

The Antimicrobial Effect of Lactobacillus Casei Culture Supernatant Against Multiple Drug Resistant Clinical Isolates of Shigella Sonnei and Shigella Flexneri in Vitro

Reza Mirnejad¹, Ali Reza Vahdati², Jamal Rashidiani³, Mohammad Erfani⁴, Vahhab Piranfar^{4,*}

¹ Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, IR Iran

² Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland

³ Nano Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, IR Iran

⁴ Department of Microbiology, Tonekabon Branch, Islamic Azad University of Tonekabon, Tonekabon, IR Iran

* Corresponding author: Vahhab Piranfar, Department of Microbiology, Tonekabon Branch, Islamic Azad University of Tonekabon, Tonekabon, IR Iran. Tel: +98-2182482558, Fax: +98-2188039883, E-mail: vahab.p@gmail.com

ABSTRACT

Backgrounds: Shigellosis remains an important public health problem in developing countries with *S. sonnei* and *S. flexneri* in US, Europe and in Asian countries being of importance.

Objectives: This study evaluates the protective effect of Lactobacillus casei cell-free culture supernatants (CFCS) against multiple drug resistance (MDR) clinical samples of Shigella sonnei and Shigella flexneri in vitro.

Materials and Methods: *S. sonnei* and *S. flexneri* was identified by common microbiological and serological methods. Antibiogram with 18 antibiotics were tested for 34 positive cultures by disc diffusion method. The Samples showed considerable resistance to antibiotics. Antimicrobial effects of CFCS were tested against *S. sonnei* and *S. flexneri* by agar-well assay and broth micro dilution methods. In addition, the antimicrobial activity remained active treatment after adjust pH 7, adding Proteinase K and heating for *L. casei*.

Results: The results implicate that *L. casei* strongly inhibits the development of pathogen samples. In contrast, via the disc diffusion method 4 out of 18 antibiogram have shown complete resistance against the pathogen samples. In addition, the natures of antimicrobial properties have been tested in different conditions such as various pH, temperature and presence of proteinase K. The MIC₅₀ (minimum inhibitory concentration) and MIC₉₀ of CFCS of *L. casei* were determined, for *S. sonnei* were 2.25 and 10.5, for *S. flexneri* were 5.25 and 5.25 respectively. The results have shown a significant resistance pattern by these four antibiotics in this case.

Conclusions: The data indicates that *L. casei* highly resistant against to antibiotics, heat, Proteinase K and so many activities against MDR Shigella pathogenic strains. *L. casei* is the best probiotics candidate.

Keywords: Lactobacillus Casei; Shigella Flexneri; Shigella Sonnei

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1. Background

Shigellosis remains an important public health problem in developing countries with *S. sonnei* and *S. flexneri* in US, Europe and in Asian countries being of importance

(1-4). Shigella is one of the most antimicrobial-resistant bacteria (3, 5) and an important cause of gastroenteritis-induced deaths in 3-5 million children aged less than five years in developing countries (6, 7). Shigella ranks the

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The results presented here provide an evidence for the fact that *L. casei* strongly inhibit the multiple drug resistance gastrointestinal pathogens *S. sonnei* and *S. flexneri*. In addition, all tested strains were found to possess desirable probiotic properties in vitro. *L. casei* good candidates for their application as novel probiotic strain.

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third among bacterial food borne pathogens (after *Campylobacter* and *Salmonella*) in the number of gastrointestinal cases according to the report of Centers for Disease Control and Prevention (8, 9). The emergence of multiple drug resistance to cost-effective antimicrobials against *Shigella* is a matter of concern in developing countries and resistant pattern of these bacteria is the cause of numerous clinical problems throughout the world. Increased resistance among pathogens causing nosocomial and community acquired infections is known to be related to the widespread utilization of antibiotics (10). Preparing the prevention and treatment protocols with natural patterns in this regard seems to be necessary (11). Recent reports have documented the role of *Lactobacillus* in prevention and treatment of some infections. *Lactobacillus* strains have commensally in the human body (12). Its beneficial effect may be associated to its ability to inhibit the growth of pathogens, apparently by the secretion of antibacterial substances including lactic acid, hydrogen peroxide and etc. (13) Now, the application of probiotics to the prevention and management of gastrointestinal disorders has received much interest (5). The antimicrobial activity of a wide range of pathogenic microorganisms by *Lactobacillus* either in vitro or in vivo has been reported, including *E. coli*, *Salmonella*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (1, 14-16). However, the antimicrobial activity against *S. flexneri* and *S. sonnei* has been little known. So the main objective of this study was to apply establishes in vitro tests to evaluate the nature of antimicrobial substances and antimicrobial properties of *L. casei* against multi-drug resistant clinical isolates of *S. flexneri* and *S. sonnei*. Furthermore, we evaluated the tolerance properties to confirm the tested *L. casei* supernatant could be potentially used as probiotic.

