ORIGINAL ARTICLE

# Comparative Evaluation of Antibacterial Activity of Probiotics SK12 and SM18: An *In Vitro* Study

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## Abstract

**Aim:** To assess the antimicrobial activity of probiotics SK12 and SM18 on *Streptococcus mutans* and also to compare the antimicrobial activity of SK12 and SM18.

**Materials and methods:** Synthetic strains of *Streptococcus mutans* were used to study the antimicrobial activity of probiotics SK12 and SM18 using various tests such as disk diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). In disk diffusion, the zone of inhibition was measured to assess the antimicrobial activity. Chlorhexidine was used as a control for this test. The MIC and MBC were assessed at different dilutions of the probiotic sample (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.12 mg/mL, 1.6 mg/mL, 0.8 mg/mL, 0.4 mg/mL, and 0.2 mg/mL).

**Result:** SM18 demonstrated 20 mm of zone of inhibition, whereas SK12 demonstrated 15 mm showing a less antibacterial activity in comparison to SM18. SM18 was found to be bactericidal and effective at a minimum concentration of 0.8 mg/mL, whereas SK12 was bactericidal and effective at a minimum concentration of 1.6 mg/mL.

**Conclusion:** Probiotics demonstrate antibacterial activity against cariogenic microflora. SM is 18 having a better antibacterial activity at lower concentrations than SK12 in reducing cariogenic microorganisms. Clinical significance: BLIS K12 and M18 both demonstrated an antibacterial effect on *Streptococcus mutans*, wherein the use of probiotic in caries prevention is found to be limited. Hence, it is suggestive to reap the bacterial effects of BLIS K12 and M18 in caries prevention.


**Keywords:** Antibacterial activity, Probiotics, SK12, SM18, *Streptococcus mutans*.

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## Introduction

The administration of adequate amounts of live organisms (probiotics) to confer beneficial effect on host is known as bacteriotherapy or replacement therapy. This concept has shown promising results in oral and general health.[1–4] The concept of bacteriotherapy was first explained by Ilya Metchnikoff in 1908 and it was Lily and Stilwell who later called it as probiotics[1,2,5] (derived from Greek meaning "prolife").

Probiotics was found to exert myriad of beneficial effects by balancing colonic microbiota and is widely used in the treatment of gastointestinal irritation candidial and urinary tract infections.[6] *Lactobacillus reuteri*, *Weisellaciberia*, *Lactobacillus acidophilus*, and *Lactobacillus fermentum* are the strains commonly used as probiotics in gastrointestinal irritation. These organisms have gastrointestinal tract as their inherent habitat whereas the use of these organisms in the oral cavity and their efficacy in oral the context are questionable.[5] Probiotics BLISK12 and BLIS M18 are other strains of microorganisms that can be used in the oral cavity. These strains are derived from gram-positive *Streptococcus salivarius*, which are oxidase and catalase-negative spherical bacterium.[7,8] These microorganisms are found to be pioneer colonizer of the human oral cavity and persist predominantly throughout the life.[9–12] They colonize on tongue dorsum and pharyngeal mucosa of infants within 2 days of birth and the source is the mother.[7,13] Up to $1 \times 10^7$ colony-forming units per mL is present in the saliva.[9,14] The strains of *Streptococcus salivarius* are producers of bacteriocin-like inhibitory substances (BLIS)[9,15] and have diverse activity, prevent overgrowth of potential pathogens, and play an important role in stabilizing oral microbiota.[3,4,9,16]

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*Streptococcus salivarius* K12—commonly used as commercial probiotic in New Zealand and was first isolated from a healthy child saliva.[17,18] K12 produces two bacteriocins, salivariacin A2 and salivariacin B, which inhibit phylogenetically related bacterial species effectively.[17,19] Due to the bacteriocin profile of K12, it was commercially produced as BLIS K12 making SK12 the first oral probiotic specifically targeting oral health.[17] SM18 differs from K12 based on the bacteriocin profile and secretes bacteriocins A2, 9, MPS, and M[9,17] and this led to commercial preparation of SM18.

