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Antimicrobial activity of *Streptococcus salivarius* K12 on bacteria involved in oral malodour

L. Masdea [a], *E.M. Kulik* [a], *I. Hauser-Gerspach* [a,*], *A.M. Ramseier* [a], *A. Filippi* [b], *T. Waltimo* [a]

[a] *Institute of Preventive Dentistry and Oral Microbiology, School of Dental Medicine, University of Basel, Switzerland*
[b] *Department of Oral Surgery, Oral Radiology and Oral Medicine and the Centre of Dental Traumatology, School of Dental Medicine, University of Basel, Switzerland*

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ABSTRACT

Objective: To investigate the antimicrobial activity of the bacteriocin-producing strain *Streptococcus salivarius* K12 against several bacteria involved in halitosis.
Design: The inhibitory activity of *S. salivarius* K12 against *Solobacterium moorei* CCUG39336, four clinical *S. moorei* isolates, *Atopobium parvulum* ATCC33793 and *Eubacterium sulci* ATCC35585 was examined by a deferred antagonism test. *Eubacterium saburreum* ATCC33271 and *Parvimonas micra* ATCC33270, which have been tested in previous studies, served as positive controls, and the Gram-negative strain *Bacteroides fragilis* ZIB2800 served as a negative control. Additionally, the occurrence of resistance in *S. moorei* CCUG39336 to *S. salivarius* K12 was analysed by either direct plating or by passage of *S. moorei* CCUG39336 on chloroform-inactived *S. salivarius* K12-containing agar plates.
Results: *S. salivarius* K12 suppressed the growth of all Gram-positive bacteria tested, but the extent to which the bacteria were inhibited varied. *E. sulci* ATCC35585 was the most sensitive strain, while all five *S. moorei* isolates were inhibited to a lesser extent. Natural resistance seems to be very low in *S. moorei* CCUG39336, and there was only a slight decrease in sensitivity after exposure to *S. salivarius* K12 over 10 passages.
Conclusion: Our studies demonstrate that *S. salivarius* K12 has antimicrobial activity against bacteria involved in halitosis. This strain might be an interesting and valuable candidate for the development of an antimicrobial therapy for halitosis.

1. Introduction

Oral malodour, also called halitosis, afflicts a significant proportion of the adult population and is of common interest due to its compromising influence in social and working environments. Most halitosis oral malodour compounds are by-products of the metabolism of certain species of oral bacteria, mainly those on the dorsum of the tongue.[1,2] These compounds consist of VSC (volatile sulphur compounds), valeric acid, butyric acid and putrescine.[2] A diverse group of Gram-negative and Gram-positive bacteria has been found to contribute to the problem. By contrast, certain bacterial species that predominate in the mouths of "healthy" subjects are noticeably absent in subjects with halitosis.[3]

Current treatments focus on the use of chemical or physical antibacterial regimens to reduce the numbers of these bacteria. The treatments typically provide only short-term relief because

* *Corresponding author at*: Institute of Preventive Dentistry and Oral Microbiology, School of Dental Medicine, University of Basel, Hebelstrasse 3, 4056 Basel, Switzerland. Tel.: +41 061 267 25 98; fax: +41 061 267 26 58.
E-mail address: I.Hauser-Gerspach@unibas.ch (I. Hauser-Gerspach).
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the offensive bacteria quickly recover after treatment is stopped.[4]

The use of probiotics has long been popular in the food industry. The World Health Organisation defines probiotics as a 'live organism which when administered in adequate amounts confers a health benefit on the host'. Their use in clinical practice has previously been discussed.[5] One potential and clinically important use of probiotics is in the prevention of dental caries.[6–10]

Preventing the re-growth of odour-causing organisms through the pre-emptive colonisation of the oral cavity with non-odorous, commensal microorganism may be a reasonable alternative to chemical or physical antibacterial regimens. Given that the dorsum of the tongue is the origin of most halitosis problems, a candidate probiotic to counter this condition should be able to persist in this particular ecosystem. The production of anti-competitor molecules such as bacteriocins also appears to confer an ecological advantage to some bacteria. A probiotic strain that efficiently colonises the tongue surface and does not produce odours metabolic by-products would be highly advantageous.

