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In Vitro Evaluation of Commercial Probiotic Products Containing *Streptococcus salivarius* K12 by Assessment of Probiotic Viability and Inhibitory Potency against Respiratory Pathogens

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Abstract: Upper respiratory infections (URI) are the most frequent illnesses, especially in children. The majority of those infections are prescribed broad-spectrum antibiotics, which are associated with various side effects and with the increase in multi-drug-resistant strains. A promising alternative approach is the administration of the probiotic strain *Streptococcus salivarius* K12 (*SSK12*) that colonizes the upper respiratory tract (URT) and produces the salivaricins A2 and B, which strongly antagonize the growth of key respiratory pathogens. However, since for food supplements no quality controls of the active probiotic ingredient are mandatory, the efficacy of commercial products containing SSK12 may vary. This study aimed to investigate the in vitro efficacy of several commercial SSK12-containing probiotics, positioned for the prevention of respiratory infections. The parameters evaluated to determine the in vitro efficacy included the viability of the probiotic bacterial strain and the minimum inhibitory dilution (MID) of the probiotic, determined by the agar spot method, against the pathogenic/potential pathogenic bacterial strains *Streptococcus pyogenes* FF22 and *Micrococcus luteus* T18. All tests were carried out both 12 and 24 months after manufacturing (AM) for each commercial product. The viability ranged from 9×10^8 to 4.4×10^9 CFU/serving at 12 months AM and from 8.5×10^7 to 2.8×10^9 CFU/serving at 24 months AM. The MID was, in general, positively correlated with the probiotic bacterium viability and varied between the commercial products, ranging from 10^{-5} to 10^{-7} at 12 months AM and from 10^{-4} to 10^{-7} at 24 months AM. Moreover, the inhibition zones related to the two indicator strains were variable in diameter for different products. The high variation of the in vitro efficacy of commercial products containing *SSK12* may explain the different results reported in the literature regarding the clinical benefits of these preparations, and the determination of this parameter may be useful to evaluate the quality of probiotic products containing this bacterial strain.

Keywords: upper respiratory infections; *Streptococcus salivarius* K12; commercial probiotics; viability; antimicrobial activity

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1. Introduction

URI such as common cold, pharyngotonsillitis and otitis media are the most frequent illnesses worldwide [1,2]. Globally, the incidence rate of URI reached more than 17 billion in 2019, accounting for almost 43% of all disease and injury cases in the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD 2019) [3]. Global data on the incidence rate of URI in 204 countries and territories from 1999 to 2019 show the highest incidence rate in children under 5 years old [3]. Moreover, a high share of those children suffers from recurrent respiratory infections (RRIs), with an incidence of 25% of all children under 1 year and of 6% of children during the first 6 years of life [4]. URI are the most important cause of hospitalization of children under 4 years of age [5]. The treatment options for

respiratory tract infections often include cough suppressants, steam inhalation and anti-inflammatories for pain relief. However, antibiotics are administered to children who have persistent bacterial infections and to those at risk of complications. In addition, a high share of those infections are prescribed broad-spectrum antibiotics [6,7], which are associated with various side effects such as gastrointestinal effects (e.g., bloating, diarrhea, abdominal pain), headache, candidiasis, rhinitis and dizziness [8]). Moreover, the high consumption of broad-spectrum antibiotics has led to an increasing prevalence of Multi-Drug-Resistant (MDR) pathogens worldwide [9]. A multinational surveillance study showed that the percentage of MDR *Streptococcus pneumoniae* and beta-lactamase-producing *Haemophillus influenzae* or *Moraxella catarrhalis*, group A β-hemolytic streptococci strains, reached in some countries 25% of all identified strains [9]. In the past 15 years, the rate of penicillin failure has dramatically increased to almost 40% in some regions of the world [10].

A promising alternative approach for the prevention and treatment of URI is the probiotic strain *Streptococcus salivarius* K12 (*SSK12*) that colonizes the URT and produces the salivaricins A2 and B [11], both strongly antagonizing the growth of key respiratory pathogens such as *Streptococcus pyogenes* and *Staphylococcus aureus,* but also the growth of *Micrococcus luteus.* Moreover, these bacteriocins are partially antagonistic to *Moraxella catarrhalis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* and may also have antiviral action [7].

