Probiotics for Everyone! The Novel Immunobiotic *Lactobacillus rhamnosus* CRL1505 and the Beginning of Social Probiotic Programs in Argentina

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Abstract: *Lactobacillus rhamnosus* CRL1505 (Lr1505) stimulates immune responses in the gut and in the respiratory tract and improves resistance against *Salmonella typhimurium* and *Streptococcus pneumoniae* infections in immunocompetent and immunocompromised mice. Considering that respiratory infectious diseases continue to be a major cause of death among preschool children in developing countries, the aim of the present study was to evaluate the effect of Lr1505 on the health of children. A randomized-controlled double-blind clinical trial in 298 healthy children (2-5 years old), attending daycare centers was performed. Yogurt containing Lr1505 was administered to children for 6 months (five times a week). Results were statistically compared with those of children from the same community that received a placebo yogurt (without probiotic). Administration of Lr1505 to young children reduced the incidence of infections: 66% of children in the placebo group presented symptoms of infection while only 34% of cases were detected in the Lr1505 group. Significant differences (P<0.05) were detected in the incidence of intestinal infections, upper respiratory tract infections and angina when placebo and Lr1505 groups were compared. Children fed Lr1505 experienced fewer fevers and needed fewer antibiotics than those receiving the placebo. The protective effect of Lr1505 was associated with increased levels of mucosal IgA antibodies. Lr1505 is a promising resource for the development of prevention strategies against mucosal infections that could be effective tools for medical application. This new probiotic strain has been included into official Nutritional Programs in Argentina and it is given to more than 200 thousand children. This project has encouraged local milk production, thanks to the constant demand of probiotic yogurt containing *L. rhamnosus* CRL1505 by provincial governments, while incorporating innovation to small and medium enterprises.

Keywords: *Lactobacillus rhamnosus* CRL1505, children, mucosal immunity, respiratory infections.

1. INTRODUCTION

Common infectious diseases continue to be a major cause of death among preschool children in developing countries [1-3]. The increase in antibiotic resistance and need for new and improved strategies to tackle infectious disease have led to an examination of the therapeutic potential of commensal induced modulation of the mucosal immune response. Consequently, it has been discovered that certain probiotic lactic acid bacteria (LAB) do have protective effects against bacterial and viral infections in the gastrointestinal tract [4]. Significant attention has been focused on the role of probiotics in the protection of gut against pathogens. However, there is steadily increasing evidence that orally delivered probiotics are able to regulate immune responses outside the gastrointestinal tract, including the respiratory mucosa [5]. In mouse studies, it has been demonstrated that oral administration of probiotic LAB protects against respiratory pathogens such as *Streptococcus pneumoniae* [6, 7], *Pseudomonas aeruginosa* [8] and influenza virus [9]. Moreover, some human trials demonstrated that administration of probiotics has been associated with lower incidence of

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ventilator-associated pneumonia [10], reduced respiratory infections in healthy and hospitalized children [11-13] and reduced duration of common cold infection [14].

With the aim to develop a new functional food able to improve both intestinal and respiratory immunity, we have evaluated the immunomodulatory capacity of several lactobacilli strains isolated from goat milk and we found that *Lactobacillus rhamnosus* CRL1505 stimulates the innate and adaptive immune response in the gut in a dose-dependent way and confers resistance to infection with *Salmonella typhimurium* in immunocompetent [15] and immunocompromised malnourished [16] mice. Moreover, we have demonstrated that orally administered *L. rhamnosus* CRL1505 is able to increase the resistance to pneumococcal infection in immunocompetent mice and that this effect is associated with improvements of innate and adaptive immune responses in the respiratory tract [15]. In addition, we recently demonstrated that the use of *L. rhamnosus* CRL1505 as a supplement in a repletion diet is able to improve the number and functionality of immune cells that participate in the response against a pneumococcal infection in malnourished mice [16].

Our studies in mice provided clear evidence that *L. rhamnosus* CRL1505 improves immune responses in the gut and beyond the gastrointestinal tract. However, the immunomodulatory effect of this strain was not tested in humans before. Therefore, in the present work we aimed to evaluate the effects of a probiotic yogurt containing the strain *L. rhamnosus* CRL1505 on the health of children attending pre-school daycare community centers. We evaluated the impact of probiotic yogurt containing *L. rhamnosus* CRL1505 on mucosal immunity and study its effect on the incidence and severity of gastrointestinal and respiratory infections.

