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Original Article

Evaluation of the Effects of Probiotics and Prebiotics on the *Salmonella typhi* Infections

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Abstract

Typhoid fever is one of the most commonly disseminated diseases and is considered to be linked to poor sanitation. It is responsible for 2-5% of all deaths, and its causative agent is *Salmonella typhi*. The current study aimed to investigate the antibacterial activity of prebiotics (inulin and starch) and probiotics against multidrug resistance of *S. typhi* bacterial isolates. Determination of the inhibitory effect of probiotics and prebiotics against *S. typhi* isolates was performed by agar well diffusion method and minimal inhibitory concentration. Body samples of all eligible patients were collected and cultured. Finally, 50 (25%) out of the total cultured samples were *S. Typhi* bacteria isolated from different samples. The bacteria were mainly found in blood, followed by stool and fluid (74%, 24%, and 2%, respectively). On differential medium, xylose lysine deoxycholate agar, the colonies appear red with black centers, while on MacConkey agar, the colonies appear smooth, pale, transparent, colorless, and raised. Regarding the inhibition zone values of bacteriocins of *Lactobacillus* from Yogurt against *S. typhi* in plate, significant differences were identified between the ones with and without prebiotic addition. Accordingly, the value of the inhibition zone for those without prebiotic addition (13.18±7.403) was significantly lower than that of cutoff values of 20 with a significant difference of -6.820 (t= -6.514, df: 49, P=0.000). Moreover, the inhibition effect of prebiotics (inulin and starch) against *S. typhi* at 37 °C for 24 h in part dish glucose as control, only the mean of inulin was found to be significantly lower than that of the cutoff value of 18 with the mean difference of -3.900 (t=-4.115, df: 49, P=0.000). Other prebiotics of glucose and starch in 24 h showed negative inhibition. Probiotics are live microorganisms that have beneficial host effects by enhancing microbial balance in the intestine, whereas prebiotics are indigestible food components having beneficial effects by enhancing the activity and growth of one or more colonic bacteria. *Lactobacillus* filtrates had considerable effects against the test *S. typhi* isolates.

Keywords: Antibacterial activity, Prebiotic, Probiotic, *S. typhi*

1. Introduction

Typhoid fever is one of the most commonly disseminated diseases and is considered to be linked to poor sanitation. It is responsible for 2-5% of all deaths, and its causative agent is *Salmonella typhi*. *Salmonella typhi* is a flagellated gram-negative rod, encapsulated, non-spore-forming, and anaerobic bacteria (1).

Antimicrobial therapy is the mainstay of typhoid treatment; accordingly, "chloramphenicol" is usually used to cure typhoid fever successfully. However, multiple drug resistance (MDR) among bacterial strains is on the rise and spreading, including resistance to older antimicrobials, such as "chloramphenicol", "ampicillin," and "trimethoprim-sulfamethoxazole" (co-trimoxazole). This problem has existed for many years and is considered the first problem in the prevention of typhoid fever (2). Consequently, "fluoroquinolone", also known as "ciprofloxacin", has

been chosen as the first-line antibiotic for treatment, especially in light of the recent rise in MDR of *S. typhi* isolates (3). As a result of the switch to ciprofloxacin, the number of *S. typhi* resistant to antibiotics has increased (4).

Probiotics are live microorganisms that benefit our health, such as lactic acid bacteria and yeasts, but *Lactobacilli* and *Bifidobacteria* are the most frequent types (5). Many studies have found that probiotics can reduce diarrhea and help with inflammatory bowel illnesses and constipation, such as yogurt, which is a good source of probiotics (5). Prebiotics are non-digestible food components (dietary fiber) that benefit the host by promoting the development or activity of a small number of bacteria in the colon, enhancing human health.

The current study aimed to investigate the antibacterial activity of prebiotics (inulin and starch) and probiotics against *S. typhi* bacterial isolates with MDR.

2. Materials and Methods

2.1. Collection of Specimen

In total, 200 clinical samples of blood, stool, and fluid were collected from patients with pyrexia of unknown origin and gastroenteritis from teaching laboratories and other laboratories in the medical city of Baghdad, Iraq, as well as private labs from November 2021 to March 2022. To be more specific, these specimens included 120 blood, 60 stool, and 20 ascitic fluid samples.

