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MISCELLANEOUS

Bacteriotherapy with *Streptococcus salivarius* 24SMB and *Streptococcus oralis* 89a oral spray for children with recurrent streptococcal pharyngotonsillitis: a randomized placebo-controlled clinical study

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Abstract

Purpose Group A beta-hemolytic *Streptococcus* (GABHS) causes a recurrent acute pharyngotonsillitis (RAPT) in children. Moreover, the repeated use of antibiotics contributes to its resistance. However, *S. Salivarius* 24SMB and *S. oralis* 89a were effective probiotics in other infections. Thus, we decided to evaluate this combination efficacy compared to placebo in RAPT.

Methods Patients with microbiologically confirmed GABHS were enrolled in this randomized, placebo-controlled trial. They received the aforementioned combination or placebo as an oral spray. We investigated episodes of frequency and duration, need for antibiotics, school days lost, the treatment impact on life quality, treatment compliance and side effects during a 90-day treatment and a 6-month follow-up.

Results We included 41 patients in each group. The mean number of GABHS infection was significantly lower during both study periods for the two groups. However, our treatment group showed a lower rate. Moreover, the probiotic group had a lower mean number and a shorter median duration of GABHS episodes during both study periods than controls. Furthermore, the mean duration of antibiotic treatment was lower in the probiotic group during the 90-day and 6-month follow-up periods. Similarly, patients in the probiotic group showed a significantly lower mean number of absence days from school but higher EQ-VAS score. Indeed, all patients included were compliant to treatment.

Conclusions We identified potential probiotics, possessing desirable features against GABHS pharyngotonsillitis. Our findings represent the first evidence which throws the light on using these probiotics that can reduce antibiotics use which did not have efficient results regarding recurrence.

Keywords *Streptococcus pyogenes* · Tonsillitis · *Streptococcus salivarius* · Probiotics

Introduction

Streptococcus pyogenes, or Group A beta-hemolytic *Streptococcus* (GABHS), is a frequent pathogenic bacterial etiology of recurrent acute pharyngotonsillitis (RAPT) in young children, that causes repeated use of antibiotics, a significant burden on both families (absences from school or work) and societies (health care costs) as well as a significant repercussion on the patients' health-related quality of life (HRQL) and their families [1–3].

Indeed, the antibiotic treatment is the most common recommended strategy for patients with GABHS-positive RAPT, however, it remains unclear which antibiotic regime is the most effective in eradicating the pathogen and preventing future episodes of APT [4]. Another problem is that only long-term antibiotic prophylaxis may give better outcomes, but of course, this strategy may be burdened by relevant adverse events [5]. Moreover, the repeated use of antibiotic agents contributes to the emergence of antibiotic-resistant bacteria, which are being reported with an increasing frequency [6]. For patients with frequent, severe episodes of GABHS pharyngitis that recur despite an appropriate antibiotic treatment, tonsillectomy is indicated but it is associated with significant costs, harms, and

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risks (e.g., pain, hemorrhage, and infection) [7]. Consequently, a conservative management strategy in terms of prevention of new cases of GABHS pharyngitis infection is increasingly promoted [6].

Indeed, several bacterial species are present in the oral cavity, preserving the microflora unaltered by interference with and/or inhibition of other pathogens through antimicrobial peptide production such as bacteriocins, having an important effect on the general health. Subsequently, it has been suggested that alterations in this environment may alter the normal microbiome balance to an infection-related species [8]. Thus, a growing interest has been directed towards probiotics and their efficacy for prevention and therapy of ear, nose, and throat (ENT) infections [9] as well as in the gastrointestinal tract, protecting the intestinal epithelium through preserving its microflora and controlling the host's immune response [10–12]. Consequently, oral probiotic *S. salivarius*, a non-pathogenic species and a prominent member of the normal oral microbiome, has been shown to be effective in reducing the repeated colonization of the main pathogens to the upper respiratory tract (URT), reducing the new attacks of acute otitis media (AOM) [13], and the frequency of recurrent pharyngeal infections in children and adult populations, besides its good safety and tolerability [5, 13, 14] which has been confirmed before in adults [14] offering a valid alternative to antibiotics in the prevention or treatment of GABHS pharyngitis [15, 16].

