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Screening, Isolation and Identification of *Lactobacillus* Species from Dental Caries of Children

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ABSTRACT

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Caries is a chronic disease that progresses slowly and is characterized by localized and irreversible destruction of the tooth. The microbial community of dental caries with many of the facultatively and obligately anaerobic bacteria, *Lactobacillus* have been consistently associated with dental caries for decades. The aim of this study was the isolation and characterization of *Lactobacillus* species, which are commonly present on the surface of dental caries of children. Forty saliva samples were collected from the surface of dental caries of children by using swabbing method, this saliva used to microbiological analysis. Physiological, morphological and biochemical test were used for identification of *Lactobacillus* species. 113 bacterial isolate were isolated from 40 saliva samples of children. In the all isolate five species of *Lactobacillus* namely *L. acidophilus* (24), *L. rhamnosus* (29), *L. casei* (17), *L. plantarum* (21), *L. fermentus* (22) were identified on the bases of biochemical tests and sugars utilization pattern. *Lactobacillus rhamnosus* was the most frequently identified species of microorganism among the populations of children with caries.

Introduction

Saliva plays an important role in oral health as it maintain the integrity of the oral hard and soft tissues, protects the oral tissue against immunogenic bacterial, fungal and viral infection (Siqueira *et al.*, 2004).

Dental caries is one of the most prevalent diseases in the world. Worldwide, approximately 2.43 billion people (36% of the population) have dental caries in their permanent teeth. The world health organization estimates that nearly all adults have dental caries at some point in time. In baby teeth it affects about 620 million people or 9% of the population (Vos *et al.*, 2012).

They have become more common in both children and adults in recent year (Bagramian *et al.*, 2009).

Dental caries can be defined as the localized destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates. In the development of caries a major role play bacteria of the *Streptococcus* and *Lactobacillus* genera which are acidogenic and aciduric (Chen and Wang, 2010).

Lactobacillus are characterized as Gram - positive, usually non-motile, non - spore

forming bacteria that produce lactic acid as a major or sole product of fermentative metabolism. The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures and configuration of the lactic acid produced ability to grow at high salt concentrations and acid or alkaline tolerance. The classification of the genus *Lactobacillus* remains uncertain and it consists of genetically heterogeneous strains which are difficult to classify by physiological and biochemical tests. Kandler and Weiss were classified *Lactobacillus* isolates from temperate regions according to their morphology, physiology and molecular characters (Kandler *et al.*, 1986).

Historically, *Lactobacilli* were the first microorganisms implicated in dental caries development (Owen *et al.*, 1949). They appear during the first years of a child's life, and are present in high numbers in saliva, on the dorsum of the tongue, mucous membranes, the hard palate, in dental plaque and, in fewer numbers, on tooth surfaces (Badet and Thebaud, 2008).

Lactobacilli absent from the oral cavity of new-borns appear during the first year of the life. McCarthy *et al.*, observed the presence of this species in 50% of new-borns during their first year with a rate from 200 to 30000 bact/sample (Carthy *et al.*, 1965).

In children without caries, the rate of salivary *Lactobacilli* varied among the different studies. Carlsson *et al.*, considered that *Lactobacilli* became regularly present in 50% of children and only since the age of 2 (Carlsson *et al.*, 1975).

In the present study, we attempted to determine the *Lactobacillus* species which are commonly present in the dental caries of children and use the biochemical tests for classification and identification of it.

Materials and Methods

Subjects

Forty patients (23 male, 17 female) ranging in age 4 to 12 years, were selected for inclusion in the study. The samples used to this study were collected from the tooth of the patients diagnosed with dental caries by Physician. Dental caries indexes were recorded at the beginning of the study using WHO criteria and those with DMFT indexes between 9- 15 were included in the study.

Sample collection

Samples were obtained between 8am and 12pm, subjects were instructed not to drunk, eat, smoke or clean their teeth for 2hours before the sampling. Samples were collected from the infected area of the tooth was swapped with sterile cotton wool and saliva transferred to a sterile screw capped tube that contained 5ml, 1% saline water (1% NaCl) with aseptic precautions, vortex mixed for 1min, to disperse the bacteria and sample were used to further laboratory examination.

Isolation of bacteria

For each sample, a 200-mL aliquot of saliva was spread on selective medium (MRS agar) that promotes the growth of *Lactobacilli* while suppressing growth of other bacteria. After incubation in incubator at 37°C for 48 hrs, a single colony with the most predominates morphology was picked and sub-cultured for 24 h on Rogosa agar under the same conditions (Rogosa *et al.*, 1960).