Probiotics anticaries activity in the oral cavity is by competing to bind to the complex ecosystem of oral microorganisms' proteins and interfere with bacteria to bacteria attachments. Thus, killing or inhibiting growth of pathogens through production of bacteriocins and interference with the signaling mechanism.[3,17,18,20–22]

*S. mutans* are gram-positive cocci which are commonly found in oral cavity and have a inevitable role in tooth decay on the

other hand SK12 and SM18 which are strains of gram-positive cocci S. salivarius are used to inhibit caries activity. Hence, a research hypothesis was stated that oral probiotics SK12 and SM18 could compete with *S. mutans* and interfere with its attachment in the oral cavity. The present *in vitro* study was designed to evaluate antibacterial effects of SK12 and SM18 against *S. mutans*.

## Materials and Methods

### Antibacterial Susceptibility Testing

Disk diffusion test was conducted to assess the antimicrobial susceptibility testing. The activity was assessed by measuring the diameter of the zone of inhibition.

#### *Preparation of Media*

Media used was Brain Heart Infusion Agar at room temperature. The colonies were transferred to the plates using a loop or swab. Visually adjusted the turbidity of broth equal to that of a 0.5 McFarland turbidity standard that has been vortexed. A sterile cotton swab was dipped into the inoculum and rotated against the wall of the tube to remove excess inoculum within 15 minutes of adjusting the inoculum to a McFarland 0.5 turbidity standard. The whole surface of the agar plate was swabbed thrice, and it was rotated approximately 60° between streaking for ensuring even distribution, avoiding hitting sides of petri plate and creating aerosols. Before making wells in the inoculated plate, it was allowed to stand for at least 3 minutes to a maximum of 15 minutes.

#### *Preparation of Stock Solution*

The stock solution weighing 10 mg of compound was dissolved in 1 mL of DMSO. A 5-mm-diameter hollow tube was heated and pressed onto the prepared inoculated agar plate and immediately withdrawn by creating a well in the plate. Likewise, five wells on each plate were made. With the help of a micropipette 75, 50, 25, 10, and 5 µL of compound were added in each well. The plates were incubated for 18–24 hours at 37°C in the incubator within 15 minutes of the compound application. Only when the growth lawn was confluent or almost confluent were the plates read. The diameter of the inhibition zone was measured to nearest whole millimeter by holding the measuring device.

### MIC: Minimum Inhibitory Concentration[23]

This test is carried out to assess the minimum concentration required to inhibit bacterial growth.

#### *Aerobic*

Nine dilutions of each drug have to be done with brain heart infusion (BHI) for MIC. Total 20 µL of drug was added into the 380 µL of the BHI broth in the initial tube. Total 200 µL of the BHI broth was applied separately to the next nine tubes for dilutions. Then, 200 µL was moved from the initial tube to the first tube containing BHI broth 200 µL. This was deemed a dilution of $10^{-1}$. In order to make $10^{-2}$ dilution, 200 µL was removed from $10^{-1}$ diluted tube to the second tube. The serial dilution was repeated up to $10^{-9}$ dilution for each drug. Total 5 µL was taken from the maintained stock cultures of required organisms and added into 2 mL of the BHI broth. Suspension was added to each serially diluted tube of 200 µL of above culture. The tubes were observed for turbidity after 24 hours of incubation.

#### *Anaerobic*

Nine dilutions of each drug have to be done with the thioglycolate broth for MIC. In the initial tube, 20 µL of drug was added into the 380 µL of thioglycolate broth. For dilutions 200 µL of the thioglycolate broth was added into the next nine tubes separately. Then from the initial tube 200 µL was transferred to the first tube containing 200 µL of the thioglycolate broth. This was considered as $10^{-1}$ dilution. From $10^{-1}$ diluted tube, 200 µL was transferred to the second tube to make $10^{-2}$ dilution. The serial dilution was repeated up to $10^{-9}$ dilution for each drug. From the maintained stock cultures of required organisms, 5 µL was taken and added into 2mL of thioglycolate broth. Total 200 µL of the above culture suspension was added in each serially diluted tube. These tubes were incubated at 37°C in an anaerobic jar for 48–72 hours and were observed for turbidity.

### MBC: Minimum Bactericidal Concentration

This test is used for assessing the bactericidal or the bacteriostatic effect of the antimicrobial.

