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Determination of Antimicrobial Effects of Probiotic Lactic Acid Bacteria and Garlic Extract Against Some Foodborn Pathogenic Bacteria

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ABSTRACT

In this study, it was investigated that the inhibition effect of some lactic acid bacteria (*Lactobacillus acidophilus* NCC68, *Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* (Ezal, commercial starter cultures)) which possessed with probiotic characteristics, against *Bacillus cereus*, *Salmonella* Enteritidis, *Escherichia coli*, *Escherichia coli* 0157:H7 ATCC 35150 and *Staphylococcus aureus* ATCC 25923. Besides, the inhibitory effect of probiotic cultures which used with meat and meat product additives that garlic extract over the antagonistic effects of sensitive pathogens were investigated *in vitro*. Consequently, the whole of lactic acid bacteria and garlic extract which were used in this study, showed inhibition effects against all selected pathogenic bacteria. *Staphylococcus aureus* ATCC 25923 was determined as the most sensitive pathogenic bacteria while *Bacillus cereus* was the most resistant bacteria against lactic acid bacteria and garlic extract. There was a distinctive increase in inhibition effects were observed by used of a combination with lactic acid bacteria and garlic extract.

Introduction

During the recent years alternative and efficient compounds for food preservation aimed at a partial or total replacement of antimicrobial chemical additives have been studied (Ranjan, 2012). Natural foods without chemical preservatives have been preferred by health-conscious consumers that will fit in their healthy lifestyle and this situation led to increase the interest in food preservation. Biopreservation is the use of natural microbiota or antimicrobials as a way of preserving food and extending its shelf life. The biopreservation of food, especially utilizing lactic acid bacteria (LAB) or herbal extract such as garlic that are inhibitory to food spoilage microbes has been practiced since early ages (Cizeikene, 2013; Reis, 2012).

Biopreservation using lactic acid bacteria (LAB) improves food safety. The antimicrobial properties of LAB are mainly related to the production of antimicrobial active metabolites such as organic acids (mainly lactic and acetic acid), hydrogen peroxide and also other compounds, such as bacteriocins and antifungal peptides (Hayaloğlu and Erginkaya, 2001; Reis, 2012). LAB has the ability to inhibit spoilage and foodborne pathogenic bacteria. As a matter of fact LAB exhibit a broad antimicrobial activity against species of *A. hydrophila*, *S. aureus*, *E.coli* and *L. monocytogenes*, *Proteus*, *Salmonella*, *Bacillus* and *Streptococcus* (Reis, 2012; Ghanbari, 2013; Siroli 2015; Argues, 2015).

Herbs have been used for many centuries by various cultures in food preservation and in the treatment of clinical ailments (Kumar, 2014). Especially garlic and its extract have been used for centuries in various countries to treat infectious disease. Garlic can be more effective as a broad-spectrum antibiotic compared with conventional antibiotics (Li, 2015). The antimicrobial activity and other therapeutic benefits of garlic have been widely recognized. The antimicrobial activity of garlic has long been recognized, with allicin, other thiosulfinates, and their transformation products having antimicrobial activity (Kyung, 2012). Garlic extracts exhibited activity against both Gram negative (*E. coli*, *Salmonella* spp. *and Citrobacter* spp., *Enterobacter* spp., *Pseudomonas* spp., *Helicobacter pylori*, *Klebsiella* spp.) and Gram positive (*S. aureus*, *S. pneumoni*, *Group A Streptococcus* spp., *Clostridium botulinum*, *Mycobacterium* spp. and *Bacillus anthrax*) (Daka, 2011; Mukhtar, 2012; Casella, 2013, Gulfraz, 2014).

E. coli O157:H7 is one of hundreds of strains of the bacterium *Escherichia coli.* Although most strains of *E. coli* are harmless, this strain produces a powerful toxin that can cause severe illness (Oz et al., 2002; Boyce et al., 1995). *Staphylococcus aureus* is responsible of food poisoning incidents in many types of food, including fermented sausages. Under suitable conditions, *S. aureus* can multiply and produce toxin during the initial stage of

fermentation (Sameshima et al., 1998). *Bacillus cereu*s is a well-known food poisoning organism that may cause illness through the production of either an emetic (vomit-inducing) toxin or at least three diarrheal toxins (enterotoxins) (Granum, 2001). In addition to, this strain is known to be responsible for a variety of spoilage problems in many processed foods and dairy products (Meer et al., 1991; Andersson et al., 1995; Larsen and Jørgensen, 1997; Mayr et al., 1999). *Salmonella* Enteritidis have been implicated in approximately 50% of the foodborne salmonellosis outbreaks in the United States. Many outbreaks are caused by *S.* Enteritidis-contaminated shell eggs, including eggs used in such traditional recipes as eggnog and Caesar salad (Pascual et al., 1999).

