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Identification and Probiotic Characteristics of *Lactococcus* Strains from Plant Materials

Hiromi KIMOTO[1]*, Masaru NOMURA[1], Miho KOBAYASHI[1], Takashi OKAMOTO[1] and Sadahiro OHMOMO[2]

[1] Laboratory of Applied Microbiology, National Institute of Livestock and Grassland Science (Tsukuba, Ibaraki 305–0901, Japan)
[2] Animal Production and Grassland Division, Japan International Research Center for Agricultural Sciences (JIRCAS) (Tsukuba, Ibaraki 305–8686, Japan)

Abstract

Probiotics are viable microorganisms that exhibit beneficial effects on the health of the host when they are ingested. In Asian areas including Japan, various fermented vegetable foods are produced and actively consumed in our meals. In the present study, we isolated lactococci from plant materials (fermented vegetables, silage and plants) and investigated their probiotic activities, such as tolerance to bile and the ability to remove cholesterol. A total of 411 lactic acid bacteria strains were isolated from plant materials. Out of the strains, 27 strains were identified as *Lactococcus lactis* subsp. *lactis* by phenotypic tests and a PCR-based method. Among them, 12 strains were subjected to further study. All lactococci tested could grow in broth containing 0.3% bile, and removed cholesterol from media during growth. These results showed that the lactococcal strains isolated from plant materials had probiotic activities.

Discipline: Food
Additional key words: lactic acid bacteria, cholesterol removal, bile tolerance

Introduction

In recent years, probiotic activity of lactic acid bacteria has been emphasized. Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance[7]. Lately, probiotics are commonly defined as viable microorganisms that exhibit a beneficial effect on the health of the host when they are ingested[16]. For example, the ability to reduce serum cholesterol levels, antimicrobial substrate production and immune modulation, etc. are considered as effective properties. Lactic acid bacteria consist of twenty-genera such as lactobacilli and lactococci. The most widely used probiotic bacteria are lactobacilli and bifidobacteria which can survive in the intestine. Extensive studies on the beneficial effects of these species on human health have been reported[1,21].

In contrast, there have been few studies on the probiotic activity of lactococci since they are traditionally not considered to be natural inhabitants of the human gastrointestinal tract[25]. However, several works showed the possibility of the presence of lactococci in the flora of the human or animal gastrointestinal tract[9,14]. Since lactococci are widely used as starter bacteria in manufacturing cheese and other fermented dairy products, establishing their effective probiotic activities could lead to development of new probiotic foods.

Lactococci can be found in milk and milk products, plant materials (i.e. fermented vegetables and fruits, etc.) and intestine of fish or insects. So far as lactococci isolated from dairy foods, previous studies have shown their probiotic activity, such as the ability to inhibit the growth of other bacteria[9,12]. In contrast, little has been published concerning the probiotic activity in lactococci from plant materials[11,26]. In Asian areas including Japan, various fermented vegetable foods are produced and actively consumed in our meals. Thus, we focused on the probiotic activity of lactococci from plant materials.

One of the beneficial health effects related to probiotics is their ability to reduce serum cholesterol levels. It has been reported that a culture of *Lactobacillus (Lb.) acidophilus* actively taking up cholesterol from growth media would function *in vivo* to exert a hypocholester-

*Corresponding author: fax +81–29–838–8606; e-mail anne@affrc.go.jp
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olemic effect[4,8]. For example, *Lb. acidophilus* ATCC 43121 can incorporate some of the cholesterol removed from media into the cellular membrane during growth[18], and it has beneficially influenced serum cholesterol levels in pig[5,8]. This is because cholesterol incorporated into or attached to cells of bacteria in the intestine is likely to be unavailable for absorption into the blood. The ability to incorporate cholesterol into or attach it to cells of bacteria has been equated with the ability to remove cholesterol from media. Many reports have been published on cholesterol removal from laboratory media containing bile and a cholesterol source in lactobacilli[2,3,27]. However, little has been published concerning this ability in lactococci[10,13]. As far as bile, absorption of cholesterol in the intestine needs bile for emulsion. In addition, probiotic bacteria must have an ability to tolerate bile because once the bacteria reach the intestinal tract, bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria.