From the MIC dilutions tubes, first three or five tubes were plated (which was sensitive in MIC) and incubated for 48–72 hours. Then the colony count was taken. The MBC is done to see whether there was bacteriostatic or bactericidal effect of the extract (drug) against the organism. If there is no growth, then it's the bactericidal effect. If there is growth, then it's bacteriostatic effect.

The data obtained from disk diffusion, MIC, and MBC were tabulated.

## Results

Among SK12 and SM18, SM18 showed a zone of inhibition of 20 mm. Whereas SK12 displayed 15 mm of the zone of inhibition (Table 1). Minimum inhibitory concentration of M18 was at 0.8 mg, whereas for K12 it was at 1.6 mg (Table 2). The minimum bactericidal concentration of K12, M18 was at 0.8 mg below which it was bacteriostatic (Fig. 1).

## Discussion

Preventive strategies for dental caries are aimed at targeting the host factor, dietary factor, and removal of plaque biofilm. This is achieved by use of topical fluorides, dietary monitoring, chemoprophylactic agents, antibiotics, caries vaccines, sugar substitutes, and restorative procedures.[16,24,25]

Various chemoprophylactic agents, such as antibiotics (vancomycin, penicillin),[9] chlorhexidine, cetylpyridinum chloride (cationic agents), sodium dodecyl sulfate (anionic agents), triclosan (nonionic agents) and plant derived (Sanguinaria extract), are used in prevention of caries.[26] Also antimicrobial peptides are used due their excellent antibacterial activity against wide spectrum of bacterial species, including drug-resistant strains.

In developing caries vaccine mucosal mediated immune system and secretory IgA in saliva was considered. As the antibody

**Table 1:** Zone of inhibition by different samples used

| *Sl. no* | *Samples* | *Zone of inhibition* |
|---|---|---|
| | *S. mutans* | |
| 1 | K12 | 15 mm |
| 2 | M18 | 20 mm |
| 3 | CHX | 25 mm |

**Table 2:** Minimum bactericidal concentration of K12 and M18

| *Sl No* | *Samples* | *100 mg/mL* | *50 mg/mL* | *25 mg/mL* | *12.5 mg/mL* | *6.25 mg/mL* | *3.12 mg/mL* | *1.6 mg/mL* | *0.8 mg/mL* | *0.4 mg/mL* | *0.2 mg/mL* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | K12 | NG | NG | NG | NG | NG | NG | NG | NG | 38 | 72 |
| 2 | M18 | NG | NG | NG | NG | NG | NG | NG | NG | 24 | 49 |