In this study, 10 ml of venous blood or ascitic fluid was obtained from each patient and added to 50 ml of brain heart infusion bottles that were loaded into BacT/Alert instruments. The bottles remained in BacT/Alert for 5 days. Afterward, a loopful was obtained of sign-positive subculture on blood agar and MacConkey agar by streaking method and then incubated at 36±1 °C for 24 h. The stool was cultured on xylose lysine deoxycholate and MacConkey agar by streaking and incubated at 36±1 °C for 24 h (6). Identification of the organisms was performed by the tests as described previously (7).

2.2. Biochemical Tests

2.2.1. Oxidase Test

The test used for the detection of the ability of bacteria to produce oxidase was saturating filter paper with an oxidase reagent. Development of purple color within 20-30 s is a positive reaction, while lack of change of the color of the colony shows a negative result.

2.2.2. Catalase Test

This test was performed to determine catalase enzyme synthesis. Accordingly, pure growth was transferred to a microscope slide using a wood stick, then a drop of 3% hydrogen peroxide was dropped on the colony, and the development of bubbles was taken as catalase-positive.

2.2.3. Kligler Iron Agar

This test was used to detect the ability to ferment the sugars of bacteria into glucose and lactose. A positive result was observed if the phenol red indicator turned from red to yellow as a result of acid formation from fermentation sugar. Moreover, in a positive result, bubbles might appear in the medium at the stabbing place due to gas production from aerobic fermentation sugar and a black residue might develop at the bottom of the tube as a result of H2S production from anaerobic fermentation with the ferrous sulfate presented on medium.

2.2.4. Indole Production Test

The test was used to detect the bacterial production ability of the tryptophanase enzyme that hydrolyzes tryptophan to indole pyruvic acid and ammonia. Moreover, peptone broth was inoculated with tested bacterial cultures that were previously grown on MacConkey agar and incubated at 36±1 °C for 24 h. Afterward, 2 drops of Kovac's reagent were added directly to the culture tube. The immediate formation of a deep red ring at the top of the broth after gentle shaking indicates a positive result, while the lack of it refers to the negative result of this test.

2.2.5. Citrate Utilization Test

The bacteria were tested by streaking on Simmon's citrate; accordingly, the transformation of the bromothymol blue from green to blue color indicated a positive (+ve) result, while the green color of the medium showed a negative result. This test determines the ability of bacteria to utilize sodium citrate as the sole carbon source.

2.2.6. Urease Production Test

This test was used to detect bacterial capacity to analyze urea and produce ammonia and carbon dioxide. Urea agar slant was inoculated by streaking the tested bacteria and incubating them at 36±1 °C for 24 h. After the incubation time, the pink color appeared which indicated a positive test. It should be mentioned that the appearance of yellow color indicates a negative result.

2.3. Analytical Profile Index

This BioMérieux analytical profile index **(**API) 20E test strip is used to identify enteric gram-negative (-ve) rods. The positive (+) sequence and test results yield a profile number, which is searched in a codebook of numbers and bacterial species (8).

2.4. Procedure of the Analytical Profile Index 20E System

This procedure included the preparation of a suspension of the bacteria in the saline tube and inoculation of a large colony of the bacterium 2-3 mm in diameter into the 0.85 % NaCl solution (a young and pure culture). This was followed by inoculation of the API strip by using the sterile pipette and inoculation of the bacterial suspension into each well while holding the strip at a specific angle up from the bench (Figure 1).

2.5. Vitek-2 System

There is a biochemical response between bacterial isolates suspended in their solutions and the media in the VITEK-2 Identification Cards that are used as an identification system. The bacterial isolates were injected on MacConkey agar plates at 36±1 °C, and a single colony was suspended after overnight incubation. The bacterial suspension turbidity was measured in 0.45% sodium chloride to match the McFarland 0.5 standard. Afterward, the gram-negative and gram-positive Vitek 2 ID card and the bacterial suspension tubes were manually loaded into the Vitek-2 system, and the software was prepared according to the instructions of the manufacturer (9).

The GN/AST system and minimum inhibitory concentration (MIC) determination were performed via ASTN084 and ASTN093 cards. The ASTN084 card contained the antibiotics extended-spectrum beta-lactamase. The MIC result must be linked to an organism identification to determine a category interpretation. A category interpretation of MIC will be reported according to the interpretations defined by the Clinical Laboratory Standers Institute (CLSI) (10).