Streptococcus salivarius is known to be a pioneer coloniser of oral surfaces and is found predominant in 'healthy' humans not affected by halitosis.[3] BLIS K12 Throat Guard lozenges (BLIS Technologies, Centre for Innovation, Dunedin, New Zealand) contain *S. salivarius* K12, which has been shown to help maintain throat health by supporting the defence against undesirable bacteria.[11] The bacterium is not genetically modified or engineered, and the product is available in three flavours (vanilla, strawberry and peppermint). The particular strain used produces two natural antibacterial peptides, salivaricin A2[12,13] and salivaricin B,[14] which are lantibiotic-type bacteriocins. In deferred antagonism studies, *S. salivarius* K12 inhibited the Gram-positive bacteria *Streptococcus anginosis* T29, *Eubacterium saburreum* and *Micromonas micros*, which are implicated in halitosis, and significantly inhibited black-pigmented colony types present in saliva samples.[4]

Based on these investigations and other promising results, *S. salivarius* K12 has an excellent potential for use as a probiotic targeting halitosis producing bacteria.

The aim of this study was to evaluate the extent of the inhibitory spectrum of *S. salivarius* K12 against three additional bacterial species recently found to be implicated in halitosis and to investigate the development of bacterial resistance against *S. salivarius* K12.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacteriocin-producing strain *S. salivarius* K12 and the nonproducer *S. salivarius* MU, were kindly provided by Prof. J. Tagg (Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand).[4]

The indicator strains used in this study included the following: *E. saburreum* ATCC 33271; *Parvimonas micra* (previously known as *Micromonas micros* or *Peptostreptococcus micros*) ATCC 33270, which served as a positive control[4] and *Bacteroides fragilis* ZIB 2800 (School of Dental Medicine, University of Basel, Switzerland), which served as a negative control. The test strains included *Atopobium parvulum* ATCC 33793, *Eubacterium sulci* ATCC 35585, *Solobacterium moorei* CCUG 39336 and four clinical *S. moorei* isolates, CH1#23, CH3A#109A, CH3#63 and CH8#20,[15] which had, to date, not yet been tested for susceptibility against *S. salivarius* K12 *in vitro*.

All bacteria were grown on Columbia agar (Columbia Agar Base [BBL Becton Dickinson, Allschwil, Switzerland]) supplemented with 4 mg/l hemin (Fluka, Buchs, Switzerland), 1 mg/l menadione (VWR International, Dietikon, Switzerland) and 50 ml/l human blood (Blutspendezentrum, Basel, Switzerland) under anaerobic conditions (Oxoid AnaeroGen Compact, Oxoid, Pratteln, Switzerland) at 37 °C for 2–4 days.

2.2. Antimicrobial activity of *S. salivarius* K12

Inhibitory activities of *S. salivarius* K12 and the salivaricin non-producer *S. salivarius* MU were analysed using a modified deferred antagonism test.[16] Sterile blotting paper (Inapa Schweiz AG, Regensdorf, Switzerland) was cut to the size of 9 cm × 1 cm and carefully immersed in a *S. salivarius* culture with a density of 4-5 McFarland standard. After removing excess fluid, the blotting paper was placed in the middle of a plate of Columbia agar containing 5% human blood and 0.1% calcium carbonate ($CaCO_3$) (E. Merck, Darmstadt) left in place for 2 s and then removed. The plates were incubated at 37 °C under anaerobic conditions for 24 h. After incubation, the growth was removed with a sterile cotton swab. To kill any residual bacterial cells on the mediums surface, the plate was exposed to chloroform (E. Merck, Darmstadt) vapours for 30 min at room temperature. The plate was then aired for 30 min.

Several colonies of each indicator strain grown on Columbia blood agar-calcium carbonate medium were suspended in 3 ml Todd-Hewitt broth and streaked at right angles to the original *S. salivarius* culture zone with a sterile cotton swab. The plates were incubated under anaerobic conditions at 37 °C for at least 48 h, and the extent of inhibition was recorded in mm (the distance between the original producer line and the inhibition line of indicator strains). Each test was performed at least three times.