2. MATERIALS AND METHODS

Study Design

A randomised, double-blind, placebo-controlled study was conducted to evaluate the effect of the consumption of a probiotic yogurt on health of children. One children group was randomly allocated to the probiotic yogurt and the other to a placebo product. The study consisted of 6 months product consumption between July and December. The trial was conducted by nutritionists (SIPROSA), pediatricians (Tucuman Children Hospital), and immunologists (CERELA). In addition, daycare center staff was previously trained and allowed to participate. The experimental protocol and informed consent for parents were previously approved by the Ethics Committee of the Faculty of Medicine of the Tucuman University (Res. Nº 1121-06).

Participants

Healthy children attending daycare centers five-days a week in Tucuman (Argentina) were recruited into the study. The purpose of the study was explained to the parents and a written informed consent was obtained. Procedure consisted of supervisor visiting the household and in presence of a third party, obtaining the consent from the mother or father after reading the consent form to them.

Our inclusion criteria were: children between 2 and 5 years old, from both sexes, adequate nutrition, attending daycares, who voluntarily agree to participate in the study, with written consent of parents or guardians. Before inclusion in the study, participants and the adults responsible for children were informed about the nature and characteristics of the study. Only those who met the eligibility criteria and whose parents or guardians gave their written consent to participate were included in the trial.

Exclusion criteria were: congenital disease affecting the digestive system (cystic fibrosis, celiac disease, allergy to cow's milk), chronic diseases (pulmonary dysplasia, COPD, chronic renal failure). In addition, children or parents which do not accept participating in the study were not included.

Intervention

Children were randomised to receive either the probiotic yogurt or the placebo product. The probiotic yogurt was sweetened, flavoured, fermented milk containing at least 10^8 colony-forming units (CFU)/100 g of the probiotic strain *Lactobacillus rhamnosus* CRL1505 with a starter culture for yogurt prepared with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains from the collection culture of CERELA. *L. rhamnosus* CRL1505, isolated from goat milk from northwestern Argentina, was previously selected by its ability to stimulate the immune system by increasing resistance to infection in the respiratory and intestinal tracts [15-17]. The placebo product was fermented milk produced with same starter culture without *L. rhamnosus* CRL1505.

The nutritional composition, appearance, taste and packaging of the probiotic yogurt and the placebo were identical throughout the study in order to maintain blinding. Enrolled children were randomly allocated to the two intervention groups and the participants were masked to group allocation. In addition, all the persons involved in the study, the different health professionals as well as the parents and guardians were unaware of the randomization codes. The secret codes were secured until completion of data entry for analysis.

The Laboratory for Experimental Foods (CERELA-CONICET) prepared the yogurt and probiotic cultures. The final products were elaborated by the dairy company "Al Pie de la Vaca" and were cold-stored (8ºC) in daycares.

The probiotic yogurt and the placebo were administered daily from Monday to Friday (five days per week). During the study, children had to ingest one bottle of 100 g/day of yogurt or placebo product.

Before initiation of the study, children underwent a clinical examination including anthropometric measures (weight, height). In addition, children and parents underwent a clinical nutrition questionnaire (which included personal data, medical history, family-inherited diseases, psychomotor, environmental and sociocultural skills).

The children were randomly allocated to probiotic yogurt or the placebo group on day zero of the study. Total of 298 children participated and were divided in two groups: 150 children were administered the probiotic yogurt while 148 children received the placebo. The clinical and nutritional monitoring was conducted by pediatricians from the Tucuman Children Hospital and by nutritionists from the SIPROSA Nutrition Division (Tucuman Government).

During the study, visual controls of both placebo and probiotic yogurt consumption were performed by nutritionists. It was recorded that 100% of the products -probiotic yogurt and placebo- were consumed by the participants throughout the studied period.

Assessment of Product Acceptability Using Hedonic Scale

We assessed the quality of the product from the subjective point of view, considering that the success or failure of a food product depends not only on its composition or nutritional value, but also on subjective reactions associated with a pleasurable sensation. A hedonic test for children was used. The scale used was the hedonic faces with different facial expressions ranging from anger (complete dissatisfaction) to happy (complete satisfaction). Children expressed their opinion concerning a pleasant character on this scale. Yogurt and placebo products were coded so that recipients could not identify the product. The assessment of overall satisfaction was greater than 90% in the strawberry- and vanilla-flavored yogurt.

