Developing Oral Probiotics From *Streptococcus salivarius*

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Abstract and Introduction

Abstract

Considerable human illness can be linked to the development of oral microbiota disequilibria. The predominant oral cavity commensal, *Streptococcus salivarius* has emerged as an important source of safe and efficacious probiotics, capable of fostering more balanced, health-associated oral microbiota. Strain K12, the prototype *S. salivarius* probiotic, originally introduced to counter *Streptococcus pyogenes* infections, now has an expanded repertoire of health-promoting applications. K12 and several more recently proposed *S. salivarius* probiotics are now being applied to control diverse bacterial consortia infections including otitis media, halitosis and dental caries. Other potential applications include upregulation of immunological defenses against respiratory viral infections and treatment of oral candidosis. An overview of the key steps required for probiotic development is also presented.

Introduction

Consumers seeking health-promoting dietary supplements have long been conditioned to the ingestion of yoghurt as a convenient source of living beneficial microbes (i.e., probiotics). The definition of the term probiotic has undergone a series of evolutionary changes[1] and is now generally accepted to be "live organisms, which when administered in adequate amounts, confer a health benefit on the host".[2] Conventional probiotics have typically comprised bacteria of intestinal origin (especially lactobacilli and bifidobacteria) and their application has principally been to provide relief for maladies of the GI tract. However, the realization that much human illness can be linked either directly (e.g., dental caries, periodontal disease and candidosis) or indirectly (e.g., cardiovascular disease and perhaps even obesity) to the development of oral microbiota disequilibria has diverted much contemporary probiotic research to the development of products that are capable of fostering a healthy oral microbiota.[3] While researchers initially tried to establish whether conventional approved intestinal probiotics could also influence the oral microbiota, these bacteria (perhaps unsurprisingly) have no oral persistence, and any oral cavity health benefits seem transitory and largely attributable to immune stimulation.[4] A more logical strategy is to utilize microbes isolated from their natural oral habitat in healthy humans as oral probiotics. The term 'oral probiotics' is used here to refer to beneficial microbes given to the (usually human) host to help maintain or effect improvements in their oral health – not intestinally derived probiotics that are delivered orally!

The scientific origins of oral probiotics can be traced to the use of mixtures of putative oral commensals producing incompletely characterized inhibitory agents with *in vitro* activity against *Streptococcus pyogenes*[5] or otitis media (OM) pathogens.[6] An alternative but, for the time being, aborted approach was the targeting of mutans streptococci using an ultra-competitive bacteriocin-producing *Streptococcus mutans*, genetically modified to attenuate its virulence.[7] Other approaches include investigations into isolates of *Streptococcus zooepidemicus*,[8]*Streptococcus oligofermentans*[9] and *Veillonella* spp..[10]

In this laboratory, we adopted the strategy of identifying an oral commensal strongly inhibitory to *S. pyogenes* – one of the principal pathogens of the human oral cavity. Key criteria sought for the ideal probiotic candidate were: nonpathogenic; large populations occurring naturally within the oral microbiota; and producer of potent *in vitro* and *in vivo* inhibitory activity against target pathogens to which resistance does not readily develop. This search led us to *Streptococcus salivarius*.[11]

S. salivarius is a pioneer colonizer of the human oral cavity and persists there as a predominant member of the native microbiota throughout the life of its human host.[12–14] In saliva, it is typically present at levels of up to 1×10^7 colony forming units (cfu)/ml and this equates to approximately 10^{10} cfu ingested daily.[15] In the healthy (i.e., immunologically competent) host, it is only extremely rarely a cause of infection. Many strains are producers of bacteriocin-like inhibitory substances (BLIS), and in these strains multiple bacteriocin loci are typically present on transmissible megaplasmids.[16] The *S. salivarius* BLIS are diverse in their activity spectra and are thought to play an important role in both stabilizing the oral microbiota and preventing overgrowth (or infection) by potential pathogens.[17] Salivaricin A, the first fully characterized *S. salivarius* BLIS, has been detected in the saliva of subjects harboring salivaricin A-positive *S. salivarius* by the application of a highly specific BLIS auto-induction assay.[15] This provides direct evidence for the production of bioactive BLIS in the human oral cavity.

This review updates the reader on some of the exciting recent research into the development of *S. salivarius* probiotics and also

outlines critical steps required in the overall process of bringing a probiotic to market.

Development of *S. salivarius* Probiotics: General Principles

The Food and Agricultural Organization and WHO have published a list of recommended guidelines for the systematic assessment and development of strains that are under consideration as probiotics.[2] While this document focuses particularly on intestinal probiotics, its recommendations can be considered generally applicable to all probiotics. Some of the key steps taken in the commercial development of a probiotic are shown in Figure 1. It is important to note that, in practice, the process does not always follow an orderly pathway (especially in the developmental stage), and that some steps may prove especially problematic and need to be repeated prior to obtaining a successful and efficacious end-product.

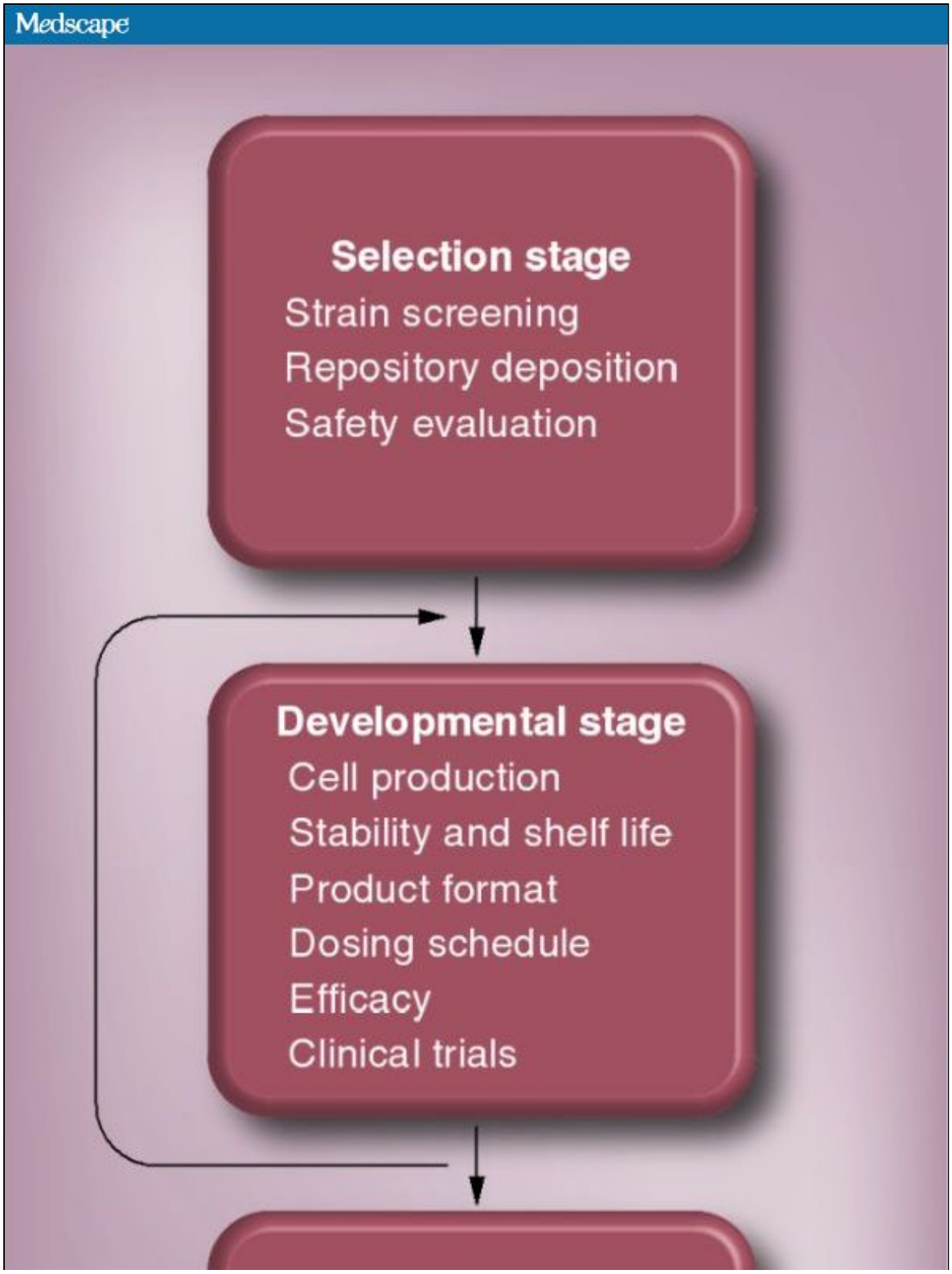

Figure 1.

