A basic study on the effect of *Streptococcus salivarius* K12 on the suppression of oral malodor in beagles

Hirotaka Matsumoto, Tomoko Okusa, Hiroki Yoshimatsu, Yohei Mochizuki, Takahiro Teshima, Hidekazu Koyama

Division of Therapeutic Sciences I, Department of Veterinary Clinical Medicine, School of Veterinary Medicine, Nippon Veterinary and Life Science University, Musashino-shi, Tokyo 180-8602

E-mail: matsumoto@nvlu.ac.jp

**Abstract**: The effects of daily administration of *Streptococcus salivarius* K12 (SSK12) on mouth odor and oral bacteria were investigated in beagles. Beagles given SSK12 (SSK12 group n=3) tablets and those given no SSK12 (control group n=3) were subjected to organoleptic tests of oral gas odor and expired gas odor and measurement of oral ammonia, methylvamine, methylmercaptan, and hydrogen sulfide by using a gas detector meter. Oral swabs were cultured anaerobically for bacteriologic examination and were also used for detection of Porphyromonas (*P. gingivalis* and *P. gulae*) by using a direct PCR method and a PCR method using black pigment-producing bacteria. Oral bacterial count was determined using real-time PCR. Organoleptic tests revealed a decrease in the assessment score of oral gas odor and an increase in the distance at which expired gas odor was detectable in beagles given SSK12. In comparison with the baseline level, the ammonia concentration decreased in the SSK12 group after 4 weeks of administration, whereas the corresponding concentration increased in the control group at 4 weeks. The concentration of methylvamine significantly increased (p<0.05) after 4 weeks in comparison with the baseline value in the control group, but there was no significant change in the SSK12 group. Oral swabs and black pigment-producing bacteria showed no presence of *P. gingivalis* and there were no distinct changes in the oral bacterial count. These findings suggest that SSK12 can be expected to exert a mouth odor-suppressing effect in dogs. However, further investigation is required for evaluating the effect of SSK12 on oral bacteria.


**Key words**: Oral malodor, Probiotics, *Streptococcus salivarius* K12

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**Introduction**

Probiotics were defined in 1989 by Fuller et al. as live microorganisms that have health-promoting effects on the host by changing the balance of intestinal resident bacteria[1]. Subsequently, in 1998, probiotics were re-defined by Salminen et al. as live microorganisms that have health-promoting effects on the host or as food products containing such microorganisms. The usefulness and safety of probiotics have been widely investigated[9], and in recent years their efficacy has been reported in the oral cavity as well as in the intestinal tract. Degraded protein products contained in the dental plaque cause...
malodor. The malodorous substances are produced by oral Gram-negative anaerobic bacteria and include short-chain fatty acids such as butyric acid and isovaleric acid; volatile sulfur-containing compounds such as monomethylamine, hydrogen sulfide, and methyl mercaptan; and nitrogen compounds such as ammonia. In particular, periodontal pathogens such as *Porphyromonas* (*P.* gulae are known to be highly active in producing these compounds[6]. Oral malodor of companion animals not only makes owners uncomfortable, but also reflects the growth of these periodontal pathogens. It is thus considered one of the symptoms of periodontal disease. Prevention of oral malodor is therefore important for the prevention of periodontal disease. In veterinary medical care, tooth brushing at home and scaling and polishing at the veterinary clinic are performed as a means of preventing oral malodor. Although there is no doubt about the efficacy of these treatments, various associated problems limit their effectiveness. For example, few pet owners are capable of appropriately performing brushing at home, and scaling requires general anesthesia, thus precluding frequent treatments.

*Streptococcus. salivarius* K12 (SSK12) is a nonpathogenic oral streptococcus isolated from the mouth of a healthy child in New Zealand[10]. In addition to its probiotic effect, *S. salivarius* is known to produce lantibiotics Salivaricin A2 and B, which suppress the growth of other bacterial species[7,11]. While its efficacy in humans has been reported, there is no report to our knowledge of the oral malodor-preventing effect in animals. The objective of this study is therefore to evaluate the effect of a SSK12-containing supplement on oral malodor, and on the change in oral bacterial flora in healthy dogs.

### Materials and Methods

This study was conducted after obtaining the approval of the animal care and use and bioethics committees of Nippon Veterinary and Life Science University, Tokyo, Japan.

**Experimental animals:** This study included 6 male beagles weighing 10.0-13.4 kg that were raised in Nippon Veterinary and Life Science University and were proven to have no abnormalities on blood biochemical examination. With regard to dental plaque and calculus in these beagles, the oral hygiene index was 18 or less, the maximum debris index was 2 or less for each block, and the maximum calculus index was 1 or less for each block. The gingival index and periodontal index for evaluation of gingivitis and periodontitis were both less than 2 points. All beagles were fed the same dry food for adult dogs twice a day at 7:00 and 19:00 since more than 2 months before the initiation to the end of this study. Each test was performed at the mid-period between the two feeding times every day. No routine oral care was conducted during this period.

