Inhibitory Potential of Metabolites from *Lactobacillus casei* against Phenotypic Characteristics of *Salmonella typhimurium* LT2

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ABSTRACT

Background and Objective: Several studies have shown the antimicrobial activity of lactobacilli against *Salmonella enterica* (serotype *typhimurium*). The aim of this study was to evaluate the inhibitory potential of metabolites produced by probiotic culture of *Lactobacillus casei* against *S. typhimurium* and its impact on *S. typhimurium* motility and biofilm formation.

Methods: In order to evaluate the impact of the metabolites, *L. casei* cell-free culture supernatant (CFCS) was collected by centrifugation of *L. casei* secondary cultures. Effectiveness of the CFCS against Salmonella was evaluated by the well-diffusion method. In addition, in vitro effect of this treatment on motility in Swarm agar and biofilm formation by the bacteria was investigated.

Results: Inhibition zone diameters of *S. typhimurium* were 0.83 and 12.1 mm at concentrations of 50 and 100 µl of Lactobacillus CFCS against the log4 of *S. typhimurium*, respectively. Moreover, CFCS treatment inhibited the motility and biofilm formation by Salmonella. Concentrations of 5% and 10% were determined as the minimum inhibitory concentrations for motility and biofilm formation by *S. typhimurium*. Furthermore, effectiveness of the CFCS against Salmonella was dose-dependent ($P<0.05$).

Conclusion: *L. casei* CFCS is able to inhibit the growth, motility and biofilm formation in *S. typhimurium*.

Keywords: Anti-Bacterial, *Lactobacillus Casei* Metabolites, Phenotypic Characteristics of *S. typhimurium*

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INTRODUCTION

Several physiological activities in gram-positive and gram-negative bacteria are regulated by cellular signals. These processes may include symbiosis, competition, conjugation, antibiotic production, motility, sporulation and biofilm formation that provide necessary defensive strategies for pathogens against the environment and hosts' immune system (1, 2). In this regard, adhesion of microbes to surfaces and the subsequent biofilm formation have been reported in different environmental conditions. Biofilm formation provides a protective mode for microorganisms in unfavorable conditions, allowing them to survive under adverse environmental conditions. However, they exhibit significantly different patterns of behavioral and physiological status compared to the free-living planktonic state. In the dairy industry, biofilms are important sources of contamination that cause food spoilage and many related public health problems such as outbreaks of foodborne illnesses. Elimination of biofilms is very difficult due to their resistant phenotype. Conventional methods of washing and disinfection may be ineffective in controlling biofilms and increase and spread bacterial resistance. As a result, new control methods such as the use of biosolutions including enzymes, phages, interspecies interactions and molecular antimicrobial compounds of microbial origin are continuously emerging (3). Biofilm cells may be released into the environment, establish on other surfaces, and act as a secondary source of contamination (4). Decreased plate heat exchanger due to increase in thickness of heat exchanger is another disadvantage of biofilms (5). On the other hand, the biological and chemical reactions of biofilm cause abrasion in metal pipes and tankers. Due to fewer adverse effects on the quality of products, physical methods have recently received a lot of attention instead of thermal methods such as pasteurization. One of these systems is the use of filtration membranes for separation of constituents and pathogens in milk that notably affects the incidence, spread of biofilms and efficiency of the filtration system, and imposes huge losses (5-7). *Salmonella enterica* is an intestinal biofilm forming pathogen that has been associated with numerous cases of foodborne infections worldwide. Controlling this bacterium has always been a challenge in food safety and public health (8, 9). *S. enterica* is able to form biofilm on various materials such as plastic, rubber, glass, stainless steel, plants and epithelial cells that create resistance and stability against host and non-host environments (10). Biofilm formation on materials commonly used in production of food processing devices and equipment (plastic and stainless steel) leads to unfavorable consequences in the food industry. The biofilm of pathogenic bacteria such as *S. enterica* on food processing equipment or food contact surfaces acts as a constant source of contamination and endangers the safety of foodstuff and human health (11-13). Thus far, the antimicrobial and anti-biofilm activity of probiotic metabolites against *Listeria monocytogenes*, Salmonella, Staphylococcus, *Escherichia coli* O157:H7, Aspergillus and Candida have been demonstrated in several studies (14-17). The present study evaluated the antibacterial potential of cell-free culture supernatant (CFCS) of *Lactobacillus casei*, and its impact on the phenotypic characteristics of *Salmonella typhimurium*.

