Original Article

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Lactic acid bacteria activating innate immunity improve survival in bacterial infection model of silkworm

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Summary **Lactic acid bacteria (LAB) have been thought to be helpful for human heath in the gut as probiotics. It recently was noted that activity of LAB stimulating immune systems is important. Innate immune systems are conserved in mammals and insects. Silkworm has innate immunity in response to microbes. Microbe-associated molecular pattern (ex. peptidoglycan and β-glucan) induces a muscle contraction of silkworm larva. In this study, we established an efficient method to isolate lactic acid bacteria derived from natural products. We selected a highly active LAB to activate the innate immunity in silkworm by using the silkworm muscle contraction assay, as well. The assay revealed that *Lactococcus lactis* 11/19-B1 was highly active on the stimulation of the innate immunity in silkworm. *L. lactis* 11/19-B1 solely fermented milk with casamino acid and glucose. This strain would be a starter strain to make yogurt. Compared to commercially available yogurt LAB, *L. lactis* 11/19-B1 has higher activity on silkworm contraction. Silkworm normally ingested an artificial diet mixed with *L. lactis* 11/19-B1 or a yogurt fermented with *L. lactis* 11/19-B1. Interestingly, silkworms that ingested the LAB showed tolerance against the pathogenicity of *Pseudomonas aeruginosa*. These data suggest that *Lactococcus lactis* 11/19-B1 would be expected to be useful for making yogurt and probiotics to activate innate immunity.**

Keywords: Lactic acid bacteria, *Lactococcus lactis*, Silkworm, Innate immunity, Infection

1. Introduction

In mammalian innate immunity, dendritic cells and macrophages produce cytokines in response to microbial pathogens (*1*). In insect innate immunity, hemocytes and fat bodies recognize microbial pathogens that induce anti-microbial response (*2*). In silkworms, immune cells produce reactive oxygen species, which activate proteases resulting in cytokine activation. Our group found an active cytokine, paralytic peptide (PP) that induces muscle contraction (*3*). We have screened innate immunity activating substances by using silkworm muscle specimens (*4,5*). This screening method has the following advantages compared to conventional screening using mammalian innate immune cells like macrophages. First, silkworm does not respond to large amounts of lipopolysaccharide (LPS). Second, insect whole body assay reflects ADMET (absorption, distribution, metabolism, excretion, and toxicity). Substances less effective on PK/PD (pharmacokinetics/pharmacodynamics) and/or are toxic on silkworm muscle contraction would be excluded from tests.

Lactic acid bacteria (LAB) have traditionally been used for fermenting dairy foods and probiotics. LAB have the following characteristics, gram-positive, catalase-negative, no spore formation, and are immotile. If an efficient method to select LAB activating innate immunity is established, it would be expected that dairy foods fermented with LAB would be helpful for human health by activating human innate immunity.

In this work, we isolated LAB and evaluated innate-immunity stimulating activity in silkworms. Isolated *L. lactis* 11/19-B1 had high activity in the silkworm contraction assay. We established an efficient method to isolate lactic acid bacteria derived from natural products. We selected a highly active LAB to activate

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innate immunity in silkworm by using the silkworm muscle contraction assay as well. The results revealed that *Lactococcus lactis* 11/19-B1 was highly active on the stimulation of innate immunity in silkworm. Silkworm that normally ingested an artificial diet mixed with *L. lactis* 11/19-B1 or a yogurt fermented with *L. lactis* 11/19-B1 showed tolerance against the pathogenicity of *Pseudomonas aeruginosa*. This LAB would be expected to be probiotics activating innate immunity. These data suggest that *Lactococcus lactis* 11/19-B1 would be expected to be useful for making yogurt and probiotics to activate innate immunity.

