenes* is bacteriostatic rather than bacteriocidal [16]. SalA inhibits the *in vitro* growth of all tested S. pyogenes, although the extent of inhibition of strains of serotype M4 (themselves producers of a SalA variant lantibiotic) is relatively reduced [15]. SalA and its variants (SalA1, SalA2, SalA3, etc.) function as cross-reactive signal peptides, capable of specifically up-regulating the production of all SalA lantibiotics [17, 18]. Perhaps even more significantly, this signal can effect inter-species communication, up-regulating production of SalA-like loci in strains of *S. pyogenes, S. agalactiae* and *S. zooepidemicus* [17]. Specific SalA auto-inducing activity has been detected in the saliva of subjects following their colonization with SalA-producing *S. salivarius*, showing that this lantibiotic activity

is produced and is biologically active *in vivo* [19]. In other studies we have demonstrated that anti-*S. pyogenes* inhibitory activity is also produced when SalA-positive *S. salivarius* are grown in saliva in an *in vitro* test system [19]. Also, the dosing of children with SalA-producing *S. salivarius* stimulated clonal expansion of their pre-existing indigenous populations of SalA-producing *S. salivarius* [20]. Moreover, when a SalA-producing *S. salivarius* population is present on the tongue, some other bacterial species on the tongue show increased levels of specific resistance to this bacteriocin [21].

Our attention soon became focused on the small proportion of *S. salivarius* isolates having P-type 777 activity (inhibitory to all nine bacteriocin indicator strains). Some of these P-type 777 strains were shown to produce in addition to SalA a second lantibiotic, salivaricin B (SalB), that unlike the bacteriostatic SalA, appeared to be bacteriocidal for all tested *S. pyogenes* [22]. SalB is a 2736 Da peptide, the production of which is controlled by a cluster of eight genes with substantial differences from the SalA gene cluster in its putative immunity (F, E, G) genes. In an interesting parallel with the antibiotic penicillin, no resistance to SalB has yet been detected in any tested strains of the species *S. pyogenes*. The prototype of the P-type 777 *S. salivarius* is strain K12. The selection of strain K12 was on the basis of its multiple bacteriocin activities against *S. pyogenes* and its origins as a representative clone from a P-type 777 *S. salivarius* lineage that had persisted in large numbers for >2 years in the oral cavity of a healthy child, during which time the child seemed not to have experienced any *S. pyogenes* pharyngitis. The designation K12 indicates that this child was study subject number 12 at Kaikorai Primary School in Dunedin.

The commercial outcome of these laboratory discoveries was the launching of the Dunedin-based company Blis Technologies Ltd in August 2000 and 2 years later the first oral probiotic product, BLIS K12 Throat Guard, appeared on the shelves of New Zealand pharmacies. A wide variety of BLIS K12 products in powder, lozenge, chewing gum and ice cream formulations have subsequent-ly been developed and many are now marketed internationally (http://blis.co.nz). A more recently released *S. salivarius* oral probiotic (strain M18) expresses the megaplasmid-encoded bacteriocins salivaricins MPS, A2 and 9 as well as the chromosomally encoded bacteriocin salivaricin M [23]. Some inhibitory spectrum differences are evident for strain M18, including increased inhibitory activity against the dental caries-associated species *Streptococcus mutans* [24].

The case for bacteriocin-producing *S. salivarius* as preferred probiotics for the oral cavity

Within hours of our birth the species *S. salivarius* has achieved numerical prominence within our oral microbiota and large populations continue to flourish throughout our lives on the healthy oral mucosae, especially the tongue surfaces [25]. When *S. salivarius* numbers are depleted, unbalanced overgrowth of relatively 'malevolent' microbes such as some of the *Candida* spp. and black-pigmented anaerobes, can sometimes occur, resulting respectively in the unpleasant clinical conditions of oral thrush [26] and halitosis [27]. It seems that due to its strategic lingual location, versatile anti-competitor weaponry and considerable numbers, *S. salivarius* is particularly well suited to execute a population surveillance and management (i.e. 'sentinel') role within the oral microbiota [28].