MATERIAL AND METHODS

Strains of *S. typhimurium* (ATCC700720) and *L. casei* (ATCC39392) were obtained from microbial collection of School of Veterinary Medicine at Urmia University. All strains were kept in suitable media containing glycerol (25%) at -80 °C. To obtain fresh cultures, lactobacilli and Salmonella strains were cultured twice in MRS broth (Merck) and Luria-Bertani (LB) broth at 37 °C, respectively. Swarm agar medium containing 1% tryptone, 0.5% salt and 0.5% agar was used to evaluate motility.

For preparation of the CFCS, secondary culture of *L. casei* was centrifuged at 10,000 rpm at 4 °C for 10 minutes (Sigma 3K30 Laboratory centrifuges, Germany). After isolation, the resulting supernatant was passed through 0.22-micron sterile filters, and then stored at 4 °C until use (18). In addition, a sample from the resulting supernatant was investigated by autoclaving at 120 °C under 15 bars of pressure to evaluate the effectiveness of induced conditions. Bactericidal activity of *L. casei* metabolites against *S. typhimurium* was evaluated by well- diffusion testing. For this purpose, secondary culture of *S. typhimurium* was grown on the surface of

Mueller-Hinton agar. Then, wells were created on the surface of culture medium under sterile conditions. Later, different levels of *L. casei* CFCS were poured into the wells. After half an hour, when the CFCS was absorbed into the agar, the plates were incubated upside down for 24 hours under aerobic conditions at 35 °C. Then, inhibition zone diameter was measured using a digital caliper. Recent single colonies of Salmonella grown on nutrient agar were transferred by a sterile rod-shaped object to swarm agar containing different concentrations of *L. casei* (v/v) CFCS. During the study, the plates were incubated in upward position at 30 °C. Motility was assessed by measuring the circular opaque areas formed because of bacteria migration from inoculated points toward the edges of the plate. Motility was defined as the ability to develop growth around the inoculation area and immotility was determined as lack of growth development on the medium (19). The Protocol of biofilm formation was performed according to the method described by Kim et al. Control Salmonella overnight cultures were washed twice with phosphate buffered saline. Approximately 10^6 CFU/ml were inoculated into the semi diluted LB broth. Different concentrations of *L. casei* CFCS were mixed with the above medium, and then incubated without stirring in polystyrene microplates for 48 hours at 30 °C. Then, they were thoroughly washed twice with sterile distilled water. A 0.1% crystal violet solution was used for staining the attached cells. After storing the plates at room temperature for 20 min, the crystal violet was discarded and the wells were washed three times by sterile distilled water. The dye absorbed by biofilms cells attached to the wall of wells were solved into 95% ethanol. Absorbance of the dye solution at 595 nm was read using Novaspec II spectrophotometer (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK) (19). All experiments were performed in triplicate. The GraphPad Prism software (Version 5.04, San Diego, CA, USA) was used for analysis of variance at significance level of $P \leq 0.05$.

RESULTS

The well-diffusion method was used to evaluate the effectiveness of *L. casei* against *S. typhimurium*. Table 1 shows the antibacterial effects of *L. casei* against *S. typhimurium* using the above method. It was found that 30 µl of *L. casei* CFCS did not have inhibitory effects on *S. typhimurium* (log2) in vitro. However, other concentrations were effective against *S. typhimurium* with a direct correlation between increase in the diameter of inhibition zone and increased concentration of the CFCS. Interaction plot showed a positive relationship between the diameter of inhibition zone and concentration of CFCS. Moreover, the correlation between high and low concentrations of CFCS was >97% (Figure 1). This indicates that increasing and decreasing the concentration of CFCS increased or decreased the pattern of inhibition zone diameter at a fixed ratio.

Table 1– Measurement of inhibition zone diameter (mm) for different concentrations of *S. typhimurium* under the influence of different levels of *L. casei* CFCS in the well-diffusion method

CFCS volume (µl)	Different doses of *S. typhimurium* based on log 10 (CFU/ml)		
	2	3	4
100	14.3±0.07a	13.2±0.05b	12.1±0.05c
50	0.94±0.14a	0.9±0.08a	0.83±0.06b
30	-*	-	-

***lack of inhibition zone**

Lowercase dissimilar letters indicate significant differences in each row (well diameter was in the range of 0.6 mm) (P≤0.05).

