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Inhibitory effect of isolated Lactobacillus from oral cavity against bacterial Pathogens and its effect on health promotion

Efecto inhibidor de Lactobacillus aislado de la cavidad oral contra patógenos bacterianos y su efecto en la promoción de la salud

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Conflicto de interés
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All Authors declare no conflicts of interest.

Declaración ética
Ethical Statement

This study has no results of experimental studies on human participants or use of laboratory animals, therefore no Ethical approve is required.

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ABSTRACT

Aims: To determine the inhibitory effect of oral Lactobacillus against bacterial pathogens and investigate correlation between presence of Lactobacillus strains and health promotion.

Method: One hundred saliva samples were collected from oral cavity of domestic dairy consumers and were investigated for the isolation and identification of Lactobacillus strain by conventional culture and sequencing of 16SrRNA. Furthermore, well diffusion assay was performed to determination of antibacterial activity of Lactobacillus strains against bacterial pathogens including *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Shigella sonnei, Shigella dysenteriae*, *Enterococcus faecalis* and *Enterococcus faecium*. Finally, association between health condition and isolation of *Lactobacillus* were investigated and obtained data using questionary form were analysed by chi-square test.

Results: Thirty Lactobacillus strains recovered from 100 hundred saliva samples. The most common isolated strain was *L. gasseri* (n=18) and followed by *L. vaginalis* (n=3) and *L. salivarius* (n=3). All Lactobacillus strains demonstrated antibacterial activity against at least one of the investigated pathogens. However, the strongest results were obtained by *L. vaginalis* against *K. pneumonia*. The correlation between the presence of thirty *Lactobacillus* strains and health promotion not found. However, only *L. gasseri* species has significant positive impact on health in their hosts ($P < 0.05$).

Conclusion: Only some Lactobacillus species have a positive impact on health promotion. Despite of weak activity against the investigated pathogens, *L. gasseri* has a positive impact on the mental problem (intense anger and depression) of their hosts.

Keywords: *Lactobacillus;* Health condition; Antibacterial activity; Oral cavity.

ABSTRACT

Objetivos: Determinar el efecto inhibidor del *Lactobacillus* aislado de la cavidad oral contra patógenos bacterianos e investigar la correlación entre la presencia de cepas de *Lactobacillus* y la promoción de la salud.

Método: se recolectaron cien muestras de saliva de la cavidad oral de consumidores de productos lácteos y se investigó el aislamiento e identificación de la cepa de *Lactobacillus* mediante cultivo convencional y secuenciación de 16SrRNA. Además, se realizó un ensayo de difusión en pocillos para determinar la actividad antibacteriana de las cepas de *Lactobacillus* contra patógenos bacterianos que incluyen *Salmonella typhimurium, Klebsiella pneumoniae, Shigella sonnei, Shigella dysenteriae, Enterococcus faecalis y Enterococcus faecium*. Finalmente, se investigó la asociación entre el estado de salud y el aislamiento de *Lactobacillus* y se obtuvieron los datos utilizando el cuestionario mediante la prueba de chi-cuadrado.

Resultados: Se aislaron treinta cepas de *Lactobacillus* de 100 muestras de saliva. La cepa aislada más común fue *L. gasseri* (n = 18), seguida por *L. vaginalis* (n = 3) y *L. salivarius* (n = 3). Todas las cepas de *Lactobacillus* demostraron actividad antibacteriana contra al menos uno de los patógenos investigados.

Sin embargo, los resultados más fuertes fueron obtenidos por *L. vaginalis* contra *K. pneumonia*. No se encontraron correlación entre la presencia de algunas de las treinta cepas de *Lactobacillus* y la promoción de la salud. Sin embargo, solo la especie *L. gasseri* tuvo un impacto positivo significativo en la salud de sus hospedadores (P <0,05).

Conclusión: solo algunas especies de *Lactobacillus* tienen un impacto positivo en la promoción de la salud. A pesar de la actividad débil contra los patógenos investigados, *L. gasseri* tiene un impacto positivo en el problema mental (ira intensa y depresión) de sus huéspedes.

