CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

THE INHIBITION OF *SALMONELLA TYPHI* GROWTH BY THE CELL FREE SUPERNATANS OF *LACTOBACILLUS ACIDOPHILUS* CULTURES

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https://doi.org/10.34302/crpjfst/2019.11.5.1

Article history:
Received:
9 March 2019
Accepted:
20 September 2019

Keywords:
Antimicrobial potency;
Cell-free supernatant;
Ciprofloxacin;
Lactobacillus acidophilus;
Salmonella typhi

ABSTRACT

Fever caused by *Salmonella enterica serovar typhi* is presently a major public health concern in developing countries. The extensive use of recommended antibiotics such as ciprofloxacin has resulted in the reduction of its efficacy in the elimination of *Salmonella typhi*. *Lactobacillus acidophilus* secretes antimicrobial compounds against pathogenic bacteria. The aim of this study was to evaluate the antibacterial activity of cell-free supernatants (CFS) of *Lactobacillus acidophilus* against the growth of *Salmonella typhi.* The efficacy of organic acids and other compounds from *L. acidophilus* CFS was tested against *Salmonella typhi* using macro dilution test and time-kill study. The results were reported descriptively. Four-fold dilutions of organic acids and two-fold dilutions of other compounds from *L. acidophilus* CFS were found to inhibit the growth of *S. typhi*. It was observed that *L. acidophilus* CFS can completely inhibit the growth of *S. typhi*. The CFS of *Lactobacillus acidophilus* especially due to its organic acid content showed an inhibitory effect against *S. typhi* growth.

1.Introduction

Typhoid fever is an ongoing health concern over the world, especially in developing countries (Zaki, 2011). Typhoid fever is an acute infectious disease caused by *Salmonella enterica* serovar *typhi* (Zaki, 2011). *S. typhi* is a gram-negative, rod-shaped, facultatively anaerobic, and motile bacterium that is pathogenic to humans. Transmission of these bacteria occurs through the ingestion of food and water contaminated with infected human waste or via carriers of the disease. An estimated 17.8 million (6.9–48.4 million) new cases of typhoid fever have been reported per year. The Central, South and Southeast Asia have a high incidence of typhoid (Garcia-Fernandez *et al*., 2014; Garcia *et al*., 2015; Warren, *et al*., 2017). The endemic nature of typhoid in developing countries is closely associated with poor hygiene and sanitation (Eng, *et al*., 2015). Serious complications that could occur in typhoid fever are intestinal bleeding, perforation, and even death (Eng, *et al.,* 2015).

The first line of antibiotics used to eliminate *S. typhi* includes chloramphenicol, ampicillin, and cotrimoxazole. However, some strains of *S. typhi* are presently resistant to these antibiotics (Zaki, 2011). Ciprofloxacin is now used to treat infections caused by multidrug resistant strains. However, various studies in Peru, Italy, Africa, and Southeast Asia have also reported that the antimicrobial activity of ciprofloxacin in inhibition of multidrug-resistant strains has also declined, probably due to its extensive use (Garcia-Fernandez *et al*., 2014; Garcia *et al*., 2015; Schellack, 2018). In Cambodia, 90% of the *S. typhi* isolates have shown a decrease in susceptibility to ciprofloxacin and azithromycin (Vlieghe *et al*.,2012). This has resulted in an urgent situation requiring the development of novel therapeutic strategies to inhibit the occurrence of antibiotic resistance in *S. typhi*

Probiotics are microorganisms that have potential health benefits to human, when ingested in an appropriate quantity (Bermudez-Brito *et al*.,2012). *Lactobacillus* spp. and *Bifidobacterium* spp. have been extensively studied, as they are the predominant groups of the human gastrointestinal microflora (Bermudez-Brito *et al*., 2012). The research on probiotics under *in vitro* and *in vivo* conditions has concluded that probiotics can improve the response to inflammation due to microbial infection, through various mechanism of action (Bermudez-Brito *et al*., 2012). Probiotics have been known to enhance the first line of defense by limiting the attachment and proliferation of microbial pathogens (Bermudez-Brito *et al*., 2012). Certain probiotics produce antimicrobial compounds and modulate the immune system, though the exact mechanism of action has not yet been fully understood (Bermudez-Brito *et al*., 2012). Lactic acid bacteria form a group of probiotics that are able to produce lactic acid. *Lactobacillus acidophilus* is classified as a lactic acid bacterium. *L. acidophilus* is a gram-positive, catalase-negative rod that produces lactic acid through carbohydrate fermentation (Bull *et al*., 2013). *L. acidophilus* can be isolated as a normal flora from various parts of the human body, such as the buccal cavity, intestinal tract, colon and vagina. *L. acidophilus* has potential benefits for human health and exerts therapeutic effects. It is known that *L. acidophilus* produces antibacterial substances, maintains barrier function, ameliorates inflammation due to infection by *Helicobacter* sp., and offers immunomodulation (Bull *et al*., 2013; Coconnier *et al*., 1998; Sengupta *et al*., 2013). The aim of this study was to evaluate the inhibitory potential of cell free supernatants of *L. acidophilus* on the growth of *S. typhi.*

