

***Limosilactobacillus reuteri* ATCC PTA 5289 and DSM 17938 as adjuvants to improve evolution of pharyngitis/tonsillitis in children: randomised controlled trial**

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Abstract

Pharyngitis and tonsillitis are the most common acute respiratory infections (ARIs) in children aged ≤ 5 years. The analysis of published data showed that some probiotics could decrease the frequency and number of days with ARIs. This study evaluated the safety and efficacy of *Limosilactobacillus reuteri* ATCC PTA 5289 and DSM 17938 to reduce the duration and severity of ARI symptoms. This randomised controlled trial included children aged from 6 months to 5 years, with pharyngitis or tonsillitis, who were randomised to receive a probiotic product containing *L. reuteri* ATCC PTA 5289 and *L. reuteri* DSM 17938 or placebo, as drops, ingested orally for 10 days as adjuvants to the use of non-steroidal anti-inflammatory drugs. The main outcomes were the duration and severity of ARI symptoms. The secondary outcomes were changes in salivary immunoglobulin A and inflammatory biomarkers. There was no fever on day 2 and subsequent days in the *L. reuteri* group (37.3 ± 0.5 °C vs 38.6 ± 0.3 °C, $P < 0.05$). Beginning on day 3, the severity of sore throat (5 ± 0.9 vs 8 ± 1.2 , $P < 0.05$) was lower in the *L. reuteri* group. Significant differences in the days with runny nose, nasal congestion, days of non-programmed visits to the medical office or emergency department, levels in tumoral necrosis factor-alpha (TNF-alpha) and related costs of treatment were observed in the *L. reuteri* group. The frequency of adverse events was similar between the groups. Therefore, *L. reuteri* ATCC PTA 5289 combined with *L. reuteri* DSM 17938 is a safe and effective adjunct to reduce the symptoms of pharyngitis or tonsillitis in children.

Keywords: *L. reuteri*, respiratory infections, paediatrics.

1. Introduction

Acute respiratory infections (ARIs) in children are a public health problem, and independent of the world region, children can have 3-10 episodes annually (Chonmaitree *et al.*, 2008; Monto *et al.*, 1971; Wald *et al.*, 1991). Approximately 85% of all ARIs are upper respiratory tract infections (URTIs), of which pharyngitis and tonsillitis are the most common (Jain *et al.*, 2001; Peasah *et al.*, 2015; Singh and Nayar, 1996; Tambe *et al.*, 1999). Moreover, 70-80% of these infections are caused by viruses, with

rhinovirus, coronavirus and adenovirus accounting for 40-60% of the total cases (Bisno, 2001). For bacterial cases, *Streptococcus* Group A (GABHS) represents one of the most important infectious agents (Shaikh *et al.*, 2010). In most cases, the management of URTIs is ambulatory, and infections are associated with remarkable direct and indirect costs and significant deterioration of quality of life (Peasah *et al.*, 2015). Unfortunately, although ARIs are generally self-limiting, and >70% are of viral aetiology, the use of antibiotics is standardised worldwide. For example, between 33 and 75% of children and adolescents aged <15

years are receiving antibiotic therapy because of URTIs in Germany (Holstiege and Garbie, 2013). This inappropriate antibiotic use is usually associated with bacterial resistance, disturbance of normal balance of human microbiota and colonisation of pathogens (Andrews *et al.*, 2012; Lange *et al.*, 2016). Practice guidelines differ considerably regarding how to initiate treatment. Some guidelines consider that bacterial URTIs, especially GABHS, must be treated with antibiotics to avoid complications (AAP, 2006; AFSS, 2003; Bisno *et al.*, 2002; Gerber *et al.*, 2009; NGCH, 2001; Snow *et al.*, 2001), while others consider that even GABHS is a benign self-limiting disease and recommend the use of antibiotics only in selected cases to prevent resistance (De Meyere and Matthys, 1999; NICE, 2008; SIGN, 2010; Starreveld *et al.*, 2008). A cohort study conducted in primary care settings with >3 million episodes of ARIs identified that the number needed to treat for antibiotics to prevent one complication (e.g. otitis media) was 4,000, concluding that it is not cost-effective to use antibiotics in all cases of URTIs (Petersen *et al.*, 2007).