The present study was carried out to isolate lactococci from plant materials and investigate their abilities to remove cholesterol from growth media and to tolerate bile.

Materials and methods

1. Isolation and maintenance of strains

Fifty-four fermented vegetables, 9 silages and 4 grasses were used as sources to isolate lactic acid bacteria (Table 1). They were gathered in Hokkaido and Okinawa area in Japan. MRS broth (Difco, Laboratories, Detroit, MI) to which 1.6% (w/v) of agar and 0.8% (w/v) $CaCO_3$ were added was used to isolate lactic acid bacteria strains from non-dairy plant materials. One-gram of each sample was homogenized with 9 mL of 0.85% (w/v) sterilized NaCl solution by shaking for several minutes. From the suspensions, serial dilutions were made in NaCl solution and plated by spreading 0.1 mL onto the surface of MRS agar. After anaerobic incubation at 30ºC for 48 h, colonies which dissolved $CaCO_3$ and formed clear zones around their own colonies on the medium plate were isolated at random. The broths inoculated with each colony were cultivated at 30ºC and tested for catalase reaction. Briefly, the cultures were centrifuged, and 15% (v/v) H_2O_2 solution was added to the pellets. Catalase-positive was determined for gas production. Isolated strains were stocked as frozen cultures in MRS broth with 15% (v/v) glycerol at –80°C.

Reference strains used for identification of isolated strains were obtained from ATCC (American Type Culture Collection, Rockville, MD) and JCM (the Japan Collection of Microorganisms, Wako, Japan). They were *Lactococcus (L.) lactis* subsp. *lactis* ATCC 19435, *L. lactis* subsp. *hordniae* JCM 1180 and *L. raffinolactis* JCM 5706. *L. lactis* subsp. *cremoris* HP and *L. lactis* subsp. *lactis* biovar diacetylactis N7 from our collection were also used as references. The strains were routinely maintained by subculturing once a week in litmus milk, incubated at 30ºC for growth and stored at 4ºC. *Lb. acidophilus* ATCC 43121 was used as a positive control for cholesterol removal ability[18]. Isolated lactococcal strains were maintained by subculturing 1% (v/v) inocula into M17 broth (Difco) supplemented with 0.5% (w/v) glucose (GM17) for lactococci or MRS broth for *Lb. acidophilus* and incubating them at 30ºC for lactococci or at 37ºC for *Lb. acidophilus* for 18 h. The cultures were stored at 4ºC between transfers and were subcultured one time before experimental use.

2. Identification methods

The generic name of isolated strains was determined based on a manual[15]. Isolates identified as Gram-positive, catalase-negative cocci were tested for the following properties. Growth at 10 and 45ºC at pH 9.6 and in 6.5% (w/v) NaCl was tested in modified Elliker broth[6] after 7 days of incubation. The medium consisted of 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) glucose, 0.4% (w/v) NaCl, 0.15% (w/v) sodium acetate, 0.05% (w/v) L-sodium ascorbate, and the pH of the medium was adjusted to pH 6.8. Gas production from glucose was also determined in modified Elliker broth. The isomer of lactic acid produced from glucose was determined by using a F-kit D, L-lactate (Boehringer Mannheim, Mannheim, Germany). Acetoin from citrate was measured by the method of Westerfeld[28].

Isolates identified as the genus *Lactococcus* were subjected to carbohydrate fermentation except raffinose and biological tests using a BBL identification kit (Boehringer Mannheim). Raffinose fermentation was determined by growth in raffinose-containing M17 broth. PCR (polymerase chain reaction) -based method was also conducted for specific *L. lactis* identification[20]. Briefly, bacterial cultures (10^7 cfu/mL) were used as templates for PCR analyses. Primers were designed from the glutamate decarboxylase gene (*gad*B) sequence of *L. lactis* subsp. *lactis* bv. diacetylactis 01-7[19]. Amplified products were electrophoresed using 1.5% (w/v) agarose for 20 min at 100 V. *L. lactis* subsp. *lactis* bv. diacetylactis N7 was used as a positive control. A 600 bp PCR fragment should be obtained from the chromosomal DNA of the strains tested if the strain belongs to *L. lactis* subsp. *lactis*[20].