Follow Up Observation

Planed visits were conducted once a week by pediatricians. In these visits, clinical examination of each child was performed as well as nutritional assessment (weight/age, height/age and weight/height ratios), according to the standards of the Provincial Health System of Tucumán (SIPROSA).

In addition, during the trial the occurrence of infectious events related to the gastrointestinal and

Table 1.

Infection	Main causative pathogen in children under 5 years	Reference
Acute otitis media	*Streptococcus pneumoniae* and *Haemophilus influenzae*	[18]
Pharyngitis and tonsillitis	Viral pathogens and β-hemolytic streptococci	[19]
Upper airway infections (common cold)	Respiratory syncytial virus, Human metapneumovirus, Influenza A virus, Parainfluenza viruses and Rhinoviruses	[20]
Pneumonia	*Streptococcus pneumoniae*, *Haemophilus influenzae* and *Mycoplasma pneumoniae*	[21]
Bronchitis	Respiratory syncytial virus, Parainfluenza viruses and Rhinoviruses	[22]
Diarrhea	Rotavirus and Adenovirus	[23]
Skin infections (impetigo and pyodermitis)	*Staphylococcus aureus* and β-hemolytic streptococci	[24]

respiratory systems was recorded. Data collection was performed by a survey performed by paediatricians. The data were entered in individual forms for each child where the date and infectious characteristics were registered. The most common infectious diseases in children under five years were analysed: Acute otitis media, pharyngitis and tonsillitis, upper airway infections (common cold), pneumonia, bronchitis, diarrhoea and skin infections (impetigo and pyodermitis) (Table **1**).

Laboratory Tests

Samples of faeces were collected at the beginning and end of the study in order to determine lactic acid bacteria counts.

In addition, levels of IgA in saliva were determined. Samples were taken the day before the beginning of probiotic yogurt or placebo administration (Basal levels) and at the end of their administration (Post treatment levels). Saliva samples were collected from 200 children (100 yogurt group and 100 in the placebo group) with sterile pasteur pipette and placed in appropriately labeled eppendorf tubes. Immediately after collection, the samples were cooled for transport to the laboratory. The samples were clarified by centrifugation at 2,500 g for 10 minutes, separated into aliquots and stored at -70 ° C until antibody assessment. Measurements were performed using the ELISA technique using anti-human IgA (Sigma # I0884). Total levels of antibody were quantified by linear regression analysis of the values of optical density (OD) of samples against a standard curve consisting of 10 serial dilutions of human IgA (Sigma # S5018). All the analytical dosages in the study were performed in blind.

Data Analysis

Statistics Division from CERELA was responsible for analysis of results. StatsDirect software was used. Appropriate parametric and non-parametric methods were employed according to the distribution of the data. The Student t test was used to compare means of the continuous variables with normal distribution. For variables with no normal distribution, the Mann-Whitney U test was used. The $\chi 2$ test or Fisher exact test were used to compare percentages. The confidence interval of 95% was calculated using the same software. The difference between groups was considered significant for $p < 0.05$.

3. RESULTS

Incidence of Infections

As shown in Figure **1**, there were 298 children enrolled in the study; 150 received the probiotic yogurt containing *L. rhamnosus* CRL1505 and 148 received the placebo product. There was no statistically significant difference between the groups in regard to age, gender, weight and height at the beginning of the study, or difference in weight and height prior to and after the intervention. Of the total of 298 children who participated, 132 (45%) of them presented infectious events during the trial (Figure **2A**). The most common infectious diseases were upper respiratory infections followed by pharyngitis and tonsillitis, lower respiratory infections (acute bronchitis) and diarrhea (Figure **2A**). In the group of children that recieved the yogurt containig the probiotic strain *L. rhamnosus* CRL1505 it was observed a significant decrease of infectious events when compared to the placebo group (34% *vs* 66%) (Figure **2B**). When the different types of infectious diseases were analyzed, we observed significant differences between the yogurt and placebo groups in the following events: upper respiratory infections (31% *vs* 69%), pharyngitis and tonsillitis (28% *vs* 72%) and acute diarrhea (26% *vs* 74%). No differences were observed in the incidence of bronchitis (Figure **2B**). In addition, the number of children suffering pneumonia, otitis media or skin infections (impetigo and pyodermitis) did not allow us to perform statistical analysis (Figure **2B**). Symptom duration of infection events for subjects in the placebo and the probiotic yogurt groups were not different (Figure **3A**). The clinical effects of probiotic supplementation on the incidence of fever and the need of antibiotic tretament were also evaluated. Subjects in the probiotic yogurt group were found to have significantly lower incidence of having fever (Figure **3B**). Furthermore, the need of antibiotic treatment among the probiotic-consuming children was significantly lower than that in the placebo group (Figure **3C**).

