

## **Probiotic Properties of Lactobacilli** **Isolated from Thai Traditional Food**

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### **Abstract**

Certain properties relevant to probiotic action, e.g. resistance to acid, bile tolerance, adhesive properties, antibacterial activity, and antibiotic susceptibility were investigated of lactobacilli isolated from four kinds of Thai traditional fermented foods. Media of pH = 2.0–7.0 and bile salt concentrations of 0.3–1.0% were used as stress conditions. The adhesive properties were assessed by determination of bacterial hydrophobicity. Antibacterial activity of the probiotic lactobacilli was determined by means of the spot-on-lawn method. Among 563 isolates, only 3 strains (two from fermented pork and one from fermented tea leaves) showed extremely high survival rates under stress caused by acid or bile salts. The identification by PCR techniques revealed that these three strains were *Lactobacillus fermentum*. The strains from fermented pork showed higher adhesive potential than those from fermented tea leaves. The three strains inhibited test pathogenic bacteria to different extents. They were sensitive to chloramphenicol, quinupristin, erythromycin, kanamycin linezolid, rifampicin, streptomycin, and tetracycline but resistant to ciprofloxacin and vancomycin.

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## Keywords

Lactobacilli • Food origin • Probiotics • Traditional fermented food • *Lactobacillus fermentum*

## Introduction

Traditional fermented food is the product of a biotechnological process. It is produced by taking advantage of the natural microbiota associated with fresh food materials. It is one of the most practical, economic, and widely applied empirical methods for preserving and often enhancing organoleptic and nutritional quality of fresh food. It has been unique to historical countries in different parts of the world, e.g. milk fermentation “yoghurt” in Bulgaria [1], vegetable fermentation “suan cai” in China [2], “kimchi” in Korea [3], “natto” and “miso” in Japan [4, 5], Gundruk, Sinki, and khalpi in India [6]. In Thailand there are famous traditional fermented products called “Nham” and “Miang”.

Nham is traditional salt-fermented ground meat, a sausage-like product commonly made of pork (fermented pork) or fish (fermented fish) with garlic, pepper, salt and cooked rice. It was reported that bacteriocin produced by bacteria isolated from nham was heat-stable even at an autoclaving temperature (121 °C for 15 min) and was active over a wide pH range [7]. Miang consists of fermented tea leaves commonly consumed in northern parts of Thailand. It is sometimes known as “eating tea”. The whole leaves of tea (*Camellia sinensis*) are used and are naturally fermented for three months in the manufacturing process without any preservatives. The product has a typical pickled flavor, sour and flowery. Okada et al. [8] had isolated lactic acid bacteria from miang and identified as *Lactobacillus plantarum* with diaminopimelic acid in the cell walls. However there are still few research data available concerning lactobacilli from nham and miang, particularly on the comparative functional properties.

Although lactobacilli show a high impact on effective protection to human health, there is obvious evidence that lactobacilli from different origins possess

probiotic properties at different levels [9]. In order to survive and colonize in the gastrointestinal tract, the bacteria should express high tolerance to acidic media and bile and should be able to adhere to the intestinal surfaces [10]. The antibiotic resistance of pathogenic bacteria is an increasing medical problem [11], and raises the question of antibiotic resistance among desired probiotic strains. Therefore, the antibiotic susceptibility test therefore should be incorporated for the safety assessment of the desired property of the promising probiotic lactobacilli. The present work was undertaken to provide practical data on lactobacilli of fermented food origin.

## **Experimental**

### ***Bacterial strains and isolation***

Four types of fermented food products, namely fermented pork, fermented fish, fermented tea leaves, and pickled garlic which are commonly distributed in the food galleries in Thailand were used as the sources of bacteria. A number of bacterial strains were isolated from such food origins by the dilution agar method. Briefly, a sample was mixed with normal saline to appropriate dilutions. A volume of 0.1 ml of the dilutions was plated on MRS agar (Merck, Darmstadt, Germany) and incubated in anaerobic conditions at 37 °C for 48 h. Isolated colonies were taken randomly for purification. The purified colonies were tested for lactobacilli by microscopic examination with gram stain and catalase production. The gram positive, catalase-negative rods were selected for further studies.