The use of probiotics as an adjuvant for treatment or prevention of URTIs in children has been evaluated in several studies, and since 2001, approximately 45 randomised controlled trials (RCTs) have been published. Recently, Wang *et al.* (2016) conducted a systematic review and meta-analysis that aimed to evaluate the efficacy of probiotics as adjuvants in children with URTIs. The analysis showed that probiotic consumption significantly decreased the number of subjects with at least one respiratory tract infection episode (relative risk, 0.89; 95% confidence interval [CI], 0.82-0.96; $P=0.004$), numbers of days of URTIs per child (mean differences [MD], -0.16; 95% CI, -0.29-0.02; $P=0.03$) and days absent from day care/school (MD, -0.94; 95% CI, -1.72 to -0.15; $P=0.02$) (Wang *et al.*, 2016). Several studies have shown the positive effect of *Limosilactobacillus* (*Lactobacillus*) *reuteri* for prevention of URTIs in children (Agustina *et al.*, 2012; Gutierrez-Castrellon *et al.*, 2014; Weizman *et al.*, 2005). Weizman *et al.* (2005) published a double-blind, placebo-controlled, randomised trial conducted in child care centres in Israel in children aged 4-10 months old. *L. reuteri* ATCC 55730, *Bifidobacterium lactis* BB-12 or placebo was administered to 68, 73 and 60 children, respectively, reporting that children who received *L. reuteri* had less respiratory infections episodes (Weizman *et al.*, 2005). Agustina *et al.* (2012) published a randomised controlled trial (RCT) in healthy children aged 1-6 years who received *Lactocaseibacillus* (*Lactobacillus*) *casei* CRL431 (n=120) or *L. reuteri* DSM 17938 (n=124). The study did not show any significant effect on URTIs (Agustina *et al.*, 2012). An RCT on healthy children, 6-36 months, attending day care centres where the subjects received *L. reuteri* DSM 17938, 10^8 cfu (n=168), or placebo (n=168), for 3 months, with an additional 3 months follow-up without supplementation, was performed by our group. The study revealed that *L. reuteri* DSM 17938 significantly

reduced the frequency and duration of respiratory tract infections at both 3 and 6 months ($P<0.05$). Additionally, the number of physician visits, antibiotic use, absenteeism from day care centres and parental absenteeism from work were significantly reduced in the *L. reuteri* Group ($P<0.05$). A cost-benefit analysis revealed significant reductions in costs in the *L. reuteri*-supplemented children. No adverse events related to the study product were reported (Gutierrez-Castrellon *et al.*, 2014).

The present study aimed to evaluate the safety and efficacy of *L. reuteri* ATCC PTA 5289 combined with *L. reuteri* DSM 17938, as an adjuvant to non-steroidal anti-inflammatory drug (NSAID), twice daily for 10 days in children with URTIs aged 6 months to 5 years. The *L. reuteri* strains DSM 17938 and ATCC PTA 5289 were selected due to their antiviral and antimicrobial properties and their effects on the immune system and mucosal integrity. Both strains have the ability to produce reuterin, a substance with both antiviral and antimicrobial activity (Ang *et al.*, 2016; Spinler *et al.*, 2008). Interestingly, the strains also produce reuterin when growing in biofilms. Strain ATCC PTA 5289 is more capable than the mother strain of DSM 17938, ATCC 55730, to form biofilms (Jones and Versalovic, 2009; Rosander *et al.*, 2008). *L. reuteri* strains have also been reported to increase the concentration of secretory immunoglobulin (Ig)A (Mu *et al.*, 2018), which could be an important factor in the defence against pathogens. Finally, different strains of *L. reuteri* have been shown to protect integrity of cultivated intestinal epithelial cells from the detrimental effect of enterotoxigenic *Escherichia coli* by increasing the expression of the longer isoform of zonulin (ZO)-1, maintaining E-cadherin expression and downregulating induction of interleukin (IL)-6 and tumour necrosis factor (TNF)- α (Karimi *et al.*, 2018).

