Original Article :

Antagonistic Activity and Probiotic Potential of Lactobacillus sp. isolated from fermented dairy products from Majmaah

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Abstract

Background And Aims:

In much scientific research, it has been found that antagonistic activity is one of the important properties of a probiotic bacterium to attach in the intestine to reduce attachment of pathogenic bacteria in the intestine. Lactobacillus sp., isolated from fermented dairy products, shows a positive impact on human health.

This study was designed to study Lactobacillus sp. isolated from fermented dairy products for their antagonistic activity and probiotic potential.

Methods:

In this study, we used the Lactobacilli strain isolated from yogurt samples by a dilution plating method and were screened for their antagonistic activities and potential probiotics. The isolates were tested for their growth in the presence of 0.3% bile salt and pH 2.0 and 3.0.

Result:

Out of the 52 strains, 30 strains (60%) had survival rates above 90% after 2 h of incubation at pH values of 2.0 or 3.0. Further screening was performed for their growth at 0.3% bile salt. From 30 strains, only ten strains showed tolerance to 0.3% bile salt. Two 2 Lactobacilli strains exhibited antagonistic activity. Moreover, all eight strains were found suitable for the potential probiotic activity, included Lactobacillus casei MU01, MU02, Lactobacillus reuteri MU 113, Bifidobacterium lactis MU85, Lactobacillus salivarius MU18, MU31, Lactobacillus plantarum MU211 3032, and Lactobacillus buchneri MU37.

Conclusion:

This study suggests that eight strains showed good antagonistic activity and probiotic potential, which can be used as supplements for good human health.

الملخص

خلفية والاهداف:

في الكثير من الأبحاث العلمية، وجد أن النشاط العدائي «المضاد» هو أحد الخصائص المهمة لبكتيريا البروبيوتيك «المعززات الحيوية» ؛ حيث أن التصاقها في الأمعاء يقلل من تعلق البكتيريا المسببة للأمراض في الأمعاء. حيث أظهرت بكتيريا اللاكتوباسيلس, المعزولة من منتجات الألبان المخمرة; تأثير ايجابي على صحة الأنسان.

تم تصميم هذه الدراسة لدراسة بكتيريا اللاكتوباسيلس. المعزولة من منتجات الألبان المخمرة؛ لنشاطها العدائي المضاد وإمكانية البروبيوتيك.

طريقة البحث:

في هذه الدراسة تم استخدام سلالة اللاكتوباسيلاي المعزولة من عينات الزبادي بطريقة التصفيح المخفف وتم فحصها بحثًا عن أنشطتها العدائية والبروبيوتيك المحتملة. ومن ثم اختبار نمو البكتيريا المعزولة في وجود ٠,٣٪ ملح الصفراء ودرجة الحموضة ٢,٠ و ٣,٠

النتيجة:

من أصل ٥٢ سلالة ، كان لدى ثلاثين سلالة أيّ (٦٠ ٪) معدلات بقاء فوق ٩٠ ٪ بعد ساعتين من الحضانة عند قيم الأس الهيدروجيني ٢,٠ أو ٣,٠ للحموضة. ثم تم إجراء المزيد من الفحوصات لاختبار نموها عند ٠,٣ ٪ من ملح الصفراء. من بين الثلاثين سلالة أظهرت عشرة سلالات فقط قدرة تحمل الملح الصفراوي بنسبة ٠,٣٪. و أظهرت سلالتين من اللاكتوباسيلاي نشاط عدائي مضاد. علاوة على ذلك ، تم العثور على ثمانية سلالات مناسبة لنشاط بروبيوتيك محتمل ، بما في ذلك:

Lactobacillus casei MU01, MU02, Lactobacillus reuteri MU 113, Bifidobacterium lactis MU85, Lac-

Key Words:
Lactobacilli, Probiotic Potential, Antagonistic activity, Lactobacillus buchneri, acid-tolerant

tobacillus salivarius MU18, MU31, Lactobacillus plantarum MU211 3032 and Lactobacillus buchneri MU37

الخلاصة:

تشير هذه الدراسة إلى أن ثمانية سلالات أظهرت نشاطًا عدائيًا جيدًا وإمكانية البروبيوتيك، والتي يمكن استخدامها كمكملات جيدة لصحة الإنسان.