According to the present literature, there are various probiotic strains from the species *S. Salivarius* with anti-*S. pyogenes* activity, such as strains K12, M18, ST3, 24SMB, and K58. However, of these, we have a clinical evidence to use as therapeutic strategies in treating a *Streptococcal* sore throat only with K12 strain [17]. It is noteworthy also that *S. oralis* 89a strain, a putative probiotic bacterium, possesses an interfering activity and a clinical effect on *streptococcal* tonsillitis [18].

In addition to that, a previous report showed the potential synergetic benefit of using *S. salivarius* 24SMB with *S. oralis* 89a in preventing recurrent otitis [5]. Notwithstanding, no previous trials investigated the efficacy and safety of this combination for RAPT in children so far.

Hence, we decided to evaluate the efficacy of *S. salivarius* 24SMB strain in a combination with *S. oralis* 89a strain as compared to placebo treatment in children with GABHS-positive RAPT and to assess them for the frequency and duration of the episodes, need for antibiotic treatment, school days lost, the impact of the treatment on HRQL, compliance and side effects of the treatment.

Materials and methods

Patients recruitment was performed from November 2016 to July 2017 by an experienced otolaryngologist not involved in the study who made daily visits to the outpatient section of the Otolaryngology Unit of the Santa Marta e Santa Venera Hospital in Acireale, Catania, Italy. Patients were eligible to participate if they are 6–11 years old and who had at least 3 episodes of microbiologically documented GABHS infections with clinical symptoms suggesting GABHS pharyngitis [19] in the period from November 2016 to July 2017. Exclusion criteria were non-completion of the entire study protocol; the presence of symptoms of another infective disease at the time of enrolment; severe respiratory and/or systemic pathologies; current antibiotics, corticoids or montelukast treatment; asthma; known immunological deficiency; had undergone tonsillectomy, adenoidectomy, or a previous reduction of tonsils; healthy carriage of *S. pyogenes*; and hypersensitivity to penicillin. A written consent was obtained from parents of children enrolled in the study.

The study protocol was reviewed and approved by the Ethics Committee of the School of Medicine—ASP 3 Catania (ID: 083/17).

This study is a prospective, randomized, single-blind, placebo-controlled pilot study to assess the effect of probiotic treatment in children with recurrent GABHS infection.

PASS 2008 software was used to calculate the minimum sample size. Parameters used for the computation was based on a previously published literature [1]. A minimum sample of 20 patients—10 for each group—achieves 93% power to detect a difference of -0.10 given that the mean number of GABHS episode for the probiotic group is 0.03 ± 0.07 and alpha of 5%. The sample size was increased to 26—13 for each group—to account for 20% potential non-response.

A statistician, not related to the study, generated the allocation schedule before the start of the study using a statistical computing web programming (http://www.graphpad.com/quickcalcs). This schedule was used to generate the list of patients who were randomly allocated to one of the two study groups (probiotic or placebo) in a one to one ratio. Participants were blinded to their treatment group throughout the study, but investigators, and study site staff were unblinded. Treatment period starts from November 2017 and ends in July 2018.

Patients assigned to the treatment group were instructed to take an oral topical device made up of a suspension of two specific bacterial strains: *S. salivarius* 24SMB and *S. oralis* 89a to be administered as an oral spray. The mix suspension consisted of a minimum of 125×10^9 colony forming units/mL (CFU/mL) in 10 mL of saline, and was delivered 2 puff once a day for 30 consecutive days each month for 3 consecutive months with an oral spray that provided 2×10^9 CFU per puff. Dosages are in accordance to the product manufacturer's instructions (DMG ITALIA SRL, Rome, Italy). Patients in the control group received a placebo—a prepared mix suspension with matching color, taste, and consistency but without any probiotics included. The placebo was administered with the same oral spray and using

the same protocol as the probiotic treatment. Preparations were administered by adequately trained parents who were instructed both on how to use and store the products. The treatment was stopped after 90 days for both the probiotic and placebo groups.

Study outcomes were assessed for a period of 6 months from the day of first treatment. The primary outcome of interest in this study is the number of new episodes of GABHS pharyngotonsillar infections. Parents of the study participants were asked to return to the clinic with their child for monthly control visits and each time their child experienced any signs or symptoms suggestive of oropharyngeal infection. To overcome the potential problem of the underreporting of GABHS pharyngotonsillar infections episodes, all of the families were systematically telephoned weekly to verify the children's status. The parents were also asked to complete a diary to record all of their children's clinical problems and the administrations of the study preparations on the planned days.