Lactic Acid Bacteria in Stools

Analysis of the amount of LAB species in stools was performed after 6 months of product consumption. Comparison between groups was analysed by using a Mann–Whitney test. The results show no statistical difference between groups in the amount of LAB species in stools (P=0.6017; Figure **4**).

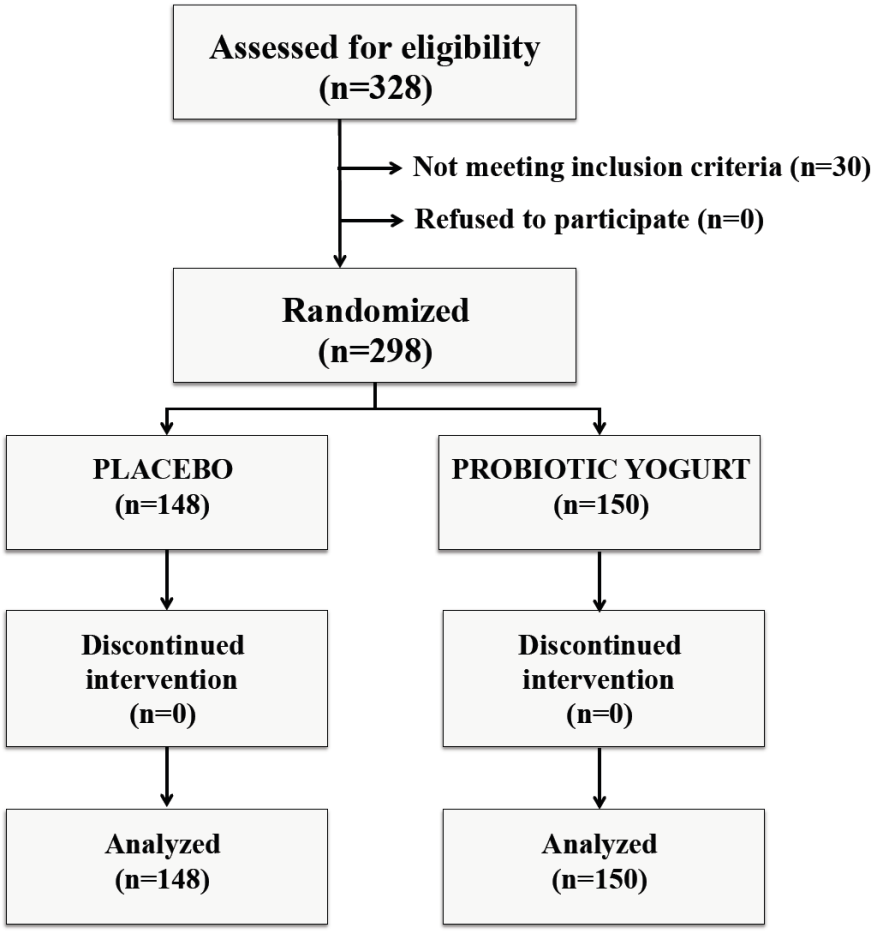

Figure 1: Diagram showing the flow of participants through the stages of the study.

