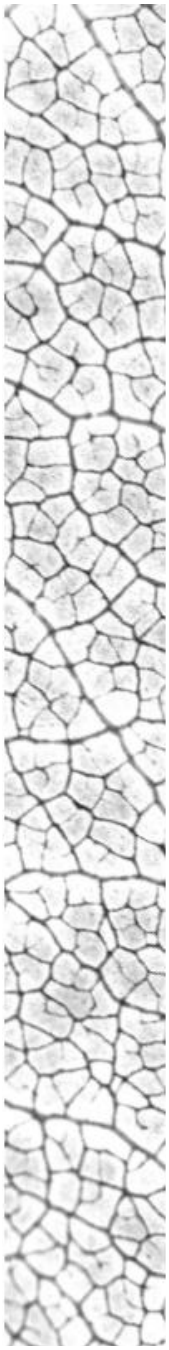

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Alteration of microbial exposure in vitro might aid in child's relief: docking of isolated oral lactobacillus fermentum with mouthwash as a potential probiotic for children oral health

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Abstract
This research was conducted to investigate the potential of using probiotic- experimental based mouthwash on the oral health of canker sores and thrush patients. In accordance, a total of forty children in the age range of one month to ten years old from both sexes were screened orally for lactobacillus isolation during 2019, where a few novel lactobacilli cultures were obtained grown in de Man, Rogosa, Sharpe (MRS) medium. Then after, single lines of lactobacilli cultures were characterized morphologically and biochemically to reveal the isolation of three novel strains of *Lactobacillus fermentum* with circular, white, glistering, convex colonies. Besides, the Gram staining had indicated, rod-shaped, short, and positive in Gram reaction staining. Besides, the biochemical tests result of the analytical profile index system (API 50 CHL) and Vitek 2 compact had revealed that all the isolates belonged to *Lactobacillus fermentum,* according to Bergey's Manual of Systematic Bacteriology. Moreover, the genotypic identification of the isolated strains unveiled the detection of the 1500 bp gene sequence of 16S rRNA for further confirmation. On the other hand, the probiotic activity of the isolated strains were demarcated according to the attributes of the absence of erythrocyte lysis of human and sheep blood in either environment, tolerance to bile salts, growth in different physiological conditions and their antimicrobial activity against *Pseudomonas gingivalis* and *Candida albicans*. Furthermore, the potential probiotic activity of the isolated strains was tested for their possible restorative effect combined in probiotic- experimental based mouthwash on the oral health of patients with a canker sore and thrush. Despite the short period for which the patients used the probiotics mouthwash, substantial improvement in the oral and gingival health of patients was observed in the study.

Keywords: *Lactobacillus fermentum*, children, oral health, RT-PCR, probiotic

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INTRODUCTION

The relationship between balanced microbiota and oral health has been well documented in several pieces of research where gut microbiota acts as a critical modulator for the child's immune system serving as an essential source of non- inflammatory immune stimulators throughout life in healthy individuals (Gosai et al, 2011; Ambalam et al, 2013; Khalesi et al, 2019). Probiotic bacteria, however, such as Lactobacilli colonize the oral cavity having either a health-promoting effect by altering the biofilm microbial composition or by stimulating the host immune response through their secretion of molecules that inhibit host pathogens by producing a range of bioactive compounds (wall et al, 2007; Kelly et al, 2007; Reza, 2020). The probiotic action of several Lactobacillus species and strains has been associated with the reduction of chronic inflammatory diseases and weight regulation (Schrezenmeir et al, 2001; Collado et al, 2006; Forsythe & Bienenstock, 2010). Recent studies showed an additional beneficial role for oral lactobacilli where, strains of *Lactobacillus paracasei*, *Lactobacillus rhamnosus,* and *Lactobacillus fermentum* isolated from infants inhibited the growth of pathogenic strains of *Candida albicans* and retrieved the healthy mouth milieu (Rossoni et al, 2018; Jang et al, 2019). Therefore, the close link between Lactobacilli, nutrition, and human health has increased interest in medical applications for health promotion in different age groups (Barzegari et al, 2020).

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Thus, this study suggested to (i) Detection of Lactobacillus spp. in the oral cavity of children at different ages, (ii) Identifying the most dominant Lactobacillus species and (iii) Evaluation of the possible probiotic effect of the isolated strains on the oral health of volunteers when applied as a mouthwash.