During every visit, information regarding any medical event that occurred to the children since the previous visit were obtained and recorded by interviewing parents. A complete physical examination with a careful oropharyngeal inspection including a pharyngeal swab for GABHS culture was also performed by trained ENT specialists. GABHS pharyngotonsillar infections were diagnosed based on the presence of McIsaac score with a clinical score ≥ 2 (adenopathy, fever > 38 °C, absence of cough, pharyngotonsillar exudate, age, and season) and confirmation of GABHS presence by throat culture or McIsaac score $= 5$ [20]. In the case of a positive diagnosis, antibiotic treatment was prescribed. The prescribed therapy was a combination of amoxicillin and clavulanic acid to be administered for 10 days for both groups equally. No other treatments were given with the exception of acetaminophen or ibuprofen in cases of fever.

Secondary outcomes include the duration of symptoms of each episode of GABHS infection, number of days under antibiotic therapy, and days of absence from school during the whole study period. The health-related quality of life (HRQL) was also measured at the end of the 90-day treatment period and the 6-month follow-up. HRQL was measured using the EQ-VAS section of the EuroQol-5 Dimension (EQ-5D) wherein patients were asked to rate how they perceive their health status using a vertical rating scale ("thermometer" design) from 0 to 100, with 0 equals "worst imaginable health state" and 100 equals "best imaginable health state" [3].

Any adverse event during the 90-day treatment period was recorded to assess the safety of the probiotics. Compliance in terms of adherence to therapy was monitored by a compliance calendar chart given to the parents to document the frequency and intake of treatments. Parents were instructed to mark one box for each day of consumption of the treatments, but if a product was not taken, the corresponding box was left unticked. Subjects were considered non-compliant if they had taken $< 80\%$ of the study treatment.

Data were encoded in MS Excel 2016 (Microsoft Corp, Redmond, Wash) the researchers. The excel file was converted to Stata file for further processing and analysis. Data were checked for completeness, consistency, and accuracy prior to the analysis. Stata MP version 14 was used for both descriptive and inferential statistics. Continuous variables were presented as mean/standard deviation (SD) or median/interquartile range (IQR) depending on data distribution. Categorical variables were expressed as frequencies and percentages.

The mean number of GABHS episodes between the previous year and study period for each treatment group was analyzed using Paired *t* test. Comparison of the primary and secondary outcomes between the two groups was performed using independent t-test or Mann–Whitney *U* test depending on data distribution. Compliance, based on the number of days of product intake by treatment group, was analyzed using Mann–Whitney *U* test. Safety was assessed using Fisher's exact test. All analyses were undertaken using the intention-to-treat principle. p values < 0.05 were considered statistically significant.

Results

There were 115 individuals assessed according to the aforementioned eligibility. Of them, 31 individuals were excluded due to not having a microbiologically documented GAHBS infections ($n = 21$) or refusing to participate ($n = 10$). Subsequently, 84 patients were randomized to probiotic or placebo ($n = 42$ for each group). However, 2 patients were lost to follow-up due to a change in residence—1 patient during the 90-day treatment period (control group) and 1 patient during the 6-month follow-up period (probiotic group), then we finally included 41 patients in each group (Fig. 1).

Table 1 presents the baseline characteristics of study participants. No significant difference was observed between the probiotic and control group in terms of age, sex, and EQ-VAS at baseline and mean number of GABHS infection episodes in the previous year (all p values > 0.05).

Additionally, Fig. 2 compares the mean number of GABHS infection episodes in the previous year versus during the study period (a 90-day treatment and a 6-month follow-up). The mean number of GABHS infection episodes was significantly lower during the study period both for the probiotic ($p < 0.00001$) and control ($p < 0.00001$) groups.

