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Probiotic Properties of Lactic Acid Bacteria and Yeasts Isolated from Korean Traditional Food, *Jeot-gal*

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젓갈로부터 분리된 젖산균 및 효모의 프로바이오틱 특성

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Abstract

In order to select probiotics having both a high survival rate and an ability to inhibit virulent pathogens, we have screened lactic acid bacteria and yeasts from *Jeot-gal* to examine their resistance to artificial gastric and bile juice. After being introduced in the artificial gastric acid for 2 hr, the isolated lactic acid bacteria and yeast were incubated for 24 hrs in the artificial bile juice. In particular, the strain ML 36, ML 128, and ML 178 survived the longest during 2 hr incubation period in the artificial gastric acid. All 3 strains of lactic acid bacteria, and 2 strains of yeast demonstrated higher growth rates than control in the artificial bile. In addition, the antimicrobial activity of lactic acid bacteria and yeasts was investigated to determine their efficiency as probiotic organisms. The lactic acid bacteria inhibited Gram positive and negative bacteria, while the yeast was marginally inhibited.

Key words : probiotic, lactic acid bacteria, yeast, gastric acid, bile juice, antimicrobial activity

Introduction

Jeot-gal, a important traditional fermentation food in Korea, is mainly produced using fish and shellfish. This product is usually fermented using certain types of microorganisms and enzymes. This is important not only as a source of protein supply, due to its peculiar flavor, and relative abundance of free amino acid and taste related components, but also a subsidiary material of *Kimchi*, or spices. Aerobic and anaerobic bacteria, which have the property of salt resistance, exist in most *Jeot-gal*. It is known that the *Jeot-gal* is largely related to otherbacteria, such as *Bacillus subtilis, Leuconostoc mesenteroids, Pediococcus halophillus*, and *Sarcina litoralis*(1).

During the intake of live strain, probiotics promote growth through the balance of intestinal microflora and the reduction of coliform bacillus. This growth promotion is achieved using an antibacteria agent, which solves the problems of residue and resistance due to the addition of antibiotics(2-4).

The conditions necessary for the use of probiotics consist of safety, functionality (viability, settlement, inhabitation, antibacteria agent creation, immunity hastening and antigenotoxic activation *etc.*), pathogenic suppression, and technical issue including the properties of organism, safety, bacteriophage resistance, viability in a production process, and a high viability in the intestine as a GRAS (Generally Recognized As Safe) microorganism(5-8).

However, the consumer demand for functional foods has increased significantly, as interest in health oriented products, and increased income. The development of a food, which utilizes the functionality and effectiveness of probiotics, has

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been officially recognized as an important field. Consequently, numerous studies for using of a probiotic organism as a functional food have been investigated(9). A probiotic agent is a live microorganism for using their products to improve the physical functions (10). The functionality of this probiotic agent was approved after determining its beneficial effects on the stabilization of the gastrointestinal microflora, reduction of saprogenic products, due to the suppression of the settlement of toxic bacteria, prevention of a disease, activation of immunity, mediate anticancer activities, lowering cholesterol, reduction of lactose intolerance, and suppression of constipation, and its prevention (11-17). Using the separation and cultivation of probiotic organism from *Jeot-gal*, this study attempted to verify its industrial utility for the purpose of resistance for artificial gastric juice, antibiotics, and pathogenics by examining the antibacterial activities.

Materials and Methods

Strain and medium

Jeot-gal was collected from a large mart or home in the area of Mokpo, Jeonnam. The strains were separated from the 10 or more collected *Jeot-gal*, such as squid *Jeot-gal*, hairtail fish organ *Jeot-gal*, small octopus *Jeot-gal,* anchovy *Jeot-gal*, and other *Jeot-gal*. A regular smear method was used to separate the strain. The applied mediums were LBS (Difco, USA) and PDA (Difco, USA) for separating a *Lactobaccillus* strain and yeast, respectively. In addition, the value of pH was configured as 3.5.

Cultivation conditions and methods

The *Lactobaccillus* and yeast, which were separated from *Jeot-gal* were inoculated into a LBS broth and PDA broth, and were precultivated at 37℃ and 150 rpm for 12 hours. Then, it was cultivated as a productive cultivation for 12 hours after inoculating it into a 500 mL baffle flask (working volumn: 100 mL)(18).