### ***Acid tolerance test***

The isolated lactobacilli were subjected to primary screening for acid tolerance in MRS broth adjusted to pH 2.5 with 1N HCl for 90 min at 37 °C. The determination of survival was performed by single streaking on MRS agar plates, and the growth was observed after 24-48 h after anaerobic incubation at 37 °C. Isolates which were growing on the agar were considered to be acid tolerant strains. These strains were

selected and cultivated in MRS broth under anaerobic atmosphere at 37 °C. Cultures ( $10^7$ – $10^8$  cfu/ml) were inoculated in 10 ml of 0.05 M sodium phosphate buffer adjusted to pH 2.0, 3.0, and 7.0 with 1 N HCl. Samples were incubated at 37 °C for 2 h. Cells were serially adjusted to 10-fold dilution by phosphate buffer pH 7.0. The dilution was plated on MRS agar for determination of viable cells after 48 h of incubation. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial cell concentration. Each experiment was performed in triplicate.

### ***Bile tolerance test***

Firstly, the screening for bile tolerance was carried out by growing the isolated lactobacilli in MRS broth containing 0.3% of bile salt (Oxgall, Difco, Detroit, USA) for 24 h under anaerobic conditions at 37 °C. Culture broths with turbidity more than 0.5 units at 600 nm were classified as bile tolerant strains. These strains were selected for exposure to broths containing higher concentrations of 0.5 and 1.0% (w/v) of bile salt. The survival rate of each strain was expressed as the percentage of viable cells in the presence of bile salt compared to that without bile salt. The experiment was performed in triplicate and the mean values were calculated.

### ***Identification of bacterial isolates***

Selected lactobacilli isolates were cultivated in MRS broth incubated under anaerobic conditions for 24 h at 37 °C. A portion of 1.5 ml of this bacterial culture was centrifuged at 8,000 rpm for 7 min at 4 °C. The pellets were washed twice with 900 µl sterile normal saline solution. The supernatant was discarded and washed the pellets washed with 900 µl of sterile EDTA (50 mM, pH 8.0). The pellet products were stored at –20 °C. The MasterPure™ gram positive DNA purification kit (EPICENTRE, Madison, Wisconsin) was used to obtain DNA samples according to *Listeria monocytogenes* performance in the instruction of the manufacturer. PCR procedure based on 16S rRNA gene sequences genus-specific, multiplex genus-specific and species-specific primers were used for validation. For genus-specific PCR, isolates were

identified to the genus level using the primers LbLMA1-rev and R16-1 according to Dubernet et al. [12]. The reaction mixture (total volume 25  $\mu$ l) contained 1  $\mu$ l of each primer (10 mM), 10 x PCR buffer (Finnzymes, Espoo, Finland), dNTP-Mix (10 mM) (Roth, Karlsruhe, Germany), Dynazyme (2 U/ $\mu$ l) (Finnzymes, Espoo, Finland), and 0.5  $\mu$ l of bacterial DNA. The PCR was conducted in a Mastercycler gradient (Eppendorf, Hamburg, Germany) with the following program: initial denaturation at 94 °C for 4 min; 35 cycles consisting of denaturation at 94 °C for 1 min, annealing temperature at 55 °C for 1 min including time increment of 2 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 8 min. The PCR products were separated by electrophoresis in 2% agarose gel. The gel was stained in ethidium bromide (5 $\mu$ g/ml) and visualized under UV light. Multiplex genus-specific PCR as described by Song et al. [13] was used to characterize *Lactobacillus spp.* in five district groups according to sizes of fragment products (300, 350, 400, 450 and 700 bp) with the group-specific primers Lac-2, Ldel-7, LU-5, LU-3, and LU-1. For species-specific, PCR with primers Lfer-3, Lfer-4, Reu-1, Reu-4, Lpla-2, and Lpla-3 were performed to identify *L. fermentum*, *L. reuteri*, and *L. plantarum*. The PCR products were analyzed by agarose gel electrophoresis and photographed under UV exposure.

