PROTOCOL

Effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* in children aged 3 to 5 years of Villavicencio and Pasto.

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1.Synopsis

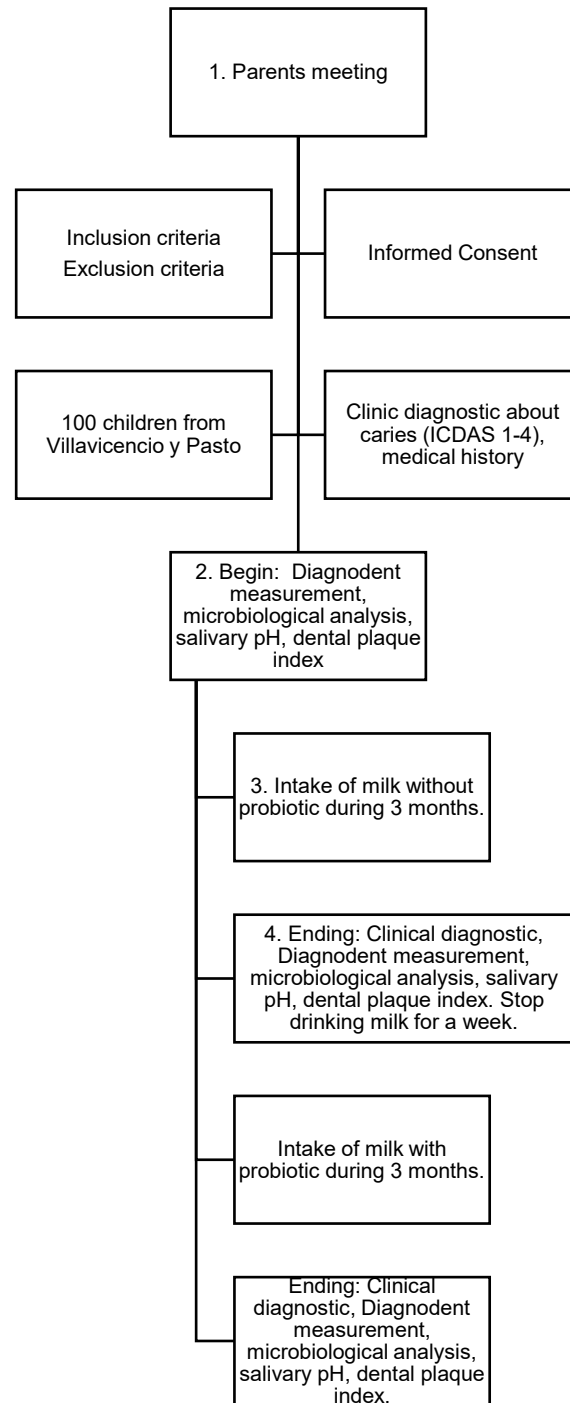| Study Type	Interventional, Pilot Study
Intervention	1. Commercial Cow Milk with Probiotic (*Lactobacillus rhamnosus* and *Bifidobacterium longum)* 2. Commercial cow milk without probiotic
Study Design:	Repeated measures design.
Allocation:	Nonrandomized
Endpoint Classification	Efficacy Study
Intervention Model:	Crossover study
Masking:	No masking
Sample size	Convenience sampling
Primary Purpose	Treatment trial
Subjects:	Three to five-year-old children
Genders:	Both
Sites	Kindergarten in Villavicencio y Pasto, Colombia
Study duration	12 months
Objective	To determine the effect of consumption of commercial milk beverages enriched with *Lactobacillus rhamnosus* y *Bifidobacterium longum* on the incidence of caries in children aged 3 to 5 years.

2.Introduction

Several bacterial strains have been studied as probiotics, either isolated or in combination, as well as the doses used in studies, intervention periods, populations, experimental designs, and routes of administration. The results often include a decrease in *S. mutans* count in saliva or plaque, but few studies conduct dental clinical examinations to assess treatment. This is confirmed by a meta-analysis conducted by Laleman et al in 2013, claiming that the scientific evidence demonstrating caries control is insufficient (1). However, the results of studies describing the effect of probiotics in the index of carious lesions (2,3) or dental plaque, are promising (4,5).