2. Materials and Methods

2.1. *Materials*

GAM broth and GAM agar were purchased from Nissui (Tokyo, Japan). MRS broth and MRS agar were purchased from Becton Dickinson (BD, USA). $CaCO_3$-MRS agar was prepared by adding a final 1% $CaCO_3$ concentration (Wako, Osaka, Japan) to MRS agar after autoclaving. ARS (Alizalin red S) milk agar contained 50% (v/v) milk (Meiji, Tokyo, Japan), 1.3% (w/v) agar (Nacalai tesque, Kyoto, Japan), and 0.044% (w/v) Alizalin red S (Wako). Milk was added after autoclaving. AnaeroPak (Mitsubishi gas chemicals, Tokyo, Japan) was used for anaerobic culture on agar plates. Saline was prepared as 0.9% NaCl (Wako). LB medium was prepared with 1% Bacto tryptone (BD), 0.5% Bacto yeast extract (BD), and 1% NaCl (Wako). LB agar plates were prepared as LB medium containing 1.5% (w/v) agar (Nacalai tesque).

2.2. *DNA sequencing*

Fragments containing 16S rDNA was amplified with PCR using KOD FX Neo (Toyobo, Tokyo, Japan), primers 9F and 1541R (*6*), and bacterial colonies. For the amplification of extended 16S rDNA sequence containing a 16S-23S rDNA spacer region, primers 9F (*6*) and 23R (*7*), and genomic DNA were used. Genomic DNA was isolated with a DNeasy Blood & Tissue Kit (QIAGEN, Germany). DNA sequence was determined with direct sequencing, BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Sequences were analyzed with NCBI BLASTN 2.2.27+ (*8*), 16S ribosomal RNA sequences database (Bacteria and Archaea, 7,545 sequences). DNA sequences are in preparation for submission to GenBank.

2.3. *Characterization of LAB*

Isolated bacteria were Gram-stained with Gram color (Merck, USA). Bacterial colonies were suspended in 3% H_2O_2 for catalase tests. *S. aureus* RN4220 and *E. coli* JM109 were used as controls. Other identification kits, Api Zym and Api 50 CHL were purchased from Sysmex BioMerieux (Tokyo, Japan), and the data was analyzed with Api web v5.1 database (Sysmex BioMerieux, France).

2.4. *Silkworm muscle contraction assay*

Silkworm muscle contraction was measured as in a previous report (*3*). Briefly, autoclaved bacterial suspension (50 μL) was injected into silkworm muscle specimens. Contraction value was determined as (prelength-postlength)/prelength. Sample amount (mg) giving a 0.15 contraction value is determined as 1 unit.

2.5. *Silkworm infection model*

Silkworm infection model was performed as in a previous report (*9*). Silkworms (*Bombyx mori* Hu•Yo × Tukuba•Ne) were reared at 27°C. Pathogens used in infection model were *P. aeruginosa* PAO1 (*10*), *S. aureus* MSSA1 (*11*), *S. aureus* MRSA4 (*12*), and *E. mundtii* 12/5-1 (*13*) from our laboratory stock. Pathogenic bacteria grown in LB medium overnight were diluted with saline (0.9% NaCl) and injected into fifth instar larva (n = 7) after feeding with Silkmate (Sysmex, Kobe, Japan) overnight. Survival of silkworm larva was counted for five days.

3. Results

3.1. *Isolation and characterization of LAB*

We developed a new and efficient selection medium for milk-fermenting LAB. ARS medium is an agar plate containing milk and Alizalin red S, which is a pH indicator developing a yellow to white color under acidic conditions after milk fermentation.

Samples from natural products containing plants, soil, and digesta of invertebrates such as slug and earthworm were screened to isolate milk-fermenting LAB (Table 1). Each sample of total 697 samples with saline was ground

Table 1. Summary of samples used in this study

Samples	*n*
Earthworm	132
Slug	13
Leach	2
Fruits	168
Vegetable	34
Wild plants	
Fruits	167
Leaf	54
Root	9
Soil	74
Small animals	44
Total	697

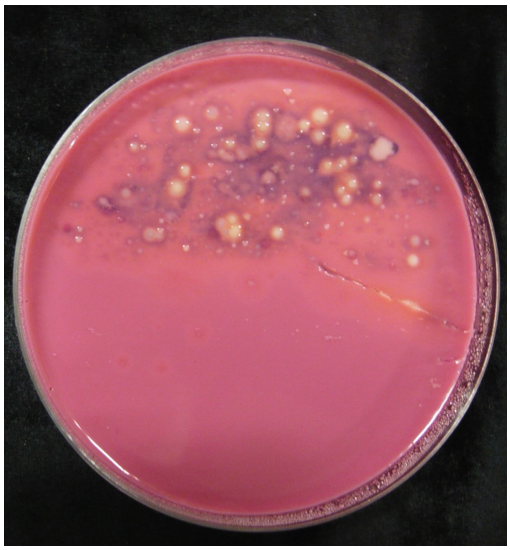

Figure 1. Isolation of LAB on ARS milk agar medium. An example of LAB isolation from soil is shown. Alizaline red S color was changed to yellow to purple by colony formation, which ferment solo nutrient milk.