Figure 1- Correlation between growth inhibition diameters and various concentrations of *L. casei* CFCS

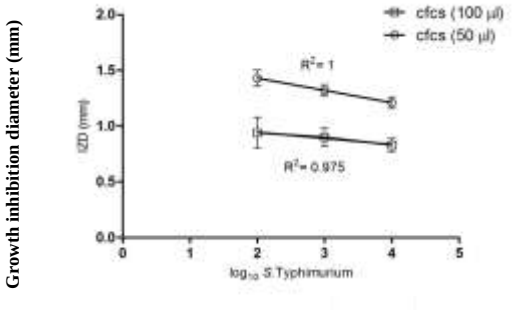
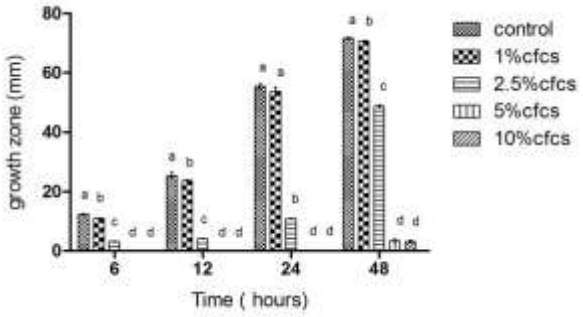
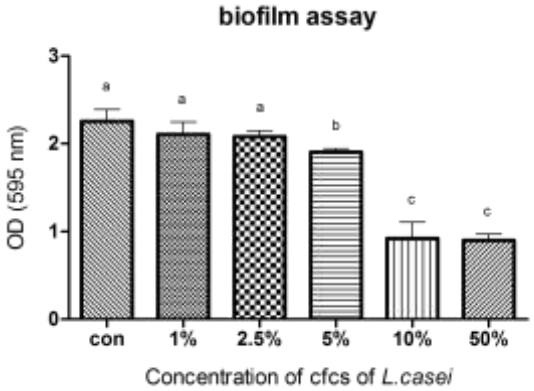


Figure 2- Interactive effects of different concentrations of non-autoclaved *L. casei* CFCS on motility of *S. typhimurium* in swarm agar at 30 °C



Letters a, b, c and d indicate significant differences at 95% confidence level of 95.

Figure 3– Effect of different concentrations of *L. casei* CFCS on biofilm formation by *S. typhimurium*



Letters a, b and c indicate significant differences at 95% confidence level.

The motility of Salmonella under the influence of 1% *L. casei* CFCS showed a clear significant difference with the control group (Figure 2). Similar to 5% CFCS, 10% CFCS significantly inhibited the motility capability until the end of the evaluation period. At concentration of 2.5%, motility of Salmonella were strongly influenced by the CFCS for up to 12 hours but after that, the motility of Salmonella had a slow upward trend for up to 24 hours. After this period, motility of Salmonella had a strong upward trend.

However, such trends were not observed for higher concentrations (5 and 10%) up to 24 hours. In the medium containing 5 and 10% CFCS, Salmonella recovery caused a 3.52 and 3.15 mm growth on the surface of swarm agar after 48 hours, which was significantly different from the 48.75 mm growth in medium containing 2.5% CFCS ($P<0.05$). Figure 3 demonstrates the equal effect of 10% and higher concentrations of CFCS on phenotypic characteristics of *S. typhimurium* biofilm. Compared with the low concentrations, the concentration of 10% can be considered as the cutoff point against Salmonella biofilm-formation ability. However, the impact of CFCS on motility and biofilm formation despite the heat and high humidity of autoclave indicates no significant difference between the two groups on the listed phenotypic characteristics of *S. typhimurium* (data not shown) ($P>0.05$).