The probiotics most commonly evaluated so far in the oral cavity are isolated microbial strains, mainly of the digestive tract, like the ones belonging to the genus Lactobacillus (*L. rhamnosus, L. reuteri, L. casei, L. brevis, L. paracasei, L. acidophilus, L. plantarum*) and Bifidobacterium (*B. bifidum, B. longum, B. lactis, B. animalis, B. infantis*) (6,7). These bacteria have proven their safety for many years, allowing clinical studies and the development of commercial products, with the added value that some have been identified in healthy individuals (DMF = 0) (L. rhamnosus, L. plantarum, L. acidophilus, L. brevis, L. paracasei) (8), suggesting their role in micro-ecologic balance.

One of the most commonly studied probiotic bacteria in the oral cavity was isolated by Gorbach and Godin in 1983 from digestive tract of a healthy adult (9). It was named Lactobacillus rhamnosus GG (ATCC53103) and is characterized by inhibiting, by means of bacteriocins, a wide variety of human pathogenic bacteria (10), including S. mutans, S. sobrinus, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia. In addition, this bacterium colonizes the microcosm of saliva, and has no cariogenic effect. This bacterium was used to conduct one of the most complete clinical studies in children (aged 1 to 6 years), reducing the incidence of caries in the age of 3 to 4 years (2).

3.Schema

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┌─────────────────────────┐
│   1. Parents meeting     │
└─────────────────────────┘

┌──────────────────────┐   ┌──────────────────────┐
│  Inclusion criteria   │   │  Informed Consent    │
│  Exclusion criteria   │   │                      │
└──────────────────────┘   └──────────────────────┘

┌──────────────────────┐   ┌──────────────────────┐
│  100 children from    │   │ Clinic diagnostic about │
│  Villavicencio y Pasto│   │ caries (ICDAS 1-4),   │
│                       │   │ medical history       │
└──────────────────────┘   └──────────────────────┘

┌────────────────────────────┐
│ 2. Begin:  Diagnodent       │
│    measurement,             │
│  microbiological analysis,  │
│  salivary pH, dental plaque │
│            index            │
└────────────────────────────┘

        ┌────────────────────────────┐
        │  3. Intake of milk without  │
        │  probiotic during 3 months. │
        └────────────────────────────┘

        ┌────────────────────────────┐
        │ 4. Ending: Clinical diagnostic, │
        │  Diagnodent measurement,    │
        │ microbiological analysis, salivary │
        │ pH, dental plaque index. Stop │
        │  drinking milk for a week.  │
        └────────────────────────────┘

        ┌────────────────────────────┐
        │  Intake of milk with        │
        │  probiotic during 3 months. │
        └────────────────────────────┘

        ┌────────────────────────────┐
        │  Ending: Clinical           │
        │  diagnostic, Diagnodent     │
        │  measurement,               │
        │ microbiological analysis,   │
        │ salivary pH, dental plaque  │
        │           index.            │
        └────────────────────────────┘
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4.Schedule of activities

Activities	Months											
	1	2	3	4	5	6	7	8	9	10	11	12
Parents meeting	■											
Informed consent		■										
Personal interview		■										
Clinical diagnostis			■									
Selection criteria			■									
Begin: diagnostic tests				■								
Intake milk without probiotic for 3 months				■	■	■						
Ending: diagnostic tests							■					
Intake milk with probiotic for 3 months							■	■	■			
Ending: diagnostic tests										■		
Analysis of information											■	■

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5.Study Description

Brief Summary: The objective of this study is to determine if the presence of bacteria with probiotic characteristics (Lactobacillus rhamnosus GG and Bifidobacterium longum) in a commercial milk, control some measures that indicate cariogenic processes. Detailed Description: Dental caries is a disease caused by a shift in pH balance, resulting in the formation of a biofilm composed mainly of cariogenic microflora. The interaction between this microflora and host factors conditions the deceleration or acceleration of the disease. Some biotechnology tools have been tried based on all this research, including probiotics, intended for a selective control of the etiological agents of caries and the maintenance of oral homeostasis. Methodology: In this study, preschool children belonging to public kindergarten in Villavicencio and Pasto, Colombia will be invited to participate. After obtaining parent´s informed consent, the children will start commercial milk intake without probiotic for 3 months (Klim fortificada Nestlé). One week after, they will start commercial milk with probiotic during others 3 months (nanPro3 Nestlé). Clinical and microbiological testing of saliva at baseline, 3 m, and 6m will be performed. Differences in salivary pH (before and after a sugar solution), remineralization and demineralization in early Stage Decay and Established decay tooth (according to ICDAS) and quantification of Streptococci of the mutans group. Expected results: Statistically significant decrease in pH drop, demineralization in decay tooth and quantification of Streptococci of the mutans group.