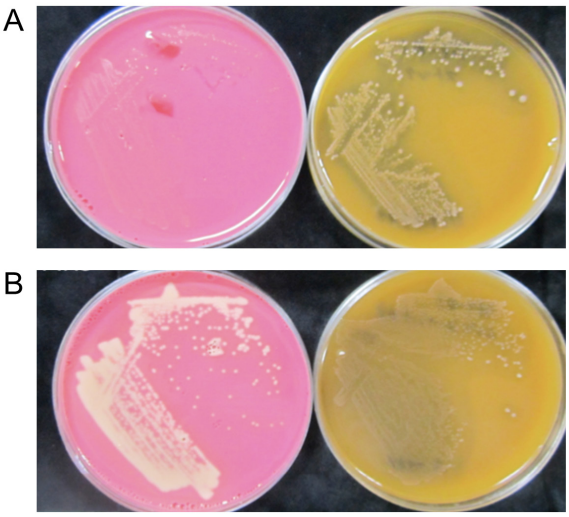

Figure 2. Growth characteristics of LAB. (A) *L. lactis* 11/19-B1 and **(B)** *S. thermopilus* A were streaked on ARS milk medium (left) and $CaCO_3$-MRS agar plate (right).

Table 2. Identification of bacteria and its activity of silkworm muscle contraction assay

Samples	Origin	Identity	ID, %	GenBank accession	Activity (U/mg)
11/19-B1	Kiwifruit	*Lactococcus lactis* IL1403	98	NR_103918.1	105 ± 5[a]
11/28-C3	Kiwifruit	*Streptococcus thermopilus* ATCC 19258	98	NR_042778.1	77
10/30-2	Earthworm	*Lactococcus lactis* IL1403	98	NR_103918.1	45 ± 32[b]
9/10#5	Slug	*Lactococcus lactis* IL1403	98	NR_103918.1	33
12/4-A12	Wild leaf	*Streptococcus salivarius* ATCC 7073	98	NR_042776.1	28
11/21-F1	Wild fruit	*Lactococcus lactis* IL1403	98	NR_103918.1	23
12/3-C11	Wild leaf	*Enterococcus gallinarum* LMG 13129	98	NR_104559.1	7.1
11/28-C8	Apple	*Streptococcus salivarius* CCHSS3	96	NR_102816.1	2.2
11/22-B7	Wild fruit	*Lactococcus lactis* IL1403	99	NR_103918.1	2.0
10/29-10	Earthworm	*Enterococcus casseliflavus*	99	NR_041704.1	0.83
11/27-D5	Soil	*Enterococcus faecium* Aus0004	94	NR_102790.1	0.45
12/3-B11	Pineapple	*Streptococcus salivarius* ATCC 7073	98	NR_042776.1	< 0.3
A	Yogurt	*Streptococcus thermopilus*			43 ± 20[b]
B	Yogurt	*Lactobacillus delbrueckii ssp. bulgalicus* OLL1073R-1			29
C	Yogurt drink	*Lactobacillus delbrueckii ssp. bulgalicus* OLL1073R-1			24
D	Yogurt	*Lactobacillus casei* YIT9029			18 ± 2[b]
E	Yogurt drink	*Lactococcus lactis* JCM5805			17
F	Yogurt drink	*Lactobacillus casei* YIT9029			5.6

[a] mean ± SE (n = 7), [b] mean ± SE (n = 2).

