# Tryptic Soy agar supplemented with bacitracin represents a remarkable advancement as a selective culture medium for *Streptococcus anginosus*

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**Purpose:** To develop a new selective culture medium specifically for *Streptococcus anginosus*.
**Materials and Methods:** Three antibiotics, sulphamethazine (s), aztreonam (a), and bacitracin (b), were tested by adding to Tryptic Soy (TS) agar at different concentrations. Initially, sulphamethazine (1.0 mg/mL) and aztreonam (0.2 mg/mL) were added to TS agar to obtain TS-sa agar. In total, 24 strains of oral bacteria including three clinical *S. anginosus* isolates were used during the entire study. A minimal inhibitory concentration (MIC) test was performed by using 96-well plate to determine the optimal bacitracin concentration for the selective medium for *S. anginosus* under anaerobic condition at 37ºC for 48 hours. Finally, bacitracin was added at a concentration of 2.0 U/mL to TS-sa agar to prepare TS-sab agar medium and selectivity was tested using all 24 strains of bacteria.
**Results:** Most strains of *S. anginosus* could grow on the TS-sab agar medium but other bacteria except for *S. intermedius* and *S. sobrinus* could not be detected on this medium. *S. intermedius* and *S. sobrinus* occasionally showed some growth on TS-sab agar medium with low detection rate. Although some clinical isolates of *S. anginosus* were not enriched like other strains of *S. anginosus,* a remarkable enhancement was achieved in this study in the development of a selective culture medium for *S. anginosus*.
**Conclusion:** TS-sab may serve as a remarkably improved semi-selective culture medium for *S. anginosus*. Continued studies are required to improve the selectivity specific for *S. anginosus.*
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**Key Words:** aztreonam, bacitracin, selective medium, *Streptococcus anginosus*, sulphamethazine

## Introduction

*Streptococcus anginosus* (*S. anginosus*) group bacteria are classified as the oral viridians streptococci and can be isolated from several parts of the human body, including the oral cavity and gastrointestinal and genitourinary tracts.[1-4] It forms part of the normal flora of the human oral cavity and upper respiratory, gastrointestinal, and female urogenital tracts.[5] *S. anginosus* is frequently isolated from purulent infections of the mouth and internal organs, including the brain, liver, lungs, and spleen,[1-8] and from cases of appendicitis, peritonitis, endocarditis, meningitis, obstetric and neonatal infections, and infections of the skin and soft tissues.[9] Although the organism is generally considered to have a low pathogenicity, evidence for the pathogenicity of these streptococci has recently been extensively reviewed[9] and indicate that it has a propensity to cause severe purulent infections at virtually all body sites. Notably, *S. anginosus* is the most common cause of brain and liver abscesses and pulmonary empyema.[5,10,11] Furthermore, it has been reported that *S. anginosus* is associated with esophageal, gastric, and pharyngeal cancer tissues.[10,12-15]

It is an important issue to develop a proper selective meium for these streptococci, due to the unusual characteristics and unclear involvement of *S. anginosus* in human infectious diseases. A proper selective medium is of high importance for optimizing the laboratory diagnosis of *S. anginosus* infections. However, conventional microbiological practices have overlooked the clinical significance of *S. anginosus*, because they are frequently underrepresented on solid media. Additionally, *S. anginosus* colonies are phenotypically indistinguishable from those of other streptococci, which traditionally have been considered to have low or no

pathogenic potential.[16,17] To overcome issues concerning the proper identification of these pathogens, several studies have been conducted to produce a selective medium.[18-20]

It is an innovative idea to examine conventionally used culture media, for example, Mitis Salivarius (MS) or Tryptic Soy (TS) agar, supplemented with antibiotics of different target spectra.[21-23] Supplementing media with antibiotics in unit volume ratios has helped to produce selective culture media to isolate certain pathogens specifically.[19,23] Bacitracin has been widely used in clinical medicine and has also been used to supplement culture media in research laboratories,[21,22] since it was first introduced in 1945.[24] Bacitracin ($C_{66}H_{103}N_{17}O_{16}S$) is also synthesized from cultures of *Bacillus licheniformis*.[25] There is evidence that *S. anginosus* is more resistant to this antibiotic than other oral bacteria (oral streptococci other than mutans streptococci), which facilitates the selection of antibiotics to be used in this study.[22]

However, a culture medium that is strictly selective for *S. anginosus* has not yet been developed.[23] Therefore, the aim of this study was to develop a selective culture medium modified for the isolation of *S. anginosus* by supplementing TS medium with antibacterial agents at optimal concentrations.