MATERIALS AND METHODS

Study group

A total of 40 children were involved in this study during the year of 2019. Twenty of them were in the age range of (1-11) months, while the rest of them were between (1-10) years old. They resided in different locales in Kirkuk city/Iraq. All parents of the children included in this study gave signed consent to participate in the study.

Detection of Lactobacillus existence in the oral cavity of the study group

Data Assortment

Parents of all participants were subjected to a questionnaire included questions about a child's gender, age, medical history, type of milk feeding (breast/formula), and eating routines according to (Siqueira et al, 2004). Oral issues such as tongue abnormalities and infection were all evaluated according to the procedure mentioned by (Badet & Thebaud, 2008; Piwat et al, 2010).

Oral samples Collection

Whole saliva sample of 2 ml of stimulated saliva collected from the mouth of the study population, transferred to a sterile screw-capped tube that contained 5ml of (1% NaCl) with aseptic precautions according to (Motisuki et al, 2005).

Isolation of Lactobacillus spp.

For each sample, a 200 mL aliquot of saliva was spread on de Man, Rogosa, Sharpe (MRS) medium as a selective growth medium of Lactobacilli, according to (Ahirwar et al, 2017). Then, cultivated plates were incubated at 37℃ for 48 h.

Identification of the isolated Lactobacillus strains

Morphological, Cultural and Biochemical Characterization tests

A single line of Lactobacillus cultures grown on de Man, Rogosa, Sharpe (MRS) medium was identified according to their morphological, cultural, Gram's staining and biochemical tests included; Catalase test, Arginine hydrolysis, Indole reaction, Oxidase Gas production, growth temperature, Citrate utilization test, and sugar fermentation according to the procedures cited by (Ahirwar et al, 2017).

Analytical profile index system (API® 50 CHL Kit)

The research strips of API® 50 CHL Kit were used in conjunction with API 50 CHL Medium (OT-50410) for the identification of putative Lactobacillus species according to their carbohydrate metabolism according to the procedure mentioned by (Blandino et al, 2016).

Testing using Vitek2 Compact system

Automated microbial identification system (Vitek2 Compact) were used to confirm the Lactobacillus isolates by the Gram-Positive Identification Card (GN ID), and the Antimicrobial Susceptibility Test Card (AST) according to manufacturer's instructions.

Genotypic characterization by 16S rRNA gene sequence analysis

Purified broth of (1.0 ml) of Lactobacillus isolates was mixed with 200 ml of Tris-EDTA buffer (pH 8.0; 10mM Tris, 1mM EDTA), and centrifuged at 12,000g for 1 min. Pellets were collected, and genomic DNA was extracted with InstaGene Matrix following the manufacturer's instructions. DNA content and purity were measured on (260-280-nm) absorbance ratios by spectrophotometer. Polymerase chain reactions (RT-PCR) were carried out in a 50-µL volume containing 10× PCR buffer, 2.5 mM MgCl2, 2.5 mM deoxynucleotide 5´-triphosphates, 20 pmol of each primer of (plb16 and mlb16) with the conserved gene sequences of (5´AGAGTTTGATCCTGGCTCAG3´) and (5´GGCTGCTGGCACGTAGTTAG 3´) respectively along with 2.5 U of Taq polymerase, and 2 µg of template DNA were used to direct the RT-PCR amplification of the 500-bp portion of the 16S rRNA gene using the method reported by (Balcázar et al, 2007; Songisepp et al, 2012). The product analysis conducted with agarose gel electrophoresis run in 1.8% agarose gel with ethidium bromide using a ladder DNA ladder of 1Kb. Gel visualization and photographing under a UV transilluminator documentary unit. The sequences of the strains in question were aligned with the 16S rRNA gene sequence of (*Lactobacillus fermentum* M58819 strain) to determine the identity of the isolated strains.