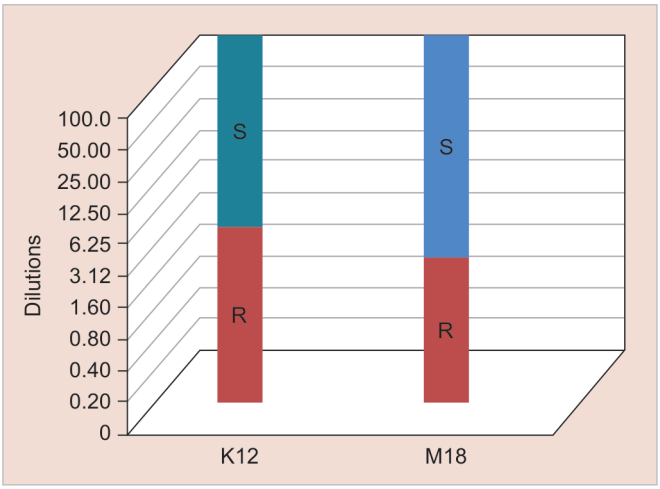


**Fig. 1:** Bar graph representing minimum inhibitory concentration of SK12 and SM18

response and allergic reactions associated with nonhuman monoclonal antibodies limit the use of caries vaccine.[26,27] Whereas, antibody response and allergic reactions associated with nonhuman monoclonal antibodies limit the use of caries vaccine. *Streptococcus mutans* produces organic acids that lead to destruction of the tooth enamel by fermenting dietary sugars such as glucose, sucrose, and lactose. Xylitolin supernatant fluids of aqueous plaque suspensions inhibits dextranase catalyzed hydrolysis of dextran and absence of lactic acid production. Thus making Xylitol not fermentable by cariogenic bacteria and prevents demineralization. Xylitol's bacteriostatic effect on *Streptococcus mutans* is explained by a futile cycle consuming cellular ATP. The antibacterial activities of the sugar substitutes (xylitol) are weak as prolonged exposure time in the oral cavity is required to be effective when compared to other agents. Requirement of novel delivery methods for prolonged exposure of sugar substitutes limits its use.[26,28] Fluorides affects the carbohydrate degradation at various levels, directly by inhibiting the enolase activity and indirectly inhibiting the uptake of sugar by the phosphor transferase systems and also its additional action as a proton carrier. It also affects the intracellular pH levels, therefore inhibiting activity of various glycolytic enzymes.[29] These actions of fluoride make it effective against caries but its limited action against caries and the toxic effects limits its use in prevention of caries.[26]

Probiotics (live microorganisms) can confer health benefits on the host when administered in adequate number.[26] A variety of beneficial effects observed on health are enhanced immune response, colonic microbiota equilibrium, vaccine adjuvant effects, reduction in enzymes initiating cancer, in travel-related and antibiotics-induced diarrhea, reduction of serum cholesterol, antagonism to food-borne pathogens and caries-inducing organism, on lactose malabsorption, candidiasis, urinary tract infections, control of rotavirus and *Clostridium difficile*-induced colitis, and prevention of ulcers related to *Helicobacter pylori*.[6]

The benefits of probiotics are based on their antagonist activity on the pathogens, either by competing with the pathogen for the binding site or by producing antimicrobial substances. Substances such a bacteriocin or bacteriocin-like substances can inhibit the growth of wide range of pathogens.[30,31] Bacteriocins are small, heat-stable, ribosomally synthesized antimicrobial peptide that is active against the pathogen and to which the producer is immune.[32] Bacteriocins produced by the probiotic help in its functionality by

aiding in the survival of the producing strain, directly inhibiting the growth of pathogens, and by serving as signaling peptide/quorum sensing molecules in intestinal environment.[32,33] BLIS K12 colonizes up to 1 month after its last administration to greater extent in the oral cavity and to some extent in nasopharynx and adenoids.[17,19] Remodels host the epithelial lining to facilitate commensal interaction after attachment of K12.[17] In children, it is found to colonize upper respiratory tract, oral cavity, adenoid tissues, and nasopharynx.[34] SK12 colonizes the pharynx, tongue, and buccal membrane within the oral cavity,[8,17] showing more predominance of colonization in the pharynx than tongue and buccal membrane.[8,17] But these colonies make up only less than 1% of the total bacterial population in these areas.[8,17] The innate defense of probiotic SK12 can be attributed to its unique interactions with oral epithelial cells that modulate the physiologic responses. SK12 allows itself to be tolerable by human host and promotes oral health by maintaining hemostasis, reducing inflammation and pathogen-induced apoptosis.[17,35] These actions are achieved by pro-inflammatory response, stimulating anti-inflammatory response and modulating genes responsible for adhesion and hemostasis.[17,35] Also affects the secretion of interlukin-8 and immunomodulatory host defense peptide during the exposure of the epithelial cells to pathogenic organisms, such as (*Pseudomonas aeruginosa*, Salmonella serovar).

Illustration of downregulation of growth-related oncogene alpha, responsible for leukocyte recruitment and proliferation by K12, demonstrates induced reduction of inflammatory response.[17,35] Also K12 stimulates anti-inflammatory response by underrepresentation of K12-modulated genes and overrepresentation of the nicotinic acetylcholine pathway. K12 modulates even the hemostatic genes involved in transcription, translation, protein trafficking, exocytosis, nucleoside metabolism, and phosphate metabolism. In summary, K12 modulates genes involved in innate response pathways and epithelial cell hemostasis making it acceptable by the human host.[17]

M18 displays a wide range of activities and is effective against *Actinomyces viscosus*, *Actinomyces naeslundii*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Listera monocytogenes*, *Hemophilus influenzae*, *Staphylococcus saprophyticus*, and *Staphylococcus cohnii* along with mutans streptococci.[8] It is seen to be effective in reducing the plaque formation, lowers *Streptococcus mutans* count in the oral cavity thereby reducing dental caries, and also reduces both moderate and severe gingivitis and periodontitis in adults.[24]