### **Cell surface hydrophobicity**

The *in vitro* cell surface hydrophobicity was determined by the bacterial adherence to hydrocarbon assay modified from the methods of Rosenberg et al. [14]. Briefly, the test bacteria were grown in MRS broth at 37 °C under anaerobic conditions. The 18–24 h (stationary phase) test culture was harvested after centrifugation at 6,000 rpm for 10 min, washed twice and resuspended in 50 mM K<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.5) to an optical density (OD<sub>560</sub>) of 0.8–1.0 (A<sub>0</sub>) measured spectrophotometrically. A portion of 0.6 ml of n-hexadecane was added to 3 ml of bacterial suspension. The mixture was blended using a vortex mixer for 120 s. The tubes were allowed to stand at 37 °C for 30 min to separate the two phases. The aqueous phase was carefully removed and the OD<sub>560</sub> of the aqueous phase (A)

was measured. Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the initial aqueous bacterial suspension due to cells partitioning into a hydrocarbon layer. The percentage of cell surface hydrophobicity (%H) of the strain adhering to hexadecane was calculated using the equation:  $\%H = [(A_0 - A)/A_0] \times 100$ .

### **Detection of antibacterial activity**

The antibacterial activity of the selected isolates was determined by agar spot-on-lawn test introduced by Schillinger and Lücke [15] with some modification. The indicator bacteria used in this study were *Escherichia coli* TISTR 780, *Salmonella typhi* DMST 5784, and *Staphylococcus aureus* TISTR 029. One  $\mu$ l of each overnight culture of selected lactobacillus was spotted on MRS plates (containing 0.2% glucose and 1.2% agar) and incubated under anaerobic conditions for 48 h to develop colonies. A portion of 0.25 ml of 1:10 dilution of an overnight culture of the indicator bacteria was inoculated in 9 ml of Brain Heart Infusion (Merck, Darmstadt, Germany) soft agar (0.7% agar). The medium was immediately poured over the MRS plate on which the tested lactobacillus was grown. The plates were incubated aerobically at 37 °C for 24 h. The antibacterial activity was related to the inhibition clear zone which calculated as the difference between the total of inhibition zone and the diameter of growth spot of selected strains

### **Antibiotic susceptibility**

The antibiotic susceptibility test used in this study was done according to the agar dilution method published by the National Committee for Clinical Laboratory Standards (NCCLS) [16]. The determination of minimum inhibitory concentration (MIC) to certain antimicrobial agents recommended by Scientific Committee on Animal Nutrition (SCAN) comprised ampicillin, chloramphenicol, ciprofloxacin, quinupristin, erythromycin, gentamycin, kanamycin, linezolid, rifampicin, streptomycin, tetracycline, and vancomycin [17] using Mueller-Hinton agar (Merck, Darmstadt, Germany) in anaerobic conditions. Briefly, the sterilized agar was

allowed to reach 50 °C in a water-bath. Dilution series of antimicrobial agents were prepared in suitable solvents and diluents. One ml of working solution was added to 9 ml of molten agar, mixed thoroughly, and poured into sterilized petri dishes. The agar plates were allowed to set at room temperature. Inoculum was prepared by suspending several bacterial colonies from a fresh agar plate in normal saline to a McFarland 0.5 turbidity standard (containing  $1-2 \times 10^8$  cfu/ml). Then, the 0.5 McFarland suspensions were diluted 1:10 in normal saline to obtain a concentration of  $10^7$  cfu/ml. A spot of 1  $\mu$ l of the inoculum was placed on the agar surface yielding approximately  $10^4$  cfu/spot. The inoculated plates were allowed to stand at room temperature for about 30 min. The plates were moved to 37 °C incubators and stored under anaerobic conditions for 24 h. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibited growth, disregarding a single colony or faint haze caused by the inoculum.