in a mortar and suspended in sterile saline. Supernatant fluids of the suspensions were spread on ARS milk agar. After incubation at 30ºC for two days, more than ten yellow to white colonies were isolated on each plate (Figure 1). We selected Gram-positive colonies from Gram staining colonies on ARS milk agar, since LAB generally recognized as safe (GRAS) are Gram-positive bacteria. Colonies were re-streaked on $CaCO_3$-MRS medium to confirm lactic-acid fermentation (Figure 2). There were 57 samples of lactate-fermenting and Gram-positive bacteria (Table 1). Bacteria subjected to silkworm contraction assays were 23 of the 57 samples (Table 1). LAB was also isolated from commercially available yogurt and silkworm contraction activity was tested for comparison. LAB tested for contraction assays was sequenced for 16S rDNA.

Silkworm contraction activity of LAB is shown in Table 2. LAB isolated from natural samples showed diverse activity from 0.3 to 105 U/mg. *L. lactis* 11/19-B1 showed the highest activity, 105 U/mL, representing higher activity than that of LAB in commercial yogurt tested in Table 2. Other *L. lactis* strains, 9/10#5, 10/30-2, and 11/21-F1 showed relatively high silkworm contraction activity, that is, 33, 45, and 23 U/mg respectively. *Streptococcus strains*, *S. thermopilus* 11/18-C3 and *S. salivarius* 12/4-A12 represented relatively higher activity, 77 and 28 U/mg respectively. LAB already reported to be activating mammal immunity had slightly high activity of silkworm contraction. Commercial yogurt strains, *Lactobacillus delbrueckii* ssp. *bulgalicus* OLL1073R-1 (*14*) and *L. lactis* JCM5805 (*15*), which activate

Table 3. Growth characteristics of *Lactococcus lactis*

Carbohydrate	11/19-B1 (kiwifruit, This study)	KF282 (mustard/cress)[a]	IL1403 (diry)[a]
Glycerol	−	−	−
Erythritol	−	−	−
D-Arabinose	−	−	−
L-Arabinose	+	+	−
Ribose	+	+	+
D-Xylose	+	+	−
L-Xylose	−	−	−
Adonitol	−	−	−
β-Methyl-xyloside	−	−	−
Galactose	±	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	−	−	−
Rhamnose	−	−	−
Dulcitol	−	−	−
Inositol	−	−	−
Mannitol	+	−	−
Sorbitol	−	−	−
α-Methyl-D-mannoside	−	−	−
α-Methyl-D-glucoside	−	−	−
N-Acetyl glucosamine	+	+	+
Amygdalin	±	+	±
Arbutin	+	+	+
Esculin	+	−	−
Salicin	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	±
Melibiose	−	−	−
Saccharose	+	+	−
Trehalose	+	+	+
Inulin	−	−	−
Melezitose	−	−	−
D-Raffinose	−	−	−
Starch	−	+	+
Glycogen	−	−	−
Xylitol	−	−	−
β-Gentiobiose	+	+	+
D-Turanose	−	−	−
D-Lyxose	−	−	−
D-Tagatose	−	−	−
D-Fucose	−	−	−
L-Fucose	−	−	−
D-Arabitol	−	−	−
L-Arabitol	−	−	−
Gluconate	±	+	−
2-Keto-gluconate	−	−	−
5-Keto-gluconate	−	−	−

[a] Data from reference 17.

murine macrophages and plasmacytoid dendritic cells respectively showed 24 and 17 U/mg silkworm contraction activity respectively.

L. lactis 11/19-B1 was further characterized for bacterial growth and identification. Growth of *L. lactis* 11/19-B1 was sensitive to temperatures higher than 42ºC. Growth of *L. lactis* 11/19-B1 in MRS medium were stimulated by addition of 0.3% glucose or 0.03% casamino acids. BLAST analysis of 16S rDNA sequence of the 11/19-B1 strain revealed that the 11/19-B1 strain had 98% similarity to *L. lactis* subsp. *lactis* IL1403

