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Action of secondary metabolites of *Lactobacillus* strains against caries causing microorganism *Streptococcus mutans*, oral care *in vitro* study

Amit Gupta [1,*], Sakshi Kaul [1], Mansi Rawat [1], Mahesh Katariya [2], Prasun Bandyopadhyay [1,2] and Ranjan Mitra [1]

[1] *Dabur India Limited, Analytical Division, Dabur Research and Development Centre, Sahibabad, Ghaziabad (U.P.), India.*
[2] *Dabur India Limited, Home and Personal Care Division, Dabur Research and Development Centre, Sahibabad, Ghaziabad (U.P.), India.*

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Abstract

Oral health has a direct impact on an individual's wellbeing and quality of life. Probiotics are increasingly being recognized as an effective preventive measure in dental hygiene. The goal of the present study was to evaluate the action of secondary metabolites of *Lactobacillus* strains against caries causing microorganism *Streptococcus mutans*. *Lactobacillus reuteri* was isolated from probiotic chewable tablet and lab reference strains *Lactobacillus plantarum* and *Lactobacillus casei* were also taken for the study. Isolated strain from chewable tablet was confirmed to be *Lactobacillus reuteri* based on colony morphology, physiological and biochemical characterization. All the strains were cultured in respective culture media and cell free supernatant was collected to observe the antagonistic activity against *Streptococcus mutans*. Antimicrobial efficacy was observed through zone of inhibition against all the strains with *Lactobacillus reuteri* 9.24±0.07 mm, *Lactobacillus plantarum* 14.42±0.02 mm and *Lactobacillus casei* 10.12±0.02 mm, Mean ± SME and no zone of inhibition was observed for MRS broth blank control. *Lactobacillus reuteri* and *Lactobacillus casei* strains were confirmed to be bacteriostatic while *Lactobacillus plantarum* was confirmed to be bactericidal. Inhibitory effect of *Lactobacillus* strains on other *Lactobacillus* strains and *Streptococcus mutans* on *Lactobacillus* strains was also observed showing no inhibition against the strains. Results confirmed that all the three strains were effective against *Streptococcus mutans* and lab reference strains *Lactobacillus plantarum* and *Lactobacillus casei* can also be used in formulations like gummies, chewable tablets and lozenges as the delivery carrier for oral probiotics products for oral care against caries causing microorganism *Streptococcus mutans.*

Keywords: Probiotics; Chewable tablets; *Lactobacillus sp; Streptococcus mutans*

1. Introduction

The use of fluoride products and xylitol chewing gum has greatly reduced the prevalence of dental caries in children [1, 2]. However, the use of these products can result in the generation of fluoride resistant bacteria [3] and the ingestion of xylitol chewing gum may be lethal for children. In China, 76.6% of children at the age of five suffer from dental caries [4] therefore, safe and effective methods for the prevention of caries needs to be explored. As probiotics can produce different antibacterial compounds, improve the oral microbial ecology, and rarely cause infections in humans, these organisms represent a safe and promising way to control caries. Dental caries, a prevalent long-lasting condition of the mouth, has the ability to impact the well-being of both adults and children [5].

Caries polymicrobial nature has been well established [6- 8], and *Streptococcus mutans* is an oral bacterium that can contribute to the onset and progression of caries [9- 11]. The cariogenic potential of *Streptococcus mutans* is mainly attributed to its capacity to: (i) form biofilms on surface of teeth, (ii) produce weak acids, mainly lactic acid, and (iii)

* Corresponding author: Amit Gupta.

adapt to environmental stress conditions such as fluctuations in pH, oxygen tension and nutrient availability [12]. Moreover, *Streptococcus mutans* can sustain growth and carry out glycolysis at pH values below the critical value to demineralize tooth enamel [13- 15]. *Streptococcus mutans* has been recognized as a primary cause of dental caries [16].