Steps required for the development of a probiotic.

Candidate Screening & Selection

Laboratory-based analyses can help provide preliminary indications of the potential beneficial attributes of a probiotic and of its safety for use in humans. The repertoire, activity spectrum and potency of bacteriocin production, as detected in deferred and simultaneous antagonism tests, sometimes provide a major criterion for probiotic strain selection. Other desirable traits include an ability of the candidate strain to adhere avidly to human epithelial cell lines and, in cobinding assays, to outcompete pathogens such as *S. pyogenes*[18,19] or *Candida albicans*.[20] For *S. salivarius* probiotics, the production of putatively beneficial enzymes, such as urease[21,22] and dextranase,[23,24] have also been evaluated. Following the strain screening (or 'auditioning') phase and prior to commitment of major developmental (or 'grooming') resources for the provisionally selected probiotic candidate, an intensive strain 'background' and 'identity check' must be performed. The Food and Agricultural Organization/WHO guidelines state that probiotics should have an official generic and species designation.[2] Strain-specific genetic and physiological characterization is important since interstrain differences within a microbial species commonly occur for characteristics that may prove critical for probiotic efficacy; for example, their bacteriocin and exoenzyme repertoires. Strain-specific characterization aids subsequent assessments of probiotic performance and also allows for accurate epidemiological surveillance of the strain following its seeding within complex indigenous ecosytems in its newly adopted human host.[25,26]

Following this predevelopmental screening phase, the probiotic front-runner candidate should be lodged in an internationally recognized strain repository, such as the American Type Culture Collection (ATCC) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Safety Evaluation

Ensuring that a probiotic is safe for human consumption should be of paramount consideration. The WHO guidelines stipulate that any bacterial species, including those having a history of human consumption, has the potential to cause disease – especially in immunocompromised individuals – and that it is the role of the manufacturer to assess the potential risk of the strain being developed.[2] While safety should be verified prior to commercial release, in practice it is an ongoing process and requires continual *in vitro* and *in vivo* analysis.

An important first step in safety evaluation is a thorough search of the literature. Identification of the history of use and reports of infection resulting from the chosen species/strain should be noted. For *S. salivarius*, although there have been several reports of infection, the majority of these have followed iatrogenic or traumatic cerebrospinal fluid contamination.[27]

In vitro safety checks should form an integral part of the early strain selection process. These tests include: metabolic profiling to

assess the production of deleterious byproducts (e.g., D-lactate); antibiogram determination to accepted standards, such as those established by the Clinical and Laboratory Standards Institute, to indicate antibiotic resistance and preferred options should there be a need to treat probiotic infection; toxicity to cell lines and blood (hemolysis);[28] and the presence of virulence factors.[29] The mutagenic ability of a strain can be assessed by the Ames test.

Many genetic techniques are available to assess the presence/absence of virulence factors and to aid strain identification. Although PCR and pulsed-field gel electrophoresis have traditionally been applied,[29] the current speed and relative cost-efficiency of entire genome sequencing now allows researchers to easily obtain genome 'snapshots' of a probiotic strain. Full genome sequencing allows for rapid and accurate identification of known virulence genes, antibiotic resistance determinants, colonization factors and genetic transfer mechanisms. At the time of writing, the genomes of six *S. salivarius* strains, namely, M18,[30] PS4,[31] JIM8777,[32] CCHSS3,[33] 57.I[34] and K12 (BioProject Accession No. ALIF01000000) have been sequenced.

Following *in vitro* testing, trials in animals (typically rats) allow for an *in situ* safety assessment of the probiotic and help predict potential toxicity for the human host. Typically, researchers will study the effect of the probiotic by analyzing changes to total body weight, individual organ weight, key biochemistry markers (e.g., enzyme fluctuations), urine and blood.[35]

Human trials should take the form of large double-blind placebo-controlled studies to reveal statistically significant outcomes. Such trials should be carried out using the anticipated commercial formulation and dosage levels to ensure its safety is evaluated in a 'real world' situation.[36]

Stability & Shelf Life

Probiotics are biological products whose viability is influenced by a variety of complex physiological and chemical factors. To ensure that the correct dose (number of viable cfu) is delivered upon consumption, knowledge of factors influencing the stability of the final product is critical. 'Stability' and 'shelf life' are closely related concerns. Stability refers to the survivability/viability of the probiotic strain in a particular format, while shelf life determines how long the product can be sold while retaining stability. Stability is affected by many different factors including manufacturing conditions (e.g., exposure to temperature and pressure, growth media, fermentation times, product blending and handling systems), auxiliary ingredients (e.g., pH and ionic strength) and composition of the final product (i.e., liquid, solid and water availability), as well as storage temperature and packaging. It may take several production 'runs' to derive the final successful product format.

Product Format

The manner of delivery is an important consideration. Factors such as palatability and effectiveness of delivery need to be optimized and, in particular, adequate contact time with host tissues needs to be achieved to foster attachment and colonization of the strain. A variety of product formats for *S. salivarius* have been considered, with current formulations including lozenges, ice cream, chewing gum, mouthwashes and yoghurt.

Dosing Schedule

In contrast with the apparent *modus operandi* of most intestinal probiotics, it is generally believed that for oral probiotics, persistent colonization is required in order to achieve optimal health benefits. Therefore, trials need to be carried out to determine the dosage regimens required to effect oral colonization. It seems that the best opportunity for successful implantation of an oral probiotic is following either rinsing with oral antiseptics, such as chlorhexidine, or a course of antibiotic therapy (i.e., when the indigenous microbiota has been reduced in numbers). Although dosing levels to determine colonization efficacy and toxicity are ideally best assessed in humans, some relevant data can also be gained from trials in experimental animals.[35]

Efficacy

By definition, a probiotic should confer a health benefit, typically measured as a reduction in symptoms or prevention of disease. Although BLIS-producing *S. salivarius* may show strong inhibitory activity in *in vitro* assays, this activity, importantly, also needs to be translated to an *in vivo* environment. Ideally, double-blind, placebo-controlled studies of the individual strain in its final product format are required to determine benefits.[2] This requirement has now attracted the attention of the major regulatory authorities such as the US FDA and European Food Safety Authority. Many current probiotics, however, have not adhered to this requirement, probably largely due to the expense and logistics involved in carrying out large clinical trials. The large regulatory hurdle of proving efficacy for a particular probiotic, while useful for ensuring products are efficacious, may also lead to consumers missing out on benefits from some newly developed products. This issue could be addressed by regulatory bodies through adoption of a staggered claim system, where low-level claims (e.g., for preventative rather than therapeutic benefits) could be

made for products that have been proven to be safe, but which have not yet been fully assessed for efficacy. High-level health claims (e.g., replacements for current treatments) should be reserved for products that have satisfied the requirements of successful outcomes from multiple double-blind placebo-controlled trials.

Probiotic Production

The production of probiotics can be achieved either in-house for companies with large infrastructure or, alternatively, be outsourced to facilities that have the appropriate fermentation capabilities and expertise. Regardless of the production process chosen, it is essential for the successful delivery of probiotics that a stringent quality assurance program is in place ensuring that the probiotic is delivered in a safe and efficacious manner. Such a program should have control over the ingredients used in the fermentation and subsequent formulation of the delivery format for the probiotic. It should also closely monitor the product for safety and ensure that throughout the stated shelf life of the probiotic an efficacious dose is delivered.