In this study, it was aimed to determine *in vitro* antimicrobial activity of probiotic lactic acid bacteria, garlic extract and their combination against some important foodborne pathogenic bacteria such as *E. coli*, *E. coli* O157:H7 ATCC 35150, *B. cereus*, *S. aureus* ATCC 25923, *S.* Enteritidis.

Materials and Methods

Microorganisms

In this study, as probiotic cultures, *Lactobacillus casei* Shirota, *Lactobacillus acidophilus* NCC68 and *Lactobacillus rhamnosus* (Ezal, commercial starter cultures) were used. Also *E. coli*, *E.coli* O157:H7 ATCC 35150, *B. cereus*, *S.* Enteritidis and *S. aureus* ATCC 25923 were used. The lactic acid bacteria and pathogen strains were grown respectively in MRS broth (Merck) at 30°C, for 48h and in BHI broth (Merck) at 37ºC for 24h. These cultures were kept as frozen stock cultures at -20ºC and propagated twice before using in inhibition assays.

Preparation of Garlic Extract

The fresh garlic was purchased from the local market. In order to obtain the garlic's extracts, 100 g of the cleaned garlic were sterilized using ethanol. The ethanol was allowed to evaporate in a sterile laminar flow chamber, and the garlic was homogenized aseptically using a sterile mortar and pestle. The extract was aseptically squeezed out using sterile cheesecloth. (Indu et al., 2006).

Preparation of Lactic Acid Bacteria Supernatant

LAB colony cells were harvested anaerobically at 30°C for 24 h in MRS (de Man, Rogosa and Sharpe) broth until the cultures reached about 10^8 CFU/ml and cell free supernatant from each LAB strain were prepared by centrifugation (Sepatech Labofuge 200, Heraeus, Germany) at 5000 ×g for 15 min. The pellet was washed twice with sterile physiological saline solution (0.85% NaCl) and resuspended in 5 ml of the same solution. The supernatants of the lactic acid bacteria cultures were filtered through 0.22µm pore-size filters (Schleicher & Sehuell, Germany) (Nieto-Lozana et al., 2002; Schillinger et al., 1989).

Determination of Inhibitory Activity

The inhibitory activities of garlic extract and supernatants were determined by using agar well diffusion analysis. For the agar well diffusion assay 1 ml of the pathogenic microorganisms (with approximately 2.9×10^8 cfu/ml) were inoculated in BHI Broth for 24h at 37ºC and transferred into BHI Agar (15 ml). The resultant mixture was poured into a Petri dish. After the solidification of the agar, 3 mm diameter wells were cut and 100µl garlic extract, supernatants and their combinations were placed into the wells. The plates were incubated at 37ºC for 24h and checked for inhibition zones (Gonzales et al., 2006). As a control, sterile BHI Broth, which does not contain any microorganism culture, was placed into the one well. All tests were performed in triplicate.

For determination of the inhibitory effect in liquid medium against foodborne pathogenic bacteria, all pathogenic bacteria (10^7 cells/ml) were planted to 10ml BHI Broth and 1 ml lactic acid bacteria supernatant with garlic extract were added to this culture and were left incubation at 37°C for 24 hours. At the end of the incubation, culture was planted to Nutrient Agar by spread method from appropriate dilution and colony counts were performed after incubation at 37ºC for 24 hours (Nieto-Lozano et al., 2002).

Statistical Analysis

Variance analysis, ANOVA was performed to evaluate the effect of the inhibitory on the samples and Duncan test was used to compare the means. In all cases the significance level considered was 1% (Del Castillo et al., 2016).

Results and Discussions

In our study, inhibitory effects of three different probiotic lactic acid bacterial strains (*L. rhamnosus*, *L. acidophilus*, *L. casei* Shirota) and garlic extract were determined by the agar diffusion method and viable cell counts method against food borne pathogen bacteria such as *B. cereus*, *S.* Enteritidis, *E. coli*, *Escherichia coli* 0157: H7, *S. aureus*. All results are evaluated statistically.