The high prevalence of upper respiratory diseases, especially during the COVID-19 pandemic, and also the efficacy of *Streptococcus salivarius* K12 make the use of oral probiotics, both for the prevention and for the treatment of URI, a good alternative for both children and adults [12].

Several commercial probiotic products containing *SSK12* have been marketed over the last decade. The clinical efficacy of the probiotic strain in reducing the incidence of respiratory infections has been reported in numerous clinical trials [7,13], although some found no significant benefits [14,15]. The clinical benefits of *SSK12* include, among others, a significant decrease of the incidence rate of bacterial and viral infections of the URT, with a concomitant decrease in antibiotic consumption, a reduction of key pathogens in the oral cavity and a significant reduction of tonsillectomies [16–21]. In addition, clinical trials conducted in both adults [16] and children [17] demonstrated the reduction of the recurrences of bacterial pharyngotonsillitis by approximately 80% and 90%, respectively.

Previous studies have shown a dose–response relationship for the colonization of *SSK12* at a critical level of 1×10^9 CFU/g [22], and there also seems to be a critical minimum concentration of *SSK12* necessary to obtain significant inhibitory activity [23]. The majority of the commercial probiotic products are marketed as food supplements in the European Union, with no mandatory tests required to prove the viability of the probiotic strains claimed in clinical studies or on the packaging. Since other studies have shown significant differences in the quality of commercial probiotics in terms of probiotic viability and strain identification [24,25], an inconsistent quality of commercial products containing *SSK12* could be an explanation for the few studies with *SSK12* which could not find any clinical benefits.

Starting from this assumption, our study investigated the in vitro efficacy of commercial probiotics containing *SSK12*, positioned for the prevention and treatment of respiratory infections in both children and adults. The in vitro efficacy was assessed by analyzing the cell viability and antimicrobial activity of *SSK12* present in the commercial products. Since water activity is a crucial aspect for the stability of probiotic products [26,27], we also determined this parameter.

2. Materials and Methods

2.1. Commercial Products Containing SSK12

Seven commercial products containing *SSK12* were purchased from different online pharmacies and were analyzed at 12 months and 24 months AM. All products were stored under room temperature (<25 °C) by strictly following the advice on the product packaging. Table 1 provides an overview of the commercial products tested, including their matrix,

packaging, direction of use and CFU counts claimed on the labels. Five of the products are delivered as a lozenge which should slowly dissolve in the mouth in order to ensure colonization of the oral cavity with the probiotic strain *SSK12*. Only two products are delivered as a powder for small children who are not yet able to consume a lozenge. The CFU counts claimed on the packaging vary between 500 million and 2.5 billion per serving. For most of the products, a daily dosage of 1 lozenge/sachet is recommended, which can be increased in times of high need. Now Foods OralBiotic® is the only product for which the daily dosage starts from 2 lozenges.

Table 1. Commercial probiotics containing *SSK12* used in this study.

Product	Manufacturer	Matrix	Primary Packaging of the Products §	Direction of Use	CFU Claimed per Serving *
Bactoblis®	Bluestone Pharma	Lozenge	alu-alu blister	1 lozenge per day	1 billion CFU/lozenge [1]
Bactoblis® Sachet	Bluestone Pharma	Powder	alu-sachet	1 sachet per day	1 billion CFU/sachet [1]
DailyDefence-BLIS K12™	BLIS Technologies	Lozenge	alu-PVDC blister	1–2 lozenges per day. In times of high need, take a maximum of 4 lozenges per day	1.25 billion CFU/lozenge [2]
DailyDefence Junior-BLIS K12™	BLIS Technologies	Powder	glass bottle	1–2 times per day	1.25 billion CFU/dose-powder [2]
ThroatGuard PRO-BLIS K12™	BLIS Technologies	Lozenge	alu-PVDC-blister	1–2 lozenges per day. In times of high need, take a maximum of 4 lozenges per day	2.5 billion CFU/lozenge [2]
Difflam® 1st Signs Defence Probiotic	Difflam®	Lozenge	alu-PVDC blister	1 lozenge, two times per day	500 million CFU/lozenge [2]
Now Foods OralBiotic®	Now Foods	Lozenge	plastic bottle	2 lozenges, 1–4 times per day	1 billion CFU/2-lozenges [2]

§ alu = aluminum; PVDC = polyvinylidene chloride; * minimum intake recommended by the manufacturer; [1] CFU claimed after 24 months shelf live; [2] CFU claimed at the date of manufacturing.