Profiles of Proposed *S. salivarius* Probiotics: Past, Present & Potential

Early Entries

S. salivarius strains TOVE-R and K58 were given preliminary consideration in the pioneering days of applied bacterial interference research for their potential to control the major *Streptococcus*-associated infections of the human oral cavity – dental caries and streptococcal pharyngitis.

S. salivarius TOVE-R

The ability of certain *S. salivarius* to interfere with the proliferation of the streptococci most commonly implicated in the etiology of dental caries was demonstrated by Tanzer *et al.*[37] Initial oral colonization of rats with *S. salivarius* strain TOVE-R (R for rough colony morphology) prevented the subsequent establishment by fecal transmission of *S. mutans* 10449S and *Streptococcus sobrinus* 6715–13WT. TOVE-R itself did not contribute to caries development and its transmission to rats already infected by 10449S or colonization of rats prior to 10449S exposure inhibited caries induction. In a further study, TOVE-R was demonstrated to colonize rat dental plaque already containing *S. mutans* or *S. sobrinus* and to persist as a prominent member of the plaque microbiota.[38] Moreover, colonization by TOVE-R effected an approximately 50% reduction in the total recoverable *S. mutans* and *S. sobrinus* populations on the teeth. The authors suggested that TOVE-R colonization may have clinical therapeutic utility for suppressing existing infection of humans by the mutans streptococci, but no follow-up clinical studies have been reported. Interestingly, the only follow-up study of TOVE-R seems to be its apparently successful application to the alleviation of periodontal disease in a beagle model.[39]

S. salivarius K58

The first *S. salivarius* specifically selected for its potential to interfere with the colonization of the upper respiratory tract by *S. pyogenes* was strain K58.[5] The bioactive agent inhibitory to the growth of *S. pyogene*s was partially purified from culture supernatants and named enocin (based on the names the investigators, Eugene and Christine).[5] Enocin was characterized as a low-molecular-weight heat-labile molecule and, since its mode of action appeared to involve interference with pantothenate utilization, it inhibited the growth of organisms requiring exogenous pantothenate such as *S. pyogenes*.[5] Interestingly, of 29 *S. salivarius* strains isolated during a series of clinical studies,[40–42] nine produced enocin-like activity against *S. pyogenes*, indicating that this agent may be frequently expressed by *S. salivarius*. Unfortunately, no further studies of this strain or of enocin have been reported. In our experience, *S. salivarius* K58 inhibits the growth of *S. pyogenes* in simultaneous antagonism tests, but not in deferred antagonism BLIS P-typing tests, indicating that the agent is produced early in the growth phase and rapidly lost/degraded [Tagg JR *et al.*, Unpublished Data].

Current Contenders

S. salivarius K12

Although *S. salivarius* K12 was initially selected on the basis of its broad inhibitory activity against *S. pyogenes*, it has subsequently been demonstrated to provide more diverse health benefits – ranging from the alleviation of halitosis to stimulation of antiviral immune defenses and the reduction of episodes of OM. This broad spectrum of potential health benefits conferred throughout the life of the human host has prompted the adoption of the colloquial moniker for this strain, "BLIS K12 – the probiotic for all ages" (Figure 2).

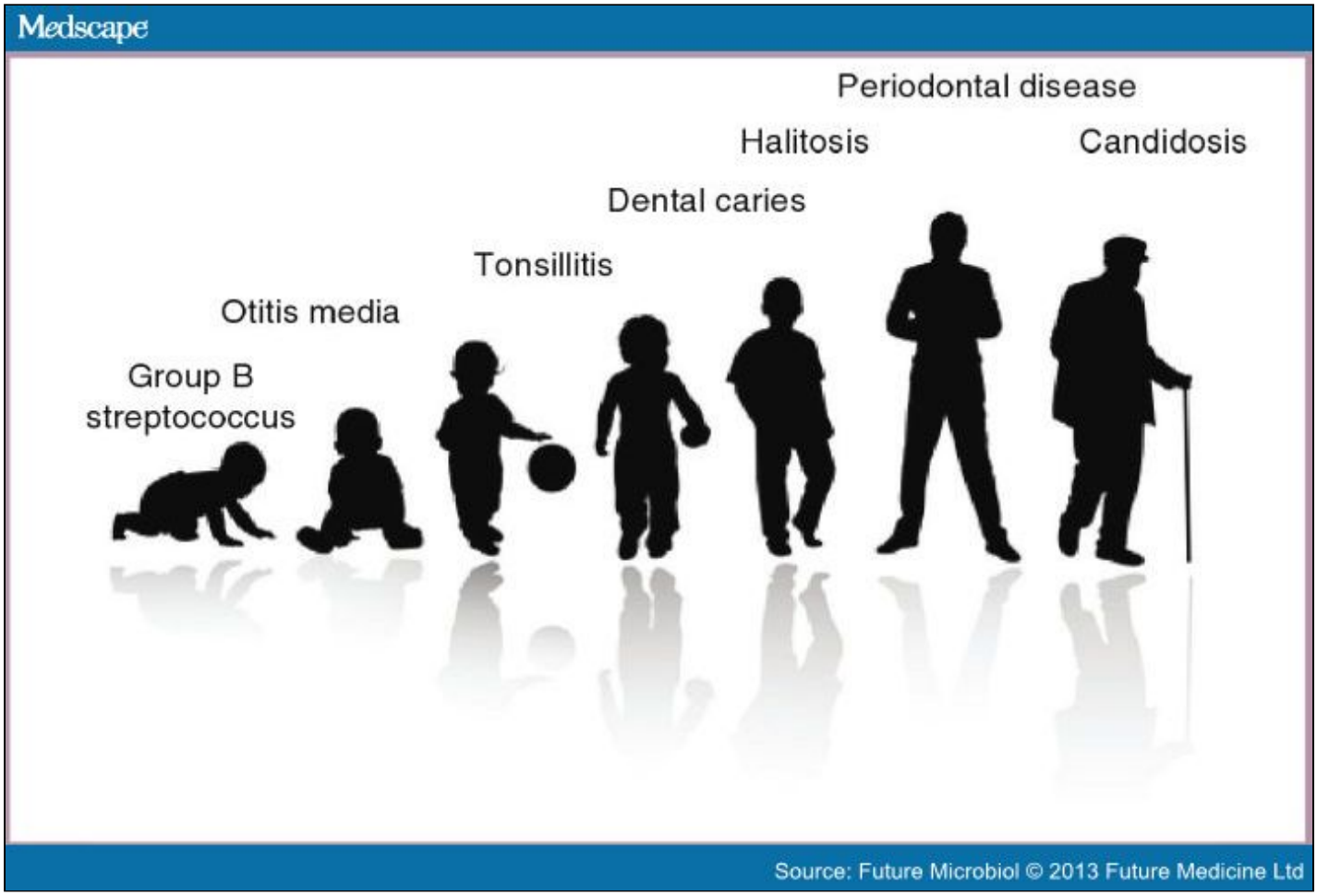

Figure 2.

***Streptococcus salivarius*: the probiotic for all ages.** Diseases that may be alleviated by *Streptococcus salivarius* probiotics and the ages at which they generally tend to manifest.
Reproduced with permission from [77].