Introduction

Lactobacilli (the species of the genus Lactobacillus) are the major residents of the vertebrate intestine, including humans. They were found in the fermentation of various food products made from dairy and plants. Microorganisms that are safe and beneficial for health are characterized as probiotics [1]. Other than Lactobacillus, probiotic strains that are considered beneficial include Pediococcus, Bifidobacterium, and Enterococcus [2]. Probiotics are defined as living microorganisms that have beneficial effects on the host and can adjust the host micro-ecological balance, improve intestinal function, and stimulate digestion and immune function. Lactobacillus was the earliest discovered probiotic of the three types of probiotics, which also include Bifidobacterium and Gram-positive cocci [3].

Use of probiotics dates back from the time human civilization eating fermented food, milk, and other products from plants and animals, but their use for health benefits is more in use from the last century, due to growing awareness about the health benefits of probiotic bacteria [4]. These bacteria not only help to maintain the gut ecosystem balanced but also help in controlling many disease conditions like inflammatory bowel disease and inflammatory bowel syndrome [1].

The antagonistic property of probiotic bacteria is an important attribute property to be evaluated as it helps in the identification of bacterial species that can adhere to the intestine walls, reduce adhesion of pathogenic bacteria. Previous research shows the number of Lactobacilli, which originate from dairy fermented products, show the antagonistic property, but not all of them are able to grow at high acidic conditions, similar to the one found in the human stomach and at low pH like 2.0 [5]. To consider any probiotic bacteria for human consumption, it must be able to withstand different pH and acidic conditions found in the human gut; also, it can attach to intestine walls and reduce the attachment of other food or waterborne pathogens [6].

Thus, the study was designed to identify the best potential probiotic Lactobacillus

isolates from fermented dairy products. The in vitro probiotic properties like bile tolerance, acid tolerance, antagonistic activity, and auto and co-aggregation property of the selected strains were studied

Material and Method

Sampling

Fifty-two different fermented dairy products were collected from different local markets. Isolation was done on MRS (Man-Rogosa-Sharp) agar as per Ananthanarayan, R [7] method and incubated at 37ºC for 24 to 48 hours to apply the phenotypic and genotypic identification[8].

Acid tolerance

Acid tolerance was studied as per the method by Chung et al.[8]. All isolates were taken in a concentration of 10 µL, incubated overnight in MRS broth into 1 mL of pH 2.0, 3.0, and 6.4 (control) MRS broths.

Culture Characters:

The streak plate method was performed to record the culture characters, and morphology was carried out according to Banson, H.J[9]. The colonies grown on MRS agar plates were carefully studied concerning size, color, opacity, form rise, and margin.

Test for Bile Toleration (pH-6.1)

For a potential probiotic bacterium, tolerance to bile concentration is an essential probiotic property. Only a few bacterial strains can resist strong bile acid, which is one of the challenges encountered by probiotics[10].

Medium:

LS broth with 0.4%- 1% bile salt (Sodium taurocholate)

Procedure:

Sodium taurocholate mixed with LS broth in different proportions of 0.4%, 0.6%, 0.8%, and 1% dispended in test tubes. Using the loop inoculation process, the tubes were inoculated and incubated at 37 ºC for 24 hours, along with a control. The result was recorded after 24 hours using the spectrophotometry method at 600 nm.

Antagonistic Activities:

To study antagonistic activity, the lactobacillus strains showing good activity for bile were grown in MRS broth at 37 ºC for 24 hours, and cell-free supernatants were collected. Strains of E. coli, Salmonella Enteritidis, and Salmonella Typhimurium isolated from food were incubated in nutrient broth for 24 hours and then diluted to 0.06 at 600 nm equivalent to McFarland standard 0.5, and to microtiter plates. An equal amount of CFS of LAB cultures was added and incubated at 37 ºC for 24 hours. The growth of pathogenic bacteria was determined using a spectrophotometer at 600 nm[11].

Autoaggregation and Coaggregation:

Activities of Auto and co-aggregation were measured using the method of Jena et al.

(2013)[11]. For auto-aggregative ability assays, LAB strains cultured overnight, harvested, washed, and re-suspended in PBS. OD (optical density) of each strain was recorded at 600 nm. The selected strains were incubated at 37 ºC without agitation, and OD readings were recorded at different time intervals (3 and 24 hours).