TESTS	SUBSTRATE	REACTION TESTED	- RESULTS	+ RESULTS
ONPG	ONPG	beta-galactosidase	colorless	yellow
ADH	arginine	arginine dihydrolase	yellow	red/orange
LDC	lysine	lysine decarboxylase	yellow	red/orange
ODC	ornithine	ornithine decarboxylase	yellow	red/orange
CIT	citrate	citrate utilization	pale green/yellow	blue-green/blue
H2S	Na thiosulfate	H2S production	colorless/gray	black deposit
URE	urea	urea hydrolysis	yellow	red/orange
TDA	tryptophan	deaminase	yellow	brown-red
IND	tryptophan	indole production	yellow	red (2 min.)
VP	Na pyruvate	acetoin production	colorless	pink/red (10 min.)
GEL	charcoal gelatin	gelatinase	no diffusion of black	black diffuse
GLU	glucose	fermentation/oxidation	blue/blue-green	yellow
MAN	mannitol	fermentation/oxidation	blue/blue-green	yellow
INO	inositol	fermentation/oxidation	blue/blue-green	yellow
SOR	sorbitol	fermentation/oxidation	blue/blue-green	yellow
RHA	rhamnose	fermentation/oxidation	blue/blue-green	yellow
SAC	sucrose	fermentation/oxidation	blue/blue-green	yellow
MEL	melibiose	fermentation/oxidation	blue/blue-green	yellow
AMY	amygdalin	fermentation/oxidation	blue/blue-green	yellow
ARA	arabinose	fermentation/oxidation	blue/blue-green	yellow
OX	oxidase	oxidase	colorless/yellow	violet

Figure 1. Reading the API 20 E system

2.6. Serological Test

A confirmatory test was performed in a central public health laboratory using polyvalent antisera and monovalent antisera for *S. typhi*.

2.7. Antimicrobial Susceptibility Testing

According to the CLSI, this test was performed according to the Kirby-Bauer 1968 (disk diffusion) technique using Muller-Hinton agar and different single antibiotic disc that was supplied commercially. Antibiotic susceptibility and resistance were determined using strain growth zone diameter (11).

2.8. Yogurt Sample Collection

Yogurt sample specimens were collected in sterile tubes under aseptic and cooling conditions from Baghdad markets Canon yogurt using *Lactobacillus* pharmacy.

2.9. Isolation and Identification of *Lactobacillus* Bacteria

De Man, Rogosa, and Sharpe (MRS) agar medium were chosen as the medium for lactic acid bacteria. Under aseptic conditions, a loopful of curd samples was streaked on a sterile MRS agar Petri plate using the streaking method. The Petri plates were streaked, incubated at 36±1 ℃ for 24-48 h, and kept at 4 ℃. The microscale topic slide fixed a loopful of *Lactobacillus* bacteria culture. Gram stain was used to examine the shape of cells and gram reaction. Moreover, non-spore-forming and biochemical tests as well as oxidase and catalase tests were used to detect *Lactobacillus.*

2.10. Preparation of *Lactobacillus* (Bacteriocins) according to Moore, DeVries

In this study, 200 ml of MRS medium was autoclaved in a flask at 121 ℃ 15 psi for 15 min according to the instruction of the manufacturing company (12). The flasks were incubated at 36±1℃ for 48 h in anaerobic conditions to ensure their sterility.

- After the incubation period, each flask was inoculated with 0.5 McFarland of *Lactobacillus* broth, and incubated at 36±1 ℃ for 48 h in anaerobic conditions. Afterward, each 10 ml was distributed in a sterile test tube for centrifugation at 5,000 rpm for 15 min.
- The precipitate was removed, and the supernatant was taken.
- The supernatant was filtered using a Millipore filter (0.22 nm pore size).
- After the filtration process, the loopful of the filtrate was streaked on the MRS agar plate and incubated for 48 h at 36±1 ℃ in anaerobic conditions to ensure the filtrate (*Lactobacillus* extract) was sterile.
- The filtrate was stored within sterile tubes and kept at 4 ℃ until use. Tubes were the last dilution of serial dilutions that does not contain turbidity, and their concentration represented MIC.