2. Material and methods

Study design, ethics and informed consent

This randomised, double-blind, placebo-controlled trial was approved by the Research and Ethical Research Committees at Hospital General Dr. Manuel Gea Gonzalez, ID 39-141-2017 and registered at [ClinicalTrials.gov](https://clinicaltrials.gov) ID NCT03377374. Each parent or legal tutor has signed an informed consent form before infants were enrolled in this study. Participants were recruited in Child Care Centre and Paediatric Emergency Department at Hospital General Dr. Manuel Gea González, Ministry of Health, México.

Participant selection

Children aged from 6 months to 5 years of both sexes, born at term with a birth weight >2,500 g and a similar socioeconomic background, were eligible if they met the study definition of pharyngitis and/or tonsillitis. Pharyngitis

was defined as presence of sore throat, fever and pain during swallowing. At visualisation, the pharynx shows swelling and redness and covered with a thick mucus material. Tonsillitis was defined as the presence of swelling or redness of tonsils with discharge, fever and pain during swallowing.

Children were excluded if they had recurrent ARIs (eight or more new episodes of otitis media, two or more serious sinus infections or two or more episodes of pneumonia within 12 months); history of two or more invasive infections (meningitis, cellulitis, osteomyelitis, septicaemia), failure to thrive, chronic diarrhoea; recurrent deep skin or organ abscesses; persistent superficial candidiasis after 1 year of age; use of antibiotics for respiratory infections for two or more months in the past 12 months; gastroesophageal reflux; atopic disease (allergic rhinitis or asthma); recent (within the preceding 4 weeks) exposure to probiotics, prebiotics or antibiotics and supplementation of probiotics 2 weeks before inclusion in the study and during the study period.

Research outcomes and sample size

The primary outcome was duration of upper respiratory symptoms & severity of sore throat. The Face, Legs, Activity, Cry, Consolability (FLACC) assessment tool provided a score for evaluation of severity of pain (sore throat), through facial expressions, leg movements, activity, crying pattern and level of consolability with a minimum score of 0 and a maximum of 10 (Merkel *et al.*, 1997). Secondary outcomes were frequency of rhinorrhea, cough episodes, nasal congestion, snoring episodes, sleep disturbances, days with fever (temperature >37.5 °C, measured four times daily, using standardised thermometers and highest value during the day reported); number of children receiving antibiotic treatment; total number of days for antibiotic use [antibiotic needs were evaluated using the Pragmatic Scoring System for Pharyngitis, considering age of children, presence of tender cervical nodes, signs of headache, petechia on the palate, abdominal pain, sudden onset, conjunctivitis, coryza or diarrhoea, with a score >3 requiring antibiotics (Joachim *et al.*, 2010)]; number of visits to medical office or emergency department; days of absence from day care centre; frequency of adverse events; aetiology of respiratory infection and change in salivary inflammatory biomarkers [inflammatory response: C-reactive protein (CRP), IL-1b, IL-8, IL-6 and TNF- α ; anti-inflammatory response: sIgA and IL-10 and immune modulation response: soluble toll-like receptor (sTLR)-2, sTLR4 and sCD14]. Compliance was measured by requesting the parents to return the investigational product at day 10 after randomisation and counting the remaining drops. Considering the primary outcome with a difference between groups of at least 24 ± 8 h on duration of symptoms, and alpha error of 5%, a power of 80% and using Stata SE version 16.0 (StataCorp LLC,

College Station, TX, USA) a minimum sample size of 30+5 children (losses to follow-up) was calculated.