Palabras clave: *Lactobacillus*; condición de salud; actividad antibacteriana; cavidad oral.

INTRODUCTION

In the past decade role of probiotic microorganisms in health promotion and as preventative agents were investigated [1,2]. Probiotics described as microorganisms which are useful if used in sufficient number [3,4]. Different bacteria such as *Bifidobacterium, Streptococcus,* and *Lactobacillus* introduced as probiotics, and known as generally recognized as safe (GRAS) and non-pathogenic bacteria [3,5,6]. Recently, *Lactobacillus spp*. considered as main probiotic in medical and industrial scope[1,2]. *Lactobacillus spp*. are heterogeneous group of bacteria including about 145 species of non-spore forming and Gram-positive rods which can be grown in anaerobic or microaerophilic conditions[3]. Also, Lactobacillus was used in dairy and fermented food for promotion of health quality [3]. However, *Lactobacillus spp*. is presented in various places like environment and human body (oral cavity, gastrointestinal and vaginal tract) [1,2,5,7].

It was previously demonstrated that consumption of *Lactobacillus* has several benefits including decrease of time and severity of diarrhea [3], antibacterial and antifungal characteristics [8], and improvement of vaginal health [7].

Inhibitory activity against the pathogens is one of the most characteristics of bacteria which known as probiotics. Probiotic inhibitory activity is related to reduce of pathogenic colonization and causing adverse growth conditions for pathogens by production of hydrogen peroxide (H_2O_2) and bacteriocin [8-10]. Moreover, it has recently been demonstrated that Lactobacillus species isolated from human are better probiotics, because these strains were adapted to the human body and also exposure to antibiotics in this place is less than other origins [11].

In recent years, the role of oral microbiome in health quality was discussed extensively. Some studies demonstrated correlation between dental caries, oral cancer and inflammation caused by oral microbiome [6,12,13]. But other study indicated that available *Lactobacillus* spp. in the oral cavity have useful role in promotion of oral and gastrointestinal health [4].

The milk products including yogurt and cheese are important dietary supplements which contain *Lactobacillus* [14]. Consumption of these products is main way to probiotic transmission to the host body. Thus, there is probably strong association between diet and commensal microorganism including probiotics bacteria [15].

The aims of this study were investigate the inhibitory activity of isolated *Lactobacillus* from oral cavity and also, determination of association between isolation rates of *Lactobacillus* and health quality in the host.

MATERIAL AND METHODS

Study population

One hundred healthy persons who use domestic dairy products were recruited in this study. The study was conducted from May to August 2017 at rural area of Bukan, Western Azerbaijan province, Iran. Also, the questioner forms were applied to all persons. These forms were included age, gender, experience of infectious disease, severe gastric pain, herpes, and mental problem (intense anger and depression). The current study was approved by the Research Ethics Committee of Tabriz University of Medical Science; also signed informed consent form was obtained from each patient prior to the initiation of sampling. Participants were subjected for sampling without any hazardous action and participation was completely volunteer. This research has been conducted in full accordance with the World Medical Association Declaration of Helsinki and was done after confirmation of ethic committee of Tabriz University of medical sciences.

Sample collection

According to Kohler and Bratthall (1979), sample collection was performed by modified spatula method [16]. Briefly, wooden spatula was inserted in oral cavity to moisten it with saliva. Then both sides of spatula placed on Rogosa SL agar (MicroMaster, India). The plates were incubated under anaerobic condition (80% N_2, 10% H_2, and 10% CO_2) provided by Anoxomat (Mart Microbiology BV) at 37°C for 72 h. Then dominant Lactobacillus colonies were selected and stored to further analysis.