2.2. Average Weight

The average weight was determined by following the method described in the Pharmacopoeia 2.9.5. Basically, 20 lozenges/sachets were measured to determine their average mass.

2.3. Water Activity

The water activity of all products was determined at 12 and 24 months AM, using the Rotronic probe type HC2-AW-(USB) (Rotronic Instrument Corp., Hauppauge, NY, USA) and following the procedure described in the product manual.

2.4. In Vitro Efficacy of SSK12 from Commercial Probiotic Products

The parameters to evaluate the in vitro efficacy included the viability of *SSK12* in the commercial products and its inhibitory activity against a typical pathogen species involved in URI, namely, *Streptococcus pyogenes*, and a strain of *Micrococcus luteus*, an opportunistic pathogen in immunocompromised patients, causing various infections, including pneumonia [28,29].

A first dilution of each product was prepared by aseptically suspending 5 times the minimum daily recommended dosage in 45 mL of PBS (Oxoid) and homogenizing for 5 min in a stomacher (MiniMix, Interscience, Saint-Nom-la-Bretèche, France). All tests were carried out at 12 and 24 months AM.

2.4.1. Viability Tests

From the first dilution of each tested product, serial 10-fold dilutions were plated on Columbia Blood Agar Base medium (BD Difco, Winnersh, UK) supplemented with 5 g/L of yeast extract, 2.5 g/L of glucose and 1 g/L of calcium carbonate [30], further referred to as CAB K12 medium, and incubated at 37 °C, 5% CO_2 in air, for 24 h. The colony-forming units (CFU) were counted in the appropriate dilution and calculated per serving.

2.4.2. Inhibitory Efficacy

The Minimum Inhibitory Dilution (MID) was determined to evaluate the antimicrobial activity of the *SSK12* strain in the commercial products against the bacterial indicator strains *Micrococcus luteus* T18 and *Streptococcus pyogenes* FF22 [31]. Both strains were purchased from the New Zealand Reference Culture Collection: Medical Section (NZRM 2283 and NZRM 2284, respectively).

The agar spot method [32] was used in order to determine the MID. Ten microliters of the serial 10-fold dilutions of each product were placed on the surface of CAB K12 agar plates and incubated at 37 °C, 5% CO_2 in air, for 24 h, to allow the growth of *SSK12*. The agar medium was then covered with a thin layer of indicator lawn, i.e., 5 mL of Brain Hearth Infusion medium (BHI, Roth, Karlsruhe, Germany), supplemented with 0.7% agar and inoculated with 100 µL of the indicator strain. The plates were then incubated for another 24 h under the same conditions.

The MID was defined as the lowest probiotic dilution that resulted in no visible growth of the indicator strains around the *SSK12* spots. The MID can therefore work as an indicator of the efficacy of the various commercial products analyzed in this study. Moreover, as a second indicator of the activity of the probiotic strain, the inhibition zones of all active dilutions of the *SSK12* samples from each commercial product, was measured with a ruler. The tests were performed for three repetitive samples of each product, with three simultaneous tests of each dilution, and the results are presented as mean values ± standard deviation (SD).

2.5. Statistical Analysis

GraphPad Prism (GraphPad Software LLC, Boston, MA, USA) was used for the statistical analysis of the results. The significance level (alpha) was set at 0.05.

3. Results

3.1. Average Weight

As seen in Table 2, the average weight was, in general, close to the net weight claimed by the manufacturer. Higher differences between the claimed and the analyzed weights (of about 10%) were detected for the products Daily Defence-BLIS K12™ and Throat Guard PRO-BLIS K12™.

Table 2. Claimed and analyzed net weights of the commercial products.

Product	Net Weight Claimed by Manufacturer	Net Weight Analyzed
Bactoblis®	950 mg/lozenge	950 ± 16 mg/lozenge
Bactoblis® Sachet	1.5 g/sachet	1.47 ± 0.01 g/sachet
Daily Defence-BLIS K12™	1 g/lozenge	902 ± 18 mg/lozenge
Daily Defence Junior-BLIS K12™	about 1 g/powder dose	n.a.
Throat Guard PRO-BLIS K12™	1 g/lozenge	901 ± 3 mg/lozenge
Difflam® 1st Signs Defence Probiotic	No information on the packaging	903 mg ± 5 mg
Now Foods OralBiotic®	500 mg/lozenge	506 ± 4 mg/lozenge

n.a. = not applicable, since the product is delivered as a powder and has to been taken with a measuring spoon provided by the distributor.