Interventions

L. reuteri (DSM 17938 and ATCC PTA 5289) oil drops were administered orally in a total daily dose of 4×10^8 cfu for 10 days. Five drops, containing 2×10^8 cfu, were obtained twice daily (in the morning and evening). The active study product consisted of freeze-dried *L. reuteri* suspended in a mixture of pharmaceutical grade medium chain triglyceride and sunflower oils. The placebo drops had identical ingredients, except that they lack bacteria. The investigational products, identical in taste and packaging, were supplied by BioGaia AB (Stockholm, Sweden). To guarantee the viability of the live bacteria, the investigational products were kept refrigerated ($+2$ to 8 °C) during the study period. Viability analysis showed that the strains in oil were stable. Batch 7DTW117 and batch 6DTW256, used to prepare oil drops for this trial, showed concentrations (cfu/5 drops) of 7.0×10^8 , 7.9×10^8 , 7.0×10^8 , 4.07×10^8 and 6.97×10^8 at 3, 6, 12, 18 and 21 months, respectively. Each child was randomly allocated to the probiotic or control group following a centralised, randomisation procedure.

Study procedures

After parents signed the informed consent, children were randomised using balanced blocking technique in the probiotic or placebo group. Blinding and randomisation were performed by the sponsor, BioGaia AB (Stockholm Sweden), using the website randomisation.com (<http://www.randomization.com>). Demographic data, medical history and physical examination, including vital signs and anthropometrics, were obtained from all eligible participants at baseline visit. Nasopharyngeal samples were obtained using nylon-specific respiratory swabs (Copan Diagnostics) to identify aetiology using Luminex (Abbott Diagnostics, Abbott Park, MI, USA). A saliva sample was obtained and prepared within 1 h of collection. Protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA), 2 μ l/ml of saliva and sodium orthovanadate (400 mM, Sigma Aldrich) and 3 μ l/ml of saliva were added to each sample, which was then centrifuged at 4 °C and $1,500 \times g$ for 15 min. Then, saliva samples were divided into aliquots and frozen at -80 °C until assayed. sIgA levels were analysed as previously described (López-Velázquez *et al.*, 2015), and salivary IL-1b, IL-8, IL-6, CRP, TNF- α , IL-10, sTLR2, sTLR4 and sCD14 were measured by ELISA. The parents or legal guardian were trained to fill out a daily diary to record the symptoms during the study period. During the treatment period, every 48 h, a designated investigator called the parents to check for progression, presence of any adverse events or other information important for the study. Ten days after randomisation, physical examination, including vital signs and anthropometrics, was performed.

A second salivary sample to evaluate sIgA and inflammatory biomarkers was obtained. When the decision, based on the Pragmatic Scoring System for Pharyngitis, was to start antibiotic treatment, the first option was amoxicillin + clavulanate 45 mg/kg/day BID for seven days. In subjects with a history of allergy to penicillin, the second choice was clarithromycin 7.5 mg/kg/day twice daily for seven days. The use of antibiotics was recorded in the daily diary and electronic case report form (e-CRF). Costs/child (USD) related to visits to emergency department or ambulatory office secondary to upper respiratory episodes during the study, and costs related to the use of anti-inflammatory drugs, antipyretic, or antibiotics were summarised and included on the outcome analysis for each group.

Statistical analyses

The intention-to-treat (ITT) statistical approach was used. Randomised subjects who fulfilled the inclusion criteria and none of the exclusion criteria that had received at least 3 days of the investigational product and from whom post randomisation data for the primary endpoints were available were included in the ITT analysis. The duration of exposure to the investigational product and compliance were summarised per group. Changes in duration and severity of respiratory symptoms, duration of fever, use of

antibiotics, number of visits to the medical office, number of visits to emergency department, number of days of absence from day care centres and costs related were compared through t-test. The frequency of adverse events in each group was compared using chi-square or Fisher test. IgA levels in the saliva and levels of salivary inflammatory biomarkers were compared between groups through t-test. The frequency of identified viral or bacterial agents in each group were compared using chi-square or Fisher test. In all sets of hypothesis analysis, differences were considered significant if the P -value <0.05 . Stata 15.0 for Mac was used to perform all statistical analyses.