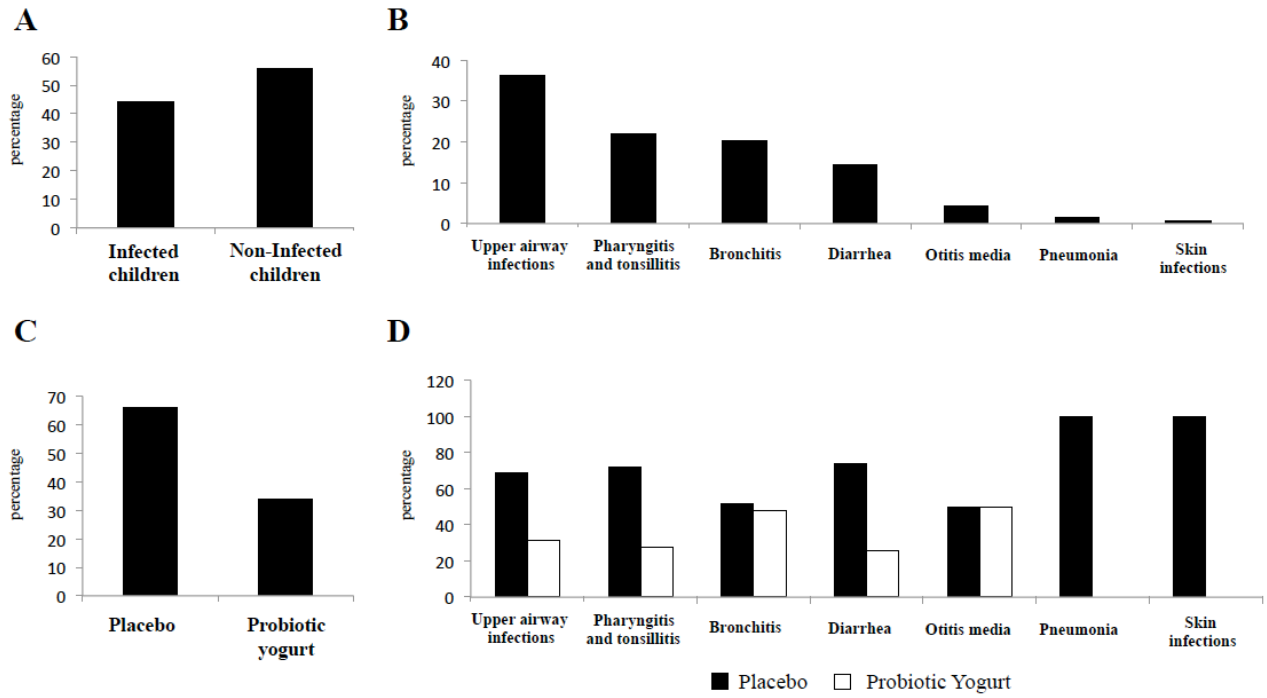

Figure 2: Effects of a probiotic yogurt containing the strain *Lactobacillus rhamnosus* CRL1505 on the incidence of common infectious diseases in pre-school children. (**A**) Incidence of infections in the group of children, (**B**) different types of infectious diseases. (**C**, **D**) Effect of probiotic yogurt consumption in the incidence of infections.