2. Objectives

This study evaluates the protective effect of *Lactobacillus casei* cell-free culture supernatants (CFCS) against multiple drug resistance (MDR) clinical samples of *Shigella sonnei* and *Shigella flexneri* in vitro.

3. Materials and Methods

3.1. Bacterial Strains and Culture Conditions

Clinical specimens (*S. flexneri* and *S. sonnei*) were collected from suspicious patients with clinical history of Shigellosis. Stool specimens were plated into plate *Shigella Salmonella* agar (Hi-Media, Mumbai, India) and deoxycholate citrate agar (DCA) (Hi-Media, Mumbai, India) as primary plates and a loop full also inoculated to Selenite Broth (Selenite F Broth) (Hi-Media, Mumbai, India). Suspicious colonies on plates, which were oxidase test negative and Gram negative, were biochemically characterized (5). *Lactobacillus casei* was provided by Microbiological Laboratory of

Clinic Detection Center of IRAN (Tehran, IRAN).

3.2. Susceptibility Testing

Susceptibility of *S. flexneri* and *S. sonnei* to 18 antibiotics including, ampicillin, ceftriaxon, chloramphenicol, cefotaxime, ceftazidime, tobramycin,, kanamycin, co-amoxiclav, ticarcillin, nalidixic acid, ciprofloxacin, tetracycline, chlorotetracycline, streptomycin, cephalothin, trimethoprim/sulfamethoxazole, gentamicin and amikacin were investigated by using Kirby-Bauer disk diffusion method and comparing their growth inhibition zones to those reported by CLSI (5, 17). The diameters of inhibition zones were measured and compared with the zones suggested by CLSI, using susceptible strains as control. From these isolates, 34 sample were selected for mix by supernatant *L. casei* culture test.

3.3. Antimicrobial Activity and Nature of Antimicrobial Substances

The inhibitory activity of supernatants of *L. casei* was screened against multiple drug resistant *Shigella* isolates using conventional Agar-well assay. Cell-free culture supernatants (CFCS) were obtained by centrifugation (13,000 ×g, 4 °C, 15 min) of *L. casei* cultures grown in 20 ml MRS broth at 37 °C for 24 h. The supernatant was filtered through a 0.22 mm filter to remove cells, and then 1 ml CFCS of *L. casei* was retained as untreated filtrate. To determine the organic acid function, 1 ml CFCS was adjusted to pH 7. In order to test the heat sensitivity, 1 ml CFCS of the *L. casei* were incubated at 100 °C for 15 min. Proteinase K sensitivity was evaluated by incubating 1 ml CFCS with a final concentration of 1 mg/ml Proteinase K at 37 °C for 4 h. The antimicrobial activity of samples was tested using the Agar-well assay (18). Briefly, indicator bacteria were grown in TSB broth overnight and spread onto the TSA agar plate after diluting to 10⁷ CFU/ml, then 5 mm-diameter wells were punched into the surface using a sterile borer. Subsequently, 50 µl samples prepared from filtrate was added to each well of the plate and incubated at 37 °C for 24 hrs. The antimicrobial activity was recorded as growth free inhibition zones (diameter) around the well. MRS adjusted at pH 7 served as control.

3.4. Determination of Minimum Inhibitory Concentration (MIC)

Minimum concentration of *L. casei* inhibitory to the growth of 50 per cent (MIC₅₀) and 90 per cent (MIC₉₀) of the isolates was determined on mueller-hinton agar (MHA) in a 9 cm plate. The agar contained concentration ranges of *L. casei* prepared by two-fold serial dilution according to the National Committee for Clinical Laboratory Standards (NCCLS). Manual inoculation with micropipette for dispensing 20 µl of standardized inoculum (10 ml) of each isolate onto the surface of *L. casei* plate was done to

obtain a final inoculum size of 10⁴-10⁵cfu/spot. CFCS free plates were inoculated at the end and were used as negative controls. The positive controls were the plates inoculated with the reference strain. MIC50 and MIC90 of each antimicrobial agent against *Shigella* isolates were evaluated after incubating the plates, containing completely absorbed inoculum, in ambient air at 37°C for 24 h (19).