## Materials and Methods

### Bacterial strains used

The bacterial strains used in this study are shown in Table 1 and include streptococci from the anginosus, mutans streptococci, mitis, and salivarius groups, as well as a staphylococcus (*Staph. aureus* Cowan 1). Among the 24 strains of oral bacteria, three clinical *S. anginosus* isolates were included. The laboratory strains have been preserved in the bacteriological facilities of our department, and the clinical isolates were kindly provided by the Department of Oral Microbiology, Tsurumi University School of Dental Medicine.

### Plate preparation

Agar plates were prepared using Tryptic Soy (TS; Becton, Dickinson and Company, Sparks, MD, USA) and agar (1.5% agarose; Wako Pure Chemical, Tokyo, Japan) and Mitis-Salivarius (MS) dissolved in distilled water as instructed by the manufacturer. The media were autoclaved and then hardened in 10-mm diameter sterile Petri dishes (Sansei Medical Co. Ltd., Kyoto, Japan).

**Table 1.** List of 24 strains of oral bacteria used in this study

| | |
|---|---|
| *Streptococcus anginosus* ATCC 33397 | *Streptococcus cricetus* ATCC 19642 |
| *Streptococcus anginosus* TU-C20 | *Streptococcus downei* ATCC 33748 |
| *Streptococcus anginosus* TU-C21 | *Streptococcus macacae* ATCC 35911 |
| *Streptococcus anginosus* TU-C25 | *Streptococcus mitis* ATCC 6249 |
| *Streptococcus intermedius* ATCC 27335 | *Streptococcus salivarius* ATCC 9759 |
| *Streptococcus constellatus* ATCC 27823 | *Streptococcus oralis* ATCC 35037 |
| *Streptococcus mutans* ATCC 25175 | *Streptococcus gordonii* ATCC 10558 |
| *Streptococcus sobrinus* B13N | *Streptococcus sanguinis* ATCC 10556 |
| *Streptococcus sobrinus* ATCC 33478 | *Streptococcus pyogenes* GTC 262 |
| *Streptococcus sobrinus* 6715 | *Streptococcus agalactiae* GTC 1234 |
| *Streptococcus rattus* ATCC 19645 | *Streptococcus pneumoniae* ATCC 33400 |
| *Streptococcus ferus* ATCC 33477 | *Staphylococcus aureus* Cowan 1 |

### Supplementation with antibiotics

Initially, sulphamethazine (s) ($C_{10}H_{10}N_4O_2S$) and aztreonam (a) ($C_{13}H_{17}N_5O_8S_2$) (Sigma-Aldrich, St. Louis, MO, USA) were added to TS and MS agar media to prepare TS-sa and MS-sa culture plates as mentioned above.

Later, three antibiotics, including the aforementioned two and bacitracin (b), were added to TS agar culture medium to prepare TS-sab agar culture medium. Sulphamethazine and aztreonam were used at 1.0 mg/mL and 0.2 mg/mL concentrations, respectively, throughout the study.

**Minimal inhibitory concentrations test**

To determine the optimal bacitracin concentration for the selective growth of *S. anginosus* on TS agar plates, a minimal inhibitory concentration (MIC) test was performed by using 96-well plate using TS broth with serially diluted bacitracin. Each bacterium was cultured in TS broth for 16 hours, and 10 μL of bacterial cell suspension was plated in each well. The plates were incubated under anaerobic (80% $N_2$, 10% $CO_2$ and 10% $H_2$) condition at 37ºC for 48 hours. Then, the turbidity of each well at 540 nm was determined by microplate reader (Bio-Rad Laboratories, Inc., CA, USA). The spot (well), in which bacterial growth was not observed, was considered as MIC. Subsequently, TS-sab agar plates were prepared using bacitracin concentrations of 3.2, 2.5, 2.4, 2.3, 2.2, 2.1 2.0 and 0 U/mL separately to determine a practically proper concentration of bacitracin. Five μL of each bacterial cell suspension was inoculated directly on the agar plate and was incubated under anaerobic condition at 37ºC for 48 hours. After this incubation, the culture plates were examined using a desktop microscope and comparative visual scale. Visible colonies were considered indicative of bacterial growth.