3. Bile tolerance of the isolated lactococci

The GM17 broth was dispensed in 4 mL volumes, and sterilized by autoclaving at 121ºC for 15 min. Bactooxgall (Difco) was dissolved in distilled water and sterilized by autoclaving at 121ºC for 15 min. Sterilized oxgall solution was added to the broths to a final concentration of 0.3% (v/v). Each broth was inoculated with 0.02 mL of a fresh overnight broth culture, and incubated at 37ºC. Although the optimum temperature of lactococci is 30ºC, we tested this experiment at 37ºC to imitate the conditions of the intestine. After 24-h incubation, bacterial growth was determined by measuring the culture as absorbance at 620 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY) against an uninoculated broth blank.

Table 1. Source of lactic acid bacteria

Isolated from	Collected place	No.	Isolated from	Collected place	No.
Fermented vegetables			Fermented vegetables		
Papaya-sake lees	Ishigaki City (store)	0	Nozawana	Obihiro City (supermarket)	9
Papaya-fruits-zuke	Ishigaki City (store)	0	Lees-1 (Kasu)	Memuro-chou (homemade)	10
Papaya-eiyou-zuke	Ishigaki City (store)	0	Lees-2	Memuro-chou (homemade)	7
Vegetable (Toubaramah)	Ishigaki City (store)	0	Lees-3	Memuro-chou (homemade)	7
Cucumber	Ishigaki City (store)	0	Lees-4	Memuro-chou (homemade)	10
Pickled radish	Ishigaki City (store)	0	Unrefined sake (Moromi)	Memuro-chou (homemade)	0
Scallion	Ishigaki City (store)	0	Miso	Memuro-chou (homemade)	0
Papaya	Ishigaki City (homemade)	0	Rice bran (Nuka)	Memuro-chou (homemade)	9
Aloe	Ishigaki City (homemade)	1	Nozawana	Memuro-chou (supermarket)	10
Papaya	Taira City (store)	0	Pickles seasoned in sake lees (Narazuke)	Memuro-chou (supermarket)	10
Sea cucumber-awamori	Taira City (store)	15	Egg plant-kimchi	Memuro-chou (supermarket)	6
Balsam pear (Nigauri)	Taira City (store)	0	Plum	Memuro-chou (supermarket)	0
Sea cucumber-kimchi	Taira City (store)	15	Cucumber-salt	Naha City (homemade)	10
Garlic	Taira City (store)	0	Scallion	Naha City (homemade)	15
Chinese watermelon	Taira City (store)	0	Cucumber-nuka	Naha City (market)	10
Melon	Taira City (store)	0	Mustard plant	Naha City (market)	10
Raw grass			Island scallion-kimchi	Naha City (market)	10
Guinea grass	Nakijin Villege	8	Turmeric	Naha City (market)	0
Setaria	Nakijin Villege	10	Papaya-soy sorce	Shuri (store)	0
Brachiaria mutica	Nakijin Villege	3	Papaya-kimchi	Shuri (store)	0
Napiergrass	Nakijin Villege	20	Papaya-kasu	Shuri (store)	0
Spoiled Cucumber	Ishigaki City	7	Balsam pear	Shuri (store)	0
Silage			Pickled radish	Urasoe City (supermarket)	0
Silage-1	Kyan Villege	10	Vegetables seasoned in vineger (Namasu)	Urasoe City (supermarket)	10
Silage-2	Ohgimi Villege	10	Celery	Urasoe City (supermarket)	10
Silage-3	Nakijin Villege	10	Chinese cabbage	Urasoe City (supermarket)	10
Silage-4	Nakijin Villege	10	Japanese radish	Urasoe City (supermarket)	6
Silage-5	Ueno Villege	0	Balsam pear	Urasoe City (supermarket)	0
Silage-6	Ueno Villege	4	Balsam pear -turmeric	Urasoe City (supermarket)	0
Silage-7	Ueno Villege	10	Cucumber-nuka	Urasoe City (supermarket)	15
Silage-8	Ueno Villege	8	Turnip-nuka	Urasoe City (supermarket)	15
Silage-9	Ueno Villege	6	Carrot-nuka-1	Urasoe City (supermarket)	10
Fermented food			Carrot-nuka-2	Urasoe City (supermarket)	10
Herring-nuka	Sapporo City	30	Chinese cabbage-kimchi	Urasoe City (supermarket)	15
			Balsam pear -sake lees	Urasoe City (supermarket)	0
			Vegetables (Ryukyu-zuke)	Itoman City (store)	0

