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Distribution and persistence of probiotic *Streptococcus salivarius* K12 in the human oral cavity as determined by real-time quantitative polymerase chain reaction

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The bacteriocin producer *Streptococcus salivarius* K12 is used as a probiotic targeting the oral cavity, so our study aimed to assess whether its dispersal and persistence could be monitored using real-time quantitative polymerase chain reaction. To this end, we designed polymerase chain reaction primers and a hybridization probe specifically targeting *salA*, which encodes for the prepropeptide of salivaricin A. Using a single individual as our subject, we administered four lozenges of K12 Throat Guard per day over 3 days, then measured *salA* gene levels for 16 different oral sites at six different intervals over 35 days. Four samples each from gingival sulci and from teeth all remained negative. In contrast, in saliva and at all mucosal membranes K12 was detected, but with varying amounts and time profiles. Relatively high *salA* gene copy numbers, calibrated on the basis of colony-forming units, were seen on the tongue (maximum 4.6×10^4/swab at day 4), in stimulated saliva (2.4×10^4/ml, day 4) and on buccal membranes (1.3×10^4/swab, day 8). K12 was present on both sides of the pharynx but asymmetrically in both quantity and duration. In conclusion, we have developed a real-time quantitative-polymerase chain reaction for counting *S. salivarius* K12 at various sites in the oral cavity. In the individual studied, K12 could be detected at the mucosal membranes for as long as 3 weeks, but with steadily decreasing numbers after day 8. Thus, K12 may have the potential to control oral bacterial infections only when the uptake is repeated frequently.

Probiotics, defined by the World Health Organization as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (3), have been used to modulate bacterial populations in several areas of the human body. Probiotic treatment was found to be successful when applied to the intestinal tract and the vagina after antibiotic therapy and for management of infections where there was a perceived imbalance in the microbial ecosystem that allowed unregulated growth of pathogenic or non-desirable bacteria (6, 15). Although even gut probiotics are still controversial (1), similar or greater success has been found using oral probiotics to prevent dental caries (2, 7, 10, 12, 13), plaque

biofilm formation (7), and – through strains of *Streptococcus salivarius* – group A streptococcal pharyngitis and malodor (3–5).

S. salivarius is a predominant inhabitant of the tongue dorsum and the pharyngeal mucosa that becomes established in the human oral cavity at a very early age – within 2 days after birth. The levels of *S. salivarius* in swab samples taken from newborn infants represent 10% of the total streptococci isolated, increasing to 25–30% after 1 month. In healthy adults, *S. salivarius* represents 2% of the total streptococci isolated from the buccal mucosae, 17% from the tongue, and 30% from the pharynx (9). In saliva samples from adults, population levels of *S. salivarius* range from 10^6 to 10^7 colony-forming units (CFU)/ml.

S. salivarius strain K12, isolated from a healthy individual, is known to produce high levels of at least two lantibiotic bacteriocins, salivaricin A (SalA) (16, 21) and salivaricin B (SalB) (20). Lantibiotics are antimicrobial peptides that are produced by, and are active against, gram-positive organisms. These peptides are ribosomally synthesized and then undergo post-translational modifications, including amino acid dehydration and thioether bridge formation (18, 21). SalA (22 amino acid residues) is a subclass A II lantibiotic produced in approximately 20% of naturally occurring isolates, including *S. salivarius* as represented by reference strains DSM 20067 and 20P3 (8). The mature SalA is generated from a 48 amino acid prepropeptide, encoded by the *salA* gene, and processed and regulated by products of the *salBCTXYKR* operon (16). By utilizing *salA* as a DNA hybridization probe it was shown that all SalA peptide-producing strains of *S. salivarius* contained *salA* sequences and that, interestingly, 63 of 65 *Streptococcus pyogenes* strains of different M-types contained a *salA* gene homolog, designated *salA*1, which could explain the high susceptibility of *S. pyogenes* against SalA (17, 21). Thus, it is not surprising that in some countries (New Zealand, Australia) *S. salivarius* K12 has been used for several years as an oral probiotic (taken particularly by school-aged children) to prevent (re-)colonization with *S. pyogenes* and to protect, at least temporarily, against group A streptococcal pharyngitis, tonsillitis and suppurative (peritonsillar cellulitis, peritonsillar abscess, retropharyngeal abscess) as well as non-suppurative (acute rheumatic fever, acute glomerulonephritis) complications (19–21). In two other recent studies it was concluded that an oral antimicrobial administration of bacteriocin-producing *S. salivarius* K12 reduces the volatile sulfur compounds responsible for oral malodor, because anaerobes that produce valeric and butyric acids or putrescine are temporarily substituted by K12 (3, 4).