3. Results

Seventy children, randomised to receive *L. reuteri* ($n=35$) or placebo ($n=35$), were included in the study (Figure 1). The number of boys and girls was similar between the groups. The age of the children was 2.1 ± 0.8 years in the *L. reuteri* group and 2.4 ± 1.1 years in the placebo group (P -value not significant). No statistically significant differences were observed when children were included in the study, in relation to the number of days of symptoms prior to study entry, duration of fever and severity of sore throat (FLACC score), symptoms observed, use of anti-inflammatory drugs and levels of sIgA and inflammatory markers (Table 1).

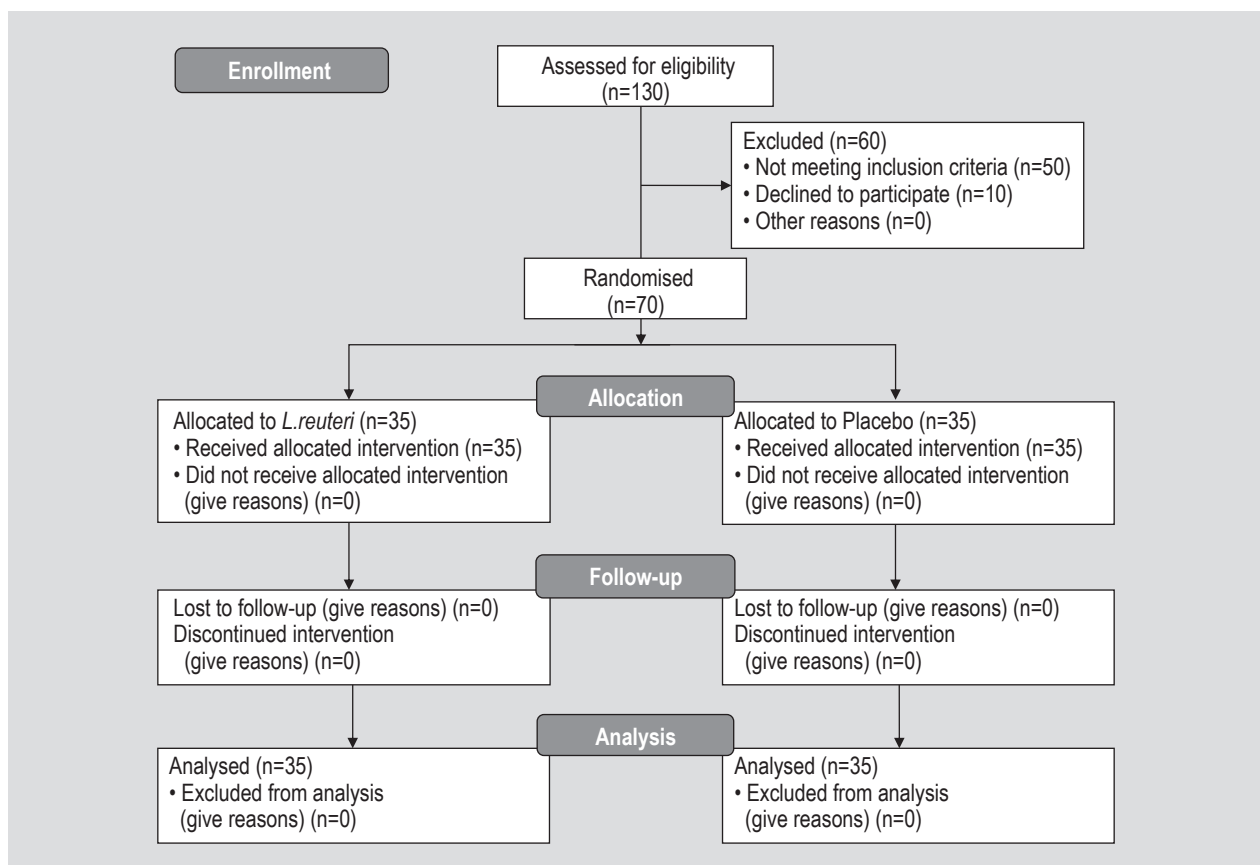


Figure 1. CONSORT flow diagram.