2.11. Determination of Total Protein of Crude Bacteriocins by UV-Visible Spectroscopy

Several methods based on UV-visible spectroscopy have been designed to quantify protein content. These methods either use the natural ability of proteins to absorb (or scatter) light in the UV-visible part of the electromagnetic spectrum or chemically or physically change proteins to absorb (or scatter) light in this region. Each of these tests is based on the same basic idea. To begin, a calibration curve of absorbance (or turbidity) versus protein concentration was created using a series of known concentration protein solutions. Absorbance (or turbidity) of the sample solution was measured at the same wavelength, and the protein concentration was calculated using the calibration curve. The main difference between the tests was the chemical groups responsible for the absorption or scattering of radiation (13).

$$\text{the concentration of bacteriocins} = \frac{\text{0. D of bacteriocins} \times \text{concentration of standard}}{\text{OD of standard}}$$

2.12. Ultraviolet Spectrum Absorption

The UV spectrum analysis was accomplished within 200-700 nm using a UV-visible spectrophotometer (Aurora Instrument Ltd- UK). In this analysis, the dried bacteriocins were examined before they were dissolved. The process using methanol whereby max was recorded for each sample (14).

2.13. Analysis with Fourier Transform Infrared Red Spectroscopy Spectral Analysis

To demonstrate the functional groups of the bioactive compound within the biosurfactant, the pellet was analyzed by Fourier transform infrared red. The functional groups and chemical bonds (post-purification) were detected. The spectrum was limited to 4000-650 cm^{-1} with a resolution of 4 cm^{-1} (15). The experiment was performed at the Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq.

2.14. Prebiotic Assay

Precultures were prepared by inoculation of a Muller Hinton broth of 5 mL containing 1×10^6 cells of each bacterium placed inside 50 ml of their respective maintenance culture media and incubation at 36±1 °C for 24 h.

An appropriate modified medium was prepared for each bacterium, substituting glucose with other carbon sources (inulin and starch) and 2 g/L glucose as control. Afterward, 200 µg/ml of each medium and the control were transferred to a 96-well micro-plate in quadruplicate and then inoculated in triplicates with 1.2×10^6 CFU of precultures, leaving one blank per assessed sample. Optical densities (OD) were measured every hour up to 1, 2, 4, and 24 h using a micro-plate Elisa Reader (16).

2.15. Identification of Metabolites Produced by the Probiotic *Lactobacillus* spp. by the Addition of Prebiotics (Inulin and Starch)

Products of prebiotics fermentation by *Lactobacillus* spp. strains, especially lactic acid, are used in prebiotic fermentation. For the comparison, inoculums of 10% v/v of probiotic strain monocultures were added to an MRS medium containing 2.0% (w/v) of each prebiotic (inulin and starch) individually, as well as an MRS with an acceptable concentration of glucose. The strains were grown for 24 h at 36±1 °C without oxygen constraint. Subsequently, the samples were centrifuged for 15 min at 5,000 rpm, and 200 µg/ml of each sample and the control was transferred to a 96-well micro-plate in quadruplicate and then inoculated in triplicates with 1.2×10^6 CFU of precultures, leaving one blank per assessed sample. The OD was measured every 1, 2, 4, and 24 h using a micro-plate ELISA Reader (17).

2.16. Biological Effects of Crude Bacteriocins with Probiotic and Prebiotic against *Salmonella typhi*

2.16.1. Agar Well Diffusion Method

Inhibitory effects of bacteriocins were determined by following a modified Kirby-Bauer disc diffusion method as briefed in the following (18):

- Bacterial culture of *S. typhi* was coordinated by spreading 100 ml of bacteria according to McFarland solution to get the proper concentration cell density; 1.5×10^8 CFU/ml of each isolate on a solid Muller Hinton agar;
- The plates were allowed to stand for 15 min. To facilitate absorption of culture, wells with diameters of 6 mm were obtained on plate sterile cork borer; subsequently, after the wells were filled with 100 µl, they were incubated for 24 h at 36±1 °C.
- After the incubation period, the size of the inhibition zone around each well was measured, and the average of the inhibition zone around the well was calculated.

2.17. Determination of the Minimal Inhibitory Concentration of *Lactobacillus* Supernatant against *Salmonella typhi*

This protocol was performed according to Wiegand, Hilpert (18). Serial dilutions were made from MRS broth to several dilutions (1/2, 1/4, 1/8, 1/16, 1/32) within sterile plain tubes and the complete volume was 2 ml. Each tube was inoculated with 100 µl of 0.5 McFarland (using Vitek McFarland standard *S. typhi*). The tubes were incubated for 24 h at 36±1 °C. Moreover, there was a control tube that contained MRS broth with bacterial inoculum. Afterward, the turbidity changes were recorded and compared with the control.