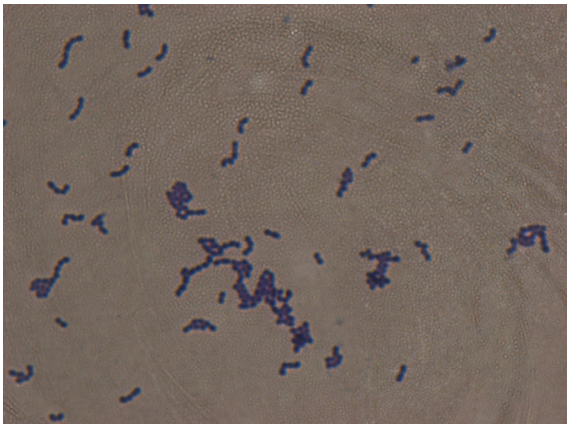

Figure 3. Gram staining of L. lactis 11/19-B1. *L. lactis* 11/19-B1 colony on $CaCO_3$-MRS agar was stained with Gram color (Merck). Image was captured with a CCD camera (Hamamatsu photonics) using an Olympus phase-contrast microscope at ×1,000 magnification.

(NR_103918), *L. lactis* subsp. *hordniae* NCDO2181 (NR_040956) and *L. lactis* subsp. *lactis* NCDO604 (NR_040955). Finally, detailed analysis of extended 16S rDNA sequence (1781 bp) of the 11/19-B1 strain showed 99% similarity to *Lactococcus lactis* subsp. *lactis* IL1403 genome sequence (16, AE005176.1).

Sugar utilization was tested using Api 50 CHL (Table 3). *L. lactis* 11/19-B1 represented a difference compared to *L. lactis* IL1403 in utilization of 6 of 49 sugars and sugar derivatives. L-arabinose, D-xylose, mannitol, esculin and sucrose were positive and starch was negative in *L. lactis* 11/19-B1 as compared to dairy strain *L. lactis* IL1403. On the other hand, *L. lactis* 11/19-B1 represented three-different sugar utilizations with *L. lactis* KF282 derived from plants (*17*). Sugar utilization of *L. lactis* 11/19-B1 matched 77.2% of *Lactococcus lactis* ssp *lactis* 1, and 21.9% of *Lactobacillus brevis* 1 in Api web v5.1 database. A possibility of *L. brevis* being identitical to 11/19-B1 was excluded because of gram-positive bacilli of *L. brevis*, in contrast to gram-positive cocci of 11/19-B1 (Figure 3). Api Zym test was also used for the identification of the strain 11/19-B1 and compared to previous data (*18*) in Table 4. *L. lactis* 11/19-B1 represented 6 and 4 different activities of 19 enzymes as compared to that of *L. lactis* CECT185^T and CECT967^T respectively.

3.2. *Probiotic effect of L. actis 11/19-B1 and its yogurt*

Next, we confirmed milk fermentation with *L. lactis* 11/19-B1 as a starter strain to form yogurt. Commercially available milk was added with 0.03% casamino acids, 0.3% glucose and *L. lactis* 11/19-B1 under sterile conditions, and incubated at 37ºC for 3 days, resulting in yogurt with a clean acid flavor. Control milk with casamino acids and glucose without *L. lactis* 11/19-B1 was not fermented after 3 days. To confirm milk fermentation with *L. lactis* 11/19-B1, colony isolation

Table 4. Enzymatic characteristics of *Lactococcus lactis*

Enzyme	11/19-B1 (This study)	CECT185^T (*lactis* genotype)[a]	CECT967^T (*cremoris* genotype)[a]
Phosphatase alkaline	−	±	−
Esterase (C4)	−	−	±
Esterase lipase (C8)	−	±	±
Lipase (C14)	−	−	−
Leucine aminopeptidase	−	+	±
Valine aminopeptidase	−	−	−
Cystine aminopeptidase	−	−	−
Trypsin	−	−	−
Chymotrypsin	−	−	−
Phosphatase acid	+	+	+
Phosphoamidase	+	+	+
α-Galactosidase	−	−	−
β-Galactosidase	−	±	−
β-Glucuronidase	−	−	−
α-Glucosidase	−	+	−
β-Glucosidase	−	+	+
β-Glucosaminidase	−	−	−
α-Mannosidase	−	−	−
α-Fucosidase	−	−	−

[a] Data from reference 18.