3.2. Water Activity

The water activity at 12 months AM varied among the commercial products containing *SSK12*, ranging from 0.099 a_w for Daily Defence Junior-LIS K12^{TM} and 0.097 a_w for Now Foods OralBiotic® to 0.245 a_w for Throat Guard PRO-BLIS K12^{TM} (Figure 1). The analysis further showed that the water activity values increased over time for all products. At 24 months AM, the Bactoblis® lozenges had the lowest water activity (0.137 a_w), while the Daily Defence-BLIS K12^{TM} product had the highest water activity (0.373 a_w) among all products tested in this study.

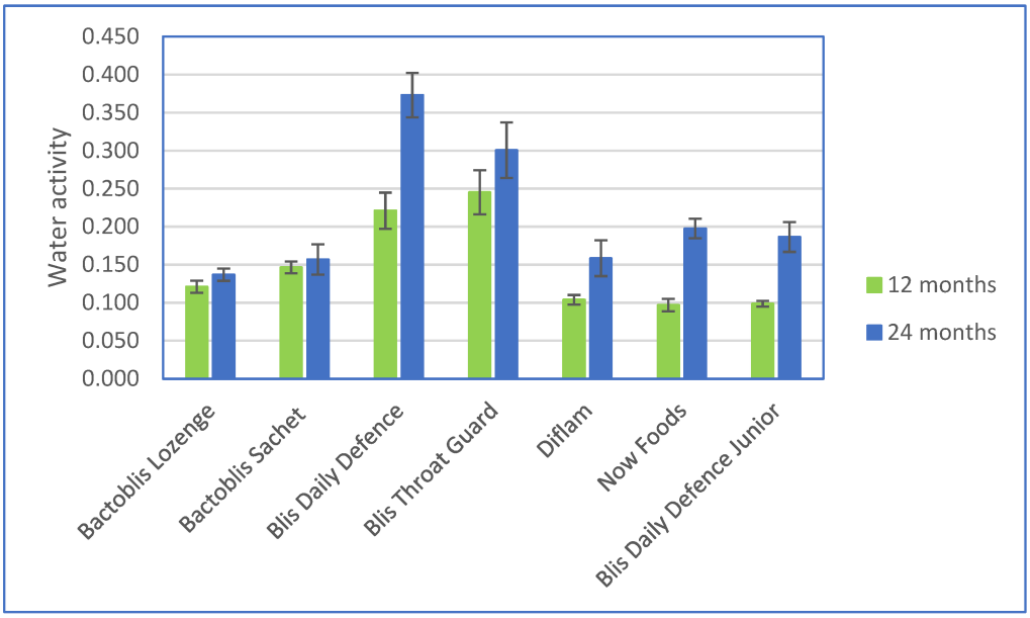

Figure 1. Water activity of the analyzed commercial products, measured at 12 and 24 months AM. The results are presented as mean values ± standard deviation.

The relative increase in water activity was, however, the highest for the Daily Defence Junior-BLIS K12^{TM} product, corresponding to about 88%, while the lowest relative increase was observed for the Bactoblis® lozenge, corresponding to only 7.5%.

3.3. In Vitro Efficacy of the Commercial Products Containing SS K12

3.3.1. Viability Tests

The enumeration of viable *SSK12* cells showed significant variations among the products ($p < 0.0001$) and at different timepoints. The viable cell counts ranged at 12 months AM from 9.0×10^8 CFU/serving for for Difflam® to 4.4×10^9 CFU/serving for Bactoblis®. In general, the viability of *SSK12* decreased over time in all the tested products, but at a variable degree. At 24 months AM, the lower viability was detected for Daily Defence-BLIS K12^{TM}, while the highest viability was found for the Bactoblis® lozenges, followed by Bactoblis® Sachet (Table 3). The latter two products were the only ones with CFU counts at 24 months AM still in the range claimed by the manufacturer.

Table 3. Probiotic viability in the examined commercial products at 12 and 24 months AM.