Measurement of the total strain numbers

The total strains were calculated by measuring the colony that formed units after diluting the cultivated solution continuously using 0.1% of peptone water, dropping it onto a planar medium with 0.1 mL, and cultivating it at the optimal temperature. The measurement of the spore number was performed by the measuring the total strains after the extinction of nutritious cells, which was achieved using heat treatment at 80℃ for 2 hours(19).

Resistance for an artificial gastric juice

An artificial gastric juice was created by adding 1% pepsin to a MRS broth, which was configured at pH 2.5 using 1N HCl, according to the Kobayashi(20) method. In order to test the resistance of the artificial gastric juice, 1 mL of each cultivated solution was inoculated into an eppendorf tube and centrifuged at 6,000 rpm for 10 minutes. Then, only the strains were collected from the centrifuge, where the supernatant was discarded. The collected strains were cultivated at 37℃ for 2 hours after adding the mixture of the same amount of artificial gastric juice and supernatant. Then the total strains were measured.

Resistance for an artificial bile juice

An artificial bile juice was created by adding a 1% of sterilized 10% oxgall (Difco, USA) solution in a sterilized broth(21). In order to test the resistance of the artificial bile juice, the cultivated solution passed through the artificial gastric juice was inoculated into test tube and cultivated at 37℃ for 24 hours. To investigate their viability, the total spore numbers were measured using the same method which was used to measure the artificial gastric juice. The comparative group was tested using the same method as previously mentioned, without adding the oxgall.

Resistance for antibiotics

A paper disc method was used to measure the resistance of antibiotics. A selected probiotic strain was cultivated at the optimal temperature for 12 hours and inoculated into a soft agar (0.7%) using 100 $\mu\ell$ of cultivated solution. Then, an agar plate was overlayed by using it. A paper disc, which was fully wet by the antibiotics for each concentration (nisin, rifamycin, streptomycin, and tetracycline), was placed on the overlayed plate and cultivated for 24 hours. Then, the resistances of the antibiotics were measured(21).

Antibacterial activities for *Listeria monocytogenes*

In order to measure the antibacterial activities, probiotics and *Listeria monocytogenes* ATCC19111 were cultivated for 12 hours, and then the cultivated solution was diluted at a level of 105 -106 CFU/mL for each strain. Then, a 5 mL of the solution was mixed in 5 different test tubes with the volume ratio of 1:1, and each solution was cultivated at 37℃ in a shaking incubator. The numbers of strain were measured

using a select medium by sampling it at 1, 2, 4, 8, and 24 hours intervals during the cultivation(22).

Results and Discussions

Resistance for the artificial gastric juice

There are several characteristics required by probiotics. Among these characteristics, the most important requirement is a high viability. The bacteria ingested from an oral cavity may reach at the intestine, which is the destination at which it thrives, through the stomach and duodenal. Most microorganisms are destructed by the stomach due to the 1.4-2.0 pH of pure gastric juice(23, 24). However, certain rates of extinction for microorganisms decreases with an increase in pH levels, due to the buffering of ingested foods. A direct examination method of *in vivo* to verify the viability and an indirect method using an artificial gastric juice were used in previous studies to test the acidic resistance. Some reports indicate that the extinction of probiotic organisms resulted from the low pH, and most tests, which were applied *in vivo*, showed nearly the same results(25-28). Given the results presented, the ML 36, ML 128, and ML 178 strain showed a significantly high viability among the lactic acid bacteria from the test, which configured at the pH of 2.5. In the case of yeasts, the YK 33 and YK 61 showed a high viability. Other lactic acid bacteria and yeasts also showed a relatively high viability, compared to the existing probiotics(Table 1).

Table 1. Survival of probiotic lactic acid bacteria and yeasts isolated from *Jeot-gal* in artificial gastric acid

Strain	Control (CFU/mL)	Pepsin(pH 2.5) (CFU/mL)	Survival (%)
Lactic acid bacteria			
ML 15	2.2×10^8	1.6×10^5	0.0
ML 23	1.7×10^8	3.4×10^6	0.2
ML 36	3.8×10^8	1.6×10^8	42.1
ML 42	7.4×10^9	1.2×10^5	0.0
ML 78	3.2×10^9	2.4×10^5	0.0
ML 102	1.6×10^8	4.1×10^7	2.6
ML 128	3.5×10^9	1.3×10^9	37.1
ML 158	2.1×10^9	3.2×10^5	0.0
ML 178	5.6×10^9	2.6×10^9	46.4
Yeasts			
YK 21	4.7×10^5	3.2×10^4	0.7
YK 33	3.2×10^6	1.2×10^6	37.5
YK 45	1.6×10^5	2.8×10^4	17.5
YK 61	4.5×10^5	2.1×10^5	46.7
YK 89	3.2×10^6	1.7×10^4	0.1