4. Analysis of cholesterol removal from growth media

Freshly prepared GM17-THIO broth (GM17 broth with 0.2% (w/v) sodium thioglycollate) for lactococci or GAM (Nissui Seiyaku, Tokyo, Japan) for *Lb. acidophilus* was supplemented with 0.2% (w/v) sodium taurocholate (as a bile salt). Sodium thioglycollate was used as an oxygen scavenger. A filter-sterilized cholesterol solution (10 mg/mL in ethanol) was added to the broth to a final concentration of 70 μg/mL. The broth was inoculated with 1% (v/v) of culture and incubated anaerobically by using GasPak® (Becton Dickinson, Cockeysville, MD) for 24 h at 37ºC. After incubation, cells were removed by centrifugation for 7 min at 5,400 × *g* and 4ºC. The method described by Rudel & Morris[22] was used to determine the amounts of cholesterol in the spent broth and uninoculated broth. Cholesterol was determined by measuring the reaction mixture as absorbance at 560 nm.

Results and discussion

A total of 411 lactic acid bacteria were isolated from plant materials (Table 1). Isolates were Gram-positive and catalase-negative. They were identified to the level of species by determining growth behaviour at 10ºC,

Table 2. Physiological and biochemical characteristics of the isolated strains

Characteristics	Isolated strains							
	G46, G47 G48, G50 G51, G53 G55, G56 G60, G61 G62, G65 H47	H45, H46 H48, H49 H50, H51 H52, H55 H60	H53, H54, H56, H57	H58	*Lactococcus lactis* subsp. *lactis* ATCC 19435	*Lactococcus lactis* subsp. *cremoris* HP	*Lactococcus lactis* subsp. *hordniae* JCM 1180	*Lactococcus raffinolactis* JCM 5706
Growth at								
10ºC	+	+	+	+	+	+	+	+
45ºC	–	–	–	–	–	–	–	–
pH 9.6	+	+	+	+	–	–	–	+
Growth in								
4% NaCl	+	+	+	+	+	–	–	+
6.5% NaCl	–	–	–	–	–	–	–	–
Gas production	–	–	–	–	–	–	–	–
Lactic acid configuration	L	L	L	L	L	L	L	L
Ammonia from arginine	+	+	+	+	+	–	+	–
Acid from								
Trehalose	+	+	+	+	+	+	–	+
Lactose	+	+	–	–	+	–	–	+
Sucrose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	–	–	–	–
Arabinose	–	+	+	–	–	+	–	–
Fructose	+	+	+	+	+	+	+	+
Raffinose	–	–	–	–	–	–	–	+
Maltotriose	+	+	+	+	+	–	–	+
Hydrolysis of esculine	+	+	+	+	–	–	–	–

+ : positive, – : negative.

45ºC, and in 6.5% NaCl, microscopic observation, gas production from glucose, and the enantiomer of lactic acid. Among the strains, 27 strains were coccoid in shape, formed L(+)-lactic acid only from glucose, and grew at 10ºC but not at 45ºC, in 6.5% NaCl. It indicated that they belonged to the genus *Lactococcus*[17]. Lactococci were isolated from one grass (Napiergrass) and one fermented vegetable food (kimchi sea cucumber, i.e. food which soaked the sea cucumber with the kimchi). Bacterial cell counts from samples on MRS agar ranged between 10^4 and 10^6 CFU/mL.