2.3. Test for resistance of *S. moorei* CCUG 39336 against *S. salivarius* K12

S. salivarius K12 or *S. salivarius* MU cells were each suspended in 3 ml Todd Hewitt broth and swabbed onto Columbia blood agar-calcium carbonate medium. Afterwards, the plates were incubated at 37 °C under anaerobic conditions for 24 h until confluent growth was observed. Bacterial cells were removed from the plates with sterile cotton swabs, and the agar surfaces exposed to chloroform vapour for 30 min and aired for another 30 min. Control plates without *S. salivarius* were also exposed to the same conditions.

To detect bacteriocin-resistant *S. moorei* isolates, several colonies of *S. moorei* CCUG 39336 were inoculated in 2 ml Todd-Hewitt broth. After incubation at 37 °C under anaerobic conditions for 24 h, 1 ml of this suspension was centrifuged at 10,000 rpm for 15 min at 15 °C and resuspended in 300 μl Todd-Hewitt broth. The exact cell density was determined by plating appropriate dilutions onto Columbia blood

agar-calcium carbonate medium. One-hundred microlitres of this *S. moorei* suspension was streaked onto the agar plate pretreated with *S. salivarius* K12 and 100 μl onto the agar plate pretreated with *S. salivarius* MU.

2.4. Test for induction of resistance in S. moorei CCUG 39336 against the bacteriocins from S. salivarius K12

Bacteriocin-producing *S. salivarius* K12 and the indicator strain *S. moorei* CCUG 39336 were grown, streaked onto Columbia blood agar-calcium carbonate medium and incubated as described above for the modified deferred antagonism test. The *S. moorei* colonies closest to the inhibition zone were subcultivated onto Columbia blood agar-calcium carbonate medium and again tested against *S. salivarius* K12. This procedure was repeated for 10 passages.

2.5. Statistical analysis

The inhibitory activity of *S. salivarius* K12 against the indicator strains was tested using a linear model. The dependent

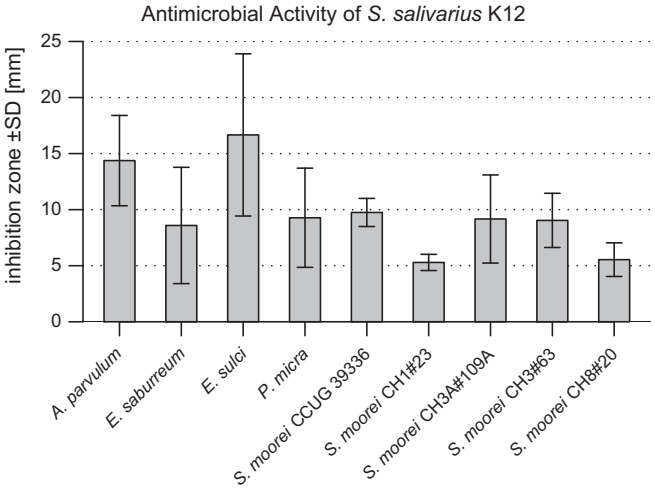

Fig. 1 – Mean inhibition zone ± standard deviation of *S. salivarius* K12 against nine Gram-positive indicator strains (*n* = 3).