6.Risk/Benefit assessment

Minimal Risk:

This study has a minimal risk because the probability and magnitude of possible harms implied by participation in the research is no greater than those encountered by participants in the aspects of their everyday life that relate to the research (TCPS Chapter 2B). The intervention is safe because the study will use two commercial milk with Sanitary Registry of health products. During the reconstituting of powdered milk, the person responsible will use bottled water and good hygienic conditions. Moreover, during the clinical diagnostic and the clinical laboratory, this research will practice principles and practice about biosafety.

Benefit:

The immediate potential benefits of this research with children, is the daily intake of two nutritive commercial milk, for 6 months. Also, the researcher gives information about the oral health, the presence of caries, and the requirements of specific treatment.

7.Objectives and Endpoints

OBJECTIVES	ENDPOINTS	Justification for endpoints
To determine the effect of consumption of milk beverages enriched with Lactobacillus rhamnosus and Bifidobacterium longum in diagnodent measurement.	Diagnodent measurement of tooth decay lesions (ICDAS 1-4). The idea is to compare the effect of probiotics in children during the intake of milk without probiotic versus during the intake of milk with probiotic.	DIAGNOdent is a laser fluorescence device used for dental caries diagnosis in occlusal and smooth surfaces. Diagnodent measurement allows knowing if the effect of probiotic suppresses the demineralization of the cariogenic lesion. The reason is, if the probiotic inhibit the cariogenic bacteria, reduce the demineralization and improve the mineralization
To determine the effect of consumption of milk beverages enriched with Lactobacillus rhamnosus and Bifidobacterium longum in the change of pH of saliva after a rinse with sugar.	For this objective, the purpose is to compare the points difference between salivary pH before and after a rinse with sugar, in children during the intake of milk without probiotic versus during the intake of milk with probiotic	The scientific literature shows when there are many cariogenic bacteria, the drop of Ph after a rinse with sugar is greater compared with people who have less cariogenic bacteria levels.
To determine the effect of consumption of milk beverages enriched with Lactobacillus rhamnosus y Bifidobacterium longum in the concentration of group mutans streptococci in saliva.	The comparison of the quantification of group mutans streptococci from saliva, during the intake of milk without probiotic versus during the	Other studies have shown the inhibition of Lactobacillus rhamnosus in Streptococcus mutans.

	intake of milk with probiotic	
To determine the effect of consumption of milk beverages enriched with Lactobacillus rhamnosus y Bifidobacterium longum in dental plaque index.	The comparison of dental plaque index during the intake of milk without probiotic versus during the intake of milk with probiotic, show us if *Lactobacillus rhamnosus* and *Bifidobacterium longum* reduce the formation of dental plaque.	Other studies, showed that some probiotics reduce the dental plaque.

8.Study Design

7.1 Hypothesis

1. The commercial milk beverages enriched with *Lactobacillus rhamnosus* and *Bifidobacterium longum* reduce the demineralization of caries lesion (ICDAS 1-4).

2. The commercial milk beverages enriched with *Lactobacillus rhamnosus* and *Bifidobacterium longum* reduce the change of salivary pH after a rinse with sugar.

3. The commercial milk beverages enriched with *Lactobacillus rhamnosus and Bifidobacterium longum* reduce the quantification of mutans streptococci in saliva.

4. The commercial milk beverages enriched with *Lactobacillus rhamnosus and Bifidobacterium longum* reduce the dental plaque.

7.2 Study design: Nonrandomized comparison, single group assignment, controlled before and after study.

7.3 Arm treatment information:

A. Experimental: Commercial Cow Milk with Probiotic. 200 ml of commercial nutritive milk fortified with probiotic Lactobacillus rhamnosus and Bifidobacterium longum once a day.

B. Placebo comparator: Commercial Dairy Cow Milk 200 ml of commercial nutritive milk without probiotic, once a day.

7.4 Dosing and administration:

Experimental: 200 ml of commercial nutritive milk fortified with probiotic Lactobacillus rhamnosus and Bifidobacterium longum (Nan Pro3, Nestlé) once a day.