**Growth assessment by colony counting**

Each bacterium was cultured in TS broth for 16 hours, and the bacterial cell suspension was diluted properly with phosphate-buffered saline (PBS) and plated on TS and TS-sab agar with a spiral plating instrument (Eddy Jet, IUL, Barcelona, Spain). All plates were incubated for 48 hours under anaerobic conditions at 37°C. After 48 hours, colony-forming units (CFUs) were enumerated with the aid of a microscope.

## Results

The results of the primary assessment test are shown in Table 2.

**Table 2.** Primary growth assessment of the oral bacteria on TS agar, TS-sa agar, MS agar, and MS-sa agar

| Bacterial strains | TS | TS-sa | MS | MS-sa |
|---|---|---|---|---|
| *S. anginosus* ATCC 33397 | + | + | + | + |
| *S. anginosus* TU-C20 | + | + | + | + |
| *S. intermedius* ATCC 27335 | + | + | + | + |
| *S. constellatus* ATCC 27823 | + | + | + | + |
| *S. mutans* ATCC 25175 | + | - | + | - |
| *S. sobrinus* ATCC 33478 | + | + | + | + |
| *S. rattus* ATCC 19645 | + | - | + | - |
| *S. ferus* ATCC 33477 | + | + | + | + |
| *S. cricetus* ATCC 19642 | + | + | + | + |
| *S. downei* ATCC 33748 | + | + | + | + |
| *S. macacae* ATCC 35911 | + | - | + | - |
| *S. mitis* ATCC 6249 | + | - | + | - |
| *S. salivarius* ATCC 9759 | + | - | + | - |
| *S. oralis* ATCC 35037 | + | - | + | - |
| *S. gordonii* ATCC 10558 | + | - | + | + |
| *S. sanguinis* ATCC 10556 | + | - | + | + |
| *S. pyogenes* GTC 262 | + | - | + | - |
| *S. agalactiae* GTC 1234 | + | - | + | - |
| *S. pueumoniae* ATCC 33400 | + | - | + | - |
| *Staph. aureus* Cowan 1 | + | - | + | - |

+: colony detected, -: no colony detected

**Table 3.** Minimal inhibition concentration (MIC) of bacitracin to resist the growth of various oral bacteria

| Bacterial strains | Bacitracin (U/mL) | Bacterial strains | Bacitracin (U/mL) |
|---|---|---|---|
| *S. anginosus* ATCC 33397 | 6.4 | *S. cricetus* ATCC 19642 | 3.2 |
| *S. anginosus* TU-C20 | 6.4 | *S. downei* ATCC 33748 | 0.8 |
| *S. anginosus* TU-C21 | 6.4 | *S. macacae* ATCC 35911 | 0.2 |
| *S. anginosus* TU-C25 | 0.8 | *S. mitis* ATCC 6249 | 0.2 |
| *S. constellatus* ATCC 27823 | 3.2 | *S. salivarius* ATCC 9759 | <0.1 |
| *S. intermedius* ATCC 27335 | 0.8 | *S. oralis* ATCC 35037 | 6.4 |
| *S. mutans* ATCC 25175 | 0.4 | *S. gordonii* ATCC 10558 | 0.4 |
| *S. sobrinus* B13N | 0.8 | *S. sanguinis* ATCC 10556 | 0.2 |
| *S. sobrinus* ATCC 33478 | 3.2 | *S. pyogenes* GTC 262 | <0.1 |
| *S. sobrinus* 6715 | 3.2 | *S. agalactiae* GTC 1234 | 0.2 |
| *S. rattus* ATCC 19645 | 3.2 | *S. pueumoniae* ATCC 33400 | <0.1 |
| *S. ferus* ATCC 33477 | <0.1 | *Staph. aureus* Cowan 1 | 12.8 |

**Table 4.** Determination of bacitracin concentration for selective growth of *S. anginosus* group on TS-sab agar

| Bacitracin (U/mL) | 6.4 | 3.2 | 2.5 | 2.4 | 2.3 | 2.2 | 2.1 | 2.0 | 1.8 | 1.0 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. anginosus* ATCC 33397 | + | + | + | + | + | + | + | + | + | + | + |
| *S. anginosus* TU-C20 | - | - | - | - | - | + | + | + | + | + | + |
| *S. anginosus* TU-C21 | - | - | - | - | - | + | + | + | + | + | + |
| *S. anginosus* TU-C25 | - | - | - | - | - | - | +/- | + | + | + | + |
| *S. intermedius* ATCC 27335 | - | - | - | - | - | + | + | +/- | + | + | + |
| *S. constellatus* ATCC 27823 | - | - | - | - | - | - | - | - | + | + | + |