In 2001, strain K12 became the first *S. salivarius* to be commercially developed as a probiotic and more than 50 million doses have now been marketed internationally by the New Zealand company BLIS Technologies Ltd (Dunedin, New Zealand). A substantial body of research was undertaken to underpin the safe and efficacious application of the strain to humans and this included a variety of clinical interventions in both animals and humans. Although *S. salivarius* is not commonly consumed as a naturally occurring food ingredient, it is nevertheless considered a low-risk organism since, in spite of its apparently invariable and plentiful presence in the human oral cavity, it is only very rarely a cause of infection in humans who are immunologically competent.[27] The safety of strain K12 has been specifically supported by a series of studies: affirming the absence of known streptococcal virulence factors and antibiotic resistance determinants; showing its low mutagenicity predisposition; acute and subacute toxicity testing in rats; and a high-dosage trial in humans.[29,35,36] The outcome of these strain-specific studies, together with recognition of the inherent safety of the species, has enabled a self-affirmed 'generally regarded as safe' (or 'GRAS') status to be granted for strain K12 in the USA. Interestingly, the species *S. salivarius* is still generally classified as a risk group 2 organism in Europe; however, on the basis of its safety profile strain, K12 has been specifically reclassified as a risk group 1 organism in Germany by the Ausschuß für Biologische Arbeitsstoffe (Translation: Committee on Biological Agents).[43]

The original source of *S. salivarius* K12 was a healthy schoolchild who had maintained a large indigenous oral cavity population of the K12 strain for a period of more than 12 months, during which time no new *S. pyogenes* infections were experienced. A distinctive (and indeed patentable) feature of strain K12 was its production of two novel lantibiotics (salivaricin A2 and B), both of which were shown *in vitro* to have inhibitory activity against *S. pyogenes*, the principal causative agent of streptococcal pharyngitis.[44] Further support, albeit indirect, for the protection offered by *S. salivarius* BLIS against *S. pyogenes* infection came from studies showing that children who harbored oral populations of salivaricin A- and/or B-producing *S. salivarius* had

significantly fewer new acquisitions of *S. pyogenes* than did children who appeared not to have BLIS-producing *S. salivarius* (17 vs 32%, respectively).[45] Another study showed that children who frequently experienced clinically confirmed sore throats were significantly less likely to have BLIS-producing *S. salivarius* than children who had not experienced sore throats in the past 3 years.[46] Furthermore, competition experiments between cocultured strain K12 and a bioluminescent *S. pyogenes* demonstrated that strain K12 binds avidly to human epithelial cell lines and can interfere with the binding of *S. pyogenes*[28,47] (Figure 3). Oral cavity colonization of humans occurs following its introduction into the mouth and the efficacy of this colonization is enhanced by prior reduction of the levels of the indigenous streptococcal population, as occurs following the use of an antiseptic mouth rinse (e.g., chlorhexidine) or after antibiotic treatment.[15,48,49] Recent, as yet unpublished, studies have also demonstrated that the use of one lozenge a day containing 1 billion viable cfu of strain K12, is sufficient to achieve oral cavity colonization in the majority of subjects [WESCOMBE PA *ET AL.*, UNPUBLISHED DATA]. Further evidence for the protection afforded by strain K12 against streptococcal pharyngitis was gathered during a small preliminary trial in which 24 children with a history of recurrent tonsillitis (0.33 episodes per month) received daily doses of either strain K12 or a placebo. The 18 children receiving strain K12 experienced fewer sore throats (0.10 per month) than did the six children in the placebo group (0.19 per month) [BURTON JP *ET AL.*, UNPUBLISHED DATA].

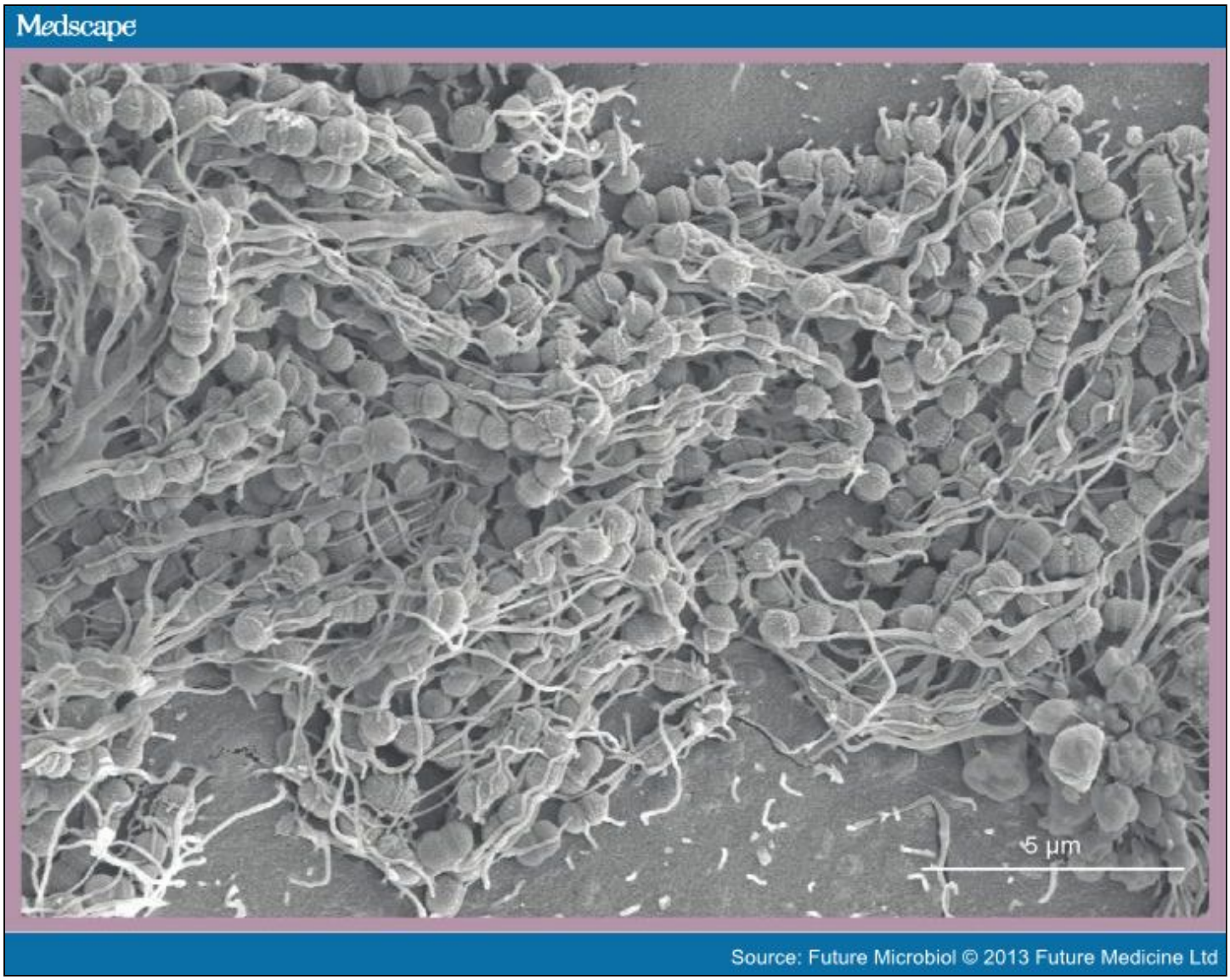

Figure 3.

Electron microscope image demonstrating the attachment of *Streptococcus salivarius* K12 to HEp-2 cells.
Image courtesy of M Rohde.

S. salivarius, *Rothia mucilaginosa* and an uncharacterized species of *Eubacterium* were identified as being present in either

relatively reduced numbers or absent in tongue dorsum populations of subjects suffering from halitosis.[50] Prompted by this observation, a trial of 23 subjects with halitosis (having breath scores for volatile sulfur compound [VSC] levels of greater than 200 ppb) undertook a 3-day regimen of chlorhexidine mouth rinsing, followed, at intervals, by the use of lozenges containing either *S. salivarius* K12 or placebo.[49] Assessment of the subjects' VSC levels 1 week after treatment initiation demonstrated that 85% of the K12-treated group and 30% of the placebo group had substantial (>100 ppb) VSC level reductions. While the majority of the subjects tested had a favorable outcome, the mechanism(s) of VSC reduction was not clearly established. *In vitro* tests showed that the inhibitory spectrum of strain K12 encompasses some of the key Gram-negative anaerobes (including *Prevotella* spp.) that have been implicated in halitosis.[49] Other mechanisms of competition (e.g., saturation of attachment sites by the newly introduced K12 cells) may also have been influential, particularly as facilitated by the chlorhexidine pretreatment step, which may have reduced populations of some critical adjunct members of the halitosis-associated consortia. Subsequent colonization of the microbe-depleted site by the incoming K12 could also limit anaerobe proliferation through specific BLIS-mediated inhibition of key members of the halitosis-associated microbiota.