The isolated lactococcal strains were subjected to a further series of experiments. Physiological and biochemical characteristics are shown in Table 2. Strains G46, G47, G48, G50, G51, G53, G55, G56, G60, G61, G62, and G65 were from Napiergrass. Strains H45, H46, H47, H48, H49, H50, H51, H52, H53, H54, H55, H56, H57, H58, and H60 were from kimchi sea cucumber. They grew at 40ºC (data not shown), grew in broth containing 4% NaCl, produced ammonia from arginine, and did not degrade citrate. In carbohydrate fermentation, differences exist between strains. All isolated strains fermented glucose, trehalose, sucrose, mannitol, fructose, and maltotriose, but did not ferment raffinose. Most strains fermented lactose. Some strains fermented arabinose, but some strains did not. The ability to hydrolyze esculine was determined by a BBL kit. The ability was detected in all isolated strains, but not in reference strains including strain ATCC 19435, type strain of *L. lactis* subsp. *lactis*. On the basis of these results, they were tentatively identified as *Lactococcus lactis*[24]. Furthermore, they were identified as *Lactococcus lactis* subsp. *lactis* by using BBL CRYSTAL software.

However, these isolated strains could grow at pH 9.6 although most lactococci can not grow at this pH[17]. Thus, the isolates were subjected to PCR amplification specific to *L. lactis* in order to identify them. A 600 bp PCR fragment derived from the *gad*B gene should be obtained from the chromosomal DNA of the strains tested if the strain belongs to *L. lactis* subsp. *lactis*[20]. Fig.1 showed that DNA from the tested strains generated fragments corresponding to the 600 bp fragment of *L. lactis* subsp. *lactis* strain N7, a positive control. Consequently, the 15 strains tested in Fig. 1 were identified to belong to *L. lactis* subsp. *lactis*. The other remainings (12 strains) had the same PCR results described above (data not shown).

The phenotypic characteristics of the isolates except the growth at pH 9.6 showed that they were considered to be lactococci although some differences exist in carbohy-

Table 3. Bile tolerance of isolated strains from plant materials

Strain no.	$A_{620\ nm}$	
	Control	Oxgall
G46	2.10	1.90
G51	1.30	1.90
G53	0.88	1.90
G55	1.18	1.86
H45	0.90	1.70
H46	0.90	1.75
H47	0.82	1.84
H49	1.00	1.75
H50	0.98	1.75
H51	1.08	1.80
H52	0.92	1.80
H60	0.80	1.78

The growth of each strain was measured after culture for 24 h at 37ºC in M17 broth supplemented with 0.5% glucose. Control: without bile. Oxgall: with 0.3% bile. All values are the means of two trials.

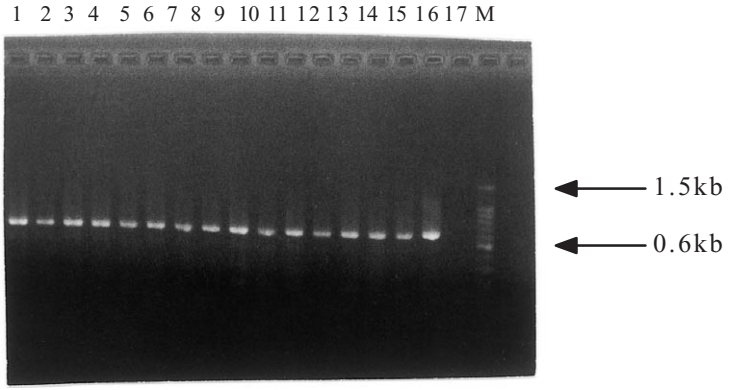

Fig. 1. PCR amplification with lactococcal primers
Lane 1 to 16: strains G46, G50, G51, G53, G55, G61, G65, H45, H46, H47, H49, H50, H51, H52, H60, and N7. Lane 17: negative control. 100 bp molecular weight ladder is located in lane M.