The formula for calculating the percentage of aggregation:

$$A\% = 100\times\left(1-\frac{A1}{A0}\right)$$

A% = 100 X (1 - At/A0)

where A0 refers to the OD 600 values at 0 hours and At refers to OD 600 values at the indicated time points

Coaggregation assay was done by taking equal volumes of the LAB strains with pathogenic bacteria isolated from food, mixed, and OD reading was taken at 600 nm. The mixed bacterial suspensions were incubated at 37 0C, and OD values were measured at 3 and 24 hours (12).

Statistical analysis

Statistical analyses were performed using SPSS 14.0 software (SPSS Inc.; Chicago, IL, USA). Significant differences among treatments were tested by ANOVA developed by Ronald Fisher in 1923 with a level of significance at $\alpha = 0.05$. Data were expressed as Mean Values ± Standard Deviation (SD). All experiments were performed in duplicate and repeated three times.

Result

Morphological and colony characteristics

Morphological and colony characteristics of the isolated bacterial culture from fermented milk products samples were carried out. Five types of different bacterial colonies with distinct morphological characters were isolated.

Identification by molecular biology method

Out of 52 strains, 38 were Gram-positive, rod-shaped, and catalase-negative using the conventional method of identification, whereas, by using molecular biology technique, isolated strains were found to be Lactobacillus casei, Lactobacillus salivarius, Lactobacillus plantarum, and Lactobacillus buchneri, Bifidobacterium lactis, Lactobacillus salivarius and Lactobacillus reuteri.

Table 1: Strains showing the highest viability (log CFU/mL) at different pH, survival percentage, and Agitation at 600 nm of strains with a high survival rate.

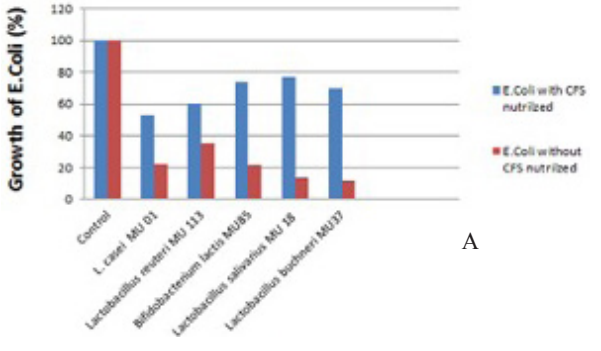
Isolates	Absorption at 600 nm (without agitation)	Absorption at 600 nm (with agitation)	pH 6.2* Viable count (log CFU/mL)	pH 3.0 Viable count (log CFU/mL)	Percentage survival (%)	pH 2.0 Viable count (log CFU/mL)	Percentage survival (%)
L. casei MU 01	0.055	0.032	6.17+0.01	5.33+0.04	90	5.70+0.02	95
L. casei MU 02	0.058	0.044	7.98+0.08	6.51+0.10	85	6.14+0.07	90

Isolates	Absorption at 600 nm (without agitation)	Absorption at 600 nm (with agitation)	pH 6.2* Viable count (log CFU/mL)	pH 3.0 Viable count (log CFU/ mL)	Percentage survival (%)	pH 2.0 Viable count (log CFU/ mL)	Percentage survival (%)
Lactobacillus reuteri MU 113	0.088	0.078	9.08+0.01	7.80+0.01	97	7.89+0.26	95
Bifidobacterium lactis MU85	0.097	0.076	8.97+0.01	8.07+0.02	92	8.99+0.21	95
Lactobacillus salivarius MU 18	0.059	0.023	9.07+0.31	8.77+0.21	96	8.47+0.09	92
Lactobacillus salivarius MU 31	0.087	0.061	7.46+0.04	6.26+0.14	84	5.96+0.21	78
Lactobacillus plantarum MU 211	0.066	0.041	8.90+0.29	7.70+0.20	86	7.20+0.11	80
Lactobacillus buchneri MU37	0.037	0.024	7.26+0.02	6.96+0.12	94	5.96+0.07	81