3.5. Turbidimetry

One ml of supernatants of *L. casei* was mixed with 1 ml of the MDR *Shigella* strains cultures (4 × 10⁵ CFU/ml) in mueller-hinton broth. The optical densities of culture media were measured at 0, 6, 12, 18 and 24 hrs after incubation at 580 nm. Also the CFU were counted by the spread-plate technique.

3.6. Statistical Analysis

All experiments were performed three times independently and each assay was performed in duplicate. Results were expressed as means ± standard deviation. The level of significance was analyzed by chi-square test and ANOVA (P < 0.05) using SPSS 17.0 for Windows (SPSS Inc.).

4. Results

S. sonnei and *S. flexneri* was isolated from clinical sam-

ples, and confirmed by phenotypic and genotypic methods. Thirty four *Shigella* were isolated from stool samples. Of these, *S. flexneri* 21 (61.77%) was the most common isolate in all age groups, followed by *S. sonnei* 13 (38.23%). CFCS of supernatant of *L. casei* displayed antimicrobial activity against 34 samples of *S. sonnei* and *S. flexneri* by the agar-well assay, respectively. These *L. casei* showed high levels of antimicrobial activity against *S. sonnei* and *S. flexneri*. When the CFCS was adjusted to pH 7, the antimicrobial activity was abolished. In addition, the antimicrobial activity remained active after Proteinase K and heat treatment for *L. casei* as shown in *Table 1*. The resistant pattern of 34 *Shigella* to antimicrobial agents is shown in *Table 2*. Moreover, all tested strains showed resistance against tetracycline, streptomycin, trimethoprim/sulfamethoxazole and ampicillin. The spread of antibiotic resistance for other potent antimicrobial agents is also shown (*Table 2*). The average MIC50 and MIC90 for *Lactobacillus casei* on *S. flexneri* and *S. sonnei*, respectively, 5.25, 5.25, 2.25 and 10.5 were recorded. The MIC tests were repeated for each *Shigella* isolates at least three times. The turbidity method survey, optimal growth was observed for standard curve of growth in the control group with *L. casei* and *Shigella* isolates in standard conditions (*Figure 1*). The second group exposed to *L. casei* supernatant with *Shigella* isolates. The Growth of pathogenic bacteria appeared to be less significant (*Figures 2 and 3*).

Table 1. The Antimicrobials Activity of *L. casei* CFCS with Several Treatments

Indicator strains	Inhibition zone, mm ^a			
	Non-treatment of CFCS	Proteinase K	CFCS adjust pH 7	Heat (100 ° C, 15 min)
	<i>L.casei</i>	<i>L.casei</i>	<i>L.casei</i>	<i>L.casei</i>
S.sonnei	+++	+++	-	++
S.flexneri	++	++	-	++

^a Symbols refer to the size of the inhibition zone diameter observed with growing cells: +, 1 mm; ++, 2 mm; +++, 2-5 mm; -, absence of an inhibitory zone.

Table 2. Resistance Percentage of *Shigella* Isolates to Various Antimicrobial Agents (Total population = 34)

Antibiotics	Shigella isolates		
	Resistant, No. (%)	Intermediate, No. (%)	Susceptible, No. (%)
Ampicillin (20µg)	34 (100)	0 (0)	0 (0)
Tetracycline (30µg)	34 (100)	0 (0)	0 (0)
Streptomycin (30µg)	34 (100)	0 (0)	0 (0)
Trimethoprim/ sulfamethoxazole(1.25/23.75µg)	34 (100)	0 (0)	0 (0)
Ciprofloxacin (5µg)	0 (0)	34 (100)	0 (0)
Chlorotetracycline(30µg)	0 (0)	34 (100)	0 (0)
Nalidixicacid (30µg)	13 (38.24)	0 (0)	21 (61.76)
Co-amoxiclav(20/10µg)	10 (29.41)	14 (41.17)	10 (29.41)
Chloramphenicol (30µg)	7 (29.59)	0 (0)	27 (79.41)
Tobramycin (10µg)	6 (17.65)	0 (0)	28 (82.35)
Amikacin(30µg)	5 (14.71)	5 (14.71)	24 (70.58)
Cefotaxime(30µg)	3 (8.83)	30 (88.23)	1 (2.94)
Cephalothin(30µg)	3 (8.83)	1 (2.94)	30 (88.23)
Ceftriaxon(30µg)	3 (8.83)	0 (0)	31 (91.7)
Ticarcillin(75µg)	3 (8.83)	0 (0)	31 (91.7)
Kanamycin (30µg)	1 (~ 3)	16 (47)	17 (50)
Ceftazidime(30µg)	1 (2.94)	1 (2.94)	32 (94.12)
Gentamicin(10µg)	1 (2.94)	1 (2.94)	32 (94.12)