The formation of oral cariogenic biofilm begins with the initial attachment of non-mutans *Streptococci* to the pellicle. This stage provides an advantageous environment for the development of *Streptococcus mutans* and the initial formation of a biofilm. *Streptococcus mutans* has certain virulence factors that play a role in the development of cavities, including: 1) Creating acid that causes damage to the hard tissues of teeth [17]. 2) Having an agmatine deiminase system and F-ATPase, which are encoded by the *agu*BDAC operon [18] and the *atpD* gene [19]. These components are important for the organism's ability to adapt to acid and contribute to its acid-resistant characteristics. The genes gtfb, gtfc, gtfd, and sacB encode for multiple glucosyltransferases (Gtfs) and a fructosyltransferase (ftf) that enable the synthesis of exopolysaccharides (EPS) from sucrose. The glucosyltransferase and fructosyltransferase enzymes are responsible for creating extracellular glucan and fructan polymers from sucrose, correspondingly [20]. The researchers believe that the EPS has two functions helping bacteria stick to surfaces and also protecting bacteria that are already embedded. These factors of pathogenicity function based on the regulation of quorum sensing systems. The comCDE and vicRKX systems are two examples of two-component signal transduction systems that play a crucial role in regulating gene expression in bacteria. These systems are vital for the survival and ability of bacteria to adapt to their environment and modulate their virulence [21, 22]. Caries management strategies involve techniques such as physically removing plaque and reducing the number of bacteria using chlorhexidine. Additional approaches involve the preservation of the mouth's ecological balance through the use of probiotics [23]. Probiotic bacteria are living microorganisms that, when given in adequate amounts, have the potential to improve the health of the individual they are administered to. The majority of probiotics are bacteria that are classified as Gram-positive and are part of the *Lactobacillus* or *Bifidobacterium* genera [24].

In the past few years, there has been a study on the efficacy of oral probiotics in the reduction and prevention of oral ailments. One option is to introduce oral probiotics in the mouth in order to maintain the balance of the microbiome. Therefore, the choice of delivery carrier for oral probiotics is crucial for effectively delivering and releasing a substantial number of oral probiotics into the mouth. The oral microbiota and the function of oral probiotics in diminishing oral ailments.

Oral probiotics are commonly found in the market in the form of chewing tablets, gummy and lozenges. Hence, the oral probiotic carriers currently used in the market and research was undertaken having probiotic strain *Lactobacillus reuteri* along with lab reference microbial strains *Lactobacillus plantarum* and *Lactobacillus casei* to observe the effectiveness of probiotic *Lactobacillus* strains on caries causing microorganism *Streptococcus mutans* that can be used as an effective probiotic microorganism in oral care against caries in formulation of chewable tablets, gummy and lozenges.

2. Material and Method

2.1. Sample collection

Product probiotic chewable tablets were procured from the local suppliers and lab reference strains *Lactobacillus plantarum* and *Lactobacillus casei* were used in the antimicrobial assessment for *In-vitro* study.

2.2. Experimental Procedure

2.2.1. Microbial culture preparation

MRS broth was prepared and *Lactobacillus plantarum* strain ATCC 8014, *Lactobacillus casei* strain ATCC 334 and *Lactobacillus reuteri* (Unique Biotech UBLRu-87strain) were inoculated from second subculture and incubated at 37 °C for 48 hrs. These cultures were used in the study.

Brain Heart Infusion broth media (BHIB) was prepared and *Streptococcus mutans* strain ATCC 25175 was inoculated from second subculture and incubated at 37 °C for 48 hrs. This culture was used in the study. All the reference microbial cultures and culture media were purchased from HiMedia Laboratories Pvt. Ltd.

2.2.2. Isolation and identification of microorganisms from the product

Sample probiotic chewable tablet was diluted in 0.85% normal saline and further serially diluted and plated in MRS agar and incubated at 37 °C for 48 hrs. Isolated colonies were selected and identified for *Lactobacillus reuteri* as claimed. Morphological, physiological, and biochemical characteristics of *Lactobacillus reuteri* isolate were done.

2.3. Antimicrobial activity

2.3.1. Plate preparation for antimicrobial activity

100 ml of Brain heart infusion agar was prepared and after sterilization cooled to 45 °C and inoculated with 100 µl culture of *Streptococcus mutans* set at 0.5 McF ($1.5x10^8$ cfu/ml) in saline (densitometer, Biosan DEN 1B, Latvia, Europe). Approximately, 25 ml of the culture media was poured into sterilized petri plate and allowed it to solidify. Wells were prepared to load the sample with the help of sterilized borer.

2.3.2. Antagonistic activity of *Lactobacillus sp.* isolates and reference lab strains *Lactobacillus plantarum* and *Lactobacillus casei* against *Streptococcus mutans*.