The inhibitory effect of probiotic lactic acid bacteria, garlic extract and their combination determined by agar well diffusion method against all tested food borne pathogen bacteria and the size of inhibition zones measured (Table 1).

As shown in Table 1 all lactic acid bacteria species showed antimicrobial effect against selected pathogenic bacteria. The antimicrobial effect differences of *L. rhamnosus* against all pathogens were significant statistically ($P \leq 0.01$). *S. aureus* was most susceptible strains against *L. rhamnosus* and the most resistant bacteria have been identified as *E. coli* and *E. coli* O157: H7. Similarly, *L. asidophilus* has an antimicrobial effect against all selected pathogens ($P \leq 0.01$). *S. aureus* was most susceptible strains against *L. acidophilus* and the most resistant bacteria have been identified as *B. cereus*. *L. casei* Shirota has an antimicrobial effect against all

pathogens, but it showed the weak inhibitory effect compared to other lactic acid bacteria. *S. aureus* was most susceptible strains against *L. casei* Shirota and the most resistant bacteria have been identified as *B. cereus.* Similar to our study, Sameshi et al., (1998) researched of lactic acid bacteria's effect against *S. aureus* growth and production of enterotoxin at 20°C and 30°C fermentation temperature. Researchers found that *L. rhamnosus* FERM P-15120 and *L. paracasei subsp. paracasei* FERM P-15121 inhibits *S. aureus* growth and production of enterotoxin. Chuayana et al. (2003) have investigated the antibacterial activity of isolated from probiotics in dairy products isolated from probiotics in dairy products *S. aureus*, *E. coli*, *P. aeruginosa*, *S.* Typhi and *S. marcescens* and they reported that *L. casei* have shown a bacteriostatic effect against these pathogens. Similarly, Aslım et al. (2005) reported that *L. acidophilus* has shown antibacterial activity on *S. aureus*, *E. coli* and *Y. enterocolitica* in their study.

Garlic extract showed a stronger inhibitory effect against all pathogenic bacteria than the lactic acid bacteria. The most sensitive bacteria have been determined as *S. aureus* of the 26 mm diameter zone. *S. aureus* followed by *S.* Enteritidis. The results are very close to each other for *E. coli* O157:H7 and *B. cereus* when analyzed inhibition effect. The differences between the inhibitory effect of *L. rhamnosus* and garlic extract combination against pathogenic bacteria was significant by statistically (P≤0.01). The combination of *L. rhamnosus* and garlic extracts (1:1 v/v) showed the inhibitory effect against all tested pathogenic bacteria. The most sensitive bacteria were identified as *S. aureus* with 30 mm zone diameter. Similarly, *L. acidophilus* and garlic extract combination (1:1 v /v) have shown the inhibition effect against all tested pathogenic bacteria and the differences between the inhibitory effect was significant by statistically (P≤0.01). It was determined that *S. aureus* were the most susceptible strains, *B. cereus* was the most resistant bacteria against *L. acidophilus* and garlic extracts combination. Also, the differences between the inhibitory effect of *L. casei* Shirota and garlic extract combination against pathogenic bacteria was significant by statistically (P≤0.01). The combination of *L. casei* Shirota and garlic extracts (1:1 v/v) showed the inhibitory effect against all tested pathogenic bacteria. And also it has been observed that the most susceptible bacteria was *S. aureus.*

Table 1 The inhibition zone diameters of probiotic lactic acid bacteria and garlic extract against different foodborne pathogenic bacteria (mm)