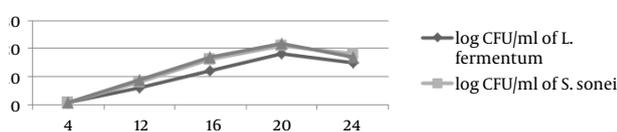


Figure 1. Growth Curve of Bacteria in Four Separate

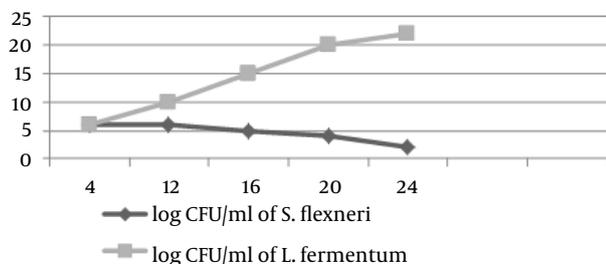


Figure 2. S.flexneri Growth Curve Presence L. Fermentum

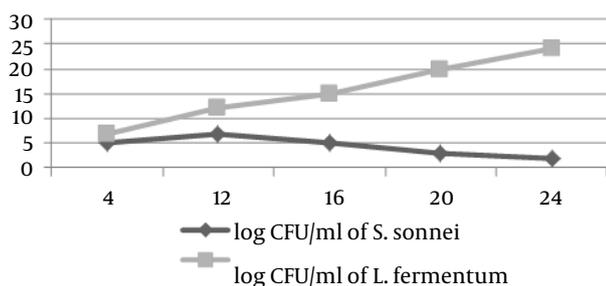


Figure 3. S.sonnei Growth Curve Presence L. fermentum

5. Discussion

S. sonnei and *S. flexneri*, are entero-invasive, cause the inflammatory destruction of the intestinal epithelium (20), leading to an acute recto-colitis causing lethal complications. High prevalence of *Shigella* with multiple antibiotic resistance isolates was observed in this study. None of the antimicrobial agents was effective against all the multi-drug tested strains demonstrating the current problem in the treatment of multiple drug resistant nosocomial infections. *Shigella* isolates strains showed complete resistance against tetracycline, streptomycin, trimethoprim/sulfamethoxazole and ampicillin. Furthermore, its susceptibility to other potent antimicrobial agents, including ciprofloxacin, chloramphenicol, cefotaxime, ceftazidime, tobramycin, co-amoxiclav kanamycin, gentamicin and amikacin, which are used was also tested. Folster et al. reported decreased susceptibility to ciprofloxacin among *Shigella* Isolates in the United States, 2006 to 2009 (21). This study showed that all MDR samples were not resistant to ciprofloxacin in IRAN. As a functional probiotic, anti-pathogen activity

is one of the important properties. The inhibitory activity of lactic acid bacteria against some resistant clinical isolates of *Shigella* has been reported. The *L. casei* tested during this study showed strong antimicrobial activity against *S. sonnei* and *S. flexneri*. However, such activity disappeared when the pH of the CFCS was adjusted to 7, and the addition of Proteinase K did not affect on the size of the inhibition zones, which indicated that bacteriocins were not involved in the antimicrobial activity of *L. casei*, so this activity was attributed to the production of organic acids. Toba et al. adjacent culture supernatant of different *Lactobacillus* with pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella* and *Staphylococcus aureus* and examined obtained Turbidity at different time intervals and concluded that turbidity levels have been studied are reduced dramatically (22). The present study also metabolites of *Lactobacillus casei* in presence of Pathogenic strains obtained from Clinical samples were exposed that descending of Turbidity is confirmed the above study suggests and shows that metabolite have been prevented the growth of pathogenic bacteria. In other study, Hirano et al. reviewed the antimicrobial effect of culture supernatant of *Lactobacillus plantarum*, *Lactobacillus sik* and *Lactobacillus kratos* and have been in plate with technique WDA and found that these substances with the creation of inhibition zone on a wide range of pathogenic bacteria such as *Yersinia enterocolitica* and *Listeria* have inhibitory effect (23). In the present study *L. casei* with well plate technique, against clinical samples of *S. sonnei* and *S. flexneri* showed antimicrobial activity and inhibition zone related metabolites of *Lactobacillus* against human pathogenic strains consideration was given. In the present study, the stability of antimicrobial metabolites of *L. casei* against the temperature was studied and it was found that until 15 minutes at 100 °C is stable and metabolites property is maintained.

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