Considering the whole study duration (a 90-day treatment up to a 6-month follow-up), the mean number of GABHS infection episodes is significantly lower in the probiotic group. When analyzed by period, the mean number of

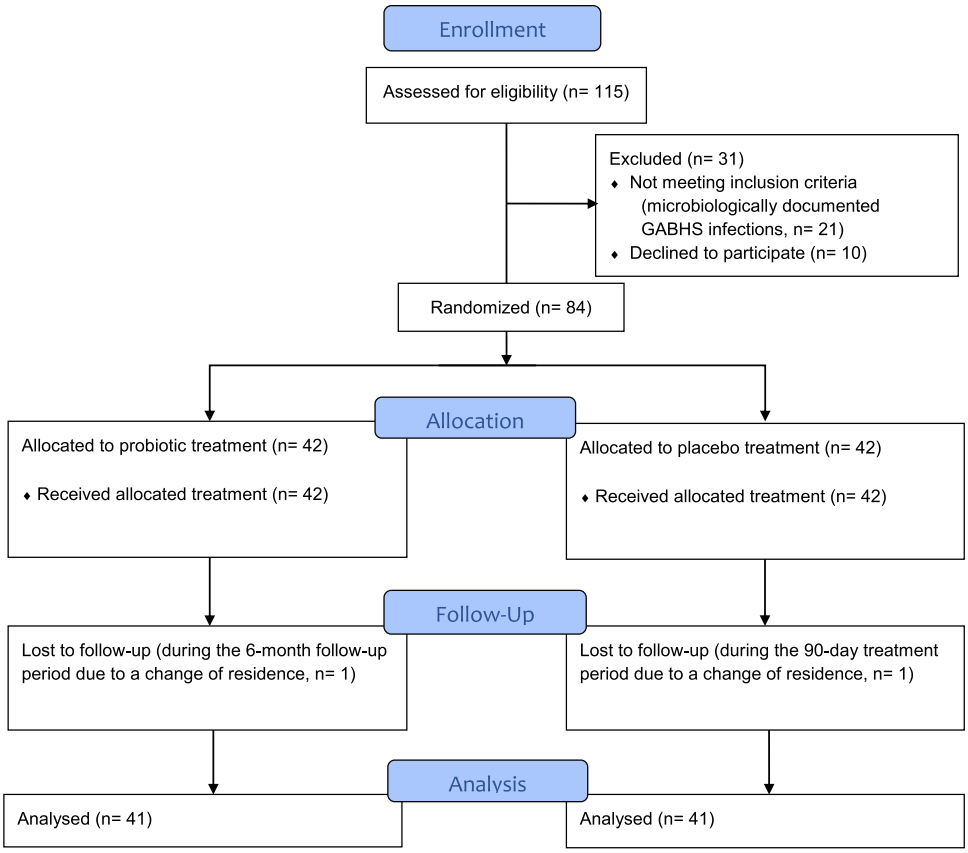

Fig. 1 CONSORT flow diagram of the study

Table 1 The baseline characteristics between the probiotic and control groups

Characteristics	Probiotic, $N=41$	Control, $N=41$	p value
Mean (±SD) age, years	8.56 ± 1.63	8.37 ± 1.55	0.5794
Gender, male (%)	24 (59)	23 (56)	0.823
EQ-VAS, mean (±SD)	84.93 ± 3.89	84.73 ± 3.81	0.819
Mean no. of GABHS infection (±SD) episode in the previous year	3.44 ± 0.55	3.51 ± 0.60	0.5653

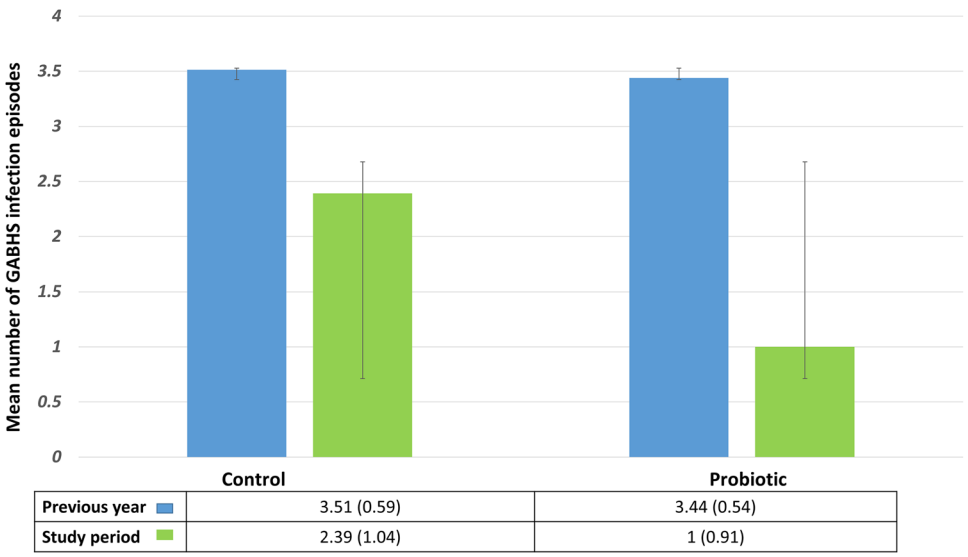

Fig. 2 The mean number of GABHS infection between the previous year and the study period between treatment groups