The isolated bacteria from product and lab reference strains were grown in MRS broth at 37 °C for 24 hrs. and further cell free supernatant of each bacterium was collected by centrifugation at 3000 RPM at 5 °C for 5 min (Eppendorf centrifuge 5804R, Germany). This process was repeated two times to collect the cell free supernatant. 100 µl of cell free supernatant was loaded into the wells of BHIA plate and 100 µl sterile MRS broth was also loaded as control blank. All petri plates containing the cell free supernatant of each bacterium and control was incubated at 37 °C for 24 hrs. The diameter of the inhibition clear zone was taken with the help of vernier caliper and noted after 24 hrs. of incubation.

2.3.3. Bacteriostatic and bactericidal activity of Lactobacillus sp.

The isolate and lab reference strains were also checked for their bacteriostatic or bactericidal activity by taking the swab near the zone area of BHIA plates and streaked on BHIA plate and incubated at 37 °C for 24 hrs.

2.3.4. Inhibition of Lactobacillus strains on other Lactobacillus strains and Streptococcus mutans on Lactobacillus strains.

Lactobacillus sp. produce antimicrobial peptides, bacteriocins, which inhibit or kill generally closely related bacterial strains and other pathogenic bacteria such as *Listeria, Clostridium*, and *Salmonella*. Bacteriocins are cationic peptides that kill the target cells by pore formation and the dissipation of cytosolic contents, leading to cell death. Inhibitory action was also performed between the combination of *Lactobacillus* strains and *Streptococcus mutans* with *Lactobacillus* strains by growing the microbial cultures in their respective culture media and cell free supernatant of each strain were collected and used in the study for antimicrobial activity through zone of inhibition as described above.

3. Result and Discussion

3.1. Isolation and identification of *Lactobacillus sp.*

One sample of a probiotic chewable tablet was collected from a local supplier. *Lactobacillus sp*. was screened and isolated from the products. After culturing for 24 to 48 hrs. the culture was later identified as *Lactobacillus reuteri* by observing their colony morphology (Fig. 1), physiological as well as biochemical characterization (Table 1).

Figure 1 *Lactobacillus casei* (a), *Lactobacillus plantarum* (b), and *Lactobacillus reuteri* (c) on MRS agar

Table 1 Morphological, physiological, and biochemical characteristics of isolated *Lactobacillus reuteri* from chewable tablet sample**.**

Species	***Lactobacillus reuteri***
Colony morphology on MRS agar	White, and creamish, smooth, mucoid, convex
Gram's stain reaction	Gram +ve rods
Cell shape	Rods
Motility	-ve
Catalase activity	-ve
Citrate reaction	-ve
Nitrate reaction	-ve
VP	+ve
Glucose	+ve
Fructose	-ve
Sorbitol	-ve
Starch	-ve
Xylose	-ve
Rhamnose	-ve
Mannitol	-ve
D-Mannose	-ve
Inositol	-ve
Lactose	+ve
Arabinose	+ve
Raffinose	-ve
Inulin	-ve
Cellobiose	-ve

+ve Positive reaction, -ve Negative reaction

3.2. Antagonistic activity of *Lactobacillus reuteri* isolate and some reference lab strains *Lactobacillus plantarum* and *Lactobacillus casei* against *Streptococcus mutans*

Lactobacillus reuteri isolate and some reference lab strains *Lactobacillus plantarum* and *Lactobacillus casei* were examined for their antagonistic activity using agar well diffusion method. The antagonistic effects of *Lactobacillus* isolates and lab strains were exposed to caries causing microorganisms *Streptococcus mutans*. *Lactobacillus* isolate strain and reference strains showed antagonistic effects against *Streptococcus mutans* showing clear zone of inhibition with 9.24±0.07 mm *Lactobacillus reuteri*, 14.42±0.02 mm *Lactobacillus plantarum* and 10.12±0.02 mm *Lactobacillus casei* but the degree of antagonism varied among the *Lactobacillus* strains and no inhibitory zone was observed for MRS broth control blank (Table 2) (Fig. 2 & 3).

To ascertain if the isolate and lab strains are bacteriostatic or bactericidal, a confirmation test was done by taking swab from each clear zone of the test organism from BHIA plates and were streaked on to BHIA plates for growth. Depending on growth, the bacteriostatic and bactericidal activities were classified (Table 3). The growth of *Streptococcus mutans* was interpreted as an inhibitory activity, called bacteriostatic, while no growth was interpreted as bactericidal.