Identification of the *Lactobacillus* species

The cultures were identified according to their morphological, cultural, physiological and biochemical characteristics (Kandler *et al.*, 1986; Sharpe *et al.*, 1979). The used tests were: Gram reaction; production of catalase,

cytochrome oxidase and hydrogen peroxide; growth at 15⁰C and 45⁰C in 1 week; acid production from carbohydrates (1 % w/v) - L-arabinose, cellobiose, D-fructose, lactose, melezitose, melebiose, mannitol, D-mannose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-xylose in MRS broth devoid of glucose and beef extract with phenol red as indicator; production of acid and gas from 1 % glucose (MRS broth without beef extract); Production of ammonia from arginine; nitrate reduction in nitrate broth; indole production in tryptone broth and growth on acetate agar.

Catalase test

A drop of 3% hydrogen peroxide was placed on a clean microscopic slide. With a nicrome wire loop pick up cells from the centre of a well isolated colony of the test culture and transfer them into the drop of hydrogen peroxide. Both were mixed and observed for gas bubble production. The strains showing gram-positive and catalase negative isolates were identified at species level.

Gas production from Glucose

In order to further define homofermentative isolates, CO₂ production from glucose test was performed. For this purpose, MRS broths and inverted Durham tubes were used. 50µl of overnight cultures were transferred into the 8 ml test media. After incubation for 5 days at 30 °C, gas accumulation in Durham tubes was taken as the evidence for CO₂ production from glucose.

Growth of isolates at (15°C and 45°C)

The isolates were tested for their ability to grow in MRS broth at 15 + 1°C for 7 days and 45°C by incubating for 24–48 h. For this, 10 ml of MRS broth tubes were inoculated @ 100µl of overnight *Lactobacilli* cultures. The

development of turbidity in culture tubes was recorded as the ability of isolates to grow at 15°C and 45°C and results were noted as positive or negative.

Arginine Hydrolysis

Autoclaved arginine hydrolysis broth tubes were inoculated with the isolated cultures (1%) and incubated at 37°C for 48 h. After incubation, 3–5 drops of the Nessler's reagent were added to each test tube and observed for the change in colour (yellow to orange colour), indicating a positive result for arginine hydrolysis.

Nitrate reduction

Nitrate reduction is an important criterion for differentiating and characterizing different types of bacteria. Therefore, the isolates were incubated at 37 °C for 24 h in trypticase nitrate broth. After incubation, 0.5 mL each of sulphanic acid (0.8%, in 5N -naphthylamine (0.5%, αAcetic acid) and in 5N Acetic acid) were added into the tubes. The appearance of red or pink colour indicated the positive test for nitrate reduction and was recorded accordingly for the isolates tested in the present study.

Citrate Utilization Test

The isolates were inoculated in Simmons citrate agar incubated at 37 °C for 24 h. After incubation, the appearance of blue coloration indicated the positive test for citrate utilization and was recorded accordingly for the isolates tested.

Sugar fermentation

Phenol red broth base medium was used as a medium for this test. Different sugar substrates namely, L- arabinose, cellobiose, D-fructose, lactose, melezitose, melebiose,

mannitol, D-mannose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose, D-xylose and glucose were used. 0.1 g (0.1 % w/v) of each sugar substrate was added to 100 ml of the medium. 5 ml of each mixture was transferred into each tube. For gas detection, Durham tube was inserted into the test tube containing glucose. All the tubes were sterilized for 15 min at 121°C. The tubes were inoculated with a single colony of the bacteria under study. The positive reaction of the bacteria was indicated by the changes in the colour of the medium.

Results and Discussion

Colonies of lactic acid bacteria were observed on the surface of MRS plates. More than one colony was observed in most of the cases (figure.1). Cultural, physiological and morphological characteristics were examined

with the help of microscope. Different types of species were observed, majority of them belonged to Gram+ve rods shaped bacteria (Table: 1). The purification of isolates was done by transferring Gram+ve rods shaped bacteria to the plates of selective media MRS respectively. These isolates were further sub cultured until pure isolates were obtained. Numbers of lactic acid bacterial cultures were isolated from saliva samples. After initial identification, most of them were determined as representative of the family *Lactobacillaceae* and genus *Lactobacillus*.

Hanging-drop wet method showed that the isolated bacteria were nonmotile. Therefore, these methods conformed that the bacteria under investigation was nonmotile. The nonmotile behaviour is a characteristic of *Lactobacillus* (Table 1).