+/- indicate 'no growth' once out of 3

**Table 5.** Growth characteristics of oral bacteria cultivated on TS-sab agar

| Bacterial strains | CFU | | Detection rate (%) |
|---|---|---|---|
| | TS agar | TS-sab agar | |
| *S. anginosus* ATCC 33397 | $2.20\times10^8$ | $2.02\times10^8$ | 91.6 |
| *S. anginosus* TU-C20 | $2.48\times10^7$ | $2.33\times10^7$ | 94.0 |
| *S. anginosus* TU-C21 | $1.83\times10^7$ | $1.02\times10^7$ | 55.7 |
| *S. anginosus* TU-C25 | $2.27\times10^7$ | $6.50\times10^4$ | 0.3 |
| *S. intermedius* ATCC 27335 | $2.24\times10^7$ | $2.80\times10^4$ | 0.1 |
| *S. constellatus* ATCC 27823 | $7.30\times10^7$ | ND | ND |
| *S. mutans* ATCC 25175 | $1.65\times10^8$ | ND | ND |
| *S. sobrinus* B13N | $3.74\times10^6$ | ND | ND |
| *S. sobrinus* ATCC 33478 | $3.74\times10^6$ | $1.22\times10^3$ | 0.03 |
| *S. sobrinus* 6715 | $4.39\times10^6$ | $4.07\times10^2$ | 0.01 |
| *S. rattus* ATCC 19645 | $2.17\times10^8$ | ND | ND |
| *S. ferus* ATCC 33477 | $1.48\times10^8$ | ND | ND |
| *S. cricetus* ATCC 19642 | $5.87\times10^6$ | ND | ND |
| *S. downei* ATCC 33748 | $1.57\times10^8$ | ND | ND |
| *S. macacae* ATCC 35911 | $1.80\times10^8$ | ND | ND |
| *S. mitis* ATCC 6249 | $1.39\times10^8$ | ND | ND |
| *S. salivarius* ATCC 9759 | $1.19\times10^8$ | ND | ND |
| *S. oralis* ATCC 35037 | $5.08\times10^7$ | ND | ND |
| *S. gordonii* ATCC 10558 | $2.19\times10^8$ | ND | ND |
| *S. sanguinis* ATCC 10556 | $2.16\times10^7$ | ND | ND |
| *S. pyogenes* GTC 262 | $9.26\times10^7$ | ND | ND |
| *S. agalactiae* GTC 1234 | $3.52\times10^6$ | ND | ND |
| *S. pneumoniae* ATCC 33400 | $4.23\times10^6$ | ND | ND |
| *Staph. aureus* Cowan 1 | $1.67\times10^8$ | ND | ND |

ND: not detected

The growth of colonies on TS and TS-sa agar media varied; most of the oral bacteria, except *S. anginosus* and a few other strains, did not show growth potential on TS-sa agar medium. Expectedly, all strains of *S. anginosus*

showed growth potential on all types of media when cultured using the same microbiological methods. Similar results were observed with MS media (MS and MS-sa agar). However, TS-sa displayed slightly more resistance to bacterial growth over MS-sa in terms of the number of bacterial strains able to grow on each medium. As a result, TS-sa agar was chosen for further modification and analysis.

Table 3 shows the results of MIC of bacitracin against various bacteria. Ten strains of bacteria out of 24 strains were resistant to bacitracin showing MIC of bacitracin at more than 3.2 U/mL. The MIC against three strains of *S. anginosus* was 6.4 U/mL. The MIC of bacitracin was found to be 2.0 U/mL, which was the concentration used to make TS-sab agar, a medium potentially more selective for *S. anginosus* (Table 4). Colony growth did not show repeatability for *S. intermedius*: although *S. intermedius* grew in some plates, it failed to grow in others. Upon follow-up growth characterization, we found that all *S. anginosus* strains showed remarkable growth with high detection rates (Table 5), especially *S. anginosus* ATCC 33397 (93.5%) and *S. anginosus* TU-C 20 (94%). Other than *S. anginosus*, *S. intermedius* and *S. sobrinus* grew on this at negligible rate (0.01-0.1%). Similar results were observed in growth assays on TS-sab agar (Fig. 1). However, as shown in Table 6, MS-sab displayed a distinct effect - no bacterial growth could be detected.