## Results and Discussion

### ***Bacterial strains and isolation***

A total of 563 strains were isolated from 4 types of the fermented food origins. Microscopic identification could determine the rod shaped cells. The gram's staining and catalase test could support the characterization of lactobacilli. After taking these criteria into account, 274 strains were found to be gram positive rod shaped, non-spore forming, and catalase negative which indicated the typical basic characteristics of lactobacilli. Among these 274 strains, 169 strains were from fermented pork, 46 strains from fermented fish, 41 strains from fermented tea leaves, and 18 strains from pickled garlic as shown in Tab. 1. These isolated lactobacilli were used for further studies.

**Tab. 1.** Number of strains obtained after purification and characterization.

Food origins	Number of Strains	
	Purification	Characterization
Fermented pork	237	169
Fermented fish	102	46
Fermented tea leaves	161	41
Pickled garlic	63	18
Total	563	274

**Tab. 2.** Number of viable strains after acid and bile tolerance screening tests.

Food origins	Acid tolerance	Bile tolerance
Fermented pork	36	66
Fermented fish	9	22
Fermented tea leaves	10	15
Pickled garlic	1	16
Total	56	119

### ***Effect of pH***

The effect of pH ranging from 2.0 to 7.0 on the survival rate of the 51 selected strains was studied. It was found that most strains could survive approximately less than 50% in pH 2.0 as shown in Fig.1 (A). Only 3 strains, 2 from fermented pork and 1 from fermented tea leaves, could survive to an extend of more than 50%. The most tolerant strain was the strain no. 2311 which was isolated from fermented tea leaves. This strain could survive in pH 2.0 at the survival rate of 62.1%. When the pH was raised to 3.0, more than 50% of isolates from all kinds of food origins exhibited a survival rate higher than 75% as shown in Fig. 1 (B). When the pH was up to 7.0, all isolates could survive 100%. Mishra and Prasad [19] reported that when pH was raised to pH 3.0, the test *Lactobacillus casei* showed higher survival