2.18. Determination of the Minimal Inhibitory Concentration of *Lactobacillus* Supernatant with Prebiotics (Inulin and Starch) and Glucose against *Salmonella typhi*

This protocol was performed according to Wiegand, Hilpert (18). Serial dilutions were made from MRS broth to several dilutions (1/2, 1/4, 1/8, 1/16, 1/32)

within sterile plain tubes and the complete volume was 2 ml. Each tube was inoculated with 100 µl of 0.5 McFarland *S. typhi*. The tubes were incubated for 24 h at 36±1 °C, and a control tube was made that contained MRS broth with bacterial inoculum. Afterward, the turbidity changes were recorded and compared with the control.

3. Results

Body samples of patients were collected and cultured according to the inclusion and exclusion criteria. In this study, 50 (25%) out of the total cultured samples were *S. Typhi* bacteria isolated from different samples, mainly in blood, followed by stool and fluid (74%, 24%, and 2%, respectively) (Figure 2).

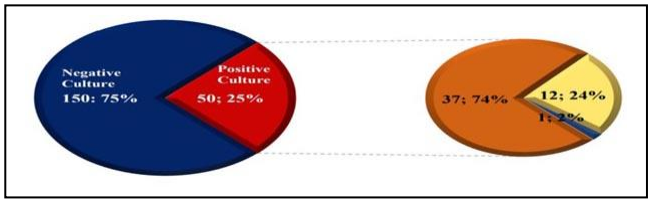

Figure 2. Prevalence *of S. typhi* isolation among sample cultures (n=200)

On differential medium, xylose lysine deoxycholate agar, the colonies appeared red with black centers, while on MacConkey agar, the colonies appeared smooth, pale, transparent, colorless, and raised (Table 1).

Table 1. Biochemical test results for *S. typhi*

Biochemical tests	Results
Oxidase	-
Catalase	+
Kligler iron	Acid /Alkaline no gas with H2S
Simmon citrate	-
Urea test	-
Semisolid mannitol	Motile /mannitol fermentation
Peptone water for indol	-

API 20 E system was used to confirm the diagnosis (Figure 3).

The slide agglutination method using polyvalent and monovalent antiserum was used for *Salmonella* somatic and flagellar antigen. The results revealed agglutination for all 50 isolates that were diagnosed earlier. The acquired results from the VITEK 2 test for *S. typhi* are illustrated in figure 4. It is worth mentioning that the GN card was used for gram-negative bacteria, which consisted of 47 biochemical examinations.

Figure 3. The results of the API 20E test used for the identification of *S. typhi*

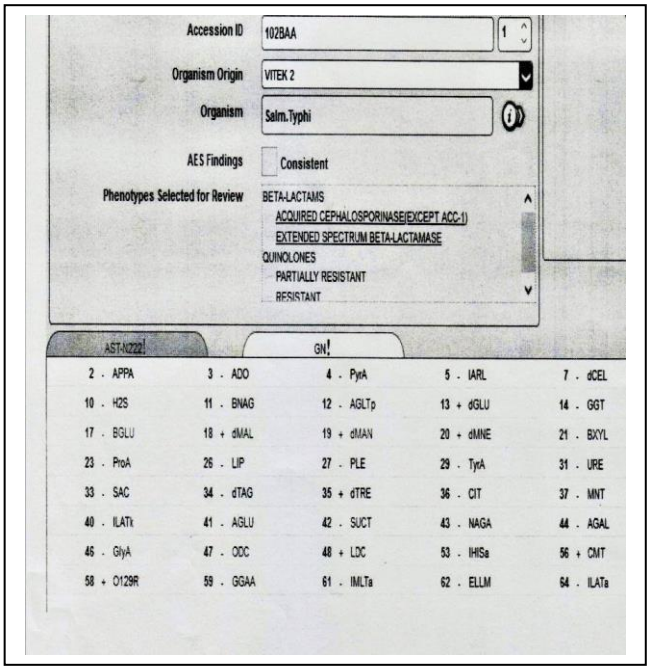

Figure 4. VITEk2 compact system for identification of *S. typhi*

Results of the phenotypic test using the disc diffusion method and VITEK2 compact system are shown in tables 2 and 3.