Evaluation of the probiotic activity of the *Lactobacillus fermentum* isolates

Hemolytic activity

A single line of *Lactobacillus fermentum* cultures grown on MRS broth was streaked onto blood agar plates containing either human or sheep blood according to the methodology cited by (Songisepp *et al*, 2012). Hemolysis of Lactobacillus fermentum was evaluated after 24 and 48 h of incubation in aerobic, microaerobic (10%CO2) and anaerobic (90% N_2, 5% CO_2, 5%H) environments. Two strains of *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (ATCC 19615) were used as positive controls

Table 1. Groups of experimental Mouthwash treatment

Groups	Treatment type	Base of Mouthwash	Amount ml/mouthwash	Duration /days
1	Probiotic-based	(10^2-10^8CFU/ml) of *Lactobacillus fermentum*	10	7
2	Normal saline	10% NaCl	10	7
3	Placebo	Distilled water	10	7
4	none	0	0	7

Antimicrobial activity against pathogens

The antimicrobial action of all isolated *Lactobacillus* species against selected indicator pathogenic bacteria was determined according to (Koll et al, 2008). Testing bacteria were strains of *Pseudomonas gingivalis* (ATCC 33277) and *Candida albicans* (ATCC 103231), where diameters of the clear zones were recorded by millimeters.

Growth characteristics of the isolated *Lactobacillus fermentum* strains under different physiological conditions

Growth conditions such as bile salts and different pH tolerance were tested according to the procedure cited by (Blandino et al, 2016).

Evaluating the *in vitro* application of *Lactobacillus fermentum* isolates as a potential probiotic for Oral Health

Preparation of probiotic- experimental based mouthwash

A single line of *Lactobacillus fermentum* cultures grown on MRS broth was centrifuged, and the pellet was diluted with distilled water where several concentrations were prepared as (10^2- 10^8 CFU/ml) of *Lactobacillus fermentum* isolates.

Design of Human Volunteer Trials

This study was directed according to the guidelines refrenced by (Songisepp *et al*, 2012). All trials were carried out following good clinical practice (GCP) and approved by the Ethics Reviews of the Scientific Committee of the Medical Laboratory Department/Technical College of Kirkuk/Northern Technical University in 2019. All participant's parents provided written informed consent at the enrollment of the study. Twenty volunteers with identified canker sores and mouth thrush were involved in this trial in the age range of (8-10) years from both sexes. Volunteers were subdivided according to the type of treatment into four groups, as mentioned in **Table 1** where each group composed of five children.

Volunteers were instructed to use the mouthwash for thirty seconds twice a day before bedtime, and a single examiner performed clinical monitoring at baseline of seven days. Then, data were collected and analyzed according to Koll et al, (2008).

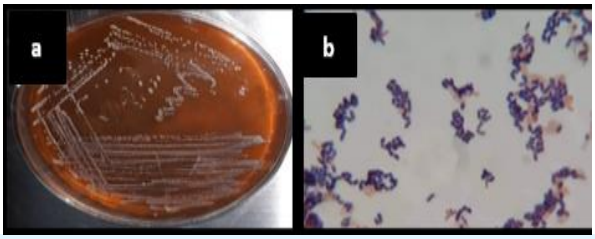

Fig. 1. (a) Typical colony characteristics of the isolates grown on MRS agar medium. (b) Microscopic view of the isolates when Gram stained

RESULTS AND DISCUSSION

Detection of Lactobacillus existence in the oral cavity of the study group

The oral cavity shelters a very numerous microbial flora (Siqueira et al, 2004). One major factor in this complex ecosystem is the presence of different species of Lactobacilli that act as a critical player in the equilibrium of the mouth's consistent environment (Badet & Thebaud, 2008; Piwat et al, 2010). However, when this equilibrium is compromised, an imbalance appears among the indigenous bacteria causing different oral suffering such as thrush, canker sores, dental caries, or periodontitis that could occur according to (Ahirwar et al, 2017). However, few limited data are available for the probiotic properties of oral lactobacilli to combat oral diseases. Thus, this study was aimed to characterize oral lactobacilli for their potential probiotic properties for children when applied as a mouthwash base.

In accordance, the cultivation of the collected whole saliva samples from forty children enrolled in the study revealed the isolation of only three single suspected strains of Lactobacillus-like isolates from three infants at the age of (3-4) months; whereas, most of the other participants showed a prevalence of Candida species in their cultivated saliva samples.

Identification of Lactobacillus-like isolates to the species level

Purified cultures were identified as species of Lactobacillus according to their cultural (**Fig 1a**) and morphological features (**Fig 1b**) according to (Koll et al, 2008) as they produced circular, white, glistering, convex colonies. Besides, the Gram staining had indicated, rod-shaped, short, and positive in Gram reaction, as indicated in **Fig. 1**.