Isolation and identification

The suspected colonies, temporary identified as Lactobacillus spp. based on phenotypically tests including colony morphology, wet mount smear, Gram staining, and catalase negative reaction[17]. DNA extraction was applied by boiling

method as previously described [18,19]. Amplification and sequencing of 16SrRNA was performed to confirmation of Lactobacillus spp. PCR amplification was performed by the following primers: Forward 5-CTCGTTGCGGGACTTAA-3 and Reverse 5 -GCAGCAGTAGGGAATCTTC-3 (Bioneer, Korea) [4]. The PCR amplification was performed in a total volume of 50 µL containing 25 µL master kit (Ampliqon, Denmark), 5 pmol of each primer and 15 ng DNA. The PCR proceed using a DNA thermal cycler (Master Cycle Gradiant, Eppendrof, Germany) programmed with initial 94°C for 5 min and followed by 35 cycles of 94°C for 30 s, 56°C for 50s and 72°C for 1 min, and with additional extension at 72°C for 10 min. PCR products were electrophoresis in 1.5% agarose gel and after staining with Safestain (Yekta tazhiz, Iran) visualized under UV light [20]. All PCR products were subjected to sequencing by Bioneer (South Korea). Finally obtained sequences were compared with available sequences in GeneBank (www.ncbi.nlm.nih.gov/blast).

Antimicrobial activity

According to earlier study [4], agar well diffusion method was performed to determination of Lactobacillus spp. antibacterial activity against various pathogenic bacteria. In fact, bacteriocin effect against growth of *S. typhimurium*, *K. pneumoniae*, *S. sonnei, S. dysenteriae*, *Enterococcus faecalis* and *Enterococcus faecium* was determinate using agar well diffusion assay. For this reason, the Lactobacillus spp. were incubated at anaerobic condition in MRS broth (37°C for 24h). Then, cell free supernatant was prepared by centrifugation at 10,000 g for 10 min [4,21].

Different antibacterial compound including organic acid and bacteriocin are produced by Lactobacillus. To remove of the organic acid effect against pathogenic bacteria, pH of supernatant should be neutralized with NaOH (2.5M) and catalase (1 mg/ml, Sigma Aldrich, Germany), respectively [4].

Briefly, then 0.5 McFarland was prepared for each pathogen and cultured on MHA (MicroMaster, India). Then 100mL of cell free neutralized supernatant was inoculated in wells and incubated at 37°C for 24 h. Finally Lactobacillus with clear zones <11 mm, 11-16, 17-22 and >22 described as negative (-), mild (+), strong (++), and very strong (+++) inhibitor, respectively. The sterile MRS broth was used as negative control [4].

Statistical analysis

Data analysis was performed using SPSS 23 software (SPSS Inc., IL, USA). Moreover, obtained data were analysed via chi-square test. Differences were considered to be statistically significant when P value was less than 0.05.

RESULT

Demographic data

In this study, one hundred oral salivary samples were investigated. Demographic data were demonstrated in Table 1. Among one hundred persons, 49 and 51 of them were male and female respectively, with mean age 36+43 years (6-81 years). Lactobacillus strains were isolated from 30 persons including 16 males and 14 females. Mean age in these persons were 34+27 years. The correlation between Lactobacillus isolation and evaluated factors (age, gender, natural or cesarean born, the experience of severe infection disease, herpes infection and gastric pain, and mental problem including intense anger and Depression) were not significant. In the other hand, the incidence of the mental problems in eighteen host *L. gasseri* significantly was low (4/18) compared to Lactobacillus-negative persons (40/70) ($P < 0.05$).

Table 1. Demographic data of persons enrolled in this study

	Lactobacillus **positive (30)**	*Lactobacillus* **negative (70)**	**Total (100)**	**P value**
Gender (male)	16	33	49	0.363
Mean age (years)	34.27	37.36	36.43	
Natural born	28	66	94	0.585
Infection disease *	8	19	27	0.584
Gastric pain *	10	20	30	0.401
Mental problem *	12	40	52	0.088
Herpes infection *	17	41	58	0.516

*: number of persons that experience severe infection disease, Gastric pain, mental problem and Herpes infection

Lactobacillus spp.