While the probiotic effects of SalA-producing K12 are generally acknowledged, it is still unknown which oral sites are preferentially colonized by this strain and for how long, information that could be important for obtaining optimum results. The aim of our study was to see if we could monitor the spatial distribution of K12 over time in a first (model) representative individual. For this purpose, after administering K12 exactly as recommended by the manufacturer, we tracked the probiotic strain in the oral cavity using a newly designed real-time quantitative polymerase chain reaction format composed of a K12-specific primer pair directed against the *salA* gene and an internal K12-specific TaqMan® probe.

Material and methods
Culture conditions

To obtain a reference culture, *S. salivarius* strain K12 was grown overnight directly from the BLIS K12 Throat Guard (BLIS Technologies Limited, Wellington, New Zealand) lozenges, which contain up to 10^{10} cells each, on Columbia blood agar at 37°C in 10% CO_2. Other *S. salivarius* strains, used for comparison (see below), were grown under the same conditions.

Clinical procedure, colonization protocol and sampling

A single individual was selected, a 40-year-old male who was otherwise healthy and who had not received antibiotic treatment during the previous 3 months, and had no previous K12 treatment. The Ethics Committee of the University Hospital, Rhine-Westfalian Technical University, approved a protocol describing the specimen collection for this investigation, and the individual signed an informed consent to participate.

A saliva sample (sample 0) was collected and analysed before the commencement of the colonization protocol to ensure that the test person did not harbor a K12 (or a BLIS K12 like-) strain before use of lozenges. On the following day (day 1), the subject brushed his teeth, used floss, and rinsed with 10 ml of 0.2% chlorhexidine gluconate for 30 s to reduce the population levels of existing oral microbiota. At 2-h intervals for 8 h, preferably after food, the subject sucked a lozenge (four in total) containing about 10^{10} CFU of *S. salivarius* K12 (BLIS K12 Throat Guard). This protocol was repeated on days 2 and 3. In addition to the recommendations on the product insert, during the colonization trial period no spirits, other anti-bacterial agents, or anti-bacterial food (e.g. garlic, onions) were allowed to be consumed. No adverse symptoms were reported during or after the trial.

To evaluate the K12 population over time, specimens were collected using polyester fiber-tipped applicator swabs (Falcon, Becton Dickinson, Franklin Lakes, NJ) in intervals at days 4, 8, 14, 21, 28 and 35 from the following sites: 1: pharynx, right; 2: pharynx, left; 3: buccal mucosal membrane, right; 4: buccal, left; 5: tongue, dorsal; 6: tongue, ventral; 7: tooth 14, buccal; 8: tooth 24, palatinal; 9: tooth 34, buccal; 10: tooth 44, lingual; paper points (ISO 45, capacity approximately 15 µl sulcus fluid and 2×10^6 cells) were used to sample the following sites: 11: sulcus gingivae of tooth 14, palatinal; 12: sulcus 24, buccal; 13: sulcus 34, lingual; and 14: sulcus 44, buccal. A cotton tampon was used to collect about 1 ml of un-stimulated saliva by chewing on it for 1 min (sample 15) and, along with a 1 ml sample of freshly paraffin-stimulated saliva (sample 16), was collected into tubes, centrifuged at 3000 *g*, and the cells were re-suspended in 200 µl brain–heart infusion (BHI) broth and frozen at −70°C. Swabs and paper points were also transferred into 200 µl BHI broth and the cells were detached and suspended using sterile glass beads. After removing the swabs, paper points and glass beads, the suspension was frozen at −70°C. The yield of bacterial cells recovered from swabs and cotton tampons was tested with a sample of saliva and corresponded to approximately 25% (swabs) and 8% (cotton tampons) relative to the amount of bacterial cells obtained directly from saliva.

DNA extraction

Microbial DNA from pure cultures and from oral samples was extracted and purified with a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's tissue protocol instructions. The DNA concentration (absorbance at 260 nm; A_{260}) and the purity (A_{260}/A_{280}) were calculated using a Gene Quant II photometer (Pharmacia Biotech, Cambridge, UK).