A high proportion of *S. salivarius* are bacteriocin producers and upon closer examination it is common to find that two or more bacteriocins contribute to the total inhibitory activity of individual strains [17]. Interestingly, most bacteriocin-producing *S. salivarius* appear to utilize very large (>100 kb) megaplasmids as the genetic receptacles for their bacteriocin loci [30]. This megaplasmid DNA (as yet unreported in any other oral bacteria) appears to be transmissible in the human oral cavity between different *S. salivarius* [30]. Sequencing of pSsalK12, the megaplasmid of *S. salivarius* strain K12, has disclosed an abundance of mobile genetic elements (putative insertion sequences),

especially flanking the loci for salivaricins A2 and B, indicating that these loci may have been acquired from other bacterial hosts via transposition events [22].

More recently, *S. salivarius* strain JH has been shown to produce at least four bacteriocins (salivaricin A3, streptin, streptococcin SA-FF22 and salivaricin E) (Walker, unpublished).

Since bacteriocin production is energetically demanding and also requires the coordinated action of multiple gene products, it seems that it must surely confer a significant benefit upon the host bacterium. Furthermore, the ubiquity of bacteriocinogenicity in the bacterial world infers that bacteriocin production and/or the corresponding specific bacteriocin immunity phenotype must have a major role in the survival of individual bacterial clones in nature. The mode of action of type-A lantibiotic bacteriocins such as those produced by strain K12, involves pore formation in the cytoplasmic membranes of target bacteria and the process of insertion only occurs if there is a sufficiently high potential difference across the target cell membrane. More specifically, these lantibiotics may modulate the composition of the oral microbiota by killing relatively rapidly multiplying competitor bacteria – such as, for example, pathogenic *S. pyogenes* during the acute stage of a streptococcal throat infection. On the other hand, when small populations of *S. pyogenes* are sequestered within the oral microbiota or even intracellularly, as occurs when they are in the relatively quiescent carriage state, they may not then be so vulnerable to lantibiotic attack. The hypothesis underlying the probiotic application of BLIS K12 is that the colonization of the oral cavity with lantibiotic-producing *S. salivarius* may help limit infection by newly acquired virulent *S. pyogenes* or shifts of indigenous *S. pyogenes* from innocuous carriage to active infection status.

Laboratory studies have shown that bacteriocin production is strongly influenced by both genetic and environmental factors [30]. In order to conserve energy, bacteriocin expression can temporarily be turned off. Alternatively, bacteriocin genetic loci may be either inactivated (via mutation) or entirely jettisoned (as in the course of megaplasmid elimination) should the production of that bacteriocin fail to confer a survival advantage to the cell as it multiplies within that particular ecosystem. Interestingly, the loci encoding homologs of some of the lantibiotics (i.e. SalA, SalB, streptin and streptococcin A-FF22) that have been identified in *S. salivarius* megaplasmids have also been detected (albeit in a chromosomal location) in various other oral streptococcal species, including *Streptococcus mitis*, *S. pyogenes*, *Streptococcus equisimilis* and *S. mutans* [28]. These observations are consistent with *S. salivarius* megaplasmids serving as flexible genetic receptacles for the acquisition and expression of bacteriocin loci that have putatively been gathered from (and then potentially donated to) a variety of other oral streptococci.

In our previous research we have been particularly interested in factors that may influence the population balance between commensal and potentially pathogenic streptococci in the human oral cavity. *S. salivarius* is principally located on the tongue surface [25]. Carriage of *S. pyogenes* (in the pharynx) is common, especially in school-aged children. When present in small numbers *S. pyogenes* causes no pathology, presumably any excessive proliferation being held in check either by competition from other members of the oral microbiota or by the host's immune defences. However, failure of these controls can result in the development of streptococcal pharyngitis. We have speculated that due to its strategic location and dominant numbers, *S. salivarius* is well-placed to help modulate the oral cavity population levels of *S. pyogenes* and various other streptococcal species [28]. Although principally located on the human tongue, *S. salivarius* can also be found in large numbers within the pharyngeal microbiota [25]. Here, bacteriocin-producing strains can more directly interact with and interfere with the proliferation of *S. pyogenes*. Moreover, the bacteriocin products of *S. salivarius* will readily saturate the saliva, which is constantly flowing over all oral surfaces and it can be anticipated that the bacteriocin concentration and thus its killing action will be especially high during our sleeping hours when the body's natural circadian rhythm slows the rate of saliva formation [19]. *S. salivarius* has also been reported to sometimes

represent substantial proportions of the microbial populations present in the intestine [31, 32], human breast milk [33] and even the lungs [34].