Totally, thirty Lactobacillus were isolated by phonotypical assay and also, confirmation was performed by PCR and sequencing of 16S rRNA gene. From 30 Lactobacillus spp. eighteen isolates (60%) was identified as *L. gasseri*. Other isolates were *L. vaginalis* (n=3), *L. salivarius* (n=3), *L. crispatus* (n=2), *L. curvatus* (n=1), *L. fermentum* (n=1), *L. kitasatonis* (n=1) and *L. panis* (n=1).

Antimicrobial activity

The growth inhibitory activity against *S. typhimurium* ATCC® 14028™, *K. pneumonia* ATCC® 13883™, *S. sonnei* ATCC® 25931™, *S. dysenteriae* ATCC® 11835™, *E. faecalis* ATCC® 29212™ and *E. faecium* ATCC® 19434™ was illustrated in Table 2. Of the 30 Lactobacillus only three isolates of them were included two *L. vaginalis* and one *L. kitasatonis* demonstrate ++ and +++ inhibitory zone. These isolates have inhibitory zone 17-22 mm and more than 23 mm against *K. pneumonia*. Thus, *K. pneumonia* was the most susceptible investigated species. In fact *L. vaginalis* was the strain of Lactobacillus that demonstrates strongest activity against *K. pneumonia*.

Table 2. Antimicrobial activity of cell-free culture supernatants of *lactobacillus* isolates

Bacterial isolates		Gram negative				Gram positive	
	Inhibitory range	*S. typhimurium*	*Sh. dysenteriae*	*Sh. sonnei*	*K. pneumoniae*	*E. faecium*	*E. faecalis*
L. gasseri (18)	-	15	10	12	11	7	18
	+	3	8	6	7	11	0
L. vaginalis (3)	-	2	2	3	1	3	3
	+	1	1	-	-	0	-
	++	-	-	-	1	-	-
	+++	-	-	-	1	-	-
L. salivarius (3)	-	-	-	3	-	3	3
	+	3	3	-	3	-	-
L. crisptus (2)	-	2	1	2	1	2	2
	+	-	1	-	1	-	-
L. curvtus (1)	-	1	-	1	-	1	1
	+	-	1	-	1	-	-
L. panis (1)	-	1	-	1	-	1	1
	+	-	1	-	1	-	-
L. fermantus (1)	-	1	-	1	1	-	1
	+	-	1	-	-	1	-
L. kitasatonis (1)	-	1	-	1	-	1	1
	+	-	1	-	-	-	-
	++	-	-	-	1	-	-

Antibacterial activity was determined using agar well diffusion assay. Interpretation of Inhibitory range, - : <11 mm (negative); +: 11-16 mm (mild); ++: 17-22 mm (strong); +++: ≥ 23 (very strong).

DISCUSSION

The probiotics bacteria were used for promotion and preservation of health quality for decades [5]. Lactobacillus species are the most well-known probiotics and the important constituent of human body flora including oral, intestinal and vaginal tract [5]. One of the main ability of probiotics is host protection against pathogen microorganisms like as Salmonella, Shigella and other invasive pathogens [1,22,23]. The previous study indicated that, in pregnant women which use from oral probiotics, colonization of Group B Streptococcus was reduced in rectal and vaginal tracts [9]. Along with listed their advantages, results of previous studies demonstrated possible association of some Lactobacillus species with host unfavorable conditions like as dental caries and glycaemia in human [5,24].