Cho *et. al*(6) reported that the main reason for the extinction of microorganism, was the low value of pH and separated the *L. fermentum* YL-3, which has a high viability, using an acidic resistance test. In these lactic acid bacteria, it presented a 10% of relatively high viability under the pH of 2.0, and after a lapse of 2 hours. Kim et. al(29) reported that the *Lactobacillus* strain showed about a 1% of viability at pH 4, due to the effect of the pH of probiotics. The ML 128 and YK 61 strains, which were separated from *Jeot-gal* in this test, showed over a 46% of viability. This result indicates that this strain has a very high viability compared to the previously reported studies.

Resistance for the artificial bile juice

The ingested probiotics reach the intestine after passing through the stomach and duodenal. The resistance to the bile juice, which is secreted in this part, is also an important characteristic of probiotics(23, 30). The artificial bile juice used in this test was applied by adding a 0.1% of sterilized oxgall and using each sterilized broth. In order to test the resistance in the artificial bile juice, the cultivated solution, which passed through the artificial gastric juice, was added to the test tube and cultivated at 37℃ for 24 hours. Next, the total strains were measured. The control group was tested using the same method, for each broth without any addition of the oxgall. After the cultivation in the artificial bile juice (by adding a 0.1% of oxgall), which passed through the artificial gastric juice (pH 2.5), the viability of lactic acid bacteria and yeasts measured in the range of 6% to over 130% (Table 2).

Table 2. Survival of probiotics lactic acid bacteria and yeast isolated from *Jeot-gal* in artificial bile juice after treated with artificial gastric acid for 2 hr at 37℃

Strain	Control (CFU/mL)	Oxgall (CFU/mL)	Survival(%)
Lactic acid bacteria			
ML 15	2.8×10^8	1.9×10^7	6.8
ML 23	3.4×10^8	4.5×10^7	13.3
ML 36	1.7×10^8	1.9×10^8	111.8
ML 102	3.6×10^8	3.1×10^7	8.6
ML 128	5.5×10^9	4.9×10^9	89.1
ML 178	1.6×10^9	2.1×10^9	131.3
Yeasts			
YK 21	4.7×10^5	3.9×10^4	8.3
YK 33	3.2×10^6	1.7×10^6	53.1
YK 45	1.6×10^5	3.8×10^4	23.7
YK 61	4.5×10^5	2.1×10^5	46.7

The ML 36, ML 128, and ML 178 strains of the lactic acid bacteria, presented 111.8%, 89.1%, and 131.3% of the viability, respectively, when the 0.3% of oxgall was added into the strains. On the other hand, Shin et. al(31) also showed that the *Lactobacillus* strain presented ranges of 1-100% of viability when the 0.3% of bile salt was added into the strain. These results were similar to the results of this study.

Table 3. Antibiotic resistances of lactic acid bacteria strains

Antibiotics (μg/mL)		Lactic acid bacteria strain		
		MK 36	MK 128	MK 178
Nisin	0	+[1)]	+	+
	25	+	+	+
	50	+	+	+
	100	-[2)]	+	+
Rifamycin	0	+	+	+
	5	+	+	+
	10	+	-	+
	20	+	-	-
Streptomycin	0	+	+	+
	5	+	+	+
	10	+	+	+
	20	+	+	+
Tetracycline	0	+	+	+
	5	+	+	+
	10	+	+	+
	20	+	+	+

[1)]Growth, [2)]No growth.

The resistance for the antibiotics

An examination of the probiotic resistance of the antibiotics can help a patient, who requires antibiotic treatment, to select the proper probiotics, and this requires the verification of the microfloral characteristics of the probiotics. It is known that probiotics have a resistance to various antibiotics. However, this resistance shows no transition. Therefore, it is not necessary to consider that the unique resistance of probiotics has a particular relationship to their safety.