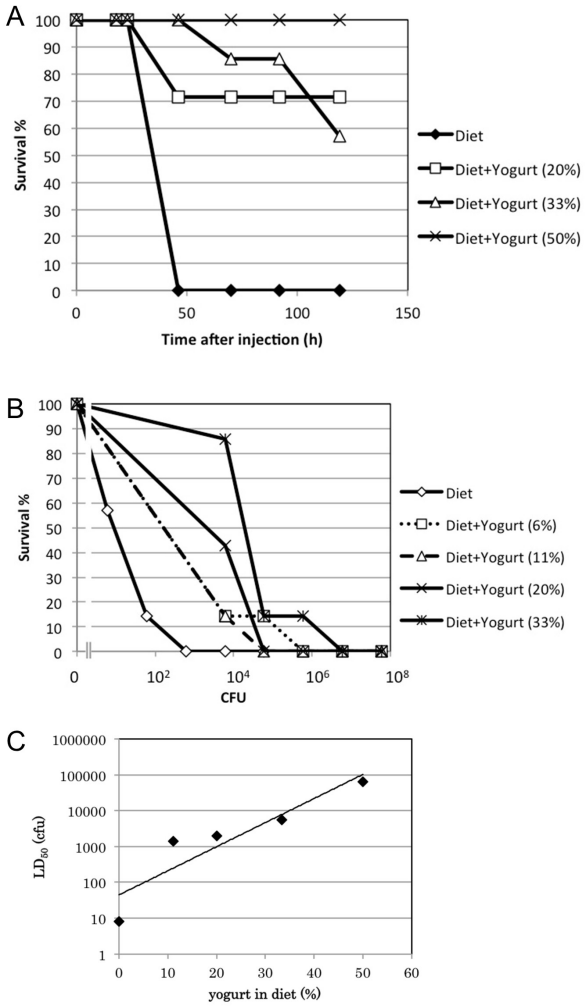

Figure 4. Probiotic effect of *L. lactis* 11/19-B1 yogurt on P. *aeruginosa* infection. (A) Time course of survival of silkworm fed a diet with or without *L. lactis* 11/19-B1 yogurt after *P. aeruginosa* PAO1 infection. **(B)** Dose response of *P. aeruginosa* PAO1 on silkworm survival after 2 days. *P. aeruginosa* PAO1 was injected into 5th instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt. **(C)** Dose response of *L. lactis* 11/19-B1 yogurt on probiotic effect in *P. aeruginosa*-infected silkworm. Data represented typical one of three experiments.

from yogurt on ARS milk agar and $CaCO_3$-MRS agar, Gram staining, and 16S rDNA sequencing were conducted.

To evaluate probiotic effect of yogurt fermented with *L. lactis* 11/19-B1, the silkworm infection model was used (Figure 4). Injection of *P. aeruginosa* to silkworm larva showed time-dependent and dose-dependent killing of silkworms. Silkworms were normally fed with a diet mixed with yogurt. Feeding of silkworms with a diet containing yogurt increased survival rate, resulting in about a 1,000-fold increase of LD_{50} for *P. aeruginosa* as compared to data in the absence of yogurt. To test if *L. lactis* 11/19-B1 has probiotic activity by itself, silkworms were fed a diet mixed with *L. lactis* 11/19-B1 viable cells (Figure 5). Feeding of silkworm with a diet containing *L. lactis* 11/19-B1 viable cells increased survival rate as well. Next, infection model with gram-positive pathogens were tested (Figure 6). Feeding of silkworms with a diet containing yogurt increased survival rate in methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) infection, resulting in about 2 and 4 fold increase of LD_{50} for MSSA and MRSA respectively. Yogurt feeding increased to a 2-fold survival rate in infection with *Enterococcus munditii*, causing flacherie disease in silkworms (*19*).

4. Discussion

4.1. *Screening LAB activating innate immunity as a yogurt starter*

In general, LAB has been thought to help human health in the gut as probiotics. Recently it has been noted that the activity of LAB stimulating the immune system is important (*14,15,20-23*). Yogurt is an example of fermented food with LAB. We studied to isolate LAB, which ferment milk to make yogurt and have an immune-stimulating activity as well. A few strains in many LAB spices ferment milk to form yogurt. In this study, we initially developed a new method to isolate milk-fermenting LAB.