Table 1. Baseline characteristics at study entry.

Parameter ¹	<i>Limosilactobacillus reuteri</i> group (n=35)	Placebo group (n=35)
Age in years (mean±SD)	2.1±0.8	2.4±1.1
Number of girls [n (%)]	19 (55)	18 (52)
Days with respiratory symptoms before recruitment (mean±SD)	0.8±0.2	1.0±0.3
Days with fever (mean±SD)	0.7±0.4	0.9±0.6
Severity of sore throat (FLACC score) (mean±SD)	8±5	9±4
Rhinorrhoea [n (%)]	25 (71)	24 (68)
Cough episodes [n (%)]	20 (58)	21 (60)
Nasal congestion [n (%)]	11 (32)	10 (29)
Snoring episodes [n (%)]	10 (29)	11 (32)
Sleep disturbances [n (%)]	11 (32)	10 (29)
Use of NSAIDs [n (%)]	31 (88)	29 (83)
Salivary biomarkers (mean±SD)		
IgA (mg/ml)	13.2±8.1	12.9±10.3
IL-1beta (pg/ml)	42±12.1	38±9.9
IL-8 (pg/ml)	223±93	211±78
IL-6 (pg/ml)	8.4±1.2	9.2±1.8
C-reactive protein (mg/l)	1.22±0.44	1.30±0.52
TNF-α (pg/ml)	7.2±1.3	6.9±0.8
IL-10 (pg/ml)	5.5±2.1	5.8±1.9
sTLR2 (pg/ml)	330±30	282±90
sTLR4 (pg/ml)	1,005±280	998±116
sCD14 (pg/ml)	2,000±305	1,892±224

¹ SD = standard deviation; FLACC = face, legs, activity, cry, consolability; NSAID = non-steroidal anti-inflammatory drug; IgA = immunoglobulin A; IL = interleukin; TNF = tumour necrosis factor; sTLR = soluble Toll like receptor.

The aetiology of pharyngitis or tonsillitis was also similar between the groups (Table 2). At the end of the study, significant differences were observed in relation to the duration of respiratory symptoms (3.1 ± 0.8 vs 5.2 ± 1.4 , $P<0.05$), days with fever (1.8 ± 0.4 vs 3.6 ± 0.6 , $P<0.05$), severity of sore throat (FLACC score 5 ± 1.2 vs 8 ± 3.4 , $P<0.05$), runny nose, nasal congestion, days of non-programmed visits to medical office or emergency department and related costs in favour to *L. reuteri* group.

There was no fever on day 2 and subsequent days in the *L. reuteri* group (37.3 ± 0.5 vs 38.6 ± 0.3 °C, $P<0.05$) (Figures 2 and 3). Significant differences were also identified for TNF-α in favour to the *L. reuteri* Group (3.8 ± 0.7 vs 5.9 ± 0.4 pg/ml, $P<0.05$) (Table 3).

The frequency of adverse events was similar between the groups (Table 4). Compliance to use the investigational

Table 2. Aetiology for upper respiratory infections [n (%)].¹

Microorganism	<i>Limosilactobacillus reuteri</i> group (n=35)	Placebo group (n=35)
Rhinovirus	30 (11)	32 (11)
Adenovirus	28 (10)	31 (10)
Influenza A	12 (4)	16 (6)
Influenza B	18 (6)	12 (4)
Syncytial respiratory virus	7 (2)	6 (2)
Coronavirus	3 (1)	2 (1)
Moraxella catarrhalis	2 (1)	3 (1)
Streptococcus pneumoniae	6 (2)	4 (2)

¹ Figures are not 100% because there were mixed infections.