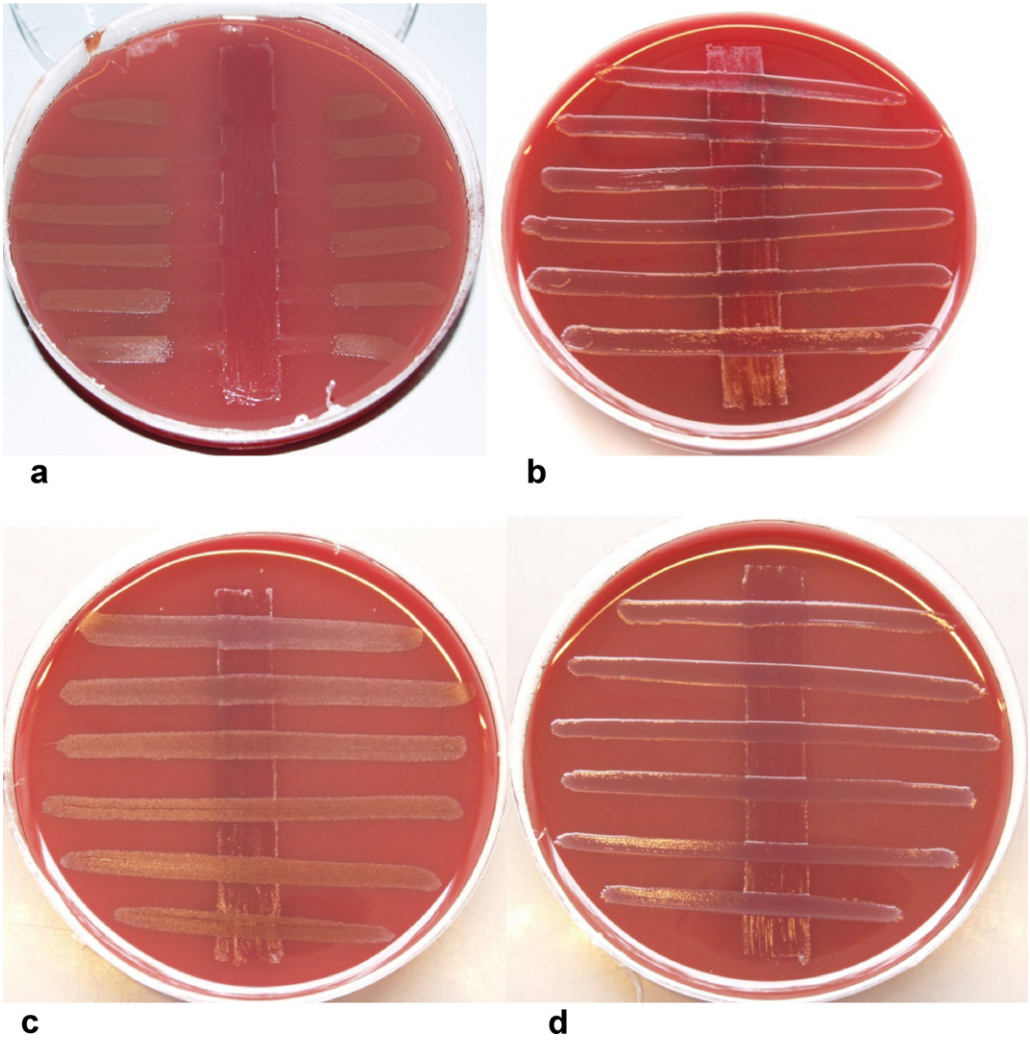

Fig. 2 – Inhibitory effect of *S. salivarius* K12 (a and b) compared to *S. salivarius* MU (c and d). A clear inhibition zone was produced against *S. moorei* CCUG 39336 culture (a), whereas growth of *B. fragilis* was not inhibited by *S. salivarius* K12 (b). The non-producer strain *S. salivarius* MU did not inhibit the growth of either *S. moorei* CCUG 39336 (c) or *B. fragilis* (d).

variable was the size of the inhibition zone, the independent variable was the indicator strain. To compare the inhibition zones of each indicator strain against the salivaricin non-producer *S. salivarius* MU, a model with no intercept term was used. Means were estimated with 95% confidence intervals with corresponding *P*-values.

To analyse the induction of resistance in *S. moorei* CCUG 39336 against the salivaricins from *S. salivarius* K12, a linear mixed effects model (LME) was used because data structures with serial dependency had to be described. The dependent variable was the size of the inhibition zones and the independent variable was the passage number. The experimental unit was treated as a random factor.

The results are presented as differences of means, with 95% confidence intervals and corresponding *P*-values. *P*-values < 0.05 were considered statistically significant.