OM is the most common bacterial infection in young children and the predominant etiological agents are *Streptococcus pneumoniae*, *S. pyogenes*, *Moraxella catarrhalis* and *Haemophilus influenzae*. As a preliminary experiment to evaluate the efficacy of probiotic interventions for the control of OM, it was shown that *S. salivarius* K12, when given to 19 young OM-susceptible children following a 3-day course of amoxicillin, led to colonization of the nasopharynx and/or the adenoid tissue of some subjects.[51] Interestingly, in that study, only 33% of the subjects achieved oral colonization with strain K12. This lower-than-anticipated level of colonization was attributed to the failure of the amoxicillin pretreatment to effect a substantial reduction in the level of the indigenous oral streptococcal populations, since most of these subjects had been preconditioned to regular amoxicillin exposure during the course of their OM therapy.[51] To determine whether delivery of the *S. salivarius* K12 probiotic to the oral cavity would have any effect on the rate of recurrence of OM, a small study was undertaken at Dunedin Hospital BURTON JP *ET AL.*, UNPUBLISHED DATA. The 13 children enrolled in the study were from the surgical waiting list for grommet implants and all had a history of recurrent acute OM (AOM). The subjects were offered a three-month treatment course of either strain K12 or placebo and nine completed the study. The children receiving the K12 probiotic (n = 6) had far fewer ear infections (0.22 per month) than they did prior to entering the study (0.50 per month, n = 13) and also by comparison with the smaller placebo group (0.55 occurences per month, n = 3) BURTON JP *ET AL.*, UNPUBLISHED DATA. The encouraging results of this study (although only preliminary) indicate that *S. salivarius* K12 dosing could potentially reduce the occurrence of OM.

An unanticipated application of *S. salivarius* K12 could be to ameliorate the development of oral candidosis. A number of early studies indirectly demonstrated that *S. salivarius* may inhibit oral candida,[52–55] but more recently Ishijima *et al.*[20] found a direct protective effect against *Candida albicans* after oral dosing with strain K12. In this latest study, K12 was shown to bind preferentially to the hyphae of *C. albicans* and to prevent its attachment to a plastic substratum. Interestingly, K12 was not able to directly inhibit *C. albicans* in a deferred antagonism assay, indicating that the bacteriocins encoded for by strain K12 do not target yeast and further supporting other observations that mechanisms other than the ability to target pathogens with antimicrobial molecules can also contribute to the health benefits of probiotics. When tested using an *in vivo* mouse model for oral candidosis, a dose-dependent improvement in symptom score was observed for mice dosed with K12 at 24 and 3 h before and at 3, 24 and 27 h after *C. albicans* inoculation, when compared with mice in a saline-treated group. Follow-up clinical evaluation of the efficacy of K12 in candidosis control in humans now seems imperative.

Although it is now well established that exposure to probiotic bacteria can impact upon the host's immune system, the outcome of these interactions can be quite strain-specific. Several *in vitro* cell culture experiments have indicated that strain K12 can help to maintain cell homeostasis. In one microarray-based study, it was demonstrated that co-culture with either strain K12 or certain bacterial pathogens differentially influenced the expression levels of 1530 genes in human bronchial epithelial cells.[56]*S. salivarius* K12 altered the expression of 660 genes (572 of which were specific to K12) and, in particular, those involved in innate immune defense pathways, general epithelial cell function and homeostasis, cytoskeletal remodeling, cell development and migration, and signaling pathways. In this same study, *Staphylococcus aureus* influenced the expression of 323 genes. The ratio of upregulated to downregulated genes was 5:2 for K12, but this ratio was reversed for *S. aureus*, further illustrating the different signaling roles of strain K12 and bacterial pathogens. Closer analysis of the affected gene pathways indicated that K12 potentially contributes to the maintainance of homeostasis between human and bacterial cells by reducing proinflammatory responses. In particular, K12 was shown, by enzyme-linked immunosorbent assay, to reduce the levels (from 318 to 5.1 pg/ml) of the cytokine IL-8 produced by the bronchial cell line in response to the presence of *Pseudomonas aeruginosa*.[56] IL-8 has been demonstrated to have a major involvement in the pathogenesis of gingivitis and so dosing with strain K12 may potentially help ameliorate some of the inflammatory manifestations of this disease. The secretion of Gro-α, an inducible neutrophil chemotactic factor synthesized in epithelial tissues during inflammation, was also inhibited by the presence of strain K12 when the epithelial cells were exposed

to flagellin (a known inducer of IL-8 secretion by epithelial cells), further emphasizing the protective role strain K12 can play for the host. The mechanism of immunosuppression by strain K12 appeared to be at least partially explained through the inhibition of activation of the NF-κB pathway (a family of transcription factors that function as dimers and regulate genes involved in immunity, inflammation and cell survival). Interestingly, the most significantly over-represented pathway in the array studies was the unified interferon signaling pathway. In this pathway, type I and II interferons signal through their specific receptors to upregulate the expression of a large number of genes responsible for innate immunity against viral infection, antitumor activity, priming of the LPS response and anti-inflammatory effects. This indicates that, while K12 cells can act to reduce inflammation, they may also 'prime' the epithelial cells through tonic signaling to respond rapidly and appropriately to the detection of viral or bacterial exposure in order to limit the spread of infection – a role that has recently been ascribed, in general, to commensal bacteria.[57]

Other preliminary studies have demonstrated that high-level oral dosing with *S. salivarius* K12 elicits increased salivary levels of IFN-γ.[58] These observations were further supported by investigations with mouse splenocytes, in which IFN-γ levels, but not the pro-inflammatory cytokines IL-1β or TNF-α, were increased in response to co-culturing with strain K12 [WALES J *ET AL.*, UNPUBLISHED DATA]. Interestingly, it seems that not all *S. salivarius* elicit similar immune responses, since *S. salivarius* strain ATCC 25975 was reported to upregulate IL-6, IL-8 and TNF-α gene expression.[59] Indeed, in that study it seemed that strain ATCC 25975 was even more efficient at inducing the release of proinflammatory mediators than was *C. albicans*. These apparently contradictory findings emphasize the importance of not extrapolating the specific findings for one probiotic candidate strain to all members of that same species. The initial findings of induction by strain K12 of an anti-inflammatory response have subsequently been independently corroborated by Guglielmetti *et al.*,[47] who showed that IL-6, IL-8 and TNF-α levels were significantly reduced when FaDu cells were co-cultured with K12. These findings will be discussed below in relationship to the probiotic candidate strain *S. salivarius* ST3.

In summary, it appears that strain K12 is well suited for use as an oral cavity and upper respiratory tract probiotic due to its natural propensity to inhabit the human oral cavity and be strongly competitive with a number of potential oral pathogens that have adapted to the same ecological niche. In addition, the immune responses of cell lines to co-incubation with *S. salivarius* K12 indicate that it elicits no proinflammatory response but rather an anti-inflammatory response, as well as modulating genes associated with adhesion to the epithelial layer and homeostasis. By these strategies, *S. salivarius* K12 appears to be well-tolerated on the epithelial surface, while also actively protecting the host by BLIS-mediated inhibition of pathogen replication and stimulation of cytokine-mediated reduction of virus replication and pathogen-induced inflammation and apoptosis.

S. salivarius M18

Some early reports indicated that certain *S. salivarius* strains (especially TOVE-R as aforementioned) may have a role in the limitation of dental caries. Following the successful discovery and introduction of the probiotic strain K12, BLIS Technologies Ltd. conducted extensive follow-up deferred antagonism testing of candidate BLIS-producing *S. salivarius* to identify strains having inhibitory spectra that included bacterial species putatively associated with the development of dental caries. In this screen, *S. salivarius* strain M18 (formerly known as Mia) was found to inhibit all tested *S. mutans* and *S. sobrinus* (collectively referred to as the mutans streptococci). Other species inhibited by strain M18 included: *Actinomyces viscosus*, *Actinomyces naeslundii*, *Streptococcus agalactiae*, *Streptococcus pneumoniae, Enterococcus faecalis, Listeria monocytogenes, H. influenzae, Staphylococcus saprophyticus* and *Staphylococcus cohnii*.[101] This unusually broad spectrum of inhibition indicated that strain M18, in addition to potentially reducing the risk of dental caries, may also have additional benefits for the host in helping to limit the growth of a variety of common bacterial pathogens of the upper respiratory tract.