Sequencing of partial salivaricin operon

A partial fragment (1560 base pairs in size) of the salivaricin operon *salAB* from K12 was amplified using primers psalF1 5′-AGATGTATTGGTTACAATAAGAGC G-3′ and psalAR3 5′-GGCTTGCAAA AATTTTCCATAG-3′. These primers were designed on the basis of the salivaricin operon information for *S. salivarius* strain 20P3 and *S. pyogenes* strain SF370 (Gen-Bank accession nos. AY005472, AE006616). The amplification was performed on an Eppendorf thermocycler (Mastercycler personal) in 50 μl containing 1x polymerase chain reaction buffer, 1.5 mM $MgCl_2$, 2 units *Taq*-polymerase, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Applied Science, Penzberg, Germany), 100 nM of each primer and 1 μl template DNA (approximately 50 ng). Polymerase chain reaction cycling conditions were 94°C for 2 min, followed by 25 cycles of 94°C for 60 s, 55°C for 1 min, and 72°C for 1.5 min, with a final extension of 72°C for 10 min. The polymerase chain reaction product was purified using the Qiagen Purification Kit according to the manufacturer's instructions. Bidirectional sequencing was performed using a Big Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an automatic capillary DNA sequencer (API PRISM 310, Applied Biosystems). The generated sequence fragment of *salAB* has been submitted to GenBank under accession no. DQ519425.

Primer design

For real-time quantitative-polymerase chain reaction, primers and a TaqMan® probe were designed based on the K12 *salAB* sequence information (see above) as opposed to *S. salivarius* strain 20P3 (GenBank AY005472). K12-specific regions were located and the primers designed were forward primer K12FP: 5′-AAGGGAGAATGATTGCCATGAA-3′, and reverse primer K12RP: 5′-GAG-TTTGGACAGTCATCAGTAATAGTTG-3′, targeting a 144-base-pair stretch including the *salA* ATG start codon. As TaqMan® probe we designed K12Taq: 6-FAM-5′-AGAGGTACAGG-TTGGTTTG-3′-MGB. The primers were tested for possible cross-amplification with the DNA of six other *S. salivarius* strains [DSM20067, AC1802 (AC: strain collection of the German National Reference Center for Streptococci, Aachen), AC2051, AC3342, AC3994, and AC4381] as well as with the DNA of two *S. salivarius* isolates (identified by rapID 32 strep, BioMérieux identification system (BioMérieux, Marcy l'Etoile, France), and given profile numbers 60077041112 and 60457041112) found initially in the oral cavity of the subject (grown from sample 0).

Real-time quantitative-polymerase chain reaction

Amplification and detection of DNA by real-time quantitative-polymerase chain reaction was performed with the aid of the ABI-PRISM 7000 Sequence Detection System (Applied Biosystems) using optical grade 96-well plates. Included in each run were four negative controls (nuclease-free water as template), and serial dilutions of DNA (corresponding to 10^2–10^6 CFU) of *S. salivarius* K12 used as standard for calibrating the *salA* gene target molecule numbers determined by real-time quantitative-polymerase chain reaction. All samples were analysed using the TaqMan® assay chemistry (TaqMan® PCR Master Mix, Applied Biosystems). Final reactions contained 100 nM of each primer and 2 μl template DNA (approximately 50 ng template DNA). The temperature profile was as follows: denaturation 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, and stringent annealing at 60°C for 1 min 45 s.

Data acquisition and subsequent analysis were performed using the ABI-PRISM 7000 SDS software (Applied Biosystems). The amount of initial template DNA was calculated by determining the threshold cycle (C_t), which is the number of polymerase chain reaction cycles required for the fluorescence to exceed a threshold value significantly higher than the background fluorescence. A threshold value of 0.2 was assumed which was approximately 10 times the background fluorescence, defined as the mean fluorescence values of the first 6–15 polymerase chain reaction cycles. All samples were run in duplicate, and the mean value was used for analysis. The coefficient of variation of the C_t-values among replicates was below 1%.

Results

To assess the validity of using a *salA* gene probe and primer set for specifically measuring the probiotic strain *S. salivarius* K12, six other *S. salivarius* strains as well as two isolates obtained from the oral cavity of the male proband were tested for contrast. All the strains tested except the target strain *S. salivarius* K12 tested negative by real-time quantitative-polymerase chain reaction, indicating the high specificity of this primer set (data not shown). As the *salA* gene levels of the probiotic strain measured by real-time quantitative-polymerase chain reaction were calibrated using serial dilutions ranging from 10^2 to 10^6 CFU, quantitative data are expressed as 'CFU equivalents of *salA* copies' (*salA*-CFU).