	Zone diameter (mm)*				
Antimicrobial	*B. cereus*	*S.* Enteriditis	*E.coli*	*E.coli* O157:H7	*S. aureus*
L. rhamnosus	9.6 ± 0.5774^{Ba}	10.6 ± 0.5774^{Cb}	9 ± 0.0000^{Aa}	9 ± 0.0000^{Aa}	12 ± 1.0000^{Dc}
L. acidophilus	9.3 ± 0.5774^{Aa}	10 ± 0.0000^{Bb}	10.3 ± 0.5774^{Cb}	10 ± 1.0000^{Bb}	10.6 ± 1.1547^{Db}
L. casei Shirota	8 ± 0.0000^{Aa}	9.3 ± 0.5774^{Cb}	8.3 ± 0.5774^{Ba}	8.3 ± 0.5774^{Ba}	9.6 ± 0.5774^{Db}
Garlic Extract	15 ± 1.000^{Aa}	16 ± 1.000^{Cb}	15.3 ± 0.5776^{Ba}	15.3 ± 1.1547^{Ba}	26 ± 1.0000^{Dc}
L. rhamnosus+G.E.	15.6 ± 0.5774^{Aa}	16.3 ± 0.5774^{Bb}	16.3 ± 1.1547^{Bb}	15.6 ± 0.5774^{Aa}	30 ± 1.0000^{Cc}
L. acidophilus+G.E.	15.6 ± 0.5774^{Aa}	18 ± 0.0000^{Dd}	17.3 ± 0.5774^{Cc}	16.3 ± 0.5776^{Bb}	31.6 ± 0.5774^{Ee}
L. casei Shirota+G.E.	15.6 ± 0.5776^{Aa}	17.3 ± 1.1547^{Dc}	16.6 ± 0.5774^{Cb}	16 ± 1.0000^{Bb}	29.3 ± 0.5774^{Ed}

* The mean values and standard error, ** A-E: The values are sorted from small to large; a-d: The differences between the values shown in different letters are significant statistically (P≤0.01)

Table 2 The inhibitory effect of lactic acid bacteria supernatants against pathogenic bacteria in liquid medium (KOB log/ml)

Pathogenic bacteria		Lactic acid bacteria*		
		L. rhamnosus	*L. acidophilus*	*L. casei* Shirota
B. cereus	Control (initial)	8.17	8.17	8.07
	Control (24 h)	8.77	8.96	8.23
	Counting (24 h)	8.57	8.43	8.11
S. Enteriditis	Control (initial)	8.34	8.23	8.44
	Control (24 h)	8.73	8.89	8.30
	Counting (24 h)	8.04	8.07	8.07
E. coli	Control (initial)	8.51	8.50	8.43
	Control (24 h)	9.00	8.63	8.68
	Counting (24 h)	8.41	8.36	8.17
E. coli O157:H7	Control (initial)	8.36	8.43	8.34
	Control (24 h)	9.00	8.77	8.65
	Counting (24 h)	8.14	8.32	7.95
S. aureus	Control (initial)	8.11	8.69	8.69
	Control (24 h)	8.20	8.14	8.20
	Counting (24 h)	6.76	8.00	8.23

* The mean values (KOB log/ml)

Table 3 The inhibitory effect of garlic extract against pathogenic bacteria in liquid medium (KOB log/ml)

Pathogenic bacteria		Garlic extract*
B. cereus	Control (initial)	8.04
	Control (24. h)	8.11
	Counting (24. h)	7.84
S. Enteriditis	Control (initial)	8.83
	Control (24. h)	>9.00
	Counting (24. h)	8.64
E. coli	Control (initial)	8.32
	Control (24. h)	>9.00
	Counting (24. h)	7.00
E. coli O157:H7	Control (initial)	8.27
	Control (24. h)	8.55
	Counting (24. h)	5.39
S. aureus	Control (initial)	8.55
	Control (24. h)	8.57
	Counting (24. h)	4.17

*The mean values (KOB log/ml)

Table 4 The inhibitory effect of lactic acid bacteria supernatants and garlic extract combination against pathogenic bacteria in liquid medium (KOB log/ml)

Pathogenic bacteria	Lactic acid bacteria + Garlic Extract*			
		L. rhamnosus+ G.E.	*L. acidophilus*+ G. E.	*L. casei* Shirota + G.E.
B. cereus	Control (initial)	8.53	8.53	8.53
	Control (24. h)	9.00	9.00	9.00
	Counting (24. h)	8.30	8.27	8.32
S. Enteriditis	Control (initial)	8.46	8.46	8.46
	Control (24. h)	8.79	8.79	8.79
	Counting (24. h)	4.00	4.30	4.00
E. coli	Control (initial)	8.30	8.30	8.30
	Control (24. h)	9.00	9.00	9.00
	Counting (24. h)	6.84	5.04	5.77
E. coli O157:H7	Control (initial)	8.17	8.17	8.17
	Control (24. h)	8.74	8.74	8.74
	Counting (24. h)	5.25	5.49	6.04
S. aureus	Control (initial)	8.89	8.89	8.89
	Control (24. h)	8.34	8.34	8.34
	Counting (24. h)	4.85	5.90	5.92