To date, four bacteriocin loci have been identified in the M18 genome: salivaricin A2,[101] 9,[60] MPS[30] and M.[30] Salivaricin A2 and 9 are well-characterized bacteriocins with broad activity against *S. pyogenes* as well as other upper respiratory tract pathogens, but not against mutans streptococci. Salivaricin MPS is less well characterized, but is known to be a large 60 kDa bacteriocin with specific activity against *S. pyogenes*.[61] Salivaricins A2, 9 and MPS have been found to be megaplasmid-encoded in strain M18.[16,30] By contrast, salivaricin M appears to be chromosomally encoded and, recently, has not only been shown to be a lantibiotic, but also to be the molecule responsible for the observed activity of strain M18 against mutans streptococci.[30] Interestingly, unlike most other *S. salivarius* bacteriocins, salivaricin M appears to be optimally produced *in vitro* on TSYCa agar (trypticase soy broth supplemented with 2% yeast extract, 0.1% $CaCO_3$ and 1.5% agar), and less effectively on BaCa (blood-containing) agar in deferred antagonism assays, an observation indicating that there is strict regulation of its locus expression.

Preliminary colonization trials have indicated that, in children who colonize well with strain M18, the salivary levels of mutans streptococci are maintained at reduced levels for significant periods (at least 27 days) by comparison with placebo-dosed control

subjects, in whom the mutans streptococci levels returned to pretreatment levels within 4–6 days.[101,62]

A variety of pathogens have been implicated in the development of gingivitis and periodontitis and it has also been shown that the etiology of these diseases is strongly linked to the inflammatory response of the host cells to the bacterial pathogens.[63,64] To determine whether strain M18 can potentially impact on pathogen-induced pro-inflammatory cytokine expression in gingival fibroblasts, strains M18 and K12 were coincubated with gingival fibroblasts both prior to and concommitantly with exposure to periodontal pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*. Strains M18 and K12 both significantly inhibited the expression of the pro-inflammatory cytokines IL-6 and -8, commonly associated with gingivitis – indicating that dosing with these probiotics may potentially be useful in the treatment of gingivitis.[65] Appropriately controlled large-scale clinical trials further investigating the potential for M18 probiotic interventions in the control of dental caries and gingivitis now appear warranted.

New Nominations

S. salivarius ST3

Using strain K12 as a model oral probiotic for comparative purposes, Guglielmetti *et al*.[18] obtained 56 probiotic candidate strains from pharyngeal sites of four healthy donors. From this initial group, 11 *S. salivarius*, established to be of separate lineages, were further investigated for their potential as probiotics targeting prevention of *S. pyogenes* pharyngitis. Assessment of their probiotic potential included tests of their binding efficacy to the human epithelial cell lines FaDu and HaCaT – an attempt to model the primary adhesion target for invading *S. pyogenes*.[66] The strains having the highest adhesion indices to FaDu cells were ST3 and K12. As another efficacy measure, the bioluminescent indicator strain *S. pyogenes* C11^{LucFF} was used to monitor the exclusion of *S. pyogenes* affected by preincubation of either FaDu or HaCaT cells with each candidate probiotic. Exclusion was strain-specific, with *S. salivarius* strains ST1 and RS1 observed to be significantly more effective at excluding the *S. pyogenes* than strain K12 in this assay. Interestingly, however, strain K12 was at least as effective as strain ST1 in competition assays, in which combinations of the bioluminescent *S. pyogenes* and *S. salivarius* probiotic were added together to the cell lines. One possible reason for the apparently superior ability of strain K12 over strain ST3 to antagonize the binding of *S. pyogenes* may be its production of the bacteriocins salivaricin A and B, both of which are known to inhibit the growth of *S. pyogenes in vitro*. By contrast, strain ST3 did not appear to produce any bacteriocin activity under the test conditions used for this study.

Guglielmetti *et al.* further characterized strains ST3, RS1 and K12 according to their ability to modulate the immune responses of FaDu cell lines.[18] Interestingly, all three strains were well tolerated on co-culture with the epithelial cells, with no strains stimulating a pro-inflammatory response – a finding that, on reflection, might indeed be anticipated for an oral cavity commensal species.[18] Co-culture with strain K12 was demonstrated to reduce baseline IL-6, IL-8 and TNF-α levels, with IL-8 and IL-6 well below the levels obtained for the other two *S. salivarius* strains (ST3 and RS1). All three *S. salivarius* were effective at reducing the levels of IL-6 and IL-8 following stimulation of the FaDu cells by IL-1β (a prototypical pro-inflammatory cytokine that plays a central role in the inflammation amplification cascade) illustrating the general anti-inflammatory activity of *S. salivarius*. IL-6, IL-8 and TNF-α are known to be mediators of the pro-inflammatory response that causes most of the tissue damage in periodontal disease and results from the host response to bacterial infection. Downregulation of the pro-inflammatory response is now a major aim of treatment regimes for periodontal disease,[67] highlighting a potential further application for probiotic therapy with *S. salivarius* strains such as K12 and ST3. A further observation was the apparent upregulation of GM-CSF and IFN-γ levels in IL-1β-stimulated FaDu cells co-cultured with K12.[18] GM-CSF stimulates stem cells to produce granulocytes, a process crucial for fighting infection, while IFN-γ is a cytokine essential for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. This stimulation of GM-CSF and IFN-γ by strain K12 indicates that another beneficial effect of probiotic treatment with this strain may be to enhance the body's natural defenses against virus infection. Immune enhancement of this nature (also described as low-level tonic signaling), enabling the body to respond more rapidly to virus infection, has also been described for some other probiotics.[57] For example, prophylactic probiotic administration has been demonstrated to limit the duration and severity of virus infections of the respiratory tract in human subjects, indicating that the effects of probiotics can extend beyond the GI tract.[68] Strains ST3 and RS1 appeared to drive a slightly different immune response to that of strain K12 in FaDu cells, in that, while they shared the ability to reduce IL-6, IL-8 and TNF-α levels, they upregulated the levels of MIP and MCP-1 – both of which are proinflammatory cytokines.[18] The authors suggest that this observed upregulation may benefit the host through upregulation of their immune defenses against pathogenic bacteria, a comment that is supported by similar observations made about the Gram-negative probiotic strain *Escherichia coli* Nissle.[69] Interestingly, strain RS1 was shown to reduce the levels of IFN-γ produced by IL-1β-stimulated FaDu cells, perhaps indicating that exposure to this strain could potentially render the host more susceptible to viral infection.

Ability to produce urease was another characteristic assessed for each of the candidate probiotic strains. Strains K12 and RS1 were demonstrated both to be strongly ureolytic, a trait considered beneficial due to its effect in reducing the acidity of dental plaque and, thereby, possibly delaying the onset and progression of dental caries.[70,71] By contrast, strain ST3 appeared non-ureolytic and, on further examination, was found to lack *ureC*, which encodes the main subunit of the urease complex.[18] The authors suggested that the inability to hydrolyze urea could be considered beneficial, in that it could result in there being less damage to the host's mucosal cells from exposure to ammonia. These observations highlight the potential for different strains to fulfill different roles in the oral cavity and, perhaps, for them to be targeted to applications in individuals with specific health needs.