A number of the beneficial outcomes from the ingestion of probiotics can be traced to their immunomodulatory activity. Probiotic cell interaction with immune cells in the mucosal environment can influence various processes dependent on the mucosa-associated lymphoid tissue (MALT) such as tolerance induction, modulation of cytokine/chemokine release and general regulation of mucosal immune responses. Since this activity is often strain-specific, it is necessary for each candidate probiotic strain to be specifically assessed for its immunomodulatory activity. Effects of probiotics such as BLIS K12 include (a) maintenance of epithelial cell homeostasis, including the down-regulation of the pro-inflammatory response elicited by pathogens [35, 36] and (b) up-regulation of the unified interferon signalling pathway which is responsible for the expression of genes involved in innate immunity against viral infection, antitumor activity, priming of the lipopolysaccharide response and anti-inflammatory effects [37].

The species *S. salivarius* has a number of innate characteristics favouring its candidature as a probiotic [38]. Of all the bacterial species known to populate the human oral and nasopharyngeal mucosa in large numbers, *S. salivarius* is perhaps the most innocuous. BLIS K12 has a very well-documented safety record [39] and has now been granted self-affirmed GRAS (generally regarded as safe) status for unrestricted food additive applications in the USA. *S. salivarius* is an early colonizer of the human oral cavity, with infants usually acquiring the mother's predominant strain of *S. salivarius* within hours of birth [40, 41]. *S. salivarius* typically then remains a numerically prominent member of the oral cavity throughout the life of the host. It has been found to be present at levels of up to 1×10^7 colony forming units (cfu) per millilitre of saliva and based on the daily estimated average consumption of saliva in adults (up to 1.5 litres), large numbers (estimates of 1×10^{10} cfu) are ingested daily. *S. salivarius* can also be isolated from breast milk and this could be an important source of the bacterium for infants in the early months of life [42].

In other studies, it has been shown that BLIS K12 (a) binds with strong avidity to human epithelial cells *in vitro* and prevents attachment of *S. pyogenes* [43] and (b) persists in the human oral cavity following ingestion [44].

Actual and potential oral probiotic applications of bacteriocin-producing *S. salivarius*

With the discovery of penicillin and the subsequent flourishing of the antibiotic era, the further development of probiotics and of bacterial interference as strategies for infection control was largely overlooked. More recently however, as the efficacy and safety of many antibiotics has diminished, together with the associated resurgence of many infectious diseases and increased numbers of immunologically compromised and aged hosts, probiotics are once again being promoted either as an alternative or as a supplement to existing therapies. Intestinal commensals, especially lactobacilli and bifidobacteria, have long been consumed by humans in a variety of dairy product formulations either to nurture gut health or to treat a wide variety of ailments of the gastrointestinal tract and vagina. With increasing interest now in probiotic interventions for the modulation of microbial diseases and disequilibria in other body tissues, attention is logically being focused upon the predominant microbial indigenes of these tissues as a prime source of novel probiotics that are best equipped to colonize and help stabilize the populations of microbes incumbent to these tissues.

The term probiotic (literally meaning 'for life') is a general term now widely used to refer to 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [45]. More specifically, 'oral probiotics' can be considered to be probiotics, the persisting action and beneficial outcomes of which are a direct outcome of their interaction with microbial and/or host cellular components of the oral cavity. Thus, bona fide oral probiotics differ from the traditional orally administered intestinal probiotics (i.e. yoghurts or other products comprising lactobacilli and bifidobacteria of intestinal origin). The latter

do not primarily or persistently localize within the oral microbiota, even though some short-term beneficial outcomes may manifest within the oral cavity following their ingestion.