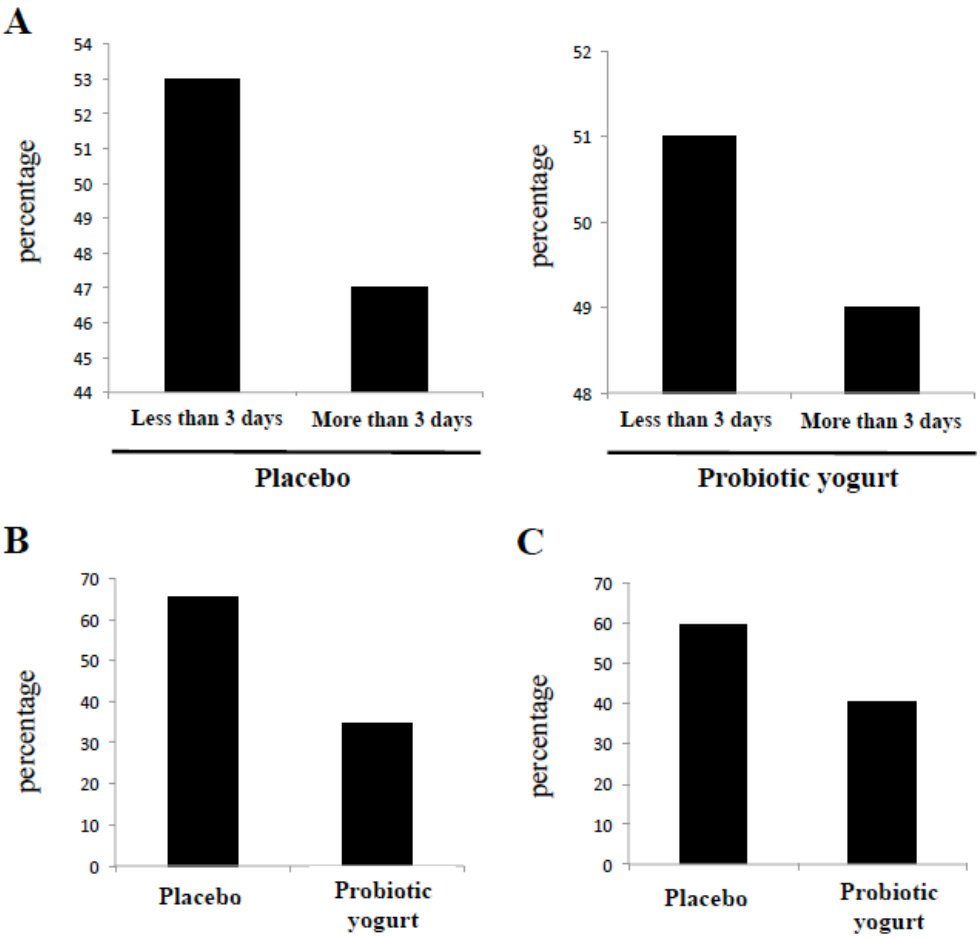

Figure 3: Effects of a probiotic yogurt containing the strain *Lactobacillus rhamnosus* CRL1505 on the severity of common infectious diseases in pre-school children. (**A**) Symptom duration, (**B**) presence of fever, (**C**) use of antibiotics.

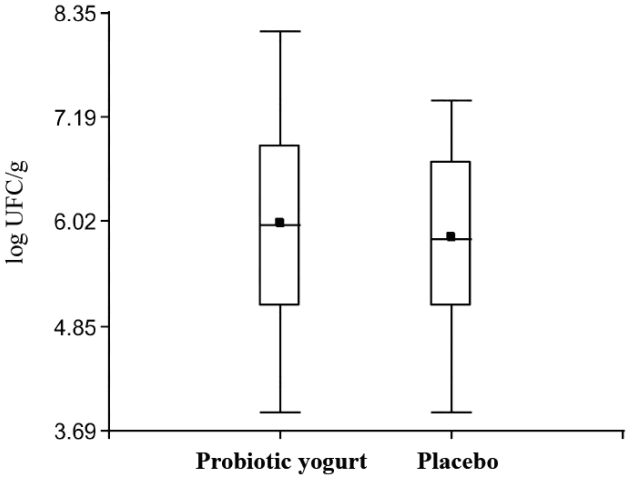

Figure 4: Effects of a probiotic yogurt containing the strain *Lactobacillus rhamnosus* CRL1505 on faecal concentration of lactic acid bacteria in pre-school children.

Levels of Immunoglobulin A in Saliva

We performed the determination of IgA in saliva as a measure of mucosal immunity. Samples were taken the day before the begining of the intervention (basal levels) and at the end of probiotic or placebo administration (post-treatment levels). No significant differences were observed in the basal levels of IgA when the probiotic yogurt and the placebo were compared (Figure **5**). On the contrary, we observed a significant increase in IgA levels in children who received the probiotic yogurt while the post-treatment levels of IgA in the placebo group were similar to those found at the beginning of the trial (Figure **5**). Therefore, the consumption of probiotic containing *L. rhamnosus* CRL1505 strain is able to improve mucosal immunity as reveled by the levels of IgA in saliva.

4. DISCUSSION

Reducing the preventable childhood illnesses among preschool children in developing countries is an important public health goal, that would not only impact mortality but would also impact better development of children. It has been estimated that 5.2 million children under five years of age die every year due to preventable infectious diseases like pneumonia and diarrhea [1]. Moreover, recent findings suggest 21% of

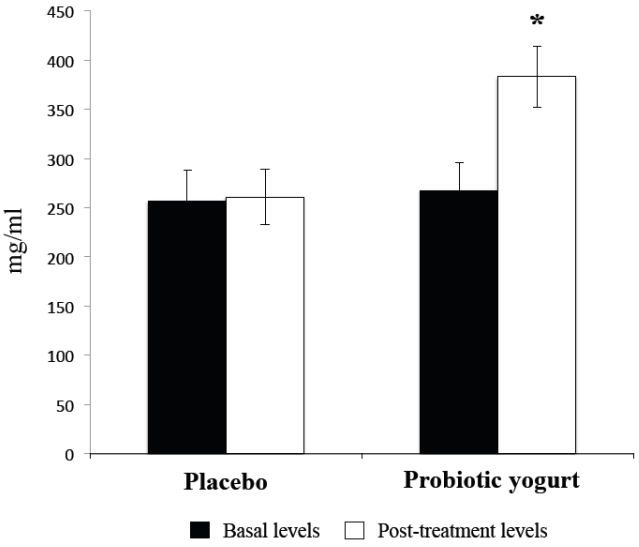

Figure 5: Effects of a probiotic yogurt containing the strain *Lactobacillus rhamnosus* CRL1505 on saliva IgA levels in pre-school children. (**A**) basal levels at the beginning of the trial, (**B**) levels of IgA after six month of treatment. * Significant differences when compared with the placebo group ($P < 0.05$).