GABHS infection episodes at the 90-day treatment period was significantly lower in the probiotic as compared to the control group. Although a slight increase during the 6-month follow-up period in both groups was observed, patients that were given probiotic still showed a significantly lower number of episodes of infection (Table 2).

The median duration of GABHS episodes was shorter in the probiotic group during the 90-day treatment and 6-month follow-up periods compared to controls. Even when only patients with ≥ 1 episode were included in the analysis, the duration of GABHS infection remained to be significantly lower in the probiotic group. In the probiotic group, the median duration of GABHS episode does not significantly differ between the 90-day treatment and 6-month follow-up period ($p = 0.1531$). Similarly, no significant difference was observed in the duration of GABHS episode in the control group between the two periods ($p = 0.9011$), (Table 2).

The mean duration of antibiotic therapy was found to be significantly lower in the probiotic group as compared to controls during the 90-day and 6-month follow-up periods.

Patients in the control group showed a significantly higher mean number of days of absence from school. This finding was observed in both the 90-day treatment and 6-month follow-up periods.

The mean EQ-VAS score was found to be significantly higher in the probiotic groups as compared to controls in both the 90-day treatment and 6-month follow-up periods (Table 2).

Table 3 shows the compliance and safety outcomes by treatment groups. All patients included in the study were compliant to the assigned intervention to them (> 80% compliance). The median number of days the product was consumed does not differ between the two treatment groups. In addition to that, only four patients reported an adverse event—three in the probiotic group and one in the control group. A mild cough was reported by two patients in the

Table 3 The compliance and safety outcomes by the treatment group

Characteristics	Probiotic, $N = 41$	Control, $N = 41$	p value
Compliance			
Median number (IQR) of days product consumed	90 (90–90)	90 (90–90)	0.4923
Safety			
No adverse event, %	38 (93)	40 (98)	0.616
Mild cough, %	2 (5)	1 (2)	
Nausea, %	1 (2)	0	

Table 2 Comparison of outcomes between the probiotic and control groups

Characteristics	Probiotic, $N = 41$	Control, $N = 41$	p value
Mean number (± SD) of GABHS infection episodes			
Whole study period	1.00 ± 0.92	2.39 ± 1.05	**< 0.00001**
90-day treatment period	0.39 ± 0.49	1.02 ± 0.61	**< 0.00001**
6-month follow-up period	0.61 ± 0.67	1.37 ± 0.80	**< 0.00001**
Median duration (IQR) of GABHS infection, days			
90-day treatment period	0 (0–3)	3 (4–4.5)	**< 0.00001**
6-month follow-up period	3 (0–3.5)	4 (3–4)	**0.0001**
Median duration of GABHS infection (IQR), ≥ 1 episode (days)[a]			
90-day treatment period	3 (3–4)	4 (4–4.5)	**0.0009**
6-month follow-up period	3.5 (3–4)	4 (3.5–4.5)	**0.0180**
Mean number (± SD) of days under antibiotic therapy			
90-day treatment period	3.90 ± 4.94	10.24 ± 6.12	**< 0.00001**
6-month follow-up period	6.10 ± 6.66	13.66 ± 7.99	**< 0.00001**
Median number (± IQR) of cycles of antibiotic therapy			
90-day treatment period	0 (0–1)	1 (1–1)	**< 0.0001**
6-month follow-up period	1 (0–1)	2 (1–2)	**< 0.0001**
Mean number (± SD) of days of absence from school			
90-day treatment period	1.49 ± 1.91	3.66 ± 2.08	**< 0.00001**
6-month follow-up period	2.24 ± 2.49	4.61 ± 2.64	**0.0001**
Mean (± SD) EQ-VAS			
90-day treatment period	83.41 ± 4.65	77.39 ± 5.54	**< 0.00001**
6-month follow-up period	82.44 ± 4.57	77.90 ± 4.66	**< 0.00001**

Statistically significant values are in bold ($p < 0.05$)

[a]Probiotic group ($n = 16$), control group ($n = 34$)

probiotic group versus one patient in the control group. Only one patient in the probiotic group reported nausea. No significant difference in the occurrence of an adverse event by treatment groups.