**Table 6.** Comparison of bacterial growth characteristics on TS-sab agar and MS-sab agar

| TS-sab agar | | | | | |
|---|---|---|---|---|---|
| Bacterial strains | CFU | | | | Detection rate (%) |
| | TS agar | | TS-sab agar | | |
| *S. anginosus* ATCC 33397 | + | $2.4\times10^6$ | + | $2.4\times10^6$ | 94.8 |
| *S. anginosus* TU-C20 | + | $1.8\times10^6$ | + | $1.7\times10^4$ | 91.9 |
| *S. anginosus* TU-C21 | + | $2.1\times10^6$ | + | $1.3\times10^6$ | 64.0 |
| *S. anginosus* TU-C25 | + | $2.0\times10^6$ | + | $7.0\times10^5$ | 35.8 |
| *S. intermedius* ATCC 27335 | + | $2.0\times10^6$ | ND | 0 | 0 |
| *S. constellatus* ATCC 27823 | + | $5.8\times10^5$ | ND | 0 | 0 |
| *S. sobrinus* ATCC 33478 | + | $1.6\times10^6$ | ND | 0 | 0 |

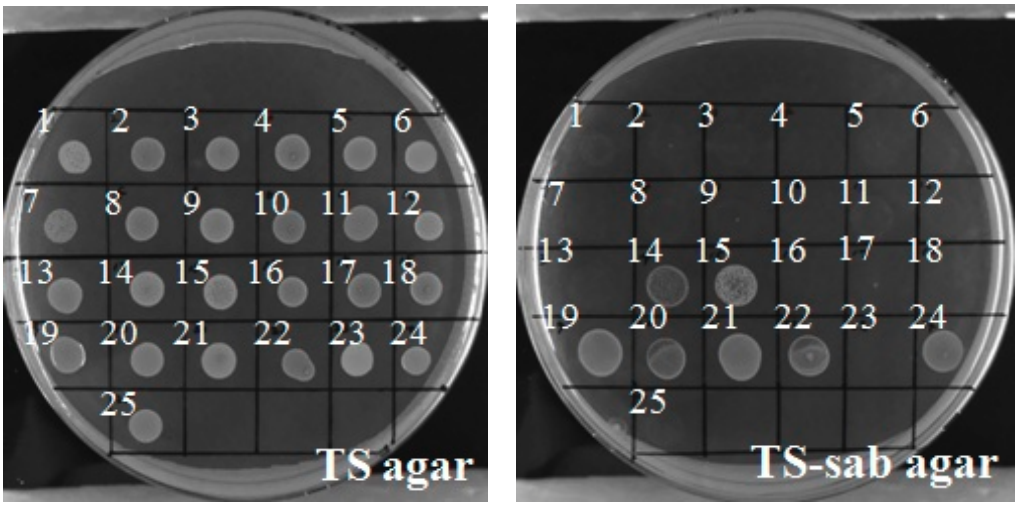
| MS-sab agar | | | | | |
|---|---|---|---|---|---|
| Bacterial strains | CFU | | | | Detection rate (%) |
| | MS agar | | MS-sab agar | | |
| *S. anginosus* ATCC 33397 | + | $2.1\times10^6$ | ND | 0 | 0 |
| *S. anginosus* TU-C20 | + | $5.0\times10^5$ | ND | 0 | 0 |
| *S. anginosus* TU-C21 | + | $1.6\times10^6$ | ND | 0 | 0 |
| *S. anginosus* TU-C25 | + | $1.1\times10^6$ | ND | 0 | 0 |
| *S. intermedius* ATCC 27335 | + | $1.6\times10^6$ | ND | 0 | 0 |
| *S. constellatus* ATCC 27823 | + | $4.4\times10^5$ | ND | 0 | 0 |
| *S. sobrinus* ATCC 33478 | + | $4.8\times10^5$ | ND | 0 | 0 |

ND: not detected

## Discussion

Selective media allow certain types of organisms to grow and inhibit the growth of other organisms. This selectivity can be accomplished in several ways. For example, organisms that can utilize a particular sugar can be easily selected by making that sugar the only carbon source in the medium. On the other hand, selective inhibition of some types of microorganisms can be achieved by adding antibiotics, salts, dyes, or specific inhibitors that affect the metabolism or enzyme systems of the organisms. For example, media containing potassium tellurite, sodium azide, or thallium acetate (at concentrations of 0.1-0.5 mg/mL) will inhibit the

growth of Gram-negative bacteria.[26] Media supplemented with penicillin (5-50 U/mL) or crystal violet (2 μg/mL) will inhibit the growth of Gram-positive bacteria.[27] Tellurite agar, therefore, is used to select for Gram-positive organisms, and nutrient agar supplemented with penicillin can be used to select for Gram-negative organisms.