global deaths in children younger than 5 years of age are attributable to malnutrition and its synergistic relationship with preventable infectious diseases [2, 3]. Our region is no exception to these global statistics. Fortification with probiotics may provide one of the potential interventions to reduce the burden of common childhood morbidities [25]. Data on the use of probiotics in preventing common childhood illnesses in a community setting from developing countries is lacking as are data evaluating the effect on illness other than diarrhea. In this study, we report the first randomized controlled trial, evaluating the effect of a probiotic yogurt on both gut and non-gut related illnesses among children from Tucuman, Argentina. Children in probiotic yogurt and placebo groups consumed similar amounts of yogurt, and yogurt in both groups was isoenergic, with identical macronutrient quality and quantity as well as quantity of vitamins and minerals. The only difference was that the yogurt for children allocated to the probiotic group delivered additionally 10^8 CFU/day of the probiotic strain *L. rhamnosus* CRL1505.

We demonstrated that administration of *L. rhamnosus* CRL1505 improved mucosal immunity and reduced the incidence and severity of intestinal and respiratory infection in children. When we studied the type of infectious events according to their location and symptoms, the frequency of them was consistent with the prevalence reported in our country. The most common infectious diseases were upper respiratory infections, followed by angina and then lower respiratory infections (acute bronchitis) and diarrhea [18, 21, 22, 23]. We registered that 34% of children who consumed the probiotic yogurt showed some type of infectious event, while in the placebo group this value was higher reaching a 66% of children. These results demonstrate a significant reduction in occurrence of infectious events associated with consumption of *L. rhamnosus* CRL1505. We also evaluated the presence or absence of fever during infectious events as well as the need of antibiotic treatment in children who had infections, as indicators of severity. There was a significant decrease in the presence of fever in children who consumed probiotic yogurt as well as a slight decrease in the need for antibiotic treatment, indicating less serious infections in relation to the placebo group.

Several studies have demonstrated that probiotics are able to improve intestinal immunity and reduce susceptibility to intestinal pathogens in children. For example, a study of young children afflicted with rotavirus gastroenteritis showed that *L. reuteri* significantly shortened the duration of diarrhea [26]. Another clinical trail demonstrated that children with mild diarrhea who consumed the combination of *L. rhamnosus* and *L. reuteri* experienced a reduction in the duration of the diarrhea [27]. More recently, a meta-analysis performed by Salari *et al*., [28] showed that probiotics administration significantly decreased the duration of diarrhea and fever in children. These studies illustrated the beneficial effects of different lactobacilli on gastrointestinal infections and they are in accordance with our results. On the contrary, few studies have evaluated whether probiotics are capable of preventing respiratory infections and reducing their severity in children. Some studies have examined the role of probiotics in the prevention of respiratory tract infections in healthy individuals [29, 30]; particularly those in children's daycare centers [11, 31]. Results from a study evaluating *Bifidobacterium lactis* or *Lactobacillus reuteri* versus placebo did not show a beneficial probiotic effect on the rate and duration of respiratory illnesses [31]. On the other hand, randomized, double- blind, placebo-controlled studies performed in daycare centers showed that administration of *Lactobacillus* GG resulted in a reduction in the number of children suffering from respiratory tract infections [11, 13]. Moreover, treatment with *Lactobacillus* GG significantly reduces the risk for developing nosocomial respiratory tract infections in children who were hospitalized on a

pediatric ward [12]. These results suggest that not all the probiotic strains that are able to stimulate intestinal defenses are capable to improve respiratory immunity. Therefore, certain LAB strains may share certain functional properties. However, some of them can perform a functional role better than others, so it is important to carry out thorough studies on specific strains, according to their therapeutic use. In this sense, our systematic studies in animals on the ability of LAB strains to improve respiratory immunity, allowed us to select a strain that is likely to have a beneficial effect in humans, which has been demonstrated in this clinical trial.