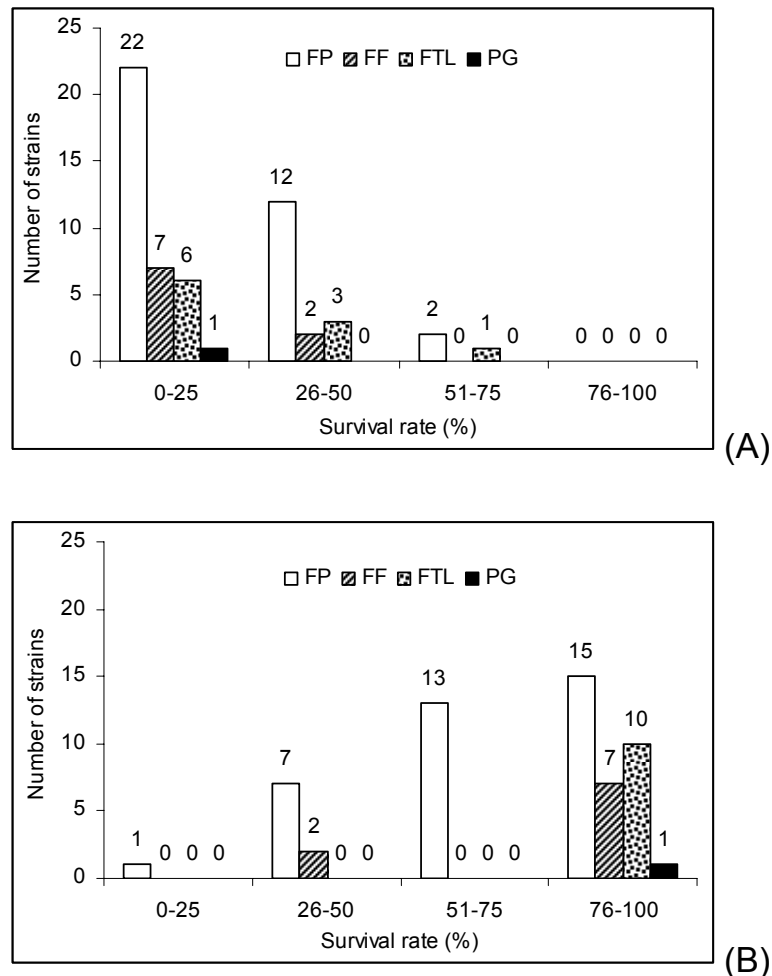


than in pH 2.0. In our experiment, the results were in agreement with the fact that the higher the pH value, the higher the percentage of viability. The effect of food source on bacterial acid tolerance was clearly observed when the percentage of viable strains from each origin was plotted against pH as shown in Fig. 2. It was shown that the isolates from different origins demonstrated different acid resistance patterns. The isolate from fermented tea leaves showed the highest acid resistance at extremely low pH (pH 2.0), followed by those isolated from fermented pork. None of the isolates from fermented fish and pickled garlic could survive at the low pH-value. This suggests that the food origin ecosystem play an important role for the bacterial cells to be able to adapt to the stress conditions.

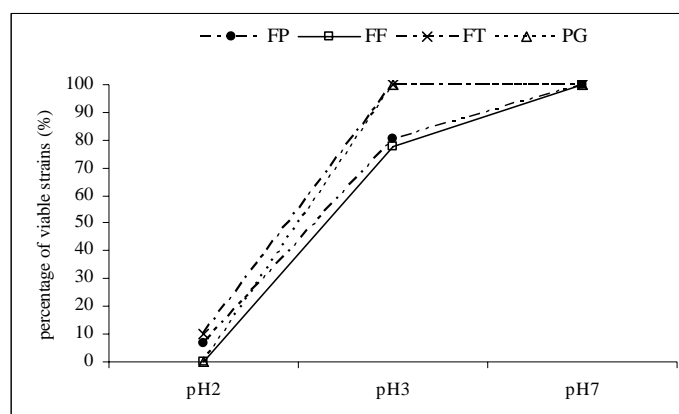
### ***Effect of bile concentration***

Beside the strong acid media in the stomach, the probiotic microorganisms taken orally have to defend against the bile salt in the gastrointestinal tract. Hence, bile tolerance is considered to be one of the important properties required for high survival and as a consequence for a probiotic activity. There is no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentration of bile salts in the small intestine is between 0.2 and 2.0% [20]. In this study, concentrations of 0.3, 0.5 and 1.0% bile salts were used. The results are shown in Tab. 3. All strains could survive more than 60% in 0.3 and 0.5% bile salt solutions. The isolates from our experiment showed stronger bile tolerance than those reported by other investigators [21]. The gradually decrease of viable cells was observed when the concentration of bile salt was increased up to 1.0%. It was considered that bile salt causes the increase in permeability of bacterial cell membranes, as the membranes are composed of lipids and fatty acids. No strain from pickled garlic could survive more than 90% in such high concentration. However, some strains isolated from other sources showed high tolerance even in the extremely high salt media. The most important probiotic property of desirable bacteria is dependent on their ability to remain viable in acid and bile in gastrointestinal tract ecosystem. Among 51 strains, only 3 strains

showed significant resistance to the acid and bile in the extremely high concentration. These 3 strains, 1 isolated from fermented tea leaves (isolate no. 2311) and 2 isolated from fermented pork (isolate no. 3007 and 3010) were selected for further studies.



**Fig. 1.** Survival rates of isolated strains from fermented pork (FP), fermented fish (FF), fermented tea leaves (FTL), and pickled garlic (PG) in incubation media having pH 2.0 (A) and pH 3.0 (B).