Besides, isolates were able to grow at pH between 4.0 and 8.0, but the optimum growth was observed at pH between 5.5 and 6.5 when grown in MRS broth at 37°C. Also, the biochemical test results revealed that all the isolates belonged to *Lactobacillus fermentum,* according to Bergey's Manual of Systematic Bacteriology, according to (Balcázar et al, 2007). Moreover, the isolates were subjected to the Analytical profile index

Table 2. Morphological and Biochemical Characteristics of *Lactobacillus fermentum* isolates of the study

Characteristics	Results
Gram's stain reaction	+ve
Cell shape	Short rods
Motility	-ve
Colony morphology	Circular, white, glistering, convex colonies
Catalase activity	-ve
Arginine hydrolysis	-ve
Indole reaction	-ve
Oxidase reaction	-ve
Growth at 37°C, 45°C	+ve
CO_2 production from sugar	+ve
Fermentation reaction to D-Glucose, D-Mannose, Lactose and Sucrose	+ve
Inositol, Cellobiose, Mannitol, Rhmnose, Salicin, Starch and Xylose	-ve
Species	*Lactobacillus fermentum*
Sample	Whole saliva from children

+ve; positive reaction result of the isolates to the test, -ve: negative reaction result of the isolate to the test

Table 3. RT-PCR products of the 16S rRNA gene sequences of three *Lactobacillus fermentum* isolates compared with the gene sequence of the standard strain of *Lactobacillus fermentum* M58819

Lactobacillus strains	Designation	16S rRNA length[b]	Similarity %
Lactobacillus fermentum[a]	M58819	1500	100%
Lactobacillus fermentum isolate	Isolate 1	1500	100%
Lactobacillus fermentum isolate	Isolate 2	1500	100%
Lactobacillus fermentum isolate	Isolate 3	1580	100%

[a]Accession number of gene bank can be retrieved from the National Center for Biotechnology Information (NCBI).
[b]Length in nucleotides

system, and Vitek 2 compact for further confirmation and results were revealed in **Table 2**.

Furthermore, the genotypic identification by the 16S rRNA gene sequence of the isolated *Lactobacillus fermentum* carried out using the real-time polymerase chain reaction aligned with the 16S rRNA gene sequence of (*Lactobacillus fermentum* M58819 strain showed (100%) similarity with the previously mentioned strain as results of the analysis were declared in **Table 3**.

Although the *Lactobacillus fermentum* isolate 3 showed the length of 1580 bp of 16S rRNA but, it is still similar to the *Lactobacillus fermentum* M58819 strain as it was published in 2007 by Balcázar *et al*, where they had Sequenced the variable regions of the 16S rRNA gene of the *Lactobacillus fermentum* isolated from the intestinal microbiota of healthy salmonids with the length of 1580 bp.

Accordingly, the morphological, biochemical, and genetic analysis test results of this study had confirmed the isolation of only three strains of *Lactobacillus fermentum* named (A1, A2, and A3) from three infants out of 40 children enrolled in this study. The result obtained, however, disagrees with the previous results published back in 2004, which recommended the procedure of the whole saliva for the isolation of (100%) of Lactobacillus species in the oral cavity of Brazilian children (Motisuki et al, 2005). On the other hand, in another study by (Vestman et al, 2013), about 47 isolates of Lactobacillus were isolated from 43 breastfed infants in 2013 with four different strains isolated from the same infant. Moreover, in 2017, Ahirwar and his colleagues were able to isolate 113 different strains of lactobacilli, including *Lactobacillus fermentum* from forty children only.

Still, the result obtained in this study was almost expected by the authors since (87.5%) of the children enrolled in the screen showed signs of severe Candidiasis. This result agrees with the results of a prospective cohort study published recently in 2019 by Tawfeeq, where a significant prevalence of Candidiasis was recorded by the researcher among school children in Kirkuk/Iraq. This research confirms the attained minimal percentage of (7.5%) of Lactobacillus encountered in the oral cavity of 40 children enrolled in this study where the low colonization of Lactobacillus in the oral cavity increases the incidence of *Candida albicans* as it was documented previously by (Vestman et al, 2013).

As far as our knowledge, no other authors had reported such a low incidence of lactobacilli in the oral cavity of children of different ages. It was initiated that two factors could be influencing the rate of salivary lactobacilli during childhood, which the dependence of infants is feeding on formula milk where de Cunha et al. in (2012) had emphasized on breastfeeding for the enhancement of the probiotic lactobacilli in the oral cavity of infants. On the other hand, the augmented intake of carbohydrates by school children instead of healthy snakes had increased the burden on the oral lactobacillus bacteria as making the oral environment unsuitable for the growth of such fastidious microorganisms as indicated by (Koll et al, 2008; Tawfeeq, 2019).