DISCUSSION

The aim of this study was to evaluate the potential inhibitory activity of metabolites produced by probiotic cultures of *L. casei* against *S. typhimurium*, and its impact on phenotypic characteristics of Salmonella including motility and biofilm formation. Several studies have indicated that the inhibitory activity of *L. casei* against Salmonella is mainly due to increased concentration of organic acids and the

subsequent pH reduction (20). However, this does not mean that other antibacterial factors produced are not involved in the inhibition of Salmonella. Nevertheless, the presence of anti-bacterial compounds in the medium produced by lactobacilli (casein known as the *L. casei* bacteriocin) is not obvious (21). Bacteriocin production may also occur in the late logarithmic phase or during the stationary phase of bacterial growth. Stability of bacteriocins that often have a protein-lipopolysaccharide nature is influenced by environmental parameters such as acidic or alkaline conditions. The Ionic strength of the environment, presence of protective molecules and pH are also among factors affecting the stability of bacteriocins (22, 23). Bleicher et al. and Mariam reported that the antibacterial effect of probiotics CFCS is due to the non-protein and non-peroxide nature so that protease K and thermal treatment (100 °C for 1 hour) do not eliminate the anti-bacterial effects of CFCS, which is consistent with our study (17, 24). However, Hartmann showed that protease K treatment of supernatant from some lactic acid bacteria eliminates their anti-Listeria activity. They also demonstrated that boiling for 20 minutes and catalase treatment have little or no effect on the anti-Listeria activity (14). Tejero-Sariñena demonstrated decreased antagonistic effects of probiotic culture supernatant by neutralizing it using alkaline substances such NaOH, which is consistent with study of Maleki on the impact of probiotic culture supernatant's pH on microorganisms. Das demonstrated the effects of probiotic CFCS on Salmonella biofilm (15). However, according to Bouttefroy, Hartmann, Das and Mariam, these inhibitory effects were temporary, which is in agreement with our study on the case of CFCS against the motility of Salmonella (14, 15, 17, 26). These phenotypic factors were chosen because motility in bacteria plays an important role in the structure and morphology of biofilms. In addition, the shape and morphology of biofilms can influence the efficacy of clean in place and mechanical cleaning methods (27-30). Biofilm formation on surfaces in contact with food can act as a permanent reservoir for pathogenic and spoilage bacteria. This could lead to microbial contamination in food processing plants and cause critical problems for the public health and economy. Most biofilms are well protected against environmental stress (disinfectants) and therefore very difficult to remove. Although high resistance of bacteria within the biofilm structure has not been well demonstrated, several possibilities are thought to be associated including 1) reduced access of antimicrobial compounds to cells within the biofilm 2) interactions between biofilms and biocide molecules 3) environmental modulators 4) production of degradative enzymes 5) genetic exchange between cells, and 6) quorum sensing. Other possibilities have been also described for the occurrence of such resistances. For example, incompatibility of biofilm cells with biocides is another cause of biofilm resistance (31, 32). Marchand et al. stated that the interspecies interaction and cooperation, and presence of extracellular polymeric substances are among factors involved in resistance of biofilm cells and the occurrence of subsequent contaminations in processed dairy products (33).

According to some scientists, the frequency of gene transfer is higher in biofilm state than in planktonic state, which is very important in transfer of drug resistance and resistance to disinfectants (34, 35). However, several biological factors occur as emerging control strategies in the prevention of biofilm formation. For example, surfactant produced by *Bacillus subtilis* cause biofilm dispersion via cellular signals, without affecting the cell growth. It also prevents biofilm formation by microorganisms such as *S. enterica*, *Escherichia coli* and *Proteus mirabilis* (3, 36). The use of some other green strategies such as enzymes, phages, antimicrobial molecules of microbial origin and intergroup interactions can also be noted, which can be utilized for controlling and inhibiting the expression of phenotypic characteristics of microbes.

It seems that a cellular and genetic adaptation is gradually evolved and developed in motility of *S. typhimurium* at concentration of 2.5% Lactobacillus CFCS. However, concentration of 5% is suggested as the cutoff point for motility of *S. typhimurium*. Due to high dose

CONCLUSION

and transient effect of lactobacilli CFCS against the phenotypic characteristics of *S.*

typhimurium (50000ppm for motility and 10000 ppm for biofilm formation), the use of hybrid system and hurdle technology is recommended to control these factors and their incidence. In this regard, it is recommended to perform a study on the role of metabolites and possible casein production by *L. casei* against other pathogenic and spoilage bacteria.

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CONFLICT OF INTEREST

The authors declare no conflict of interest regarding this manuscript

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