Every human harbors a personalized oral cavity microbiome, the presence of which is essential to heath maintenance, but also having a dark side capable of causing harm to host tissues, both oral and systemic. The biofilms coating every surface of the oral cavity comprise complex ecosystems that help to maintain our health when they are in equilibrium. Ecological shifts within the microbiome can, however, sometimes allow pathogens that are either intrinsic to the biofilm (in a carriage state) or newly introduced to the oral cavity to become manifest and induce disease. The intentional seeding of the oral microbiome with harmless probiotics equipped with the capability of exerting targeted bacterial population control (i.e. bacterial interference or 'germ warfare') provides an approach to infection prevention that has as its basis the augmentation of naturally occurring processes. Within the oral cavity the principal bacterial infections that have to date been identified for probiotic intervention have been streptococcal pharyngitis, otitis media, halitosis and dental caries.

Successful implantation of the oral microbiota with BLIS K12 provides a potential means of preventing streptococcal pharyngitis and also an alternative to antibiotic prophylaxis for the prevention of rheumatic fever recurrences. Acute *S. pyogenes* infections and their non-suppurative sequelae continue to exact a severe toll on susceptible populations. Prior to the development of BLIS K12 the only available strategy has been treatment of the acute streptococcal infections as they occur by administration of therapeutic doses of a broad-spectrum antibiotic such as penicillin. Studies by di Pierro and colleagues in Italy have shown that the taking of BLIS K12-containing lozenges by children [46] and adults [47] can effect a reduction in the occurrence of streptococcal pharyngitis. Otitis media-prone infants given a paediatric formulation of strain K12 for 2 weeks prior to ventilation tube placement displayed colonization of the adenoid tissue [48], and more recently the taking of BLIS K12 was found to reduce the occurrence of secretory otitis media in children [49]. A relative deficiency of *S. salivarius* in the oral cavity has been linked to the overgrowth of malodorous bacteria on the tongue and the development of bad breath (halitosis) [27]. Administration of BLIS K12 can help alleviate the symptoms [50]. The more recently developed probiotic *S. salivarius* strain M18 (BLIS M18) (initially referred to as strain Mia) differs from BLIS K12 in having additional bacteriocin activity against the mutans streptococci, bacteria that are commonly implicated in the development of dental caries. Strain M18 also produces strong dextranase activity. Successful colonization of young schoolchildren with strain M18 has been shown to reduce both the levels of mutans streptococci and the plaque index [24]. Oral persistence of BLIS M18 was shown to be dose-dependent [51].

Oral cavity microbial disequilibria can not only lead to the development of dental caries, periodontal disease and candidosis, but also can facilitate initiation of the transition between carriage state and active infection by bacteria such as *S. pyogenes* as well as possibly influencing the onset of preterm labor and a wide variety of non-transmissible diseases ranging from cardiovascular disease and diabetes to obesity. In other studies, *S. salivarius* K12 has been shown to outcompete *Candida albicans* and *Streptococcus agalactiae*, respectively, in the rat oral cavity [52] and the mouse vagina [53], opening up prospects for future applications to the control of candida in the elderly and *S. agalactiae* infections in the newborn.

One practical issue concerns how best to facilitate probiotic colonization of the host tissues. Clearly the attachment and flourishing of a newly introduced probiotic bacterium will be enhanced when the native microbiota of the host is relatively sparse, as occurs in the first days of life or during the course of antibiotic treatment. Indeed, in order to increase the prospects for probiotic implantation it can be recommended that any course of prescription antibiotics should be linked to a follow-up regimen of probiotics, with probiotic dosing beginning on the last day of antibiotic administration.

In conclusion, the time now appears opportune for the accelerated development and application of bacteriocin-producing *S. salivarius* probiotics to the control of a wide variety of specific bacterial infections or of other diseases of more complex aetiologies that reflect undesirable population structures within the indigenous microbiota. The bacteriocin approach to infection control is cost-effective, easily implemented and does not present many of the complications associated with immunization (hypersensitivity or antigenic cross-reactivity) or chemotherapy (resistance development and direct toxicity). The considerable scope for their potential applicability throughout the life of their natural human host encourages consideration of BLIS K12 and BLIS M18 as the probiotics for all ages.

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