**Fig. 2.** Percentage of viable strains isolated from fermented pork (FP), fermented fish (FF), fermented tea leaves (FT), and pickled garlic (PG) of fermented foods showing a survival rate of 50% at various pH-values.

**Tab. 3.** Effect of bile concentration on the viability of the strains isolated from fermented pork (FP), fermented fish (FF), fermented tea leaves (FTL), and pickled garlic (PG).

Bile concentration	Percentage of viable strains at survival rate >60%			
	FP	FF	FTL	PG
0.3%	100	100	100	100
0.5%	100	100	100	100
1.0%	96.8	100	100	100
	Percentage of viable strains at survival rate >90%			
	FP	FF	FTL	PG
0.3%	38.7	77.8	70	100
0.5%	29.0	66.7	20	100
1.0%	19.4	22.2	20	0

### **Identification of bacterial isolates**

The results from gel electrophoresis of genus-specific PCR, multiple PCR, and species-specific PCR (data not shown) were used to identify the three selected strains. From genus-specific PCR, all of selected isolates were identical to *Lactobacillus* species. The results from multiple PCR suggested that DNA from the tested isolates generated fragments corresponding to the 350 bp fragment of Lb95, a positive control for 350 bp group that comprise *L. curvatus*, *L. reuteri*, *L. plantarum*, *L. parapantarum*, *L. pentosus*, *L. keferi*, *L. fermentum*, *L. animalis*, *L. mucosae*, *L. aviaries* ssp. *aviaries*, *L. salivarius* ssp. *salicinus*, *L. salivarius* ssp. *salivarius*, *L. hilgardii*, and *L. panis*. From species-specific results, the typical bands for *L. fermentum* of the three selected strains were observed. Hence, it was considered that the selected strains no. 2311, 3007, and 3010 are *L. fermentum*.

### **Cell surface hydrophobicity**

The ability to adhere can give information about the possibility of probiotics to colonize and may modulate the host immune system. Several mechanisms were reported about the adhesion of microorganisms to intestinal epithelial cells [22]. Cell hydrophobicity is one of factors that may contribute to adhesion of bacterial cells to host tissues [23]. This property could indicate an advantage and importance for bacterial maintenance in the human gastrointestinal tract [24]. In this study, the in vitro determination of microbial adhesion to hexadecane droplets was carried out. This method was reported to be qualitatively valid to estimate the ability of a strain to adhere to epithelial cells [25]. The results revealed that the highest value of 68.7% hydrophobicity was found for the strain no. 3007 followed by 53.71% in the isolate no. 3010 as shown in Tab. 4. It is interesting that both strains were from fermented pork origin. The isolate no. 2311 isolated from fermented tea leaves showed a very low hydrophobicity value (20.9%). This indicates the influence of food source on the potential of bacterial adhesion to gut epithelial cells of human intestine.

**Tab. 4.** Hydrophobicity of the selected *Lactobacillus fermentum* strains.

Isolate no.	Food origins	Hydrophobicity <sup>a</sup> (%)
2311	Fermented tea leaves	20.9 ± 1.6
3007	Fermented pork	68.7 ± 6.2
3010	Fermented pork	53.7 ± 8.2

<sup>a</sup> mean ± standard deviation., n=3

**Tab. 5.** Antimicrobial activity of the selected *Lactobacillus fermentum* strains.

Strain	Inhibition zone (mm) of indicator strains <sup>a</sup>		
	<i>S. aureus</i> TISTR 029	<i>E. coli</i> TISTR 780	<i>S. typhi</i> DMST 5784
2311	5.43 ± 2.15	7.30 ± 1.59	6.70 ± 1.60
3007	4.67 ± 1.35	4.97 ± 1.31	5.90 ± 0.69
3010	6.30 ± 0.83	6.87 ± 0.87	7.33 ± 2.31