Recently, strain ST3 was examined for its possible use as an oropharyngeal probiotic in combination with a second putative probiotic bacterium, *Lactobacillus helveticus* strain MIMlh5.[19] The two strains were investigated for their ability to coordinately modulate the host innate immune system in a manner beneficial to the host. Although exposure to this strain combination significantly induced expression of the proinflammatory cytokine TNF-α, the levels achieved were never above the co-induced levels of the anti-inflammatory molecule IL-10, indicating that the effect was probably regulatory rather than pro-inflammatory. However, a further observation that did support the potential immune benefits of prophylaxis with this strain combination was the induced expression of *COX-2*, the gene encoding the isoform of prostaglandin synthase H, which is known to be induced by several stimuli including bacterial components. Prostaglandin synthase H synthesizes prostaglandins, which are known to contribute to the protection of the gastrointestinal mucosa and are also involved in both the induction of oral tolerance, by guiding T cells towards an immunosuppressive phenotype,[72] and the resolution of inflammation.[73] A rapid upregulation of COX-2 expression in response to injury or inflammation has, furthermore, been reported to restore mucosal integrity, thus reducing the time available for pathogens to penetrate the innate defenses of the intact mucosa.[74] In addition to these immunological effects, the probiotic strain combination was shown to interfere with the binding of *S. pyogenes* to FaDu cells, while not interfering with the binding characteristics of each probiotic partner strain, indicating that they were compatible for use in a combined probiotic product. It is of interest to note that *L. helveticus* strain MIMlh5 was able to antagonize *S. pyogenes* better than strain ST3 in the luminescence assay deployed. However, since *S. salivarius* is more commonly located in the oral cavity, it is to be expected that it may perform better *in vivo*, emphasizing the need for clinical studies on human subjects to confirm the benefits of each probiotic strain. Both strains were also found to grow efficiently in milk, indicating that fermented milk products may be suitable delivery vehicles for the probiotics.[19]

S. salivarius 24SMB

Santagati *et al.* recently screened 81 α-hemolytic streptococci isolated from nasal and/or pharyngeal swabs of healthy children, with the intention of identifying commensal bacteriocin-producing bacteria for use in the prevention of upper respiratory tract infections.[28] The principal selection criteria were: safety for the host, strong adhesion to HEp-2 cells and inhibitory activity against *S. pneumoniae*. *S. salivarius* strain 24SMB had an inhibitory spectrum apparently specific for *S. pneumoniae* when tested on Columbia agar base supplemented with 5% horse blood and 0.1% $CaCO_3$. When tested on TSYCa, the activity spectrum increased to also include three *S. pyogenes* strains. The authors concluded that, since strain 24SMB appeared to contain no known bacteriocin loci, it may express one or more as yet uncharacterized bacteriocins. Interestingly, one of the main criteria for the selection of 24SMB was its ability to bind to HEp-2 cells, which were originally thought to have been derived from an epidermoid carcinoma of the larynx but, subsequently, have been established to have arisen from HeLa (derived from cervical cancer) cell contamination.[75] Since HEp-2 cells are positive for keratin by immunoperoxidase staining and therefore do share this characteristic of human tissue surfaces, they may have some relevance for evaluation of oral probiotic attachment. However, for oral probiotic selection, it seems that cell lines that are established to have oral cavity origins should preferably be used. In any case, although strain SMB24 was shown to bind well to HEp-2 cells (50–57%), strain K12 was also found to bind to a similar extent (50–60%), indicating no benefit in this regard for strain SMB24 over the currently established K12 probiotic. The safety of strain SMB24 was evaluated through a screen for potential streptococcal virulence genes and testing for sensitivity to antibiotics. Strain SMB24 was negative for all potential virulence genes tested and, additionally, was sensitive to all antibiotics used in the screen. Overall, although the investigations are still preliminary, strain SMB24 appears to have good potential for use as a probiotic to prevent upper respiratory tract infections.

S. salivarius T30

An important target for *S. salivarius* probiotics is AOM, the most common bacterial infection in growing children – the key causative agents of which are *S. pneumoniae*, *S. pyogenes*, *M. catarrhalis* and *H. influenzae*. It is presumed that the infection originates from the nasopharynx with the bacteria entering the middle ear via the Eustachian tube. Current treatments include the use of broad-spectrum antibiotics and insertion of tympanostomy tubes. Antibiotic dosing fosters resistance development and also weakens the natural defenses through a reduction in the numbers of the normal healthy microbiota. Tympanostomy tube insertion

is costly and carries with it the associated risks of general anesthesia (in some cases) and possible membrane damage. Some probiotic-based strategies to reduce the incidence of OM have been explored, including the successful use of inhibitory α-hemolytic streptococci (a mixture of *Streptococcus mitis*, *Streptococcus sanguinis* and *Streptococcus oralis*) as a nasal spray.[6] A complication of this is that the three species utilized in the formulation are all recognized human pathogens, with *S. mitis* associated with lung infection and abscess formation, and both *S. oralis* and *S. sanguinis* implicated in endocarditis. Therefore, a *S. salivarius* probiotic, if also shown to be clinically efficacious, may be viewed more favorably by regulatory bodies for use in young children. With this in mind Walls *et al.*[76] looked at the nasopharyngeal microflora of 20 children with recurrent AOM (having more than six episodes of AOM in the previous year) and 15 healthy controls (having no more than one AOM episode). While no significant correlation was observed between the groups for the presence of BLIS-producing streptococci, three *S. salivarius* isolates (two from the control group) were inhibitory to representative strains of at least three of the four major species of AOM pathogens *in vitro*.[76] Interestingly, for some subjects, salivaricin A- and B-producing *S. salivarius* were isolated from the nasopharynx, but not the saliva, indicating that *S. salivarius* producing these bacteriocins may be particularly well-adapted for growth in the nasopharynx. One isolate (strain T30) was characterized further and demonstrated to have broad inhibitory activity against all four major AOM pathogens when grown on TSYE agar under anaerobic conditions, but not on BaCa medium or when incubated with a 5% CO_2 in air atmosphere.[76] This strain was patented for use in the prevention or treatment of OM but was withdrawn in 2006.[102] However, the concept of using a strongly competitive nasopharyngeal-localized *S. salivarius* for the probiotic-mediated control of AOM certainly seems meritorious.

Future Perspective

With the recent rapid expansion in the variety of available probiotics and in their delivery vehicles, consumers are developing a growing enthusiasm for the health benefits to be derived from their consumption. While the majority of probiotics have been designed for use in the GI tract, it is clear that there is now an impetus to progress the field to encompass other regions of the body, including the oral cavity. As this review has shown, there are now a number of candidate probiotic strains from the species *S. salivarius* that have been proposed for application to the control of microbial diseases of the oral cavity. While the safety of *S. salivarius* for application to humans appears to have been well established, there is still only relatively limited clinical evidence to support claims of health benefit. Most current supporting evidence has been based on either *in vitro* studies or the results of clinical trials that have been limited in size. There is no doubt that, in the next few years, the benefits to be gained from these probiotics (both in terms of health and commercial gain) will provide the incentive for clinical studies of sufficient magnitude to clearly establish the roles that *S. salivarius* probiotics can play in the human oral cavity, the upper respiratory tract and beyond (Figure 4). It is also apparent that, due to strain variation (even in the immunological responses that they evoke), individual strains will be selected for their specific health benefits which could include the prevention of: dental caries; OM; streptococcal sore throat; halitosis; oral thrush; general immune priming and potentially many more (). This promises to be a rapidly evolving and rewarding research area to observe and to participate in over the next decade.

Table 1. *Streptococcus salivarius* strains investigated for their potential as probiotics.