All statistical evaluations and graphs were done with the publicly available R software v. 2.14.0 for Windows®.[17] The linear model was calculated using function lm( ) and corresponding confidence intervals were calculated using the function confint( ) (package stats). The linear mixed effects model was calculated using the function lme( ) and confidence intervals were estimated using the function intervals( ) (package nlme). Tests for normality of distribution were conducted using the function qqPlot( ) fom the package car. No systematic deviations from normal distribution were observed. For creation of graphs, packages plotrix and gplots were used.

3. Results

3.1. Antimicrobial activity of S. salivarius K12

All Gram-positive indicator strains were inhibited by *S. salivarius* K12 (Fig. 1), while *B. fragilis*, a Gram-negative bacterium, was not inhibited (Fig. 2b). The mean size of the inhibition zones for the five *S. moorei* isolates were between 5.3 mm for *S. moorei* CH1#23 and 9.8 mm for the type strain *S. moorei* CCUG 39336. The zones of inhibition for *E. saburreum* and *P. micra* were in the same range, whereas *A. parvulum* and *E. sulci* where more susceptible to *S. salivarius* K12 with inhibition zones of 14.4 mm and 16.7 mm, respectively. Variability was evident within most species. *E. saburreum*, *E. sulci* and *P. micra* showed the biggest variation in the results, whereas *S. moorei* CCUG 39336 and *S. moorei* CH1#23 had the smallest variation.

The bacteriocin-nonproducing strain *S. salivarius* MU did not inhibit any of the indicator strains (Fig. 2c and d). The inhibition of all Gram-positive indicator bacteria by *S. salivarius* K12 was statistically significant ($P < 0.001$) when compared to the bacteriocin-nonproducing strain *S. salivarius* MU (Table 1).

Table 1 – Results of the statistical analysis of the inhibition of the Gram-positive indicator bacteria by *S. salivarius* K12 compared to the bacteriocin-nonproducing strain *S. salivarius* MU. Shown are the respective indicator strain, the estimated mean differences (est. mean difference) in mm, the upper and lower 95% confidence intervals (95% confint) in mm and the corresponding *P*-values.

Indicator strain	Est. mean difference	95% confint		*P*-Value
		Lower	Upper	
A. parvulum	14.38	12.40	16.35	<0.001
E. saburreum	8.59	6.52	10.66	<0.001
E. sulci	16.67	12.71	20.63	<0.001
P. micra	9.28	6.99	11.56	<0.001
S. moorei CCUG 39336	9.75	7.33	12.17	<0.001
S. moorei CH1#23	5.29	3.31	7.27	<0.001
S. moorei CH3A#109A	9.17	6.88	11.45	<0.001
S. moorei CH3# 63	9.04	7.06	11.02	<0.001
S. moorei CH8#20	5.54	3.56	7.52	<0.001

3.2. Test for resistance of S. moorei CCUG 39336 against S. salivarius K12

To test for an intrinsic resistance of *S. moorei* against the bacteriocins produced by *S. salivarius* K12, up to 9.1×10^7 *S. moorei* CCUG 39336 cells were streaked on plates previously seeded with *S. salivarius* K12 or *S. salivarius* MU. No growth could be detected on the plates pretreated with *S. salivarius* K12 even after prolonged incubation, while there was confluent growth of *S. moorei* CCUG 39336 on plates pretreated with *S. salivarius* MU.

3.3. Test for induction of resistance in S. moorei CCUG 39336 against the bacteriocins from S. salivarius K12

The inhibition zones of *S. moorei* CCUG 39336 decreased slightly with each passage, from 8.2 ± 0.6 mm at the beginning to 6.2 ± 0.3 mm after 10 passages (Fig. 3). Comparing inhibition zones of successive passages with those of the first passage, the linear mixed-effect model indicated that this reduction was statistically significant (Table 2). From the 5th passage on, all differences were highly significant ($P < 0.001$).

4. Discussion

To compete with other species for nutrients in the same ecological niche, many different bacterial species produce

Table 2 – Results of the statistical analysis comparing the size of the inhibition zones of the first passage with those of the following passages. Shown are the respective passage number, the estimated mean differences (est. mean difference) in mm, the upper and lower 95% confidence intervals (95% confint) in mm and the corresponding *P*-value.