The study of the mechanisms responsible for the beneficial role of probiotics on the gut have documented direct anti-microbial effects and improvement in mucosal barrier function as a result of the effects of probiotics on both innate and adaptive immunity. However, the mechanisms responsible for the improvement of defenses against upper respiratory infections have not been fully elucidated. We have made some progress in the knowledge of the immunological mechanisms involved in the protective effect of *L. rhamnosus* CRL1505 against respiratory pathogens [32].

Our laboratory has demonstrated that the oral administration of certain LAB strains is able to induce the IgA cycle and increase the IgA^+ cell population in the respiratory tract [7, 33]. In the case of *L. rhamnosus* CRL1505 we found that this strain was capable of increasing the number of IgA^+ cells in intestine and bronchus of mice [15]. We also found that *L. rhamnosus* CRL1505 was able to improve the production of anti-pneumococcal IgA in the airways [15]. The production of IgA in the respiratory tract during an infectious process is important because it prevents colonization of mucosal tissues and subsequent spreading into the systemic circulation [34]. Additionally, IgA antibodies can bind antigens and minimize their entry with a consequent reduction in inflammatory reactions, which prevents potentially harmful effects on the tissue. In this study we found improved levels of IgA in children that received the probiotic, therefore we assume that the stimulation of the IgA cycle and the improvement of the levels of IgA induced by the *L. rhamnosus* CRL1505 could partly explain the greater resistance of children to respiratory infections.

On the other hand, it is known that the symptoms associated with common cold are a result of the inflammatory response by the host towards the infection. Therefore, compounds with the capacity to improve immunity and control unproductive inflammation are supposed to be effective antivirals. In this sense, we have demonstrated in animal studies that treatments with *L. rhamnosus* CRL1505 prior to pneumococcal infection induced a significant increase in IL-10 in lung and serum [15]. Consequently, in agreement with other reports [35], IL-10 was valuable to attenuate inflammatory damage and pathophysiological alterations in lung infected with pneumococci. According to our results, *L. rhamnosus* CRL1505 treatment would beneficially regulate the balance between pro- and anti-inflammatory cytokines, allowing a more effective inflammatory response against infection. This controlled inflammatory response induced by *L. rhamnosus* CRL1505 could explain the beneficial effect of this strain in the incidence and severity of common cold observed in our study.

Although we did not evaluate etiology of diarrhea in this study, previous evaluations have shown rotavirus and adenovirus to be responsible for most cases of intestinal infection in children [23]. In addition, viral pathogens, such as respiratory syncytial virus, human metapneumovirus, influenza A virus, parainfluenza viruses, and rhinoviruses are considered the major viruses that can cause respiratory tract diseases in children [20]. Therefore, the findings of this study suggest that administration of *L. rhamnosus* CRL1505 may provide one of the potential interventions to reduce the burden of common childhood morbidities, especially those associated to viral infections. The precise cellular and molecular mechanisms involved in the improvement of antiviral immunity induced by *L. rhamnosus* CRL1505 is an interesting topic for future investigations.

In this work, we demonstrated that consumption of a fermented dairy product containing *L. rhamnosus* CRL1505 is associated with a significant decrease in the duration and severity of mucosal infections providing the first evidence that a dairy fermented product containing this immunobiotic strain may have a beneficial effect against respiratory infections in young children. Based on the results summarized in this work and given the high morbidity and mortality in children especially associated with airways infectious diseases, dietary intervention using a dairy product containing the probiotic strain *L. rhamnosus* CRL1505 can be useful to improve health status of this vulnerable population. From the above, this new probiotic strain has been

included into official National Nutritional Programs in Argentina. Since 2008, the probiotic yogurt containing *L. rhamnosus* CRL1505 (YOGURITO®) is given daily to more than 200 thousand children in Tucumán city thanks to the Government actions (Social Development Ministry). The use of the immunobiotic CRL1505 strain to improve children health has already transcended the limits of Tucuman province since other provinces in Argentina are also participating in this Social Program and more children are being benefited with the probiotic YOGURITO®.

The social project YOGURITO® has multiple positive impacts at different levels, in the regional economy by encouraging both the local milk production and the competitiveness in small and medium enterprises (SMEs) through innovation and dairy products differentiation; in the children education and health status by improving wellness and the immune system from the basis that “more health is equal to less school desertion”.

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