**Feeding of SSK12:** Dentalbio™ (Kyoritsu Seiyaku Corporation, Tokyo) containing $8.0 \times 10^9$ cfu/g of SSK12 per tablet was used. In the SSK12 group ($n=3$), the recommended dose (two tablets per day) was mixed with the morning diet and given to the dogs. The administration period was 4 weeks. Control dogs ($n=3$) were given no SSK12.

**Test for malodor in oral cavity gas:** Two types of sensory tests were performed: (i) a test for the odor of oral cavity gas in which the tester took a sniff of odor in the oral cavity by bringing his nose as close to the open mouth as possible, and (ii) a test for breath odor in which the longest distance to detect breath odor was measured. The same tester performed both tests. The evaluation criteria for this sensory test for the odor of oral cavity gas are shown in Table 1. The head of each test animal was manually retained in the upright position. Oral gas components were measured in the following manner: a detector tube connected with a gas detector meter

<table>
<thead>
<tr>
<th>Score</th>
<th>Odor Intensity</th>
<th>Odor Quality</th>
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<tbody>
<tr>
<td>0</td>
<td>No odor</td>
<td>No smell above the threshold of olfac-tory sensation</td>
</tr>
<tr>
<td>1</td>
<td>Very light</td>
<td>Abnormal smell not recognized as mal-odor</td>
</tr>
<tr>
<td>2</td>
<td>Light</td>
<td>Smell recognized as slight malodor</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>Smell easily recognized as malodor</td>
</tr>
<tr>
<td>4</td>
<td>Strong</td>
<td>Strong but tolerable malodor</td>
</tr>
<tr>
<td>5</td>
<td>Very strong</td>
<td>Intolerable, violent malodor</td>
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Table 1 Criteria for sensory test for oral gas odor
(GASTEC CORPORATION, Kanagawa) was inserted into the oral cavity on the left buccal side, and oral gas aspirated by the detector tube was measured immediately. Four types of detector tubes were used to detect the following gases: Ammonia (No.3 L Ammonia Detector Tube, Gastec Corporation, Kanagawa), methylamine (No.180 Amines Detector Tube, Gastec Corporation, Kanagawa), methyl mercaptan (No.70L Mercaptans Detector Tube, Gastec Corporation, Kanagawa), and hydrogen sulfide (No.4 L hydrogen sulfide detector tube Gastec Corporation, Kanagawa). These measurements were conducted before beginning the experiment and after 4 weeks of administration.

**Bacteriological test:** An oral swab sample was taken from the outside of the upper right canine using a sterilized swab, which was then added to 5.0 mL of TE buffer (10 mM Tris, 1 mM EDTA) (pH 8.0). This was considered the sample solution, and was immediately smeared onto brain heart infusion agar supplemented with 5% defibrinated sheep blood (BHA blood agar medium). Bacteria were then cultured at 37°C for 48 hours under anaerobic conditions. After the culture, the number of black colonies that grew on BHA blood agar medium was counted (Fig. 1). Direct PCR method using oral swabs was used to detect *P. gingivalis*, a bacterium reportedly involved in causing mouth odor[5], and *P. gulae*, a major oral bacterium in dogs[6]. As specific PCR primers, 5’-CCGCATACACTTGTATTATTGCGATTATT-3’ (Forward) and 5’-AACAGTTTACATCCATTGACTG TCT-3’ (Reverse) were used for *P. gingivalis* and 5’-TTGCTTGGATGATCGTGG-3’ (Forward) and 5’-GCTTATCTTAGTGATATTTCAC-3’ (Reverse) were used for *P. gulae*. A swab sample solution (1.5 mL), prepared by floating an oral swab on TE buffer, was placed in an Eppendorf tube and centrifuged at 12,000 rpm for 2-3 min. The precipitate was suspended in Insta Gene matrix and the resulting solution was incubated for 15 to 30 min in a thermostat bath at 56°C, followed by thorough mixing for 10 sec in a Vortex mixer. The mixture was centrifuged again for 2 to 3 min at 12,000 rpm, and the supernatant obtained was used as a DNA sample. Black-pigmented bacteria that grew on BHA blood agar medium under anaerobic conditions were subjected to PCR to identify *P. gingivalis* and *P. gulae*. The number of bacteria in the oral cavity was assessed by real-time (RT) -PCR. Oral swabs were used as samples, and a calibration curve was drawn using the following universal primers for *Escherichia coli*: 5’-TCCCTACGGGAGCGAGGTGT-3’ (forward) and 5’- GACTACAGGGTATCTGAATCCGT-3’ (reverse).