* The mean values (KOB log/ml)

The inhibitory effect of lactic acid bacteria supernatants on pathogenic bacteria in liquid medium has shown in Table 2. As shown in Table 2. when initial alive cell count of *S. aureus* was 8.11 log cfu/ml the number of viable cells are fallen to 6.76 log cfu/ml after 24 h of incubation in the sample was added *L. rhamnosus* supernatant. When considering to the inhibitory effect of *L. acidophilus* supernatant in a liquid medium on *S. aureus*, initial alive cell count of *S. aureus* was 8.69 log cfu/ ml the number of viable cells are fallen to 8.00 log cfu/ml after 24 h of incubation. When evaluated on inhibition effect on pathogen bacteria of *L. acidophilus* supernant after 24 hour incubation at broth medium, the most resistant bacteria is *B. cereus* similarly well diffusion method results. In a study investigating the inhibitory effect of lactic acid fermentation on *B. cereus* growth and sporulation, it has been reported L. acidophilus has no effect on *B. cereus* sporulation and the sport remains alive (Rossland et al., 2005). When evaluated on inhibition effect on pathogen bacteria of *L. casei* Shirota supernant after 24 hour incubation at broth medium, when the most resistant bacteria is *B. cereus* similarly, it was shown a similar inhibition effect on well diffusion method results *E. coli* and *E. coli* O157: H7.

When considering the inhibition effect of garlic extract in the liquid medium, the number of viable cells of *S. aureus* is 8.55 log kob/ml initially, but the number of viable cells of *S. aureus* decreased to 4.17 log kob/ml while adding garlic extract substituted after 24 h incubation (Table 3).

The resistant bacteria have been identified as *B. cereus* like agar well diffusion method when assessing the inhibition effect of garlic extract on pathogenic bacteria after 24 h incubation. Kumar and Berwal (1998), in a similar study, demonstrated that 5% ratio of garlic extract using against *S. aureus* has shown an inhibition effect 80% success. Also, 10% ratio of garlic extract using for inhibition of *S.* Typhi and *E. coli* has shown a success rate of 90% success when the ratio of inhibition success against *L. monocytogenes* has demonstrated 85%.

Fig 1 The inhibition effect of *Lactobacillus rhamnosus* against *Staphylococcus aureus* by agar well diffusion method (K: Control)

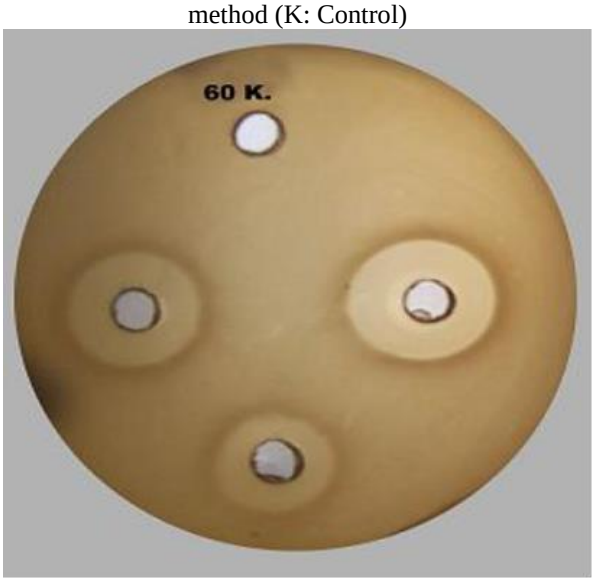

Fig 2 The inhibition effect of garlic extract against *Staphylococcus aureus* by agar well diffusion method (K: Control)