Product	CFU/Serving at 12 Months AM	CFU/Serving at 24 Months AM
Bactoblis®	$4.4 \times 10^9 \pm 2.8 \times 10^8$	$2.8 \times 10^9 \pm 1.4 \times 10^8$
Bactoblis® Sachet	$2.9 \times 10^9 \pm 1.1 \times 10^8$	$2.4 \times 10^9 \pm 1.5 \times 10^8$
Daily Defence-BLIS K12™	$1.4 \times 10^9 \pm 5.0 \times 10^7$	$8.5 \times 10^7 \pm 2.6 \times 10^6$
Daily Defence Junior-BLIS K12™	$1.2 \times 10^9 \pm 6.9 \times 10^7$	$7.0 \times 10^8 \pm 6.9 \times 10^7$
Throat Guard PRO-BLIS K12™	$1.3 \times 10^9 \pm 7.8 \times 10^7$	$3.7 \times 10^8 \pm 2.5 \times 10^7$
Difflam® 1st Signs Defence Probiotic	$9.0 \times 10^8 \pm 5.0 \times 10^7$	$8.5 \times 10^8 \pm 9.7 \times 10^7$
Now Foods OralBiotic®	$1.6 \times 10^9 \pm 9.5 \times 10^7$	$2.6 \times 10^8 \pm 8.7 \times 10^6$

3.3.2. Inhibitory Efficacy

Figure 2 shows two examples of the results obtained by the agar spot method used in order to determine the MID. A clear inhibition of the two indicator strains was detected for the Bactoblis® product up to a dilution of 10^{-7}, both at 12 and 24 months AM. For the Daily Defence-BLIS K12™ product, a clear inhibition could only be observed at a dilution of 10^{-6} at 12 months AM and of 10^{-4} at 24 months AM, respectively.

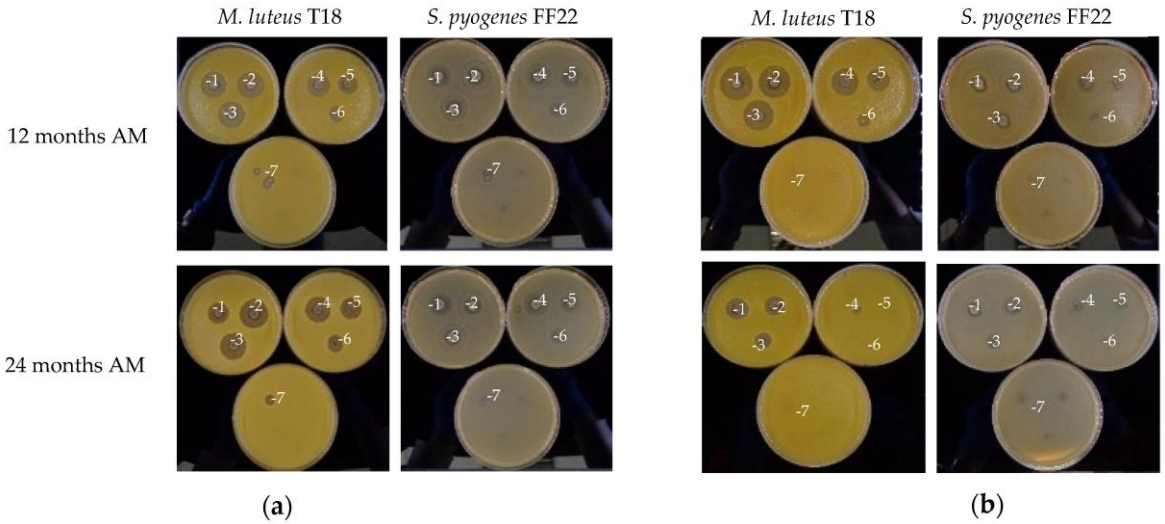

Figure 2. Inhibitory activity of Bactoblis® (**a**) and Daily Defence-BLIS K12™ (**b**) at 12 and 24 months AM. The inhibitory activity was determined by the agar spot method, using *Micrococcus luteus* T18 and *Streptococcus pyogenes* FF22 as indicator strains. The numbers represent the decimal dilutions of each tested sample.