The lactic acid bacteria that revealed a resistance against the artificial gastric juice, such as the ML 36, ML 128, and ML 178, presented a resistance at the concentration of 0-100 μg/mL from the examination using the treatment, which was applied by the antibiotics of nicin, rifamycin, streptomycin, and tetracycline, even though each case presented a little difference according to the antibiotics. Paik et. al(19) reported that resistance was observed in the concentration of 0-100 μg/mL from the resistance test of antibiotics for the *Bacillus polyfermenticus* SCD in which they applied the same antibiotics as those previously mentioned were applied. In addition, this study ascertained that the test, which was applied using the same probiotic strains as in this study, showed a resistance to antibiotics.

Growth inhibiton activities on *Listeria monocytogenes*

Probiotic strain can also be used to normalize the gastrointestinal microflora and to suppress gastrointestinal infection or growth after dosing antibiotics. *Listeria*, which is a kind of pathogenic and non-spore bacilli of gram-negative, causes an infective disease in cattle (cow, horse, sheep, pig, antelope, and so on), poultry (chicken, duck, and so on), and even in human beings. People can largely be infected after direct contact with an infected animal or meat product as a peroral infection. However, infection through breath or dust can also occur. The incubation time ranges from three days to a couple of weeks, and symptoms include meningitis, septicemia, and endometritis (a pregnant woman)(19).

Fig. 1 presents the antibacterial activities against *Listeria monocytogenes* ATCC 19111 of the three lactic acid bacteria strains that were separated from *Jeot-gal* in this study. As shown in Fig. 1, the ML 36 and ML 128 strains, which were a kind of lactic acid bacteria, showed a perfect inhibitory effect before 24 hours of cultivation. However, the ML 178 strain showed a relatively low inhibitory effect. This bactericidal inhibitory seemed to result from the operation of various enzymes and bacteriocin, which were produced by probiotic strains.

Consequently, it was partially recognized that lactic acid bacteria and yeasts, which were separated from one of the traditional foods, *Jeot-gal*, presented the possibility of being useful as probiotics. In addition, the investigation of microfloral behaviors, their settlement in the intestine, and the antimutation of the separated strains has been advanced through the present study.

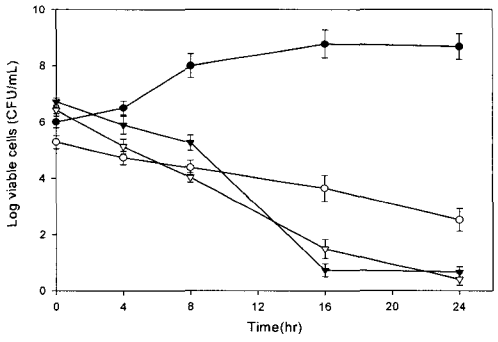

Fig 1. Growth inhibition of *Listeria monocytogenes* ATCC 19111 by lactic acid bacteria strain isolated from *Jeot-gal*.

-●- non-treated(control), -○- lactic acid bacteria ML178
-▼- lactic acid bacteria ML36 -▽- lactic acid bacteria ML128

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요 약

젓갈은 전남 목포지역의 대형마트 및 가정에서 수집하였으며 오징어젓, 갈치속젓, 꼴뚜기젓, 멸치젓 등 10여종의 젓갈로부터 균을 분리하였다. 이들 분리 유산균 중 3균주가 인공위액과 인공담즙액에서 내성을 나타내었다. 이들 분리된 균주 중 생존력이 왕성한 3균주는 모두 인공위액에서 2시간, 인공담즙액에서 24시간 생존 가능하였다. 특히 유산균 ML 36, ML 128, ML 178 외 2종의 효모는 초기 생균수와 인공위액에서 2시간 배양 후 생균수의 변화가 거의 없어 가장 강한 내성을 나타내었다. 인공위액에서 분리된 균주의 생존율은 낮은 pH로 인하여 낮은 경향을 나타내었으나, 인공담즙액에 대해서는 높은 생존율을 나타내었다. 인공위액 및 인공담즙에 내성을 나타낸 3종의 유산균이 항생물질인 nicin, rifamycin, streptomycin, 그리고 tetracycline에 대하여 내성을 나타내었다. 또한 이들 분리 유산균은 병원성 미생물인 *Listeria monocytogenes* 대해 뚜렷한 항균 효과가 있는 것으로 나타났다.

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