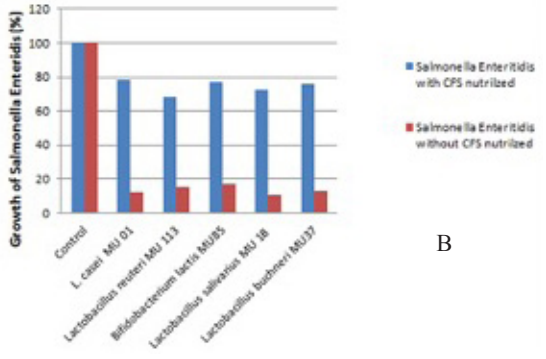
Antagonistic Effect of Strains on Food-borne pathogens in the presence of CFS

Antagonistic activity of strains having survival rate higher than 90 % was measured in the presence of CFS with neutralization using 1M HCl in Figure 1A E. coli, 1B for Salmonella Enteritidis, and 1C for Salmonella Typhimurium with and without neutralization was studied.

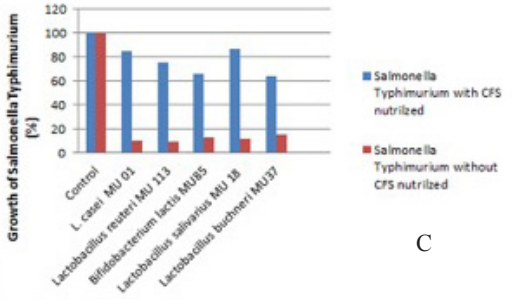
Figure 1: Inhibition of foodborne pathogens in the presence of CFS with and without neutralization using different LAB strains isolated from fermented milk products. A) E.coli B) Salmonella Enteritidis , and C) Salmonella Typhimurium.



A



B



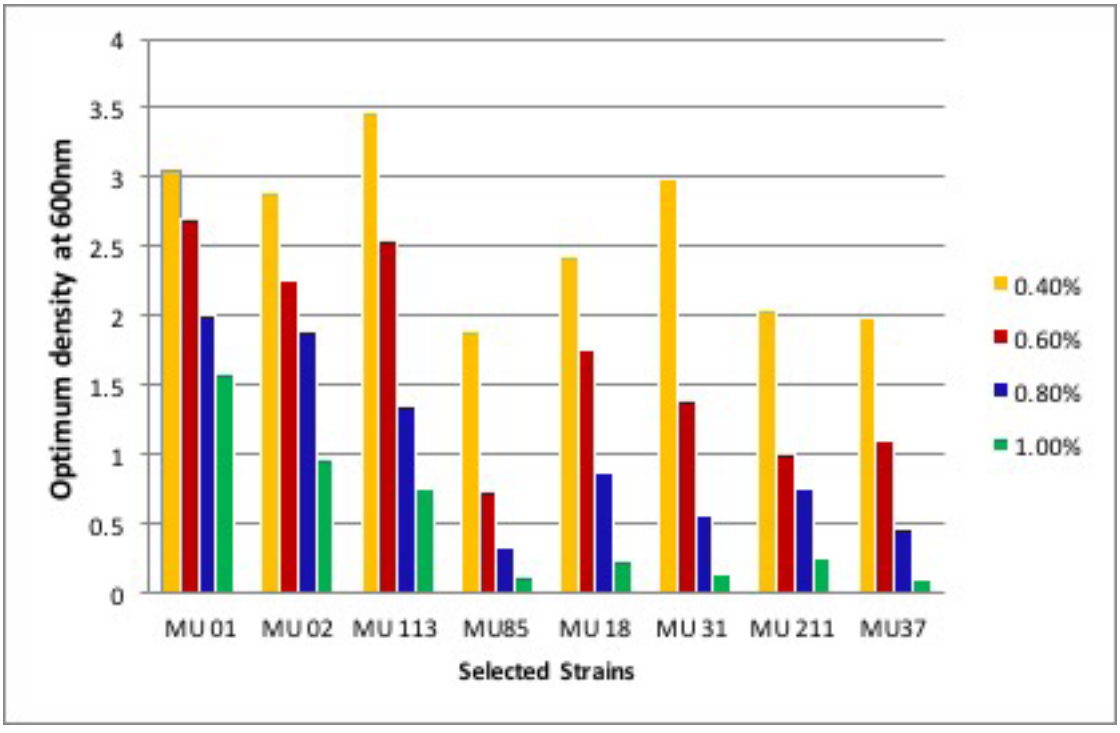
C

Bile salt

Bile salt concentration 0.4% -1% showed different degrees of inhibition on eight strains selected with more than 90% survival rate. The result obtained was analyzed for bile salt tolerance using Gilliland et al. (1984)[13]. Two strains; Lactobacillus reuteri MU 113 and Lactobacillus salivari-