Table 2 Antagonistic activity of *Lactobacillus* isolate from sample and lab strains against *Streptococcus mutans*

Lactobacillus sp.	**Zone of inhibition (in mm)**
Lactobacillus reuteri	9.24±0.07*
Lactobacillus plantarum	14.42±0.02*
Lactobacillus casei	10.12±0.02*

* Data is presented in Mean ±SEM (Standard Error of Mean)

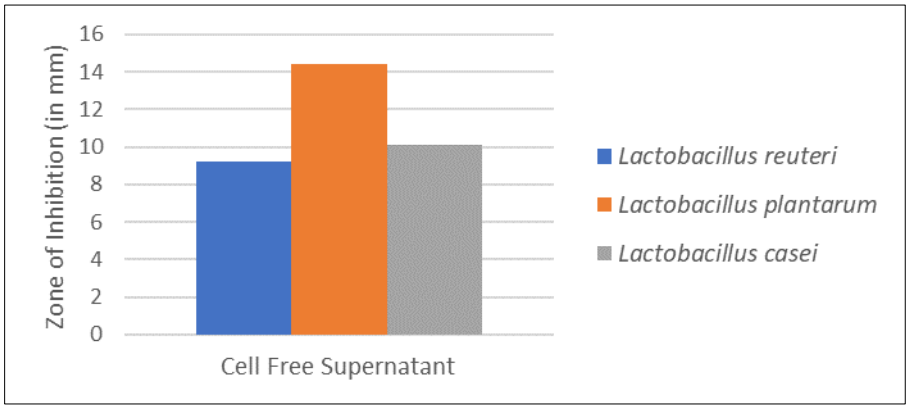

Figure 2 Column diagram showing zone of inhibition of cell free supernatant of *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus casei* against *Streptococcus mutans*

Figure 3 Plates showing zone of inhibition of *Lactobacillus plantarum* (a), *Lactobacillus casei* (b), and *Lactobacillus reuteri* (c) against *Streptococcus mutans*

Table 3 Bacteriostatic and bactericidal activity of *Lactobacillus sp.*

Caries causing microorganism	***Lactobacillus reuteri***	***Lactobacillus plantarum***	***Lactobacillus casei***
Streptococcus mutans	+	-	+

+ bacteriostatic, - bactericidal

3.3. Inhibition of *Lactobacillus* strains on other *Lactobacillus* strains and *Streptococcus mutans* on *Lactobacillus* strains

The inhibitory effect of *Lactobacillus* strains was observed as some *Lactobacillus* strains inhibit related *Lactobacillus sp.* and was also observed inhibition for *Streptococcus mutans* against *Lactobacillus* strains. The observation was tabulated in Table 4.

Table 4 Inhibitory effect of *Lactobacillus* strains on other *Lactobacillus* strains and *Streptococcus mutans* on *Lactobacillus* strains

Organism	ZOI against	Observation
Lactobacillus plantarum	*Lactobacillus casei*	No zone observed
	Lactobacillus reuteri	No zone observed
Lactobacillus casei	*Lactobacillus plantarum*	No zone observed
	Lactobacillus reuteri	No zone observed
Lactobacillus reuteri (Unique Biotech strain)	*Lactobacillus casei*	No zone observed
	Lactobacillus plantarum	No zone observed
Streptococcus mutans	*Lactobacillus plantarum*	No zone observed
	Lactobacillus casei	No zone observed
	Lactobacillus reuteri	No zone observed

The concept of employing probiotic therapy for oral health has emerged due to the widespread occurrence of bacteria becoming resistant to antibiotics. Various studies have been carried out and it has been asserted based on the findings that certain types of probiotics, specifically *Lactobacillus* and *Bifidobacterium* strains, can reduce the occurrence of dental caries, which are linked to bacterial presence in the saliva. A chewable tablet containing probiotic *Lactobacillus reuteri* and strains such as *Lactobacillus plantarum* and *Lactobacillus casei* can be advantageous in combating dental caries and related oral conditions by promoting the restoration of a healthy microflora. The aim of this work was to isolate and characterize probiotic bacteria from chewable tablet sample and to assess the anti-bacterial activity against *Streptococcus mutans* against isolated strain *Lactobacillus reuteri* and two probiotic reference lab strains *Lactobacillus plantarum* and *Lactobacillus casei*. Based on colony morphology, physiological as well as biochemical characterization it was confirmed that the isolate was *Lactobacillus reuteri* from sample. The results confirm that the microbial strain claimed in product chewable tablet was the same *Lactobacillus reuteri*.
The antagonistic effects of *Lactobacillus* isolates and lab strains *Lactobacillus plantarum* and *Lactobacillus casei* were exposed to caries causing microorganisms *Streptococcus mutans*. *Lactobacillus* isolate strain and reference strains showed antagonistic effects against *Streptococcus mutans* showing clear zone of inhibition.