Placebo comparator: 200 ml of commercial nutritive milk without probiotic (Klim Fortificada Nestlé), once a day.

7.5 Study intervention duration:

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3 months intake the placebo comparator, and 3 months intake the experimental treatment.

7.6 Sites:

Kindergarten in Villavicencio, Colombia

Kindergarten in Pasto, Colombia

7.7 Phase of the trial:

1. Invitation to participate in the trial: at the kinder garden during a meeting, the researchers invite parents to participate.

2. To request the informed consent of the parents of the children.

3. Diagnosis, selection, and collection of samples. Each child will be informed of the process in a simple way.

First Day:

3.1 First selection: the dentist checks up the oral health condition. Children without caries or with advanced caries will not continue in the process. Children who enter the criteria continue in the process.

3.2 Second selection: Clinical assessment of demineralization with Diagnodent, after of dental prophylaxis. The children with measure upper to 7, are included.

3.3 ICDAS: dentist with calibration in ICDAS take this measure.

Second day:

3.4 Index dental plaque Sillness and Löe.

3.5 Collection of saliva: Each child will accumulate saliva and then collect it in sterile containers (approximately 1.5-2 ml).

3.6 1 ml of saliva with glycerol (10%) will be frozen (-20°C) in microcentrifuge tubes (1.5ml). This sample is for microbiological analysis of S. mutans).

3.7 With the other 1ml is to determine the pH. The Salivary pH will be determined with pHmeter Fisherbrand Accumet Portable AP110.

3.8 Rinse with 10% sugar solution. The children will make a mouthwash with sugar solution and they will wait 5 minutes. Finally, the pH of the saliva is will be determined again.

4. Microbiological Analysis of S. mutans

The 100µl of saliva sample will be dilute with 900µl of sterile normal saline. Then will be performed decimal dilutions of saliva were made up to 10-3. 100µl of every dilution will be uniformly spread in Agar Mitis Salivarius with tellurium and bacitracin. Finally, the plates will be incubated via anaerobically system with 5% CO_2 for 48 hours at 37°C. The confirmation of morphology of the bacterial colonies will be determined under a stereo microscope. Also, the CFU will be count under the stereomicroscope.

5. Interview with the parents to know the information about the children (state of health, diet, teeth brushing frequency)

6. INTERVENTION

Children were given 200 mL of milk with after the breakfast every day, for five days a week. First, the children will drink milk without probiotic, during the 3-months period. And then the children drink milk with probiotic during the 3-months period (L. rhamnosus and B. longum). The nursery school staff prepared the commercial milk with researcher supervision. The milk will be prepared with safe water (packaged water).

6. After the intervention with each milk, the next outcome measures:

6.1 Index dental plaque Sillness and Löe.

6.2 Collection of saliva: Each child will accumulate saliva and then collect it in sterile containers (approximately 1.5-2 ml).

6.3 1 ml of saliva with glycerol (10%) will be frozen (-20°C) in microcentrifuge tubes (1.5ml). This sample is for microbiological analysis of S. mutans).

6.4 With the other 1ml is to determine the pH. The Salivary pH will be determined with pHmeter Fisherbrand Accumet Portable AP110.6.5 Rinse with 10% sugar solution. The children will make a mouthwash with sugar

solution and they will wait 5 minutes. Finally, the pH of the saliva is will be determined again.

6.6 Dental prophylaxis

6.7 ICDAS

6.8 Clinical assessment of demineralization with Diagnodent.

8 Study Population

8.1   General information: children from 3 to 5 years of kindergartens

8.2   Inclusion criteria:

•   Pre-school children from age 3 to 5.

•   Healthy children from a general health perspective, with early stage decay and established decay

8.3   Exclusion criteria

•Children with systemic disorders who need special care and/or who have the intolerance to milk drinks or allergy to any of the components of the experimental and/or the placebo beverage.

•Children who don't like milk.

•Children whit severe decay

8.4 Lifestyle consideration:

The children's parent will inform when:

-The child uses antibiotic

-The child visits another dentist and the dentist use Fluor.

-The child uses an antiseptic or mouthwash.

-The child improves the brush frequency.

9 Study intervention

Primary Outcome Measure:

1.Change in remineralization of tooth decay

DIAGNOdent peak reading in three moments,at the beginning and at the end of the milk without probiotic (after 3 months), and at the end of the milk with probiotic (after 3 months) (Table 1).