Evaluation of isolated Lactobacillus as potential probiotics for oral health

Since the isolated strains of *Lactobacillus fermentum* (A1, A2, and A3) did not cause the lysis of erythrocytes of both human and sheep blood. Whereas, complete lysis was produced by both strains of *Streptococcus pyogenes* and *Staphylococcus aureus* in addition to the isolates tolerance to the bile salts and their antimicrobial activity against *Pseudomonas gingivalis* and *Candida albicans*; therefore, single lines of each isolate was mixed with normal saline in an experimental mouthwash to be used for the aid of oral health of children with canker sores and thrush. The results of the *in vitro* application of the mouthwash were declared in **Table 4**.

Moreover, the quantifiable improvements in the oral issues of canker sores and mouth thrush among children volunteered in the study were acknowledged in **Figs 2** and **3**).

Table 4. *In vitro* application of experimental mouthwash based on the isolated *Lactobacillus fermentum* strains of the study

Group No.	No. of children /group	Treatment type	Base of Mouthwash	Duration /days	[a]Positive results%
1	4	Probiotic-based	(10^2-10^8CFU/ml) of *Lactobacillus fermentum*	7	100%
2	4	Normal saline	10% NaCl	7	0
3	4	Placebo	Distilled water	7	0
4	4	none	0	7	0

[a]Positive results referred to children with healed oral issues

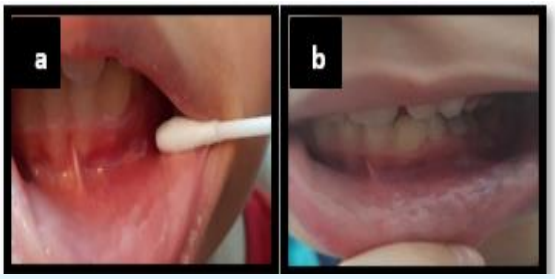

Fig. 2. (a) Canker sore in the mouth of an eight years old child enrolled in the study. (b) Relief of canker sore of the same child volunteered to use mouth wash containing Lactobacillus fermentum for three days

It could be noticed from the results of **Table 4** and **Figs 2** and **3** that volunteered children of group1 who received the probiotic-based mouthwash for seven days had more advantageous improvements in their oral health issues than the children of the other groups.

Likewise, the normal saline-based mouthwash did not show any improvements in the canker sores or thrush conditions of children of group2, approving the probiotic activity, especially the antimicrobial of the isolated strains of the study. This antimicrobial activity

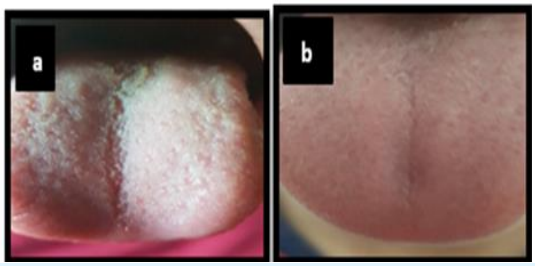

Fig. 3. (a) Mouth thrush of the tongue of a ten years old child enrolled in the study. (b) Mouth thrush relief of the tongue of a ten years old child relief after voluntarily use of the mouth wash mixture of *Lactobacillus fermentum* for five days of treatment

probably justified the restorative effect of *Lactobacillus fermentum* – based mouthwash on the canker sore and thrush of voluntary children where it was previously found that Lactobacilli do not interfere with the fungal adhesion. However, they could suppress their filamentation and translocation Similar results were documented by (Malik et al, 2017) upon their application of mouthwash for periodontitis treatment.

CONCLUSION

A significant reduction was recorded in the rate of Lactobacillus counts in the oral cavity of children at different ages, probably due to increased formula-fed infants and carbohydrates in junk foods. Besides, the reduced colonization of Lactobacillus increased the incidence of oral infections in children. These findings might explain the significant increase in the epidemics and decreased aptitude of our bodies to fight pandemics such as CONVID-2019. Nevertheless, the application of probiotic mouthwash could impose beneficial health potentials on the child's health.

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