In different studies, Lactobacillus species isolated from oral cavity were diverse. In the current study, like of previous studies *L. gasseri*, *L. salivarius*, and *L. vaginalis* were isolated from oral cavity [11,25]. But predominant isolates recovered from oral cavity in different studies were diverse too. In agreement reported paper [26] results of this study demonstrated that, *L gasseri* was the predominant isolated strain, while in other studies performed by Strahinic *et al.* and Ahirwar *et al*, *L. rhamnosus* and *L. fermentum* introduced as predominant recovered strains, respectively [25,27]. It is likely that main reasons related to this diversity are oral health and wealth condition, diet, kind of samples (saliva or dental plaque), the age of patient and performed methods to species identification. For example, in contrast with an earlier study, in this study PCR and sequencing of 16SrRNA (the most reliable method) was performed for species identification and confirmation [27]. Also, Piwat *et al* (2010) demonstrated that *L. plantarum* and *L. mucosae* were recovered in patients with moderate to high dental caries, but the most frequent species in the low-dental caries group were *L. gasseri, L. vaginalis*, and *L. oris* [6]. However, in accordance with this study, previously reported literature illustrated that, in contrast with dental samples in oral saliva, *L gasseri* was predominant isolated strain [26].

In agreement with previous works, this report demonstrated that Lactobacillus strains were able to inhibit the growth of enteropathogen bacteria [9,28]. In accordance with previous study, among the tested *Enterobacteriaceae*, *S. typhimurium* were the most resistant species against CFS of *L. gasseri* [29]. The previous study performed by Koga *et al.* demonstrated that *L. gasseri* was unable to inhibit growth of Salmonella species, while in current study few strains of *L. gasseri* isolates were able to inhibit growth of *Enterobacteriaceae* including *S. sonnei* (6/18), *S. dysenteriae* (8/18), *K. pneumoniae (7/18) and S. typhimurium* (3/18). This diversity is related to several factors including isolation origin, performed methods and applied Lactobacillus species. For example, Lactobacillus strains with oral and gastrointestinal origin are more effective against *Enterobacteriaceae* compared to strains with other origins. This is related with the high adaptability of Lactobacillus strains.

Previously showed that antibacterial activities of Lactobacillus strains against *S. typhimurium* is related to production of different compounds which inhibit motility and penetration of these bacteria into enterocyte-like Caco-2/TC7 cells [30].

The *Lactobacillus* strains have wide range of inhibitory growth effect from absent to very strong against *Enterococcus* species. Shim *et al.* (2016) demonstrated mild inhibition effect against *E. fecalis* [31]. But in this work, none of *Lactobacillus* isolates were able to inhibit growth of *E. fecalis*, while 61% of *L. gasseri* isolates demonstrate mild inhibitory effect against *E. faecium*.

In agreement study performed by Zhang *et al.* (2001), the current work showed that growth of Salmonella was inhibited by treatment of *L. salivarius* culture supernatants [32].

Until now, different studies discussed the impact of *Lactobacillus* genus on protection and promotion of the human health. Some studies illustrated beneficial effect on human health, but in contrast, some studies have not found these impacts and rejected this theory [22,24,33]. For example, an earlier study demonstrated relation of dental caries and the prevalence of Lactobacillus strain in oral cavity [5]. Their ability of acid production by *Lactobacillus* was expressed for dental caries [5]. In disagreement this statement, some studies demonstrated that oral health was promoted due to presence of Lactobacillus, and this is related to activity of Lactobacillus against cariogenic Streptococcus, and periodontal pathogens [1,5,34].

In this study, any relation between the presence of *Lactobacillus* and investigated health factors was observed. However, interestingly in accordance with other studies, presence of *L. gasseri* is contributed to health promotion in current study [9,35,36]. This contradictory result is related to the investigated species and showed that *L. gasseri* has a positive impact on human health in comparison to other species. Until now, it is difficult to explain how the Lactobacillus strains have effects on host health.

In summary: in this study, most Lactobacillus strains isolated from oral cavity able to inhibit growth of gastrointestinal pathogens. This ability is related to high adaptability of *Lactobacillus* strains. Moreover, non-association between the presence of different Lactobacillus species and investi-

gated health factors was observed. However, only *L. gasseri* has a positive impact on the mental problem (intense anger and depression) of their hosts. These results indicated that only some species of Lactobacillus genus has the positive impact on their host.

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