Strain	Target area(s)	Commercialized	Antibacterial agents	Safety	Efficacy
K12	Oral health Halitosis Otitis media Strep sore throat Oral thrush Anti-inflammatory	Yes	Salivaricin A Salivaricin B	GRAS High-dose human trials Absence of streptococcal virulence genes Antibiotic sensitivity Low mutagenicity Acute and subacute studies in rats More than 50 million doses sold over 10 years	Clinical: Colonization trials in humans Small-scale clinical trials Otitis media, recurrent tonsillitis Clinical studies on halitosis Bacteriocin detection in human oral cavity Increased salivary levels of IFN-γ *In vitro*: Anti-inflammatory effect that reduces inflammation by a range of pathogenic bacteria Anti-*Candida* exclusion studies Good adhesion to FaDu and HEp-2 epithelial cell lines Inhibition of many pathogenic bacteria

				Genome sequence	including *S. pneumoniae, M. catarrhalis* and *S. pyogenes* *S. pyogenes* exclusion assays
M18	Dental caries Gingivitis Periodontal disease Anti-inflammatory Strep sore throat	Yes	Salivaricin A Salivaricin M Salivaricin MPS Salivaricin 9	Human trials Antibiotic sensitivity Genome sequence	Clinical: *S. mutans* reduction for colonized individuals *In vitro*: Inhibition of *S. mutans* and *S. pyogenes* Immunological studies – anti-inflammatory effect – protection against periodontal pathogen-induced inflammation Dextranase production Urease production
ST3	Strep sore throat Anti-inflammatory	No	Not known	Antibiotic sensitivity Inability to hydrolyze urea	*In vitro*: Good adhesion to FaDu epithelial cell line Inhibition of *S. pyogenes* *S. pyogenes* exclusion assays Immunological studies – anti-inflammatory effect
24SMB	Otitis media Strep sore throat	No	Not known	Antibiotic sensitivity Absence of streptococcal virulence genes No harmful enzymatic activity	*In vitro*: Inhibition of *S. pneumoniae* and *S. pyogenes* Adhesion to HEp-2 cells
T30	Otitis media	No	Not known	No specific testing	*In vitro*: Inhibition of *S. pneumoniae*, *M. catarrhalis*, *H. influenzae* and *S. pyogenes* Isolated from nasopharynx
Tove-R	Dental caries	No	Not known	No specific testing	Clinical: Rat model demonstration of efficacy to prevent *S. mutans* colonization and reduce existing numbers Has capability to colonize dental plaque
K58	Strep sore throat	No	Enocin	No specific testing	Inhibitor capable of interfering with pantothenate utilization – active against *S. pyogenes* Isolated from a child resistant to *S. pyogenes* colonization

GRAS: Generally regarded as safe; H.: Haemophilus species; M.: Moraxella species; S.: Streptococcus species.

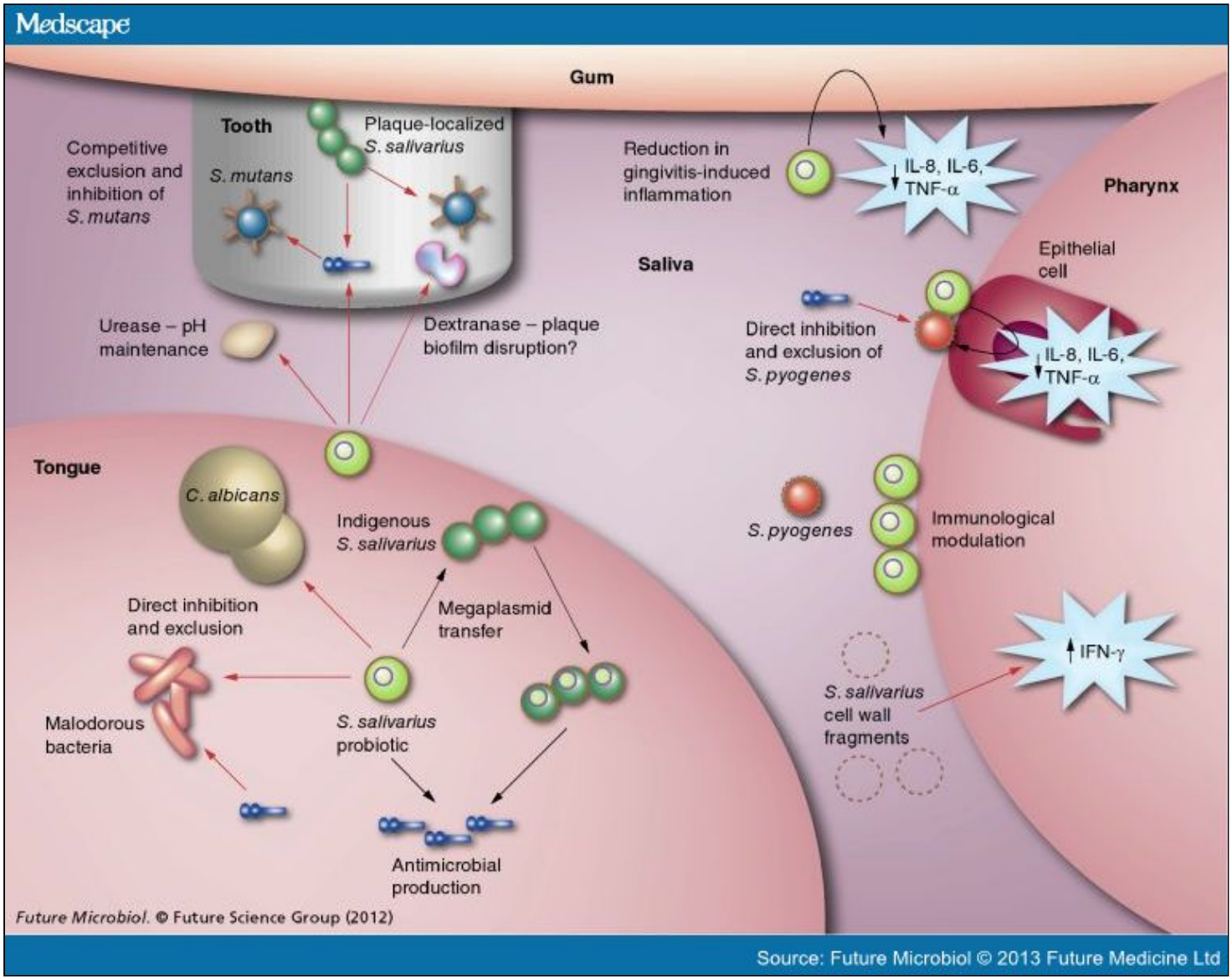

Figure 4.

Influence of *Streptococcus salivarius* probiotics in the oral cavity. Health benefits can occur through the direct inhibition and exclusion of pathogens, modulation of the human immune system to reduce pathogen-induced inflammation or by 'priming' the immune system to respond rapidly to viral or bacterial infection.

Sidebar

Executive Summary

Probiotics

- Live cells, the use of which benefits the health of the consumer.
- Some probiotics effect beneficial modulation of the indigenous microbiota via:
    - Colonization to occupy space or to effect increased anticompetitor activity within the native biofilm;
    - Megaplasmid transmission to modify the genetic repertoire of the existing microbiota;
    - Immune stimulation – a transient effect not requiring probiotic colonization, but potentially helping to control virus infection by upregulating the host's immune defenses.

Streptococcus salivarius

- Pioneer oral cavity commensal that remains a predominant component of the oral microbiota throughout life.
- Rarely disease-associated in healthy (immunologically competent) humans.
- Can harbor transmissible megaplasmids.
- Some strains produce multiple bacteriocins, sometimes referred to as anticompetitor molecules or bacteriocin-like inhibitory substances (BLIS).

S. salivarius *K12*

- The prototype oral probiotic, originally selected for its strong anticompetitor activity against *S. pyogenes* .
- Shown to have a role in the control of consortia bacterial infections, such as otitis media and halitosis, and also possibly in the reduction of oral candidosis and infections by certain upper respiratory tract viruses.

Other Candidate* S. salivarius *Probiotics: Past & Present

- Early contenders, TOVE-R and K58, had nonbacteriocin-mediated anticompetitor activities.
- Strain M18 selected because of its uncommon BLIS activity against mutans streptococci.
- New contenders ST3 and 24SMB were selected for their binding efficacy to epithelial cells and *in vitro* antipathogen activity, but neither produce characterized bacteriocins.
- The anti-AOM candidate strain T30 was selected for its BLIS activity against otitis media pathogens and was originally isolated from the nasopharynx of a healthy child.

Bringing an Oral Probiotic From Benchtop to Marketplace

- Producing a safe product is of paramount importance.
- Significant investigations of the potential benefits of a strain need to be conducted before commencing efficacy trials.
- Oral cavity probiotics require different considerations than intestinal probiotic products.
- Clinical trials evaluating the efficacy of the final product are becoming increasingly important.
- The road to market is not linear and may require several attempts to create an efficacious formulation.

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Papers of special note have been highlighted as:
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