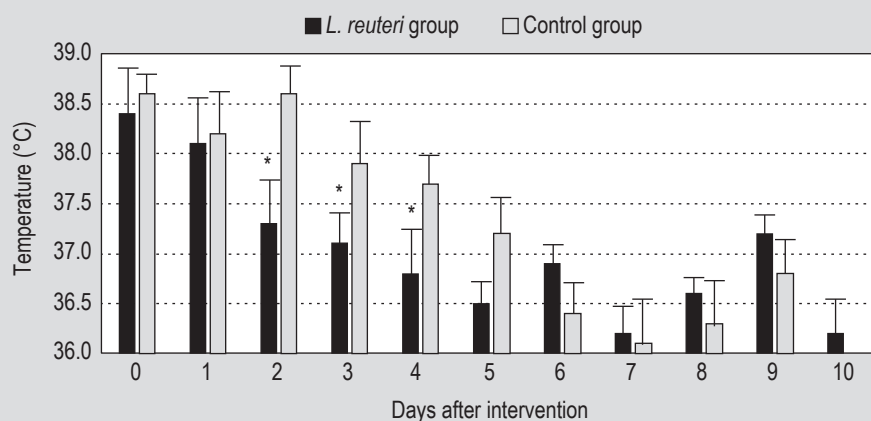


Figure 2. *Limosilactobacillus reuteri* and evolution of fever in children with pharyngitis/tonsillitis. * $P < 0.05$

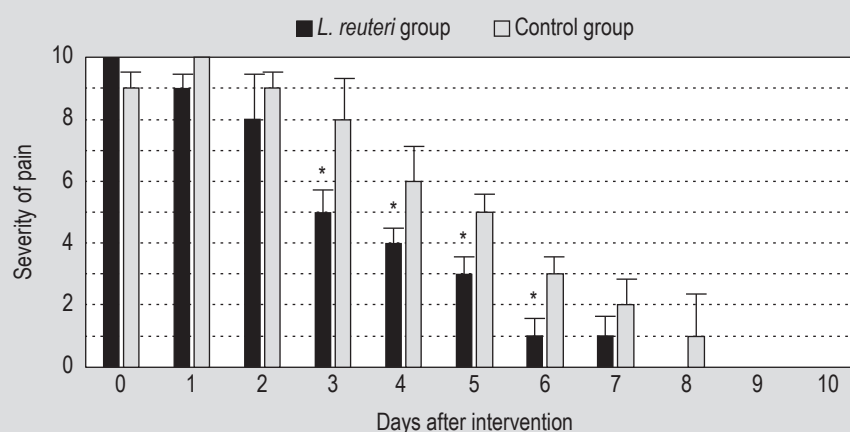


Figure 3. Face, legs, activity, cry, consolability (FLACC) score in children with pharyngitis/tonsillitis. * $P < 0.05$

products was 98% and 97% for the *L. reuteri* and placebo groups, respectively.

4. Discussion and conclusions

URI is the most frequent acute infection in children aged <5 years, with a significant impact on public health worldwide and a significant economic burden on the patients with the disease. The frequency of URIs is significantly increased in children who attend day care centres due to the greater burden of pathogens they are in contact with (Thrane *et al.*, 2001). A significant associated problem is related to the use of antibiotics even though most of these diseases are caused by viruses, as observed in our study and in correlation with what have been observed in other studies. Although we did not observe significant differences in the use of antibiotics in our study, the use of probiotics in this study, could reduce the use of antibiotics and therefore decrease the high frequency of adverse events observed due to their inappropriate use. In the control group of

children in our study, we observed the persistence of fever and pharyngeal pain until the fifth day of supplementation. This type of behaviour usually moves the parents to look for therapeutic alternatives that could contribute to improve symptoms, such as fever, pain and inflammation. Recently, a systematic review with meta-analysis was published, with the aim of evaluating the efficacy of different specific strains of probiotics as adjuvants in the treatment of URIs in children attending day care centres. The analysis of 12 clinical trials with >4,500 children showed that some specific strains significantly reduced the duration of URIs (Pilmann-Laursen and Hojsak, 2018). Our proof-of-concept (PoC) RCT showed that the product with the combination of two strains of *L. reuteri* (ATCC PTA 5289 and DSM 17938) reduced not only the duration of days of fever and pharyngeal pain but also other respiratory symptoms, such as rhinorrhoea and nasal congestion, which results in extreme discomfort in young children. The importance of this PoC lies not only on the clinical impact observed but also on the reduction of related costs, which undoubtedly