Passage number	Est. mean difference	95% confint		*P*-Value
		Lower	Upper	
2	−0.67	−1.20	−0.14	0.017
3	−0.83	−1.36	−0.30	0.004
4	−0.67	−1.20	−0.14	0.017
5	−1.83	−2.36	−1.30	<0.001
6	−2.00	−2.53	−1.47	<0.001
7	−2.33	−2.86	−1.80	<0.001
8	−1.67	−2.20	−1.14	<0.001
9	−2.00	−2.53	−1.47	<0.001
10	−2.00	−2.53	−1.47	<0.001

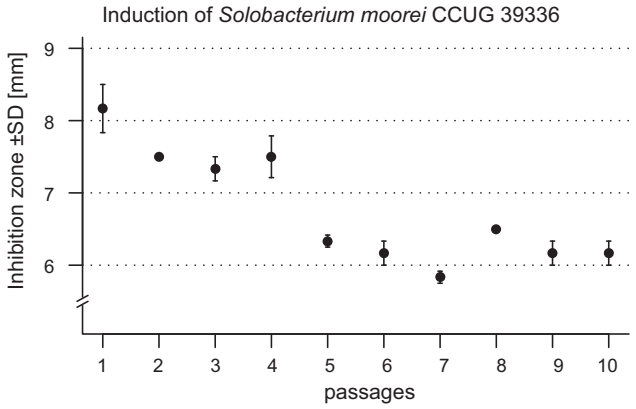

Fig. 3 – Test for induction of resistance in *S. moorei* CCUG 39336 against the bacteriocins from *S. salivarius* K12. Shown are levels of inhibition (mm) in different passages.

bacteriocins. These ribosomally synthesised peptides or proteins have antibacterial activity.[18,19] Due to their potential as food preservatives and their antagonistic effect against important food pathogens, the bacteriocins that have been studied most extensively are derived from lactic acid bacteria. The lantibiotic nisin, which is produced by different *Lactococcus lactis* spp., is the best studied bacteriocin and, so far, the only one that is used as a food additive.[20,21] However, bacteriocin-producing starter cultures are commonly used in food fermentations, and probiotic bacteria have recently gained increased interest and acceptance due to their potential health benefit. Production of antimicrobial substances against pathogens has been proposed as an important mechanism by which probiotic bacteria may improve human health.[22,23]

There have been few attempts to examine the effects of probiotic bacteria in the oral cavity.[24] This complex ecosystem is inhabited by more than 700 bacterial species,[25] some of which have been shown to produce antimicrobial substances, including bacteriocins. The caries pathogen *Streptococcus mutans* produces several kinds of bacteriocins called mutacins. The efficient replacement of indigenous cariogenic mutans streptococci by a genetically modified *S. mutans* strain is based on the production of the broad-spectrum lantibiotic mutacin 1140. Animal testing indicates that an avirulent *S. mutans* strain producing ethanol instead of lactic acid and harbouring mutacin 1140 can successfully displace other *S. mutans* strains and lead to significantly reduced level of caries.[26] Phase I safety trials using an auxotrophic strain are planned to determine the level of transmission of this bacterium.[27]

A few other studies have examined the effect of probiotic bacteria, mainly lactic acid bacteria, on salivary bacterial counts and caries prevention. These initial studies yielded promising results; a reduction of salivary *S. mutans* counts and a reduced caries risk was found in most studies.[6,7]

S. salivarius, one of the predominant commensal bacteria of the oral cavity, is known to produce bacteriocins and bacteriocin-like inhibitory substances, which makes *S. salivarius* strains promising candidates for the development of oral probiotics against oral infectious diseases. It has already been shown that *S. salivarius* can antagonise the action of *Streptococcus pyogenes*, the main etiological agent of bacterial pharyngitis in children; indeed, lozenges containing *S. salivarius* K12 are sold in some countries as an oral probiotic to maintain throat health.[11,28–31]

Therefore, the possibility of screening probiotics against several bacteria implicated in halitosis seems very promising. The experiments in the present study were performed to elucidate the inhibitory effect of the probiotic *S. salivarius* K12, which produces at least two lantibiotic bacteriocins, on strains of several species of Gram-positive bacteria.