**Statistical analyses:** Results are expressed as mean± standard deviation. Statistical analyses were performed using the SPSS® tabistical software. Time-course changes in the same group were subjected to Tukey’s multiple comparison test. Comparisons between the control group and the SSK12 group at the same time point was performed using the Student- t test. In both tests, the significance level was set at 5%, with (p<0.05) considered significant.

**Results**

**Test for oral cavity gas odor:** In the control group, the maximum odor level of the oral cavity gas was 4.7±0.6 before the experiment and the minimum level was 3.3± 0.6 3 weeks after the start of the experiment. There were no significant changes in these observed values during the course of the experiment. In the SSK12 group, the starting odor level was 4.0±1.7, which decreased over time to 2.3±0.6 3 weeks. There were no significant differences within or between the two groups (Fig. 2).
Test for breath odor: In the control group, the distance was 10.0±0.0 cm before the start of the experiment, which increased over time, reaching a maximum level of 15.0±0.0 cm 4 weeks after the start of the experiment. The distance at which expired gas odor was detectable in the SSK12 group was 13.3±7.6 cm at the baseline and remained at 10 cm or more at 3 weeks, but decreased to 8.3±5.8 cm at 4 weeks. However, the ammonia concentration in this group decreased to 1.3±0.6 ppm at 4 weeks as compared with the baseline value of 2.0±0.5 ppm (Fig. 3).

Measurement of oral gas components: Neither methyl mercaptan nor hydrogen sulfide was detected in any group either before or 4 weeks after the start of the experiment. However, the observed ammonium level in the control group was 2.0±0.0 ppm before the experiment, and increased to 3.0±2.0 ppm 4 weeks after the start of the experiment. In contrast, in the SSK12 group, the ammonium level was 2.0±0.5 ppm before the experiment, but decreased to 1.3±0.6 ppm 4 weeks post treatment. Furthermore, there were no significant differences either within or between groups (Fig. 4). The methyamine levels in the control group were 4.2±2.3 ppm and 11.3±3.1 ppm before and 4 weeks after the start of the experiment, respectively, showing a significant increase ($p<0.05$) at the 4 week time point compared with the baseline level. On the other hand, the methyamine concentrations at baseline and at 4 weeks in the SSK12 group were 4.5±1.8 ppm and 8.3±2.9 ppm, respectively. Although the value was higher at 4 weeks, the difference between these values was not statistically significant (Fig. 5).

Bacterial culture from oral swabs with black pigment-producing bacteria as the index: No black pigment-producing bacteria were detected in either group from baseline to 3 weeks after beginning the experiment. However, at 4 weeks, these bacteria were detected in 3 dogs, i.e., 2 dogs in the control group and 1 dog in the SSK12 group. There was, however, no distinct difference in the bacterial count between the two groups (Table 2).

P. gingivalis and P. gulae detection by direct PCR: P. gingivalis was not detected in any of the dogs during the
Identification of isolated bacterial species by PCR: No PCR fragments specific to *P. gingivalis* or *P. gulae* were detected in any of the black-pigmented bacteria colonies.

**Measurement of oral bacterial count by RT-PCR:** The oral bacterial count increased after the beginning of the experiment compared with the baseline count in 2 of 3 dogs in both the control and *S. salivarius* K12 groups. Individual rates of increase of oral bacterial count were similar in the two groups (Fig. 6).

### Discussions

Since both the tests for the odor of oral cavity gas and breath odor are sensory tests that require a specific skill set, the same tester performed the sniff tests upon confirming the extent of the intertester error. These findings indicated that administration of SSK12 caused a decrease in the intensity and the quality of oral gas odor, resulting in an increase in the distance at which breath odor was detectable, thereby indicating the mouth odor-decreasing effect of SSK12. Gram-negative anaerobic bacteria including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* (*F. nucleatum*), and *P. gingivalis* are reported as representative periodontal pathogens in humans. However, in addition to *P. gingivalis*, other bacteria of the genus *Porphyromonas*, including *P. denticanis*, *P. salivosa*, and *P. gulae*, are also periodontal pathogens in dogs[4]. *F. nucleatum* and *P. gingivalis* both produce hydrogen sulfide from cysteine, and methyl mercaptan from methionine via the enzyme methioninase. In the present study, the presence of *F. nucleatum* was not assessed, however *P. gingivalis* was not detected in any of the experimental animals, as assessed by PCR analysis. In

#### Table 2 Culture bacteria in oral swabs using black-pigmented bacteria that grew on BHA blood agar medium as the index