Table 3. Primary and secondary outcome analysis at day 10.

Parameter ¹	<i>Limosilactobacillus reuteri</i> group (n=35)	Placebo group (n=35) ²
Primary outcomes		
Duration of symptoms (days) (mean±SD)	3.1±0.8	5.2±1.4*
Severity of sore throat (FLACC Score) (mean±SD)	5.0±1.2	8.0±3.4*
Secondary outcomes		
Rhinorrhea [n (%)]	6 (17)	15 (43)*
Cough episodes [n (%)]	6 (17)	7 (21)
Nasal congestion [n (%)]	3 (8)	10 (28)*
Snoring episodes [n (%)]	4 (12)	5 (14)
Sleep disturbances [n (%)]	3 (8)	4 (11)
Days with fever (mean±SD)	1.8±0.4	3.6±0.6*
Use of antibiotics [n (%)]	2 (6)	4 (12)
Number of non-programmed visits to medical office or emergency department (mean±SD)	0.8±1.1	1.4±1.2*
Days of absence to day care centre (mean±SD)	3.2±0.9	2.9±1.1
Average cost/child (USD\$) (mean±SD)	71±10	101±19*
TNF-α (pg/ml) (mean±SD)	3.8±0.7	5.9±0.4*

¹ SD = standard deviation; FLACC = face, legs, activity, cry, consolability; TNF = tumour necrosis factor.
² * $P < 0.05$, otherwise non-significant.

Table 4. Frequency of adverse events divided by age groups.

Adverse event	<i>Limosilactobacillus reuteri</i> group	Placebo group
6-12 months (n=28)	Worsening of rhinorrhoea [n (%)]	7 (25)
	Change of stool consistency [n (%)]	3 (11)
	Abdominal pain [n (%)]	1 (3)
1-3 years (n=42)	Worsening of rhinorrhoea [n (%)]	5 (12)
	Change of stool consistency [n (%)]	1 (2)
	Abdominal pain [n (%)]	7 (17)

adds a significant value from a public health point of view, considering the frequency of ARIs observed in children aged <5 years and the impact on the quality of life. In 2014, our research group demonstrated in a similar study conducted in day care centres that the administration of one of the strains (*L. reuteri* DSM 17938) represented a cost-effective intervention by reducing the expenses related to the integral care of these infections (Gutierrez-Castrellon *et al.*, 2014). In this study, we demonstrate that the use of this combination of *L. reuteri* strains can represent a cost-effectiveness strategy. One of this studies strengths is that we analysed not only the clinical manifestations and etiological profile of the URTIs but also the immunomodulatory profiles of this type of adjuvant. Within the limitations is the fact that this study is a PoC with a small sample size, which limits the extrapolability of the results to other populations. Although we have identified significant differences between the treatment groups, strong enough to establish some

recommendations, it is advisable to corroborate the identified findings in a second clinical trial, with a larger sample size, which allows evaluation of the impact of some covariates that could generate different impacts on the outcome variables. Based on our results, we conclude that *L. reuteri* ATCC PTA 5289 combined with *L. reuteri* DSM 17938 is an adjuvant to reduce the duration and severity of clinical symptoms in children with pharyngitis or tonsillitis. Due to its anti-inflammatory effects, this combination of strains reduces the duration of fever and intensity of sore throat.

Conflict of interests

All authors have no potential conflicts of interest to disclose.

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