Discussion

ENT infections have a big burden since they require a medical consultation, prescription of anti-pyretic treatments, anti-inflammatory, and/or antibiotics. Besides the direct costs, URT infections involve high indirect burdens due to the parents absence from their work and a negative effect on the HRQL of children and their families which augment in recurrent infections. Indeed, the commensal microbiota such as *S. salivarius, S. oralis, S. mutans, S. mitis*, and *S. sanguis* is able to inhibit the growth of the pathogens due to the interaction and competition between both of them [21–24] because pathogenic organisms have to compete with normal flora for their nutrition and adhesion receptors on the mucosa. Thence, if that pathogen is capable of attaching to these receptors, it will replicate and consequently, cause the infection [25]. Even alpha-*streptococci* absence or lower levels which are directed to otopathogens for AOM is associated with more recurrent infections in patients with *Streptococcal* pharyngotonsillitis [23]. Another mechanism of pathogenic organisms competition with other commensal flora is antimicrobial substances production such as *S. pneumoniae* produces hydrogen peroxide, which is bactericidal for *S. aureus* [21–25].

Moreover, previous reports showed that *S. salivarius*, which has a low pathogenic potential, restored the nasopharyngeal flora balance due to being the main producers of bacteriocins, which possesses an antimicrobial efficacy [26], thus reducing new attacks of URT infections and AOM as well [13, 27], as a result, probiotics can yield in a natural guard against other pathogenic organisms. It is noteworthy as well that *S. salivarius* K12 has a good bacteriocin-like inhibitory substance activity towards *S. pyogenes*, which is a frequent etiology for bacterial pharyngitis [28]. Tagg and Burton et al., disclosed the first *S. salivarius* K12 use for *S. pyogenes* and halitosis infections treatment due to producing the salA/B bacteriocin which inhibits *S. pyogenes* strains [29, 30]. Another study investigated the role of *S. salivarius* 24SMB, in children with AOM, which showed a significant reduction in children with AOM and they showed no more AOM attacks [13]. As a further matter, a previous literature review suggested that intra-nasal steroids and anti-inflammatory drugs may provide relief in mild sleep apnea, even though their long-term efficacy is restricted, non-invasive ventilation is limited by poor compliance; moreover, weight loss is effective in obese individual only. Besides, Viciani et al., disclosed good findings, whereas GABHS induced epithelial damage and streptolysin O release and cysteinyl leukotrienes production by granulocytes in recurring tonsillitis, subsequently that induces T- and B-cells proliferation, hence it causes tonsillar hyperplasia in children with sleep apnea. Indeed, these findings show alterations in the flora of the URT for pathogenic organisms which cause recurrent infections. Thus, *S. salivarius* 24SMB nasal spray could serve as an adjunct therapy for recurrent URT infections, AOM, and sleep apnea in children, fundamentally due to having its substantial inhibitory activity against *S. pneumoniae* which causes recurrent AOM [15]. Even children having more risk of developing AOM showed a lower concentration of *Streptococci* (such as *S. oralis, S. mitis, S. sanguis*, and *Lactobacillus rhamnosus*) compared to other children [13], suggesting the pivotal role of them in prophylaxis [13, 24, 31]. In addition to that, *S. oralis* 89a interferes with GABHS in *Streptococcal* tonsillitis and otitis media [23, 24, 32] due to having colicin V, a peptide antibiotic [18]. Bernstein et al. [33], revealed that *S. oralis*, in patients with hypertrophy or recurrent otitis media and undergo adenoidectomy, have the ability to antagonize and inhibit the growth of pathogenic organisms (such as *S. pneumoniae, H. influenza, Mor. catarrhalis* and *S. pyogenes*) in the nasopharynx.