To isolate LAB which grow and ferment milk, we developed a selection medium containing agar, milk as a nutrient, and ARS as a pH indicator, which show red in neutral conditions and yellow in acidic conditions. Because of its hydrophobicity and indiffusibility, ARS is useful to stain an acidic colony by fermentation. Since LAB has been known to colonize natural products including leaves and fruits of plants and gut in animals, we isolated LABs as starter candidates by screening acidic colonies on ARS agar from natural products.

As LAB belongs to Gram-positive bacteria, we

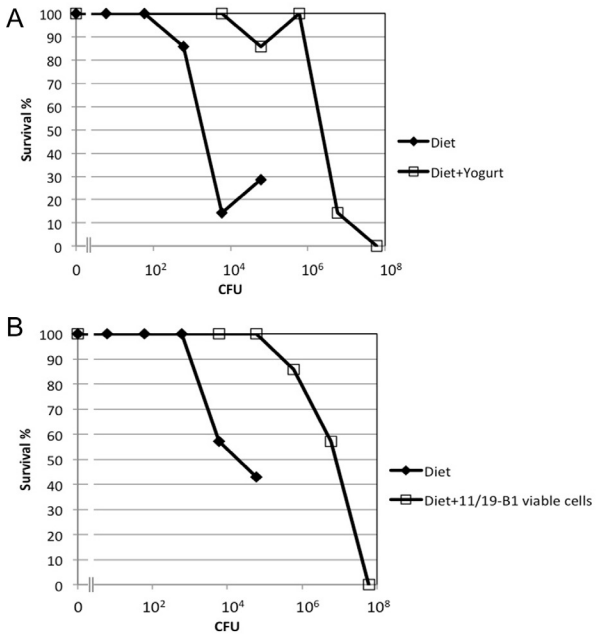

Figure 5. Probiotic effect of *L. lactis* 11/19-B1 yogurt on *P. aeruginosa* infection. Dose response of *P. aeruginosa* PAO1 on silkworm survival after 2 days. *P. aeruginosa* PAO1 was injected into 5th instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt **(A)** or viable cells (4×10^7 cfu/larva) **(B)**. Data represents a typical one of three experiments.

excluded Gram-negative bacteria isolated on ARS agar. We selected lactic-acid producing isolates on $CaCO_3$-MRS agar, in which lactic acid solubilizes $CaCO_3$ to form a clear spot around a colony. Then, we determined 16S rDNA sequences of each isolate. The level of 16S rDNA sequence similarity between *Lactococcus lactis* subsp. *lactis* and other *Lactococcus* species is 90-93% and it is 98-99% between *Lactococcus lactis* subsp. *lactis* and other subspecies of *Lactococcus lactis* (*24*). The identity of the 11/19-B1 strain as *L. lactis* was suggested by 99% similarity between the 11/19-B1 strain and *L. lactis* subsp. *lactis* IL1403 (*16*).

4.2. *Innate-immune activation in silkworm*

We determined the activity of LAB to stimulate innate immunity in silkworms using a muscle contraction assay (*3*). In this assay, insect cytokine PP upon innate immune activation induces muscle contraction in silkworms. Compared to a conventional method using macrophages, the muscle contraction assay does not require cell culture but is insensitive to LPS. Isolated LAB showed a variety of muscle contraction activity ranging from 0.3 to 105 U/mg. *L. lactis* 11/19-B1 had the highest activity. *L. lactis* 11/19-B1 fermented milk to form a yogurt with good flavor, which we used for further investigation.