Table.1 Morphological and simple physiological characteristics of *Lactobacillus* isolated from the saliva

| Species | <i>L. acidophilus</i> | <i>L. rhamnosus</i> | <i>L. casei</i> | <i>L. plantarum</i> | <i>L. fermentum</i> |
|------------------------------|-------------------------------------|-------------------------------------|---|-------------------------------------|-------------------------------------|
| Gram's stain reaction | Gram's +Ve | Gram's +Ve | Gram's +Ve | Gram's +Ve | Gram's +Ve |
| Motility | - | - | - | - | - |
| Cell shape | Rod | Rod | Rod | Rod | Rod |
| Catalase activity | - | - | - | - | - |
| Citrate reaction | - | - | - | - | - |
| Nitrate reaction | - | - | - | - | - |
| Colony morphology | Circular, large, smooth, glistening | Circular, large, smooth, glistening | White, and creamish, smooth, mucoid, convex | Circular, white, glistening, convex | Circular, white, glistening, convex |

Table.2 Biochemical characteristics of *Lactobacillus* species isolated from dental caries of children's

| <i>Lactobacillus</i> species | <i>L. acidophilus</i> | <i>L. rhamnosus</i> | <i>L. casei</i> | <i>L. plantarum</i> | <i>L. fermentum</i> | |
|-------------------------------------|-----------------------|---------------------|-----------------|---------------------|---------------------|---|
| No. of isolates | 24 | 29 | 17 | 21 | 22 | |
| CO2 from glucose | - | - | - | - | + | |
| NH3 from Arginine | - | - | - | - | - | |
| Growth | at 15°C | - | + | + | + | - |
| | at 37°C | + | + | + | + | + |
| | at 45°C | - | + | - | - | + |
| Arabinose | - | - | - | - | 8 | |
| Cellobiose | + | + | - | + | - | |
| D-Mannose | + | + | + | + | 11 | |
| Inositol | - | - | - | - | - | |
| Lactose | 10 | + | + | + | + | |
| Melezitose | - | + | + | 17 | - | |
| Melibiose | 16 | 6 | - | + | + | |
| Maltose | + | + | 9 | + | + | |
| Mannitol | - | + | + | + | - | |
| Raffinose | 11 | - | - | + | + | |
| Rhamnose | - | 18 | - | + | - | |
| Ribose | - | + | + | + | + | |
| Salicin | + | 22 | + | + | - | |
| Starch | - | - | - | - | - | |
| Sucrose | + | + | + | + | + | |
| Xylose | - | - | - | - | - | |
| Glucose | + | + | + | + | + | |

+: Positive reaction of all the isolates, -: Negative reaction. Numbers are the positive isolates. All the isolates fermented D- Glucose, D- Mannose, Lactose, Sucrose.

Fig.1 *Lactobacillus* on MRS agar plate



Fig.2 Sugar fermentation



The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. In performing catalase test, no bubble was observed indicating that the isolated bacterium is catalase negative and could not mediate the decomposition of H_2O_2 to produce O_2 (Table 1). Arginine hydrolysis, nitrate reduction, citrate utilization tests were performed, all isolates gave negative results (Table 1).

The main task of Carbohydrate fermentation test is to investigate the ability of bacteria to ferment different types of carbohydrate. Phenol red broth base medium was used as an indicator to differentiate the bacteria

according to their patterns of carbohydrate utilization. Table 2 shows the sugar fermentation patterns, total 113 bacterial strains were isolated. In total, 15 sugars were used for conformational identification of *Lactobacilli* and these were: L-arabinose, cellobiose, D-fructose, lactose, melezitose, melebiose, mannitol, D-mannose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-xylose. Different isolates showed different types of sugar utilization patterns where some tubes containing isolates and sugar turned yellow whereas the other remained brown colored, indicating the positive and negative tests, respectively for sugar fermentation (Figure: 2). When these

sugar utilization patterns were compared with those given for *Lactobacillus* species in the Bergey's Manual of Determinative Bacteriology, the isolates were tentatively identified as *L. acidophilus*, *L. rhamnosus*, *L. casie*, *L. plantarum*, *L. fermentum*. Hence, five different species within *Lactobacillus* genera in the saliva sample obtained from dental caries were confirmed in the present results.

In conclusion, in the current study we are isolate five species of *Lactobacillus* namely *L. acidophilus*, *L. rhamnosus*, *L. casie*, *L. planturam*, *L. fermentum* from dental caries of children. *Lactobacillus rhamnosus* is predominant in all isolated species, and play important role in dental caries progration.

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