To our best of knowledge, it has not been investigated the efficacious role of *S. salivarius* 24SMB together with *S. orals* 89a strains in children with recurrent GABHS-pharyngotonsillitis. Hence, our prospective, randomized, placebo-controlled study is the first study to throw the light on the synergistic impact of this combination in that disease due to having a good efficacy and safety profile. It is noteworthy that one of the promising facts about *S. salivarius* is its close relation with *S. thermophilus* which is involved in cheese and yogurt production [15]. Thereby, it has the "generally regarded as safe" status [30, 34]. Furthermore, a recent study, comparing 13 various alpha-*streptococci* strains, showed that only *S. salivarius* 24SMB has a proper probiotic role due to its great adhesion to HEp-2 cells, anti-inflammatory as well as immunomodulatory activity, high plasmid-encoded bacteriocins production, safety in human [14, 15], and a potential inhibitory effect against *S. pneumoniae* [15]. Therefore, these features demonstrate the association between the mucosal microbiome and innate immunity in keeping a resistant epithelial barrier [35]. Another mechanism may be due to increasing the phagocytic activity as well as the capacity of peripheral blood leucocytes [36], higher specific immunoglobulins [37] and the increased production of cytokines [38]. Indeed, the characteristics of probiotics differ and are specific for each strain with specific mechanical properties for each location. Therefore, they cannot treat all infections [39]. For example, in comparing our probiotics against other probiotics, a previous study showed that the inhibitory capacity of *S. Salivarius* K12 is due to the

action of the lantibiotics salivaricin A2 and B [40] which is more effective against the infective GABHS but not the GABHS which present normally in the oral microbiota [17, 40]. On the contrary, *S. salivarius* K58 produces pantothenic acid antagonist, enocin, against *S. pyogenes* infections [41] while *Lactobacillus fermentum* VRI-003 in athletes lead to a great decline in the number of days of respiratory illness and other symptoms [42]. Indeed, pathogenic bacteria could get rid of resistance to infection; hence it stimulates asymptomatic inflammation, which facilitates further bacterial invasion such as invasion by *H. influenzae* which induces changes in the epithelial barrier and, subsequently, produces early pro-inflammatory cytokines in chronic rhinosinusitis [43]. However, normal flora like *Prevotella* resists against pathogen-mediated signaling, hence shares in keeping the mucosal homeostasis in respiratory diseases such as asthma associated with bacteria and chronic obstructive pulmonary diseases [44].

Although our control group showed a reduced rate of GABHS infections compared to the previous year, our treatment group showed a lower rate than the other group. A possible explanation for that is probably due to the immune competence that physiologically increases over time as children become older. Moreover, the treatment group showed still some episodes of GABHS infections after treatment in the follow-up period, although that rate was significantly reduced, this may be due to a less effective inhibitory activity of *S. salivarius* 24SMB against some *S. pyogenes* strains, despite the use in combination with *S. oralis* 89a.

Lastly, this study is limited by being conducted in a single center and unblinded to investigators and study site staff; hence this could be a potential source of bias and a potentially limiting to the external validity. In addition, our study does not provide any information about the colonization of the oral microbiota by our bacterial probiotics, therefore, any association between the colonization degree and the event of a new case of GABHS pharyngotonsillitis cannot be made, as well as it is unknown the persistence of our probiotic over time, and despite antibiotic treatment, we urge future studies to investigate the utility of additional doses of our probiotics following the use of antibiotics or in follow-up for maintaining colonization and assuring long-term protection against GABHS pharyngitis due to their high sensitivity to penicillin and many other antibiotics which are commonly prescribed to treat acute GABHS pharyngitis episode [15] because antibiotics might paradoxically damage an essential portion of the defense system of patients [45]. Therefore, it is recommended to include microbiologic monitoring of probiotics colonization in future studies to add essential data about the efficacy over time of the probiotics treatment. It is noteworthy as well that a cost-effective study is needed to determine the cost of the probiotics in the 90-day treatment course.

Conclusions

We identified two strains as potential oral probiotics, possessing desirable features for bacteria-therapy against GABHS pharyngotonsillitis and being non-pathogenic for children as demonstrated by our safety assessment. Our findings represent the first evidence which throws the light on using these probiotics, in children with GABHS pharyngotonsillitis, that can reduce the antibiotics use which did not have effective results regarding recurrence.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional committee of the School of Medicine—ASP 3 Catania (ID: 083/17) and with the 1964 Helsinki declaration.

Informed consent Informed consent was obtained from all parents of children included in the study.

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