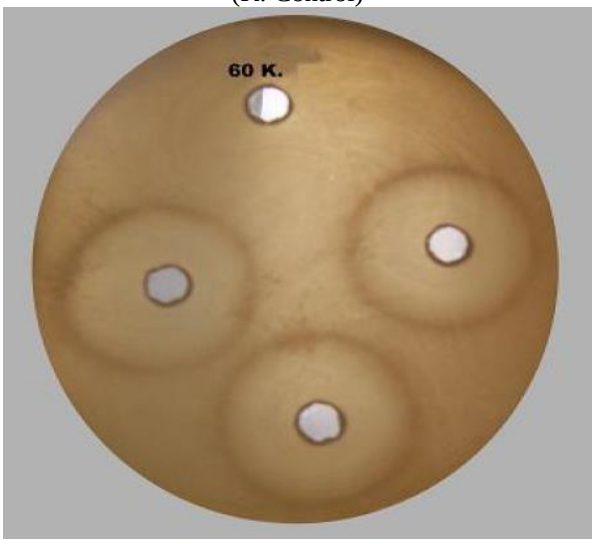

Fig 3 The inhibition effect of *Lactobacillus rhamnosus* and garlic extract combination against *Staphylococcus aureus* by agar well difussion method (K: Control)

In another study, researchers have reported that garlic extract have shown an effect against *S. epidermidis* after 1 hour incubation with the rate of 93%. Similarly, garlic extract has shown a bactericidal effect against *S.* Typhi after 3 hour incubation (Arora and Kaur, 1999). Benkeblia (2004) has researched that the inhibition effect of garlic extract with different ratio against *S.* Enteritidis. The researcher reported that garlic extract has an inhibition effect all tested concentrations and red onion and garlic extracts have shown a very strong inhibitory effect against *S.* Enteritidis.

As shown in Table 4. *L. acidophilus* and garlic extract showed the maximum inhibition effect against *B. cereus* between other lactic acid bacteria, whereas *L. casei* Shirota showed the weakest inhibition effect. Similarly, *L. acidophilus* and garlic extract showed the maximum inhibition effect against *E. coli*, whereas *L. rhamnosus* and garlic extract showed the weakest inhibitory effect. Also *L. rhamnosus* and garlic extract showed the maximum inhibition effect against *E. coli* O157:H7, whereas *L. casei* Shirota and garlic extract showed the weakest inhibitory effect. *L. rhamnosus* and garlic extract showed the most inhibitory effect against *S. aureus*, whereas *L. casei* Shirota and garlic extract showed the weakest inhibitory effect. A significant increase is concerned about antimicrobial effect by using garlic extract and lactic acid bacteria as a combination of all strains. *S. aureus* was determined as the most sensitive bacteria against which using in our study lactic acid bacteria and garlic extract combination (Fig. 1, Fig. 2 and Fig. 3)

Other studies are reported that lactic acid bacteria have more inhibition effect against Gram positive bacteria than Gram negative bacteria (Sobrino the ark., 1991; Makras and De Vuyst; 2006). On the other hand it is stated that Gram negative bacteria came to the vulnerable against bacteriocins when deteriorated of the integrity of the outer membrane of bacteria (Philiphs and Duggan, 2001). Also, it is described that using nisin with combination garlic extract increase to the antimicrobial effect of Nisin (Singh et al., 2001). *B. cereus* has been identified as resistant bacteria against the combination lactic acid bacteria and garlic extract which using in our research. Rossland et al. (2003; 2005) reported that the inhibitory effect of lactic acid bacteria against *B. cereus* depending on the amount of acid produced is due to the pH change. And it also described that in same study *B. cereus* retains viability by forming endospores at low rate of pH change (Rossland et al., 2003; Rossland et al., 2005).

Conclusions

According to the results of this study, *L. rhamnosus* showed the maximum inhibition effect against *B. cereus* and *S.* Enteritidis, and also observed that *L. casei* Shirota showed the weak effect. *L. acidophilus* showed the maximum inhibition effect against *E. coli* and *E. coli* 0157:H7 whereas *L. casei* Shirota showed the weak antimicrobial effect. And *L. rhamnosus* showed the

maximum inhibition effect against *S. aureus* whereas *L. casei* Shirota showed the weak antimicrobial effect in all tested lactic acid bacteria. According to the obtained results, it was observed that *S. aureus* was the most sensitive bacteria and *B. cereus* was the most resistant bacteria. However, it was determined that *L. rhamnosus*, the lactic acid bacteria used in this study, have the strong inhibition effect whereas *L. casei* Shirota have the weakest effect.

Consequently, this study must be expanded and replicated with other pathogenic bacteria as known Gram positive and Gram negative. In addition, it should be investigated whether lactic acid bacteria show the inhibitory effect against yeast and fungi which cause problems in foods. It should be investigated that usage possibilities of probiotic lactic acid bacteria in meat and meat product in future studies.

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