A summary of the results for all the tested products is presented in Table 4. At 12 months AM, the highest inhibitory activities were detected for Bactoblis® and Bactoblis® Sachet. The MID in these cases was 10^{-7}, and the inhibition zones were the largest recorded among the products, in relation to both indicator strains. These two products also maintained a MID of 10^{-7} up to 24 months AM, although the diameters of the inhibition zones were slightly smaller at the end of their shelf life.

Most of the other products showed clear inhibition zones up to a dilution of 10^{-6} (MID = 10^{-6}) at 12 months AM, except for Now Foods OralBiotic®, with an MID of 10^{-5} at this time point. However, there were significant differences ($p < 0.005$) in the diameters of the inhibition zones among the products as well as in relation to the indicator strains used. In general, the inhibition zones obtained with *Micrococcus luteus* T18 were larger compared to those obtained when using *Streptococcus pyogenes* FF22.

Table 4. Minimum inhibitory dilution (MID) of the commercial products and diameters of the inhibition zones they produced when tested against *Micrococcus luteus* T18 and *Streptococcus pyogenes* FF22 at 12 months and 24 months AM.

Product	Diameter of the Inhibition Zones (mm) *							
	12 Months AM				24 Months AM			
	M. luteus T18		*S. pyogenes* FF22		*M. luteus* T18		*S. pyogenes* FF22	
	10^{-6}	10^{-7}	10^{-6}	10^{-7}	10^{-6}	10^{-7}	10^{-6}	10^{-7}
Bactoblis®	14 ± 0.3	10 ± 0.7	15 ± 0.6	6 ± 0.5	15 ± 0.5	6 ± 0.6	14 ± 0.9	5 ± 0.9
Bactoblis® Sachet	20 ± 1.1	14 ± 0.8	17 ± 0.4	11 ± 1	15 ± 0.2	9 ± 1.2	15 ± 1.1	4 ± 1.8
Daily Defence-BLIS K12™	12 ± 2.7	n.a.	5 ± 1.2	n.a.	MID = 10^{-4}			
Daily Defence Junior-BLIS K12™	13 ± 0.4	n.a.	13 ± 0.1	n.a.	MID = 10^{-5}			
Throat Guard PRO-BLIS K12™	13 ± 1.2	n.a.	9 ± 0.9	n.a.	MID = 10^{-4}			
Difflam® 1st Signs Defence Probiotic	4 ± 0.4	n.a.	5 ± 0.8	n.a.	10 ± 1.8	n.a.	9 ± 2.8	n.a.
Now Foods OralBiotic®	MID = 10^{-5}				MID = 10^{-4}			

* n.a. = no activity (no inhibition could be observed at this dilution); MID = minimum inhibitory dilution. * for a comparison, the diameters of the inhibition zones at the dilutions of 10^{-6} and 10^{-7} are shown in the Table; when no inhibition was detected at any of these two dilutions, the MID value is mentioned in the table; no inhibition could be detected at dilutions higher than 10^{-7} for any of the tested products.

At 24 months AM, except for the two Bactoblis® products, only Difflam® 1st Signs Defence Probiotic had the same MID as that at 12 months AM (MID = 10^{-6}). For all the other products, we could observe a decrease in inhibitory activity over time, in terms of both the MID value (1–2 points lower compared to the MID at 12 months AM) (Table 4) and the diameter of the inhibition zones (results not shown).

A statistical analysis of all the results (Figure 3) showed that the alu–alu packed Bactoblis products, both at 12 and 24 months AM, grouped together, having the highest CFU values of *SSK12* and the lowest MID, but also showing the lowest increases in water activity at 24 months. For the two products packed in plastic/glass bottles, namely, Now Foods Oral Biotics and Daily Defence Junior, we observed a high increase in water activity at 24 months, with a concomitant decrease of probiotic viability and an increase in MID. A similar pattern was observed for Daily Defence and Throat Guard PRO, packed in alu–PVDC. In the case of Difflam, however, an increase in water activity did not have a significant effect on probiotic viability and MID.