Lactobacillus isolates were collected from yogurt samples and *Lactobacillus* pharmacy. Figure 5 shows the *Lactobacillus* growth on a selective medium MRS. The colonies appeared white/creamy, large, smooth, and round with an entire margin.

Isolates reacted positively with the Gram stain and appeared as long purple bacilli or short rod coccobacilli. It should be mentioned that the cells were arranged singly or in pairs (Figure 5).

Table 2. Number and percentage of resistant and sensitive isolates against tested antibiotics

NO	Name of antibiotic	Code	Disc potency (µg/disc)	Salmonella typhi isolates resistance	Salmonella typhi isolation sensitive
1	Ampicillin	AM	10 µg	47	3
2	Azithromycin	AZM	15µg	24	26
3	Ciprofloxacin	CIP	5 µg	41	11
4	Levofloxacin	LEV	5 µg	37	13
5	Ofloxacin	OFX	5 µg	40	10
6	Trimethoprim –sulfamethoxazole	SX T	25 µg	2	48
7	Cefotaxime	CXT	30 µg	48	2
8	Ceftriaxone	CRO	30 µg	47	3
9	Tetracycline	TE	30 µg	34	7
10	Chloramphenicol	C	30 µg	19	31

Table 3. The number and percentage of resistant and sensitive isolates against tested antibiotics using the Disc diffusion test (n= 10) depend on clinical and laboratory standards institute (CLSI)

NO	Name of antibiotic	*Sal.typhi* isolates resistance (blood)	*Sal.typhi* isolation resistance (stool)	*Sal.typhi* isolation sensitive (blood)	*Sal.typhi* isolation sensitive (stool)	*Sal.typhi* isolation sensitive (fluid)
1	Ampicillin	34	13	2	0	1
2	Azithromycin	15	9	22	3	1
3	Ciprofloxacin	32	9	4	4	1
4	Levofloxacin	29	8	7	5	1
5	Ofloxacin	31	9	4	5	1
6	Trimethoprim-sulfamethoxazole	1	1	35	12	1
7	Cefotaxime	35	13	1	0	1
8	Ceftriaxone	34	13	2	0	1
9	Tetracycline	29	14	3	3	1
10	Chloramphenicol	7	12	20	10	1

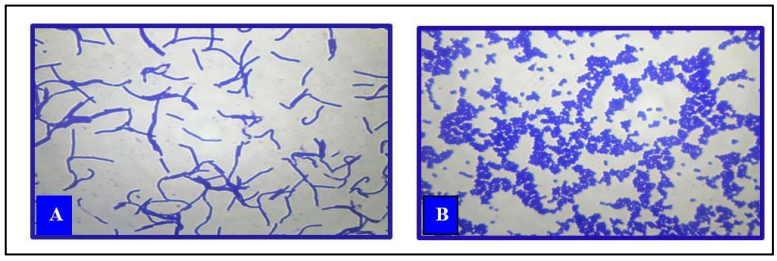

Figure 5. Gram stain (A) *Lactobacillus* Yogurt purple long bacilli, (B) *Lactobacillus* treatment purple short rod coccobacilli

The biochemical characteristics of *Lactobacillus* are summarized in table 4.

It was noticed that the bacteriocins synthesized by *Lactobacillus* had increased activity following UV light exposure for 30 and 15 min and were described by two peaks at 443 and 291 nm as shown in figure 6.

The infrared spectrum of the crude lyophilized protein sample analyzes the spectrum of the four significant peaks at 3387.00, 12923.1, 2681.05, and 798.53.59 Cm^{-1} (Figure 7). It should be mentioned that the rest of the spectrum consists of very close values.

The activity was determined using the well diffusion method. Diameters of inhibition zones were recorded within the range of 10-20 mm (Figure 8). Furthermore, the data obtained from *Lactobacillus* spp. bacteriocins extracted in *Lactobacillus* spp. treatment showed no inhibition zone (Figure 9).