Secondary Outcome Measures:

1. Change of salivary pH after a rinse with sugar.

Change pH after a rinse with sugar (point of difference), in three moments, at the beginning and at the end of the milk without probiotic (after 3 months), and at the end of the milk with probiotic (after 3 months) (Table 1).

2. Change of Dental Plaque Index

Silness-Löe plaque index n three moments,at the beginning and at the end of the milk without probiotic (after 3 months), and at the end of the milk with probiotic (after 3 months) (Table 1).

3. Change of concentration of group mutans streptococci in saliva

Colony Forming Units (CFUs) determination in three moments,at the beginning and at the end of the milk without probiotic (after 3 months), and at the end of the milk with probiotic (Table 1).

TABLE 1.

Variable	Scale of measurement	Data	Type of variable	Measure Type	Measure of dispersion
BEFORE (Control and Intervention)					
Diagnodent measurement	Quantitative	Values 0-99	Dependent	-Mean -Median	-Standard deviation -Inter-Quartile range
Change of salivary pH after a rinse with sugar.	Quantitative	Point of difference	Dependent	-Mean -Median	-Standard deviation -Inter-Quartile range
Dental Plaque Index	Qualitative	0=absent 1=present	Dependent	Frequency	NA
Colony Forming Units of the group mutans streptococci	Quantitative	UFC	Dependent	-Mean -Median	-Standard deviation -Inter-Quartile range
AFTER (Control and Intervention)					
Diagnodent measurement	Quantitative	Values 0-99	Dependent	-Mean -Median	-Standard deviation -Inter-Quartile range

Change of salivary pH after a rinse with sugar.	Quantitative	Point of difference	Dependent	-Mean -Median	-Standard deviation -Inter-Quartile range
Dental Plaque Index	Qualitative	0=absent 1=present	Dependent	Frequency	NA
Colony Forming Units of the group mutans streptococci	Quantitative	UFC	Dependent	-Mean -Median	-Standard deviation -Inter-Quartile range

10 Statistical considerations

Statistical hypothesis

Primary Efficacy

H01. There is no significant difference of consumption of commercial milk beverages enriched with Lactobacillus rhamnosus and Bifidobacterium longum in diagnodent measurement in comparison with commercial milk without probiotic.

Secondary Efficacy

H02: There is no significant difference of consumption of commercial milk beverages enriched with Lactobacillus rhamnosus and Bifidobacterium longum in change of pH of saliva before and after a rinse with sugar, in comparison with commercial milk without probiotic.

H03: There is no significant difference of consumption of commercial milk beverages enriched with Lactobacillus rhamnosus and Bifidobacterium longum in concentration of group mutans streptococci in saliva in comparison with commercial milk without probiotic.

H04: There is no significant difference of consumption of commercial milk beverages enriched with Lactobacillus rhamnosus and Bifidobacterium longum in dental plaque index in comparison with commercial milk without probiotic.

11 Statistical analysis

Data will be organized with Microsoft Excel. The analyses will be performed using the program SPPS® Statistics 25.0 version. Univariate descriptive

analyses: means, standard deviations, medians, and interquartile ranges according to the normality. Data comparisons will be performed with Students t-test for related samples. A p-value <0.05 was considered as statistically significant. If there are not data normally distributed, the comparison will be with Wilcoxon test. In the case qualitative variable (Dental plaque index), the comparison will be with McNermar's test.

11. Regulatory, ethical, and study oversight consideration

According to the classification in the article 11 of the Scientific, technical and administrative norms for the investigation in health (Resolution 008430 of 1993 of the Ministerio de Salud de Colombia), it is a research with minimum risk since it treats of a commercial milk. In addition, only a dental examination will be performed and plaque and saliva will be collected, without implying any harm to the child. The examination and taking of the dental plaque will be done by an expert in the area. The study will be endorsed by the university's bioethics subcommittee. The informed consent will be applied to the children's parents.

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The work will be carried out in the laboratory following all the norms set out in the Biosafety Manual in the WHO laboratory (WHO, 2005) and does not represent any risk to humans, since it is a controlled in vitro study. In addition, the project will not represent negative effects on the environment during the execution of the project, since the waste management of the reagents will be carried out following the waste disposal regulations.

12.References

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