us MU 31, were considered to be resistant (d≤ 15 min): Out of8 strains, four strains were found to be tolerant strains (MU01, MU02, MU113, MU31; 15<d≤40 min); other four strains (MU18, MU85, MU37 and MU211; 40<d<60) were found to be weakly tolerant strains as shown in Figure 2.

Figure 2: Bile acid tolerance (0.4% to 1.0%) of different strains at an optimum density of 600 nm.



Autoaggregation and Coaggregation activity:

To measure co-aggregation activity, the strains showing the best activity in other parameters are selected. The co-aggregation property of these strains with food-borne pathogens was determined at 37 0C at a time interval of 3 and 24 hours (Table 2).

Figure 2: Bile acid tolerance (0.4% to 1.0%) of different strains at an optimum density of 600 nm.

Co-aggregation activity (%)						
Strains	E. coli		Salmonella Enteritidis		Salmonella Typhimurium	
	3 hour	24 hour	3 hour	24 hour	3 hour	24 hour
MU 01	13.6±0.4	42.6± 0.2	11.7±0.8	53.4±0.7	16.3±0.3	40.5±0.2
MU 02	7.5±0.4	30.1±0.5	10.6±0.4	44.6±0.3	11.5±0.2	33.6±0.1
MU 113	15.7±0.6	60.4±0.8	12.3±0.2	56.9±0.6	13.1±0.3	48.9±0.3
MU 31	11.4 ±0.5	46.7±0.2	13.2±0.4	44.6±0.3	12.6±0.5	38.7±0.6

Discussion

The bacterial species isolated from fermented milk products were subjected to general bacteriological isolation tech-

niques. Among the biochemical test, the important results showed by the isolates were catalase-negative, lactic acid production from glucose, and heavy growth on Tomato juice agar[14]. All the isolates showed pigmentation on Tomato juice agar, which ranges from orange to brown color. The growth of isolates on MRS agar plates at pH 6.5 confirmed the lactobacillus sp.[15]. For a probiotic bacterium to work efficiently, it must have the capacity to survive in the gastrointestinal tract.

In many studies, the acid resistance for Lactobacillus at pH 2.0 and 3.0 was studied with MRS broth. In a study on 52 strains of lactobacilli, a favorable resistance at pH 2.0 was observed among 72% of isolate[16]. In the present study, more than 60% of strains showed the survival rate of ≥90% at pH 3.0, whereas 62.5% of the strains showed the survival rate of ≥90% at pH 2 (Table 1). Antagonistic activity of different LAB strains isolated from plant and animal products showed CFS to be a complete inhibitor without neutralization against many foodborne pathogens [17, 18]. In our study, it was found that the MU18 strain inhibited more than 60% growth of all three foodborne pathogens with CFS without neutralization.

In the human gut, NaCl is an inhibitory substance that can inhibit the growth of the microbe, so; the isolates were grown in a medium with NaCl concentration ranging between 0.4-1% bile concentrations [5, 19]. It was found that all 30 strains were able to survive at 0.5% bile salt, but only eight were found to be tolerant to all concentrations between 0.4-1.0 percent, whereas the rest were found to be weakly tolerant (figure 2). The highest optimum density was shown by MU113, whereas the least OD was recorded by MU 85.

The co-aggregation properties are another important factor in selecting a probiotic strain[20]. Most LAB strains exhibited significant co-aggregation activity with foodborne pathogens after 24 hours of incubation. As per the results of the present study (Table 2), MU113 displayed the highest co-aggregation E.coli after 24 hours of incubation, which was similar in the study by Zhang et al. [21, 22] .

Conclusion

In conclusion, out of 52 strains, 30 strains were selected as appropriate probiotic potential strains, out of which eight were found suitable for promoting hosts' intestinal health and to maintaining healthy natural micro-flora during antibiotic treatment. Also, there is a need for an in vivo study to verify the effectiveness of selected strains.

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