The *salA*-CFU varied considerably among the samples from different locations and time periods (see Table 1). Teeth and sulci (samples 7–14) were negative for K12 over the whole testing period. As these locations are known to harbor a

Table 1. Levels of probiotic strain *Streptococcus salivarius* K12 over time (4–35 days) at various oral sites monitored by real-time quantitative-polymerase chain reaction and referred to as CFU equivalents to *salA* gene copy numbers (*salA*-CFU)

Time (days)	PxR	PxL	BuR	BuL	ToD	ToV	SaC	SaS
Total aerobes, day 4	9000×10^3	4000×10^3	3000×10^3	7000×10^3	$120,000 \times 10^3$	$24,000 \times 10^3$	$300,000 \times 10^3$	$200,000 \times 10^3$
Total anaerobes, day 4	$29,000 \times 10^3$	$14,000 \times 10^3$	4000×10^3	5000×10^3	$250,000 \times 10^3$	$96,000 \times 10^3$	$380,000 \times 10^3$	$210,000 \times 10^3$
K12								
Day 4	1600	400	100	1000	2500	45,800	700	23,900
Day 8	32,900	123,700	3100	12,800	0	7800	6000	1700
Day 14	300	100	0	100	0	0	0	0
Day 21	500	0	0	0	200	800	900	0
Day 28	100	0	0	0	0	0	0	0
Day 35	100	0	0	0	0	0	0	0

PxR, pharynx, right; PxL, pharynx, left; BuR, buccal mucosal membrane, right; BuL, buccal, left; ToD, tongue, dorsal; ToV, tongue, ventral; SaC, saliva collected by chewing cotton tampon for 1 min; SaS, paraffin-stimulated saliva. Further samples (teeth: 14, buccal; 24, palatinal; 34, buccal; 44, lingual; sulci gingivae of teeth 14, palatinal; 24, buccal; 34, lingual, and 44, buccal) are not listed, as they remained K12-negative over the whole test period.

variety of bacteria, including various streptococcal species, in high cell numbers (10^8–10^9/mg plaque), negative findings here indirectly confirm the specificity of the K12 directed real-time quantitative-polymerase chain reaction assay.

K12 was detected at all other sites, but at different levels and with individual time profiles (Fig. 1). The amount of salA-CFU per sample ranged from 1×10^2 (detection level) to a maximum of 1.24×10^5. In the pharynx K12 had an asymmetric presence: at the right side we found K12 until day 35 with a maximum of 3.3×10^4 salA-CFU at day 8. In contrast, at the left side K12 was found for a shorter period (until day 14), but with a maximum of 1.24×10^5 salA-CFU at day 8. Relatively high levels of salA-CFU were also detected on the ventral side of the tongue (4.6×10^4/swab maximum at day 4), in paraffin-stimulated saliva (2.4×10^4/ml maximum at day 4), and on the buccal mucosal membrane, left side (1.3×10^4/swab maximum at day 8). In terms of probiotic effect (health benefit) or microbial impact, the amount of K12 detected after day 14 (see Table 1) appeared negligible.

Discussion

The aim of this study was to design a real-time quantitative-polymerase chain reaction (TaqMan® format) to determine the potential colonization sites of the SalA-producing probiotic S. salivarius K12 in the human oral cavity.

The target sites for S. salivarius K12 are the pharynx, tongue and buccal membranes, habitats of agents causing pharyngitis and/or halitosis. The maximum K12 cell numbers (salA-CFU) per swab found at these sites were 3.3×10^4 and 1.24×10^5 (pharynx), 3.1×10^3 and 1.3×10^4 (buccal), 2.5×10^3 and 4.6×10^4 (tongue) and 6×10^3 and 2.4×10^4/ml saliva. Taking into account that the swabs contained a total number of bacterial cells between 3×10^6 (buccal) and 1.2×10^8 (tongue, dorsal) aerobes, and between 4×10^6 (buccal) and 2.5×10^8 (tongue, dorsal) anaerobes, the proportion of K12 can be calculated and reached only a maximum of 1% (pharynx, left side, day 8) relative to the total microbial flora.

Burton et al. measured the prevalence and abundance of K12 in 13 subjects (and 10 controls) after chlorhexidine rinse and K12 lozenges (in that case K12^{Str}, a variant resistant to 100 µg/ml streptomycin) (3). In contrast to our study, only saliva was investigated and enumeration was performed by plating 10-fold dilutions onto Mitis-salivarius-agar (for S. salivarius) and Mitis-salivarius-streptomycin agar (for strain K12^{Str}). As a result, the mean S. salivarius cell counts were between 4.5 and 7.8×10^7 CFU/ml and the cell counts of strain K12^{Str} were between 1.8 and 5.8×10^7 CFU/ml, measured at days 7 and 14 after commencing treatment. In our study, the CFU equivalents per ml saliva were much lower (between 2.4×10^4 on day 4 and 1.7×10^3 on day 8), a difference that was most likely because, after the initial 3-day period of four K-12 lozenges per day, in the study by Burton et al. the subjects continued taking the K12 morning and night for the next 2 weeks. Thus, even if tested on only a single subject the study shows that the introduction of K12 (four times a day for 3 days) into the oral cavity does not necessarily result in high numbers or long-term colonization of K12, and therefore repeated administration of this strain might be required at certain intervals.