M18 role in reducing dental caries is explained through a molecular mechanism that increases oral pH and reduces plaque formation.[17] Benefits of these probiotics are seen to be reaped by individuals with high plaque score, as they demonstrate superior levels of plaque reduction.[17,36] Along with this there is greater reduction in the levels of *S. mutans*.[17] Higher the level of concentration of M18, greater is the reduction in the caries causing bacterium, thereby leading to reduction in dental caries itself.[17,36] The reduction of these caries causing bacterium can be attributed to the release of several proteins released by the strains. M18 releases salivaricin M, which is said to limit *S. mutans* and *S. sobrinus*, i.e., the caries-causing microorganisms.[17] The dextranase and urease released former leads to breakdown of dextran and latter facilitates hydrolysis of urea. Plaque being rich in dextran gets solubilized due to dextranase activity. Urease increases pH of the oral cavity by breaking down urea to carbon dioxide and ammonia.[17] Probiotics reduce the plaque formation by competing with the other microorganisms for substrate available and involves itself in its metabolism.[26]

The mechanisms of action of probiotics (SK12 and SM18) were suggestive of antimicrobial at large. The initial research conducted on probiotics focused on its benefits on the gastrointestinal tract, but probiotics displays a wide array of benefits even in the oral cavity. When compared to probiotics used for the gastrointestinal tract, oral probiotics are relatively new formulations showing the capability of fighting pathogenic microorganisms in the oral region.[17,37] In this study, antibacterial effects of (SK12 and SM18) against common cariogenic organism (*S. mutans*) were evaluated.

To test the antibacterial effects of SK12 and SM18 against *S. mutans* disk diffusion method was chosen to assess the antimicrobial susceptibility testing by measuring the diameter of the zone of inhibition. The MIC was opted to study the minimum concentration required to inhibit the bacterial growth. The MBC was opted to understand the lowest concentration required to kill the bacteria over an extended period of time under specific set of conditions.

The ability of SM18 to increase oral pH and reduce plaque formation through molecular mechanism would reduce dental caries.[17] Benefits of these probiotics are seen to be reaped by individuals with high plaque score, as they demonstrate superior levels of plaque reduction.[17,36] Along with this, there is greater reduction in the levels of *S. mutans*.[17,37] Higher the level of concentration of M18, greater is the reduction in the caries-causing bacterium, thereby leading to reduction in dental caries itself.[17,36]

The result of this study demonstrated a zone of inhibition of 15 mm by K12 with a minimum concentration at 1.6 mg/mL and bactericidal effect at 0.8 mg/mL. M18 demonstrated 20 mm of inhibition with a minimum concentration at 0.8 mg/mL and bactericidal effect at 0.8 mg/mL

The reduction of these caries-causing bacterium can be attributed to the release of several proteins released by the strains. M18 releases salivaricin M, which is said to limit *S. mutans* and *S. sobrinus*, i.e., the caries-causing microorganisms.[17,37]

## Conclusion

- SM18 demonstrated better antibacterial activity when compared to SK12.
- SM18 was effective at minimum concentration than SK12.
- SM18 demonstrated better antibacterial activity than SK12 against *Streptococcus mutans*.

Probiotics are commonly used in gastrointestinal disturbances, candidiasis, and urinary tract infections. Whereas its use against caries organisms is very limited. Before drawing conclusion on use of probiotics on *Streptococcus mutans*, it is recommended for randomized control trial involving larger population.

## Clinical Significance

Individuals have 200–300 variant species of microorganism in the oral cavity. *Streptococcus mutans* is one of the opportunistic pathologic cariogenic microorganisms and has established strong correlation with caries experience. Probiotics are microorganisms that confer health and are used to replace pathogenic microorganisms with health-conferring microorganism. BLIS K12 and M18 both

demonstrated antibacterial effect on *Streptococcus mutans* and its use in caries prevention was found to be limited. Therefore, from the above findings it is suggestive to reap the beneficial effects of BLIS K12 and M18 in oral health.

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## Manufacturer's Names

- BLIS M18® Bio-Pro PerioFresh
- BLIS K12® Throat Guard Pro

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