2. Materials and methods

2.1. Microorganisms

The strain of *Salmonella typhi* used in this study was isolated from a hospitalized patient that was diagnosed with typhoid fever at the Immanuel Hospital, Bandung, Indonesia. The isolate was identified using standard bacteriological methods. The purified strain was grown on Trypticase Soya Agar (Oxoid) and sub cultured in Trypticase Soya Broth (Oxoid) at 37 ^{o}C for 24 h. Before the use in the experiment, the culture was inoculated in Tryptic Soya Agar at 37 ^{o}C for 24 hours. Bacterial density was measured by suspending 5–10 colonies in 0.9% NaCl (Sigma-Aldrich-Merck) to the equivalent of the 0.5 McFarland standard.

Lactobacillus acidophilus ATCC 4356 was purchased from Microbiologics^{TM} Fisher scientific. The strain was identified using gram staining and biochemical tests (Branch,2015). The culture was stored in de man Rogosa Sharpe slant agar (Oxoid) at 4–8^{o}C and subcultured in MRS broth at 37 °C for 24 h before the use in the experiment.

2.2. Preparation of Organic Acid Cell free supernatant (OACFS)

L. acidophilus ATCC 4356 was anaerobically cultured in de man Rogosa Sharpe broth at 37 ^{o}C for 24 h. CFS was obtained by centrifuging the bacterial culture at 6,000 rpm and 4 °C for 15 minutes (Eppendorf, Centrifuge 5424 R, Germany). The supernatant was filter-sterilized using a 0.2-micron filter (Minisart, Sartorius Stedim biotech). The filtrate was then placed in UV light Biosafety Cabinet (Telstar Bio II Advance) for 40 minutes.

2.3. Preparation of Other Compunds Cell Free Supernatant (OCCFS)

CFS from the *L. acidophilus* was obtained as described above and was neutralized to pH 7.0 using 2N NaOH (Sigma-Aldrich-Merck) and sterilized using 0.2-micron filter (Minisart, Sartorius Stedim biotech) (Kaur, 2015).

2.4. Determination of Minimal Inhibitory Concentration (MIC).

The MIC values were determined by the tube macro dilution technique. Two-fold serial dilutions of ciprofloxacin, OACFS, and OCCFS (crude bacteriocin) were prepared volumetrically in Mueller-Hinton (MH) broth (Oxoid). The bacterial suspension was added to the broth containing the dilutions and incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of the compound that showed no visible growth of *S. typhi,* demonstrated by the absence of turbidity in the broth.

2.5. Time-kill Study

The Time-kill curves of *S. typhi* were constructed by evaluating the reduction in the number of colony forming units (CFU)/mL following exposure to CFS in MH broth at 37ºC for 48 h. An inoculum of 10^8 CFU/mL of *S. typhi* was used for this test. The samples were collected from the culture of the test bacteria at time intervals of 0, 4, 8,12, 24, 28, 32, 36 and 48 h, diluted serially, and cultured in triplicate in Plate Count Agar at 37 °C for 24 h (Del, 2017). The numbers of viable cells were counted as CFU/mL.

3.Results and discussions

The strain of *L. acidophilus* used in this study was tested for its ability to produce lactic acid and other compounds which inhibited the growth of *S. typhi*. Upon inoculating *L. acidophilus* on de man Rogosa Sharpe Agar (MRSA) plates containing $CaCO_3$, zones of inhibition were obtained around the colonies, which indicated that *L. acidophilus* could produce lactic acid. OACFS and OCCFS were prepared from the culture of *L. acidophilus* and their antimicrobial activity was evaluated. The pH of OACFS was 4.1. The antimicrobial activity of CFS was compared with the recommended antibiotics.