Recently, the use of *S. salivarius* K12 as a probiotic in clinical practice has been tested.[5] In a deferred antagonism test, Burton et al. reported strong inhibition by *S. salivarius* K12 against Gram-positive halitosis associated species, including *E. saburreum* and *P. micra* (*M. micros*).[4] The procedures followed in our study were related to the test used by Tagg and Bannister.[16] The results demonstrated an inhibition of *E. saburreum* and *P. micra*, indicating adequate culture conditions for the bacteriocin-production of *S. salivarius*. In addition, Gram-negative bacteria showed no inhibition, which was consistent with the corresponding literature. Thus, the applied test arrangement could be routinely used to study further bacterial species implicated in halitosis.

S. moorei has recently been identified in specimens from patients suffering from halitosis.[3,15] Inhibition by *S. salivarius* K12 was demonstrated against type strain CCUG 39336 and four clinical isolates of *S. moorei* (CH1#23, CH3A#109A, CH3#63, CH8#20)[15] that originated from samples taken from the human oral cavity. *A. parvulum* ATCC 33793 and *E. sulci* ATCC 35585 were used for this study because they are known to be implicated in halitosis and because they had not been tested with the deferred antagonism test. The variation in inhibition zones of *E. saburreum*, *E. sulci* and *P. micra* could be attributed to their demanding growth conditions and challenging cultivation.

The development of strain resistance is of major concern for the *in vivo* application of probiotic strains, and the emergence of resistance against bacteriocins has been best documented for nisin. In laboratory settings, nisin-resistant bacteria can be obtained by repeatedly exposing sensitive strains to increasing amounts of nisin. Gram-positive and Gram-negative bacteria can exhibit resistance against nisin. The molecular mechanisms leading to nonsusceptibility have been shown to involve changes in the bacterial cell membrane or cell wall, although the precise nature of the factors involved in resistance development remains elusive, and bacteria may employ several strategies simultaneously to acquire nisin resistance (reviewed in Ref. 21).

A possible mechanism leading to the acquisition of resistance is horizontal gene transfer wherein genes are transferred between bacteria. This mode of gene transfer was demonstrated in *S. salivarius* K12 where the large plasmid harbouring the loci for bacteriocins production could be transferred *in vivo* into a plasmid-negative *S. salivarius* strain by oral transmission.[32,33] So far, no studies have been conducted to determine the host range of this plasmid or whether there is transmission to other oral streptococci or even to potential pathogens.

No resistance against the bacteriocins produced by orally administrated *S. salivarius* K12 have been reported so far. In our study, no intrinsic resistance of *S. moorei* CCUG 39336 against *S. salivarius* K12 could be detected, although there was a decrease in sensitivity when *S. moorei* CCUG 39336 was repeatedly exposed to *S. salivarius* K12 over 10 passages.

Further studies are needed to determine if resistances might also occur *in vivo*.

In contrast to the situation with antibiotics in which there is currently no antibiotic in clinical use to which resistance has not developed, bacteriocin resistance does not yet pose a serious problem. However, cross-resistance between bacteriocins have been observed and thought to represent a general mechanism of resistance, and this emphasises the need for efficient and safe probiotics.[34,35]

5. Conclusions

In conclusion, our study demonstrated that the bacteriocin-producing strain *S. salivarius* K12 displayed antimicrobial activities against several halitosis bacteria including *S. moorei*, which has recently been found to be a major contributor to oral malodour. Additionally, the type strain *S. moorei* CCUG 39336 did not seem to have a natural resistance against *S. salivarius* K12, and there was only a slight decrease in sensitivity after repeated exposure to *S. salivarius* K12. Based on these results, *S. salivarius* K12 might be an interesting and valuable candidate for the development of an antimicrobial therapy to treat oral malodour.

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Competing interests

None declared.

Ethical approval

Not required.

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