Antibacterial substances produced by probiotic *Lactobacilli* include lactic acid, which can inhibit microbial growth by lowering the pH; hydrogen peroxide, which can inhibit bacterial DNA synthesis [25] and bacteriocins, which can destroy bacterial cell membranes to kill gram-positive bacteria. As *Streptococcus mutans* is acid-tolerant and hydrogen peroxide production by *Lactobacillus* is low, it is possible that the antibacterial substances in probiotics may be primarily bacteriocins or bacteriocin-like proteins. However, at present the antimicrobial mechanism of probiotic *Lactobacilli* against *Streptococcus mutans* is still not fully understood and requires further research.

Bacteriostatic and bactericidal activity of *Lactobacillus sp.* was also observed for the isolated strain and lab strains by taking swab from each clear zone of the test organism from BHIA plates and were streaked on to BHIA plates for growth. *Lactobacillus reuteri* and *Lactobacillus casei* strains were confirmed to be bacteriostatic as growth was observed on the BHIA plates while *Lactobacillus plantarum* was confirmed to be bactericidal as no growth was observed on the BHIA plates.

Inhibitory effect of *Lactobacillus* strains on other *Lactobacillus* strains and *Streptococcus mutans* on *Lactobacillus* strains was also performed to confirm that *Lactobacillus* strains do not inhibit other closely related *Lactobacillus* strains and for *Streptococcus mutans* that do not inhibit *Lactobacillus* strains.

Study reported that probiotic supplementation (fermented milk containing 5×10^{10} CFU/ml of *L. rhamnosus* SD11 or condensed milk powder containing $7.5 \pm 0.20 \times 10^{7}$ CFU/g of *L. rhamnosus* SD11) significantly reduced caries lesions, the percentage of caries progression and number of counts of *Streptococcus mutans* in children. Both the fermented milk and condensed milk powder had a positive effect compared to baseline and the respective placebo controls [26]. In another report the supplementation of probiotic milk containing 7.5×10^{5} CFU/ml of *L. rhamnosus* GG and 4.5×10^{5} CFU/ml of *B. longum* for 3 months showed no positive effect on the oral health status in children. The consumption of probiotic milk increased the salivary acidity and reduced the remineralization of dental caries but did not reduce *Streptococcus mutans* count. The results indicated undesirable effects on the oral health status of children, and the study warned that the selection of potent probiotic strains is a very critical step in developing probiotic-based dental care products [27]. So, the present study also suggests that the selection of probiotic strain is also becomes the important factor to get the desirable benefit.

4. Conclusion

Oral diseases are prevalent worldwide and are typically experienced by individuals at some point in their lives. *Streptococcus mutans* is regarded as the primary species responsible for causing dental caries. There is an anticipation that probiotics can have positive effects on oral health. This is because they can regulate the collection of microorganisms in the intestines and decrease the presence of harmful bacteria. These health benefits are particularly evident in the mouth, as it is an integral part of the gastrointestinal system. As a result, multiple studies have been carried out in laboratory and living organism environments to investigate how probiotics can prevent tooth decay. In this study, while investigating the effect of different strains of probiotics *Lactobacillus reuteri, Lactobacillus plantarum* and *Lactobacillus casei* on oral diseases, dental caries caused mainly by *Streptococcus mutans* it was found that all the strains were effective against *Streptococcus mutans*. Probiotics play an important role in the improvement of oral health. Additionally, they can be used as an adjunct to fluoride in dental caries prevention or control in high caries risk children. With the above results it was confirmed that *Lactobacillus reuteri, Lactobacillus plantarum* and *Lactobacillus casei* are effective against caries causing *Streptococcus mutans* and lab reference strains *Lactobacillus plantarum* and *Lactobacillus casei* can be used in formulations like gummies, chewable tablets and lozenges as the delivery carrier for oral probiotics products for oral care against *Streptococcus mutans.*

Authors Contributions

Amit Gupta: Research planning executing, writing manuscript. Amit Gupta, Sakshi Kaul, Mansi Rawat: Research work and optimization of experimental work. Mahesh Katariya: Providing probiotic chewable tablet sample. Mahesh Katariya, Ranjan Mitra: Reviewing manuscript, Supervision. Prasun Bandyopadhyay, Ranjan Mitra: Providing the facility to carry out the research work and valuable inputs. All authors read and approved the final manuscript.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest related to this article.

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