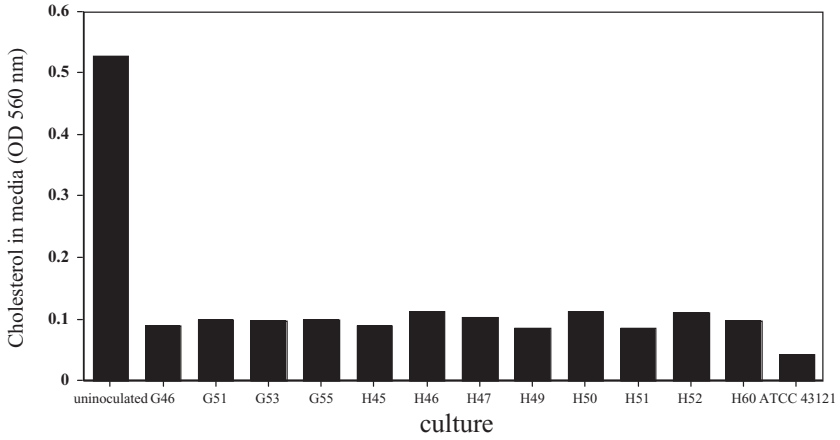

Fig. 2. Cholesterol removal from media during growth of lactic acid bacteria

Cells were incubated for 24 h at 37°C in M17 broth or GAM broth supplemented with 0.5% glucose and 0.2% sodium taurocholate and cholesterol (final concentration in broth = 70 μg/mL). Cholesterol in media removed by the culture was measured as absorbance at 560 nm. Data are representative of two separate experiments. G46, G51, G53, G55, H45, H46, H47, H49, H50, H51, H52, H60: *Lactococcus lactis* subsp. *lactis*. ATCC 43121: *Lactobacillus acidophilus*.

drate fermentation pattern between the isolates and reference strains. So far as the growth at pH 9.6, Uhlman et al.[26] succeeded in isolating strains of *L. lactis* from vegetables, and the strains could grow at pH 9.6. It is suggested that plant-derived strains have some different characteristics from dairy product-derived strains. It is of great interest to investigate other phenotypic differences between dairy and non-dairy lactococci.

Next, the isolates were investigated for effective probiotic activity, their tolerance to bile in GM17 broth (Table 3). All strains tested could grow well in the bile-containing broth at 37ºC. The growth with bile was better than that without bile in some strains.

The isolated lactococci tested could remove cholesterol from growth media (Fig. 2). The amount of cholesterol removed by the isolates was slightly smaller than that by strain ATCC43121, which had been shown to beneficially influence serum cholesterol levels in pigs[5,8]. A high level of cholesterol in blood is generally considered to be a risk factor for coronary heart disease. Further study is needed to evaluate the effect of oral administration of the isolated strains on serum cholesterol levels *in vivo*.

The present study showed that lactic acid bacteria which have probiotic activities exist in fermented vegetable foods like pickles currently consumed in Asia including Japan from ancient times. This leads us to raising the nutritional worth of fermented food in the Asian area. There are many traditional fermented vegetable (or plant) foods which are not investigated in Asia, and lactic acid bacteria promising as probiotics may be found out in the future. Should Asian traditional foods be focused on more? In European and American probiotic food markets, dairy products have mainly been consumed. However, in Asia, in addition to dairy probiotic products, we want to propose development of new probiotic foods using the lactic acid bacteria originated from fermented vegetable foods which are employed in the eating habits in Asia. Of course, this would not only benefit Asian food but the traditional fermented foods of other countries.

The development of new probiotic strains, which are more actively beneficial organisms, is awaited in the dairy food industry. Lactococci are promising species because of their extensive usage in manufacturing fermented milk products. They can grow well in milk while some of the probiotic cultures, such as *Lb. acidophilus* strains, do not grow well during production of fermented milk products. The isolated lactococcal strains can grow in milk although they were not isolated from milk or milk products. In future, milk products that have probiotic activity by the utilization of lactococcal strains may be produced. As described above, there seems to be differences between dairy lactococci and non-dairy strains in phenotypic properties. Milk products made by non-dairy strains may differ from those made by dairy strains in terms of taste or flavor. It may lead to the development of new types of probiotic milk products. In the selection of new probiotic organisms, their safety has been of prime importance[23]. The safety of the isolated strains in this study should be severely assessed.

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