<sup>a</sup> mean ± standard deviation, n=4

### Detection of antibacterial activity

The good probiotics should present their antimicrobial actions particularly to the pathogens in the GI system. In this study, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* were used as the test bacteria because they are occasionally found as food borne microorganisms that might cause gastroenteritis. The results revealed that the antibacterial activity of the three selected lactobacilli could inhibit all test pathogenic bacteria however at different inhibition levels as shown in Tab. 5. The isolate no. 3010 showed the most antibacterial potency to *S. aureus* and *S. typhi* whereas the isolate no. 2311 demonstrated the highest potency to *E. coli*. The production of organic acid and hydrogen peroxide by lactobacilli was reported to inhibit both gram positive and gram negative bacteria, whereas bacteriocin affects only the growth of gram positive bacteria [26]. The difference in

inhibition potential among the three selected strains was considered to be due to the different intrinsic factors induced by different food origins.

### ***Antibiotic susceptibility***

Antibiotic susceptibility of lactobacilli is one of the crucial criteria for the safety point of view of potential probiotics since bacteria used as probiotics may serve as host of antibiotic resistance genes, which can be transferred to pathogenic bacteria. The isolates no. 2311 and 3010 showed resistance to gentamycin and linezolid whereas isolate no. 3007 was susceptible to gentamicin but resistant to linezolid as shown in Tab. 6. All tested isolates were susceptible to the protein synthesis inhibitors chloramphenicol, quinipristin, erythromycin, kanamycin, rifampicin, streptomycin and tetracycline since the MIC to the antibiotics was lower than the breakpoints. Fons et al. [27] reported the erythromycin resistance of *L. fermentum* isolated from human gut whereas Ahn et al. [28] reported the resistance to chloramphenicol of *Lactobacillus* spp. isolated from dairy origin. These reports in addition with our results demonstrated the effect of food origin on properties of bacteria. The difference in antibiotic susceptibility of *L. fermentum* from different food origins indicated the role of bacterial sources which might influence the bacteria in the genetic level of antibiotic resistance. For trimethoprim, the isolate no. 2311 and 3010 showed resistance, whereas isolate no. 3007 was susceptible to this drug. Trimethoprim inhibits the synthesis of folic acid which is necessary for the synthesis of purines, essential substance in bacterial nucleic acid. Resistance of strain no.2311 and 3010 to trimethoprim was considered to be due to a trimethoprim-insensitive dehydrofolate reductase [29]. The results to a cell wall synthesis inhibitor showed that the strain no. 2311 and 3010 were susceptible to ampicillin whereas the strain no. 3007 was resistant to this drug. All selected isolates were resistant to ciprofloxacin and vancomycin. Resistance to ampicillin, ciprofloxacin, and vancomycin are commonly found in the genus *Lactobacillus*. The high frequency of vancomycin resistance found among lactobacilli might not pose a problem as this type of vancomycin resistance is different from the inducible,

transferable mechanism observed in enterococci [30]. Moreover, the absence of resistance transfer genes, *vanA*, *vanB*, or *vanC1-3* in *L. fermentum* was previously reported [31]. Therefore, all three selected strains of *L. fermentum* in our study are considered as safe.

**Tab. 6.** Antibiotic susceptibility of the selected strains.

Antibiotics (breakpoint <sup>a</sup> µg/ml)	MIC (µg/ml)		
	2311	3007	3010
ampicillin (2)	<1	2	<1
chloramphenicol (16)	<8	<8	<8
ciprofloxacin (4)	4	8	4
quinipristin (4)	<2	<2	<2
erythromycin (4)	<0.5	<0.5	<0.5
gentamycin (1)	1	<0.5	1
kanamycin (32)	<16	<16	<16
linezolid (4)	4	4	4
rifampicin (32)	1	1	1
streptomycin (16)	<8	<8	<8
tetracycline (16)	<2	<2	<2
trimethoprim (32)	32	<16	32
vancomycin (4)	>8	>8	8

<sup>a</sup> The breakpoints for *Lactobacillus* sp. by SCAN category.  
Strain with MIC equal to or higher than the breakpoint is considered as resistant.

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