Nonetheless, as salA-CFUs were higher on day 8 than on day 4 in most of the mucous membrane samples, K12 cells must have multiplied in situ, indicating the potential for starting colonization of the oral surfaces. In any case it is important to note that the numbers of probiotic cells present in probiotic lozenges, drinks, or washes are not necessarily the same numbers that actually colonize the target, e.g. mucous membranes. To achieve optimal probiotic effects it would be important to monitor the amount and persistence of K12 in several individuals and link these data with treatment effects. Questions worth considering could include: (i) the influence of the pre-treatment regimen (type, concentration, and potency of antimicrobial agent as well as duration and frequency of treatment) on achieving an adequate initial reduction of pharyngitis- or halitosis-associated bacteria (11, 14); (ii) formulation (lozenges, chewing gums, mouth-wash, etc.) of the probiotic and timing of its administration; and (iii) the influence of anti-microbial (onions, garlic, spices) or other pro-microbial (probiotic yoghurt) foods and beverages consumed during treatment.

Since S. salivarius K12 is a non-traditional probiotic species, it generally evokes greater concerns about potential adverse effects but the most recent study by Burton et al. documented the absence of (i) adverse reactions in 14 subjects actively ingesting S. salivarius K12, (ii) major streptococcal virulence factors (sagA for streptolysin S, scpA for C5a peptidase, smez-2 for SMEZ-2, speB for SPE-B and emm for M-Proteins), and (iii) relevant antimicrobial resistances or acquisition of
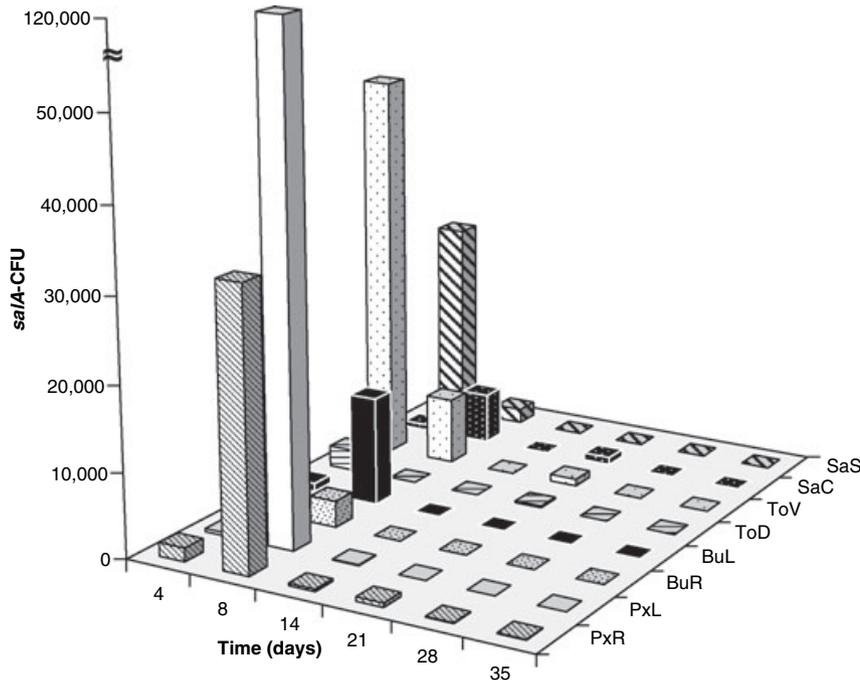

Fig. 1. Levels of probiotic strain *Streptococcus salivarius* K12 over time (4–35 days) at various oral sites monitored by real-time quantitative-polymerase chain reaction and referred to as CFU equivalents to salA gene copy numbers (salA-CFU). For abbreviations see Table 1.

corresponding resistance genes (5). Added to this is New Zealand's 5- to 6-year history of commercial probiotic K12 distribution and application with no known adverse side-effects (more than 150,000 doses administered, mainly to children). The probiotic *S. salivarius* K12 can therefore be referred to as 'safe' and unlikely to cause disease in healthy humans (5).

In conclusion, the real-time quantitative-polymerase chain reaction assay developed in this study enabled us to portray a heterogeneous distribution pattern for probiotic K12 that may or may not be unique to the individual that was monitored. In any case, we now have the tools for more extensive research and a method for monitoring other individuals to test for the consistency of such profiles and ascertain a correlation between K12 prevalence and treatment efficacy.

Acknowledgments

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