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<thead>
<tr>
<th>Group</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Control</td>
<td>+++ 0</td>
<td>+++ 0</td>
<td>+++ 0</td>
<td>++ 0</td>
<td>+++ 0</td>
</tr>
<tr>
<td><em>S. salivarius</em> K12</td>
<td>+++ 0</td>
<td>+++ 0</td>
<td>+++ 0</td>
<td>++ 0</td>
<td>++ 0</td>
</tr>
</tbody>
</table>

this study, two sulfated compounds, hydrogen sulfide and methylmercaptan, were not detected in the oral gas. This seems to be related to the fact that no \textit{P. gingivalis} was detected. Although the ammonia concentration increased over time in the control group, there was a decrease in the ammonia concentration in the SSK12 group. In addition, the increase in the methylamine concentration was markedly suppressed by administration of SSK12. It is known that the recognition threshold of ammonia is 46.8 ppm, whereas the recognition threshold of methylamine is low, at 0.021 ppm\cite{3}. Methylamine was considered the main cause of the mouth odor recognized by the organoleptic test, and the results of this study suggest that the production of methylamine in the mouth was inhibited by SSK12. Since \textit{P. gingivalis} and \textit{P. gulae} are known to produce black pigments when cultured in blood agar medium, cells were cultured using black-pigmented bacteria as the index in the present study. The direct PCR method using oral swabs at 4 weeks after beginning the experiment showed presence of \textit{P. gulae} in all dogs, but \textit{P. gingivalis} was not detected in any of the dogs examined. In addition, neither \textit{P. gingivalis} nor \textit{P. gulae} was detected from black pigment-producing colonies. Therefore, the major bacterium responsible for mouth odor in this experiment may be \textit{P. gulae}. There were no distinct differences in the total oral bacterial count between the SSK12 group and the control group, as determined by the real-time PCR method. In addition to \textit{Porphyromonas} bacteria, oral anaerobic bacteria that produce compounds that cause mouth odor include \textit{Prevotella} sp., \textit{Fusobacterium} sp., and \textit{Peptostreptococcus} sp. It is possible that SSK12 exerts the mouth odor-suppressing effect by acting on these bacteria\cite{2}. However, the present study did not investigate the possible involvement of these bacterial species, therefore future studies on the effect of these bacteria are warranted.

In humans, it is shown that antibiotics produced by SSK12 decrease the number of periodontal pathogens by suppressing their growth and, as a result, decrease the causative substances of oral malodor, which are metabolites produced by these pathogens\cite{8}. This raises the possibility that no marked suppression of oral malodor can be achieved if periodontal pathogens are present at low levels.

The oral cavity and expired gas are cited as the sites of origin of mouth odor. It is known that ammonia and amines are increased in the expired breath in persons who have liver failure, whereas amines are increased in those with renal failure. These components are produced by enteric bacteria and absorbed in the blood\cite{10}. In humans, SSK12-containing tablets are generally chewed and then swallowed. On the other hand, in this study, the dogs swallowed the tablets without chewing. Therefore, it is necessary to conduct clinical investigations in patients who have periodontal disease and to evaluate the useful effect of SSK12 on expired breath.

References

H. Matsumoto, T. Okusa, H. Yoshimatsu, Y. Mochizuki, T. Teshima, H. Koyama


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ビーグルにおける*Streptococcus salivarius* K12による口臭抑制効果についての基礎的検討

松本浩毅・大草朋子・吉松宏基・望月庸平・手嶋隆洋・小山秀一

日本獣医生命科学大学獣医学部獣医学科臨床獣医学部門治療学分野  東京都武蔵野市 180 - 8602

要約：ビーグルに対して*Streptococcus salivarius* K12（SSK12）を連日給与することにより口臭や口腔内細菌に対する影響を検討した。SSK12の給与群（n=3頭）と無給与の対照群（n=3頭）を用いて口腔ガス臭と呼気臭の官能検査そして気体検知管測定器を用いて口腔内のアンモニア、メチルアミン、メチルメルカプタンおよび硫化水素を測定した。口腔内スワブを唾液培養し黒色素産生菌集落の数を求めた。

Porphyromonas (P.) gingivalisとP. gulaeの検出は、口腔内スワブと黒色素産生菌を用いてPCR法により行った。口腔内細菌数の測定はReal-time PCR法で行った。官能検査ではSSK12給与群の口腔ガス臭の評価ポイントの低下と呼気臭輸血までの距離の延長が認められた。また、対照群のアンモニア濃度は、実験前に比べその4週後は増加したがSSK12給与群ではSSK12給与後に低下した。対照群のメチルアミンは、実験4週後には有意（p<0.05）に増加したが、SSK12給与群では給与前後で有意差は認めなかった。口腔内スワブと黒色素産生菌の両者からはともにP. gingivalisは検出されず、口腔内細菌数の変化には明らかに差異は認められなかった。これらのことから、SSK12は犬の口臭抑制効果を期待できることが示唆されたが、口腔内細菌に対する効果についてはさらなる検討が必要である。

キーワード：Oral malodor, Probiotics, *Streptococcus salivarius* K12