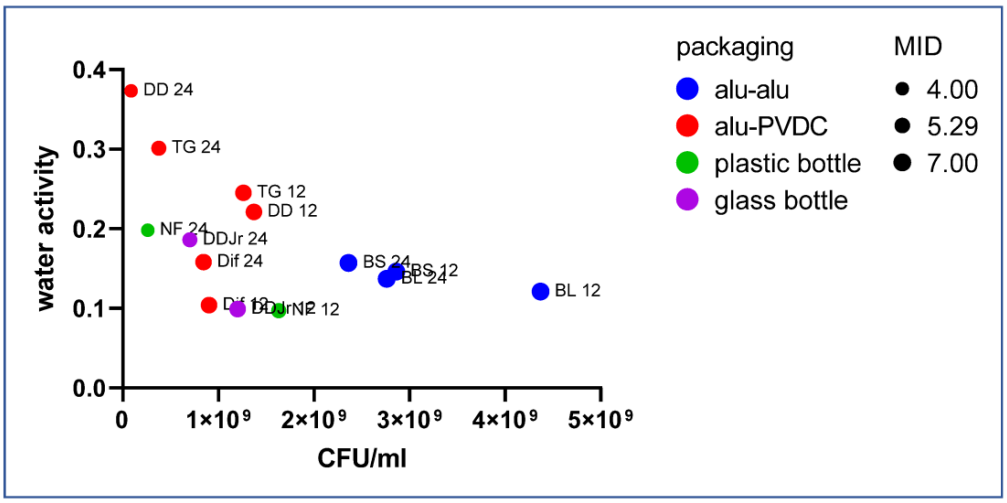

Figure 3. Principal component analysis (PCA) of the probiotic products at 12 and 24 months after manufacture (AM). BL = Bactoblis Lozenge; BS = Bactoblis Sachets; DD = Daily Defence; DDJr = Daily Defence Junior; Dif = Difflam; TG = Throat Guard PRO; NF = Now Foods Oral Biotics. The numbers (12/24) indicate the time of testing, namely, 12/24 months AM.

4. Discussion

The number of clinical trials using oral probiotics is still limited, but the field is rapidly expanding. In general, the published studies recognize that *S. salivarius* K12 has a low pathogenicity and is well tolerated, but the results regarding its clinical benefits differ from one study to another [7,13,14,33]. For instance, in the presence of acute pharyngotonsillitis treated with penicillin, *SSK12* probiotics are unlikely to be effective [14] but may be used as an alternative to antibiotics in non-severe cases or for the prevention of disease recurrence [7]. On the other hand, the prophylactic administration of *SSK12* seems to reduce streptococcal and viral infections in children with a history of recurrent oral streptococcal disease [17].

The variable results obtained in different clinical trials may be explained by the use of different *SSK12* formulas. Since all these probiotics contain the same active bacterial strain, the reason for the variable efficacy may be found in the different efficacy of this strain in the particular formulation of each product, but also in the concentration and stability of the product. In this context, we aimed to study the differences among several commercial probiotics containing *SSK12* in terms of in vitro efficacy. Probiotic viability and inhibitory efficacy were evaluated both in the middle (12 months AM) and at the end of the claimed products' shelf life (24 months AM).

Recent studies showed that stability is a critical issue for commercial products containing live probiotics, and often only freshly produced products contain the number of viable cells claimed on the product packaging [34,35]. In our study, for most products, the number of viable cells claimed by the manufacturer could be confirmed at 12 months AM. The number of live probiotic bacteria decreased over time in all products. However, the decrease varied among the products, and we could observe an inverse correlation between the viability of the active strain and the water activity of the product ($r = -0.54$). It was previously suggested that water activity is one of the main factors influencing a probiotic stability over the shelf life of a product [27]. The lowest *SSK12* viability at 24 months AM was observed for DailyDefence-LIS K12^{TM}, which also appeared to be the product with the highest water activity (0.373 a_w). In general, the water activity highly differed between the commercial products and increased over time. In order to reach a high stability of a probiotic, a low water activity of the finished product is one of the most crucial aspects. According to Fenster et al. (2019) [36], once a low-water-activity probiotic product has been produced, a low water activity can be maintained by choosing a type of packaging with adequate moisture vapor transmission rate (MVTR). The lower the MVTR, the slower the moisture increase, and the better can the viable cell count be maintained over the shelf life of the product [36]. Therefore, ensuring a low water activity during manufacturing and throughout the shelf life of a probiotic product is a key factor for the viability of the product. Moreover, the oxygen transmission rate (OTR) is the second crucial aspect when discussing different packaging options. The packaging of the commercial products tested in this study was very diverse, ranging from high-quality alu–alu-blisters to alu–PVDC blisters or plastic bottles for the lozenge products and from alu sachet packaging to glass bottles for the powder products. The products with the highest *SSK12* viability at 12 months AM were Bactoblis® and Bactoblis® Sachet, with CFU counts of 4.4×10^9 and 2.9×10^9, respectively. These products also presented the highest cell counts at 24 months AM, namely, 2.8×10^9 and 2.4×10^9, respectively. Both products are packed in alu–alu blisters/sachet, a packaging option which is usually more expensive but offers low MVTR and low OTR and guarantees a high viability of the probiotics over the product's shelf life [37]. For all the other products, the viability at 12 months AM was, in general, close to that mentioned by the producer, but it decreased in the following 12 months below the critical level of 1×10^9. The viable cell counts of DailyDefence-BLIS K12^{TM} even dropped to 8.5×10^7 CFU/serving. The alu–PVDC packaging of this product, with its higher MVTR and OTR, might be an explanation for this significant drop in CFU counts over time.