4.3. *Acquisition of tolerance to bacterial infection by ingesting L. lactis 11-19-B1 yogurt*

We used silkworms as a surrogate animal to test

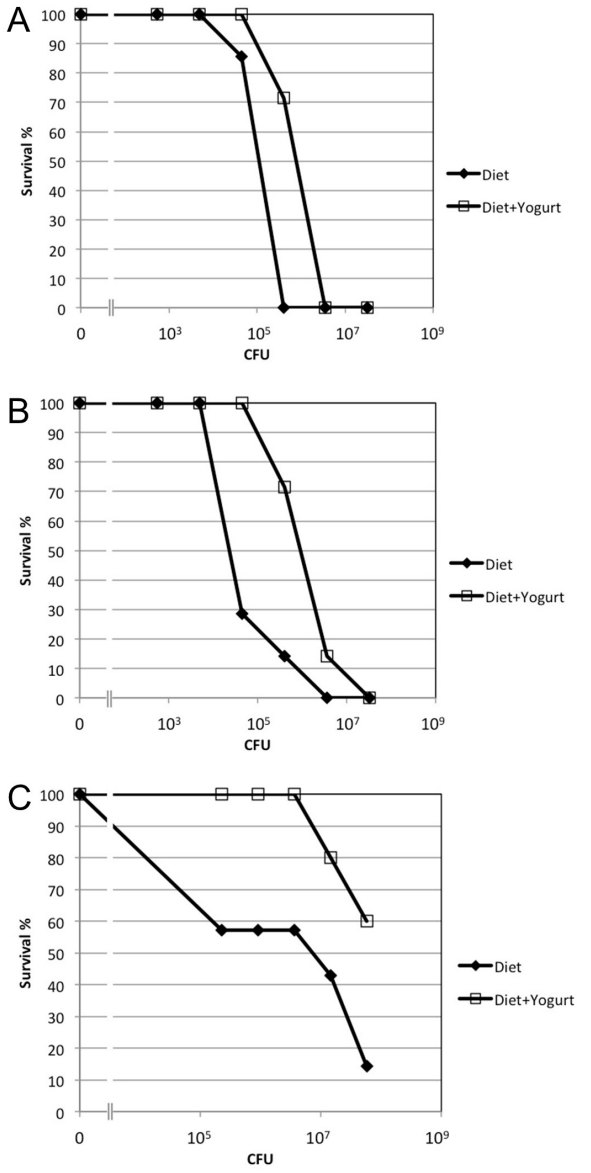

Figure 6. Probiotic effect of *L. lactis* 11/19-B1 yogurt on gram-positive pathogen infection. Dose response of gram-positive pathogens on silkworm survival after 2 days. Fifth instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt or viable cells . *S. aureus* MSSA1 **(A)**, MRSA4 **(B)**, or *E. mundtii* 12/5-1 **(C)** was injected into 5th instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt. Data represents a typical one of three experiments.

probiotic effect of yogurt. Silkworms were fed a diet containing yogurt without any problems. Silkworms fed yogurt had tolerance to lethality on infection with *P. aeruginosa*, *S. aureus*, and *E. munditii*. A thousand-fold decrease of LD_{50} of *P. aeruginosa* suggests that tolerance of yogurt-fed silkworm to *P. aeruginosa* was significant. Our previous results reported that silkworms acquire tolerance to *P. aeruginosa* infection by ingesting a diet mixed with the peptidoglucan of *P. aeruginosa* or *Lactobacillus plantarum* (*25*). In this study, we reported that an ingestion of viable cells or yogurt of *L. lactis* 11/19-B1 improved the survival of silkworms in *P. aeruginosa* infection. These data

suggest an innate-immune activation induced a primed immunity, an apparent acquired immunity, to microbial infection.

LAB would be expected to be an application for dairy products to help human health. Reports of a probiotic effect of LAB in an infection model has been limited. It was reported that *Bifidobacterium* protected *E. coli* O157 infection in germ-free mice (*26*). Oral administration of *Bifidobacterium longum* prevented *P. aeruginosa* gut-derived sepsis in a mouse model (*27*). Heat-killed *L. casei* protected against *P. aeruginosa* infection in mice (*28*). *L. lactis* is a nonpathogenic LAB known as not colonizing the mouth and gut, and not belonging to human gut flora (*29*). The *L. lactis* IL1403 genome was sequenced and a recombinant technique was established to construct a strain expressing specific antigens (*16,30,31*). Another recombinant expressing IL-10 was used to treat a mouse colitis model (*32-34*). Our data suggest that silkworms are a useful model animal to evaluate probiotic effects of LAB and isolated LAB would be expected to be an application for dairy products to help human health as well, even though a probiotic effect of isolated LAB on mammals including humans is unknown and needs further investigation.

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