1) *S. cricetus* ATCC 19642 2) *S. ferus* ATCC 33477 3) *S. rattus* ATCC 19645 4) *S. agalactiae* GTC 1234
5) *S. pueumoniae* ATCC 33400 6) *S. oralis* ATCC 35037 7) *S. macacae* ATCC 35911 8) *S. downei* ATCC 33748
9) *S. sanguinis* ATCC 10556 10) *S. gordonii* ATCC 10558 11) *S. mutans* ATCC 25175 12) *S. mitis* ATCC 6249
13) *S. salivarius* ATCC 9759 **14) *S. sobrinus* ATCC 33478** **15) *S. sobrinus* 6715** 16) *S. sobrinus* B13N
17) *S. pyogenes* GTC 262 18) *S. pyogenes* GTC 262 19) *S. anginosus* ATCC 33397
**20) *S. anginosus* TU-C20** **21) *S. anginosus* TU-C21** **22) *S. anginosus* TU-C25**
23) *S. constellatus* ATCC 27823 **24) *S. intermedius* ATCC 27335** 25) *Staph. aureus* Cowan 1

**Fig. 1.** Photograph showing growth of bacteria after cultivating on TS and TS-sab. All the strains showed active growth on TS but only few (mainly *S. anginosus)* grew on TS-sab (bold and underlined).

In this study, three different antibiotics were chosen to develop a selective culture medium for *S. anginosus* because this group of bacteria has been reported to be involved in multiple infectious diseases of delicate organs of the human body and, most importantly, has been found in sarcomas and oral and esophageal cancers.[28,29] The development of easy, efficient, and cost-effective methods to obtain a confirmatory diagnosis in the clinic are of the utmost importance, even in modern-day medicine and dentistry. This is no different for the confirmatory diagnosis of *S. anginosus*, for two reasons: the presence of this species of bacteria is inconsistent, and there is no established detection method for a confirmatory etiological diagnosis or, more specifically, there is no selective culture medium available. Although bacitracin is well known for its antibacterial potency, the addition of this antibiotic to conventional culture media to develop a selective culture medium for confirmatory diagnosis has not been reported before.

The addition of bacitracin, sulphamethazine, and aztreonam produced a modified TS agar medium for *S. anginosus* that showed considerable selectivity and reproducibility in this study. Bacitracin has been widely used in MS agar medium[21,22] to achieve selective growth of mutans streptococci. The addition of the other two antibiotics is probably responsible for the change in selectivity, as reported previously.[22] In contrast, it has been reported that bacitracin can be used to distinguish *Streptococcus pyogenes* from other bacteria.[30] To develop a new selective culture medium for *S. anginosus*, three antibacterial agents (sulphamethazine, aztreonam, and bacitracin) were added to tryptic soy medium to form TS-sab in this study. At this stage, reliable selectivity was

observed only for *S. anginosus* ATCC 33397 at concentrations of bacitracin from 2.0 to 2.3 U/mL. Nevertheless, bacitracin at 2.0 U/mL represented the point at which most strains of *S. anginosus* survived, but the growth of other bacteria could not be detected. Therefore, remarkable progress was achieved in the development of a selective culture medium for *S. anginosus* in this study. However, some clinical isolates of *S. anginosus* were not detected at the same high rates as the lab strains. In addition, *S. sobrinus* ATCC 33478 from the mutans streptococci group showed strong resistance to bacitracin in this study. Variations in the results might be obvious as the *S. anginosus* species comprises 16S rRNA ribogroups that differ in phenotypic characteristics and clinical relevance.[31] Therefore, TS-sab may serve as a semi-selective culture medium for *S. anginosus*. Continued studies are required to develop an appropriate selective medium for confirmatory clinical diagnosis by using a series of antibacterial agents and peptides at various concentrations and also using various bacterial species. In the oral cavity, antimicrobial peptides, such as defensin, histain, and cathelicidin, play a pivotal role as a first line of defense against a succession of invading bacteria.[32] The β-defensins are small, cationic, antimicrobial peptides. The peptides hBD-1 and -3 were detected in the salivary gland and gingival, tongue, and buccal mucosa. Such peptides should be considered to supplement TS-sab for the specific identification of *S. anginosus*.

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