The MID against the two bacterial strains *Micrococcus luteus* T18 and *Streptococcus pyogenes* FF22 was the second parameter we examined to evaluate the in vitro efficacy of

the tested commercial products. Since *Streptococcus pyogenes* and *Micrococcus luteus* are among the pathogenic/opportunistic pathogenic species causing bacterial URI, the MID can be used as an indicator of the antimicrobial activity of commercial probiotic products against bacterial pathogens involved in URI. The lower the MID is, the more potent may the *SSK12* strain be in the probiotics. We observed that the MID was, in general, directly related (r = 0.82) to the viability of *SSK12* in the commercial products. The two Bactoblis® products, with a viability of over 2.4 billion CFU/dose, both at 12 and 24 months AM, had the lowest MID, of 10^{-7}, during the entire shelf life. In addition, the inhibitory activity of *SSK12* evaluated by measuring the diameters of the inhibition zones was the highest for these two products. Decreased functional properties, such as the inability to inhibit a certain pathogen, have been previously described and related to the manufacturing processes and the probiotic matrix [38].

Having in mind that previous studies showed a dose–response relationship for the colonization of *SSK12* at a critical level of 1×10^9 CFU/g [22] and that a critical minimum concentration of *SSK12* seems to be required to obtain significant inhibitory activity [23], it is very likely that most of the commercial products tested in this study do not provide clinical benefits associated with *SSK12* such as a reduction in the incidence rate of bacterial and viral URI (pharyngitis, tonsilitis, otitis media, influenza, COVID-19) and consequently a reduction in antibiotic and anti-inflammatory intake, when taken later than 12 months AM.

Moreover, for two of the products, namely, Daily Defence-BLIS K12™ and Throat Guard PRO-BLIS K12™, a difference of about 10% between the claimed and the measured net weight was observed, which is above the maximum percentage deviation of 5% for lozenge products of 250 mg or more, defined in the European Pharmacopoeia (European Pharmacopoeia 10.0; 2.9.5 Uniformity of Mass of Single Dose Preparations).

5. Conclusions

The results showed a high variation in the concentration and inhibitory activity of *SSK12* found in commercial products. Many of the products provided, at 24 months AM, a concentration of live *SSK12* below the critical level of 1×10^9, which may be an explanation for the lack of benefits reported by the few clinical trials with *SSK12* for the prevention of URI. Moreover, most of the commercial products showed a low in vitro inhibitory activity of *SSK12*, which again may explain the different clinical efficacies reported.

This study clearly demonstrates that the quality of commercial supplements containing *SSK12* highly varies, as it has been shown for many other commercial probiotic supplements [39]. In order to ensure the outstanding clinical efficacy reported for the probiotic strain *SSK12* in more than 20 clinical trials, the commercial products containing *SSK12*—or any other commercial probiotic—should be tested much more rigorously in terms of quality before being marketed. More rigorous quality control is also required to ensure that the products contain the viable cell counts claimed by the manufacturer over the period they are available in the pharmacy.

In this respect, the probiotic viability and antimicrobial potency might be important criteria to evaluate the quality and clinical efficacy of probiotic products containing *SSK12*. They are simple parameters that may be evaluated in systematic quality control procedures of commercial probiotics which claim a mode of action based on an antibacterial activity.

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