Two-fold serial dilutions of ciprofloxacin, OACFS and OCCFS were prepared for the broth macro dilution method. The MIC was 0.2 µg/mL of ciprofloxacin (Table 1), 4-fold dilution of OACFS, and 2-fold dilution of OCCFS (Table 2). For the time-kill study, normal *S. typhi* growth curves were compared with that in the OACFS, OCCFS, and ciprofloxacin at their respective MIC (figure 1).

Table 1. Minimal Inhibitory Concentration (MIC) of ciprofloxacin

	Concentration (µg/ml)									
	25	12.5	6.25	3.12	1.56	0.78	0.39	0.2	0.1	0.05
Ciprofloxacin	No growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth	Growth	Growth

Table 2. Minimal Inhibitory Concentration (MIC) of organic acid cell-free supernatant (OACFS) and other compounds cell-free supernatant (OCCFS)

	Concentration of CFS dilution				
	2-fold	4-fold	8-fold	16-fold	32-fold
Organic acid	No growth	No growth	Growth	Growth	Growth
Other Compounds	No growth	Growth	Growth	Growth	Growth

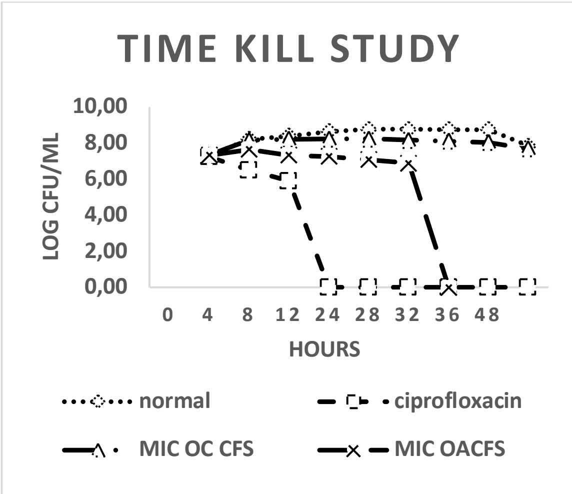

Figure 1. The comparison of *S. typhi* growth in the presnce of ciprofloxacin, OACFS, and OCCFS.

Lactobacillus spp. are probiotics that can be found in the gastrointestinal tract. *L. acidophilus* is used extensively in research to develop probiotics as a complementary therapy. In this study, OACFS showed the bactericidal effect after 36 hours of incubation. The possible mechanism of the bactericidal effect of these organic acids especially acetic acid and lactic acid involves their ability to enter the bacterial cell and disrupt cytoplasm (Bermudez-Brito *et al*., 2012). *S. typhi* is an acid-sensitive bacterium. Organic acids with low pH can directly lead to the death of *S. typhi*. The bactericidal effect of organic acids appears to accelerate the typhoid fever recovery process. In this study, the antimicrobial activity of OCCFS appears to be limited against *S. typhi*. The inhibition of the OCCFS may be attributed to the presence of bacteriocin and other peptides (having antimicrobial activity) that are produced by the lactic acid bacteria. Bacteriocin is an antimicrobial substance that could disrupt the bacterial cell membrane permeability, which may cause perforation in the bacterial cell wall (Gogineni et al., 2013). Further studies by using other isolation OCCFS method is needed to evaluate its *in vitro* effect in inhibition the growth of *S. typhi*.

Ciprofloxacin is a quinolone antibiotic, which acts by inhibiting the bacterial DNA gyrase and topoisomerase IV during bacterial replication (Abde-daim *et al*., 2013). The isolate studied in this work was sensitive to ciprofloxacin based on recent CLSI MIC recommendation (sensitive: ≤ 1 μg/mL, Intermediate: 2 μg/mL, Resistant ≥ 4 μg/mL (Scellack *et al*., 2018). However, this strain required a higher concentration of ciprofloxacin to show growth inhibition.

The decrease in susceptibility to ciprofloxacin is defined when an isolate of *Salmonella* showed an MIC of 0.12–2 μg/mL (Inical *et al*., 2011). Further studies are required to ascertain the decrease in susceptibility of this strain to ciprofloxacin.

4. Conclusions

Our study found that the cell-free supernatants of *L. acidophilus* especially those containing organic acids have an inhibitory effect in the growth curves of *S. typhi*.It appears that *L. acidophilus* exerts an antimicrobial effect on *S. typhi* and it may offer a promising therapeutic approach for the elimination of *S. typhi*

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Acknowledgment

The authors thank Dr. Wahyu Widowati from Biomolecular and Biomedical Research Center for laboratory support. The authors also thank to Ms Shinta Asarina and Mr Ubaydillah Munshy for technical assistance.