Table 4. Biochemical tests results for *Lactobacillus* SPP

Test	Result
Catalase	Negative
Oxidase	Negative
Motility	Negative
Gram stain	Gram-positive purple long bacilli or short rod coccobacilli

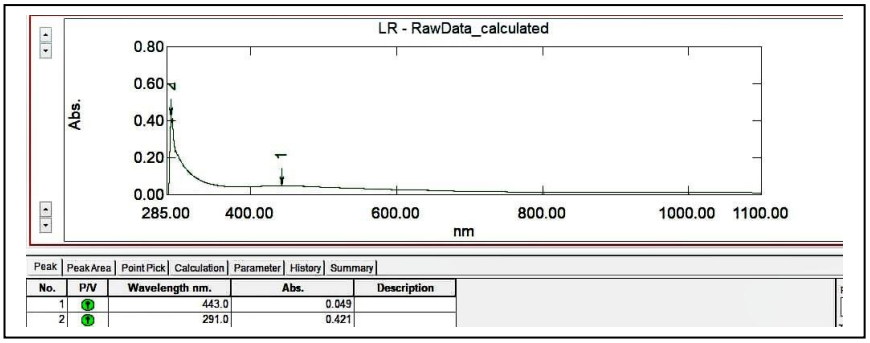

Figure 6. UV spectrum absorption of Bacteriocins in peak 443.0, 291.0

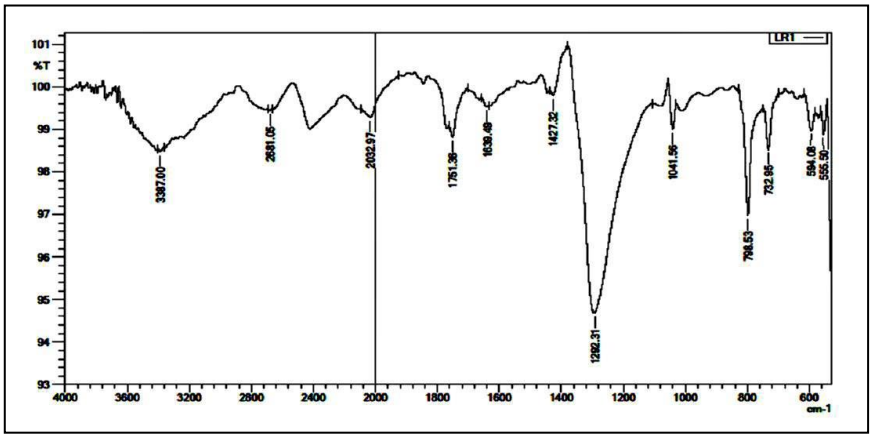

Figure 7. Fourier transforms - infrared FTIR in Bacteriocins of *Lactobacillus*

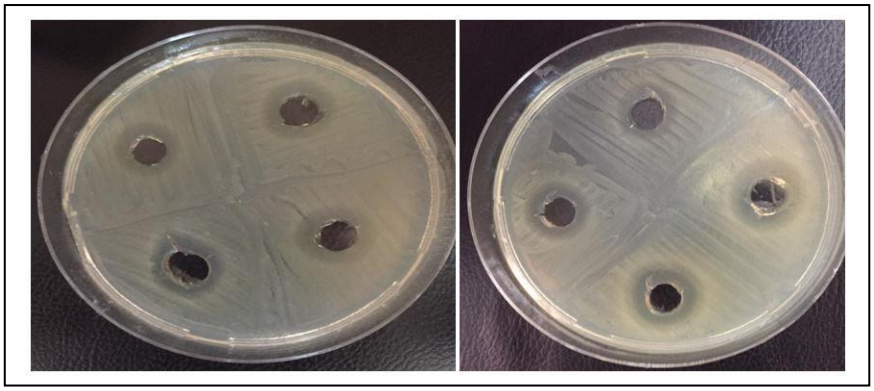

Figure 8. Different inhibition zone of bacteriocins production from *lactobacillus* yogurt against *S. typhi*

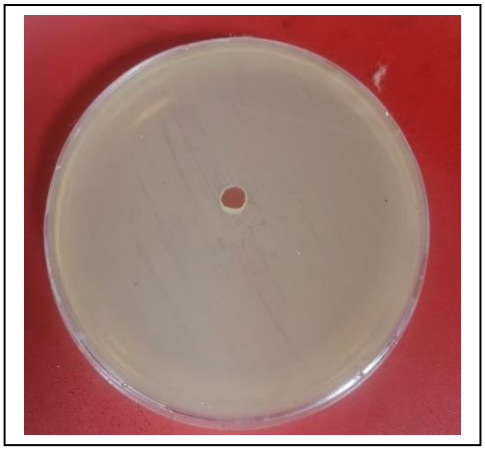

Figure 9. No inhibition zone of Bacteriocins

This experiment was conducted to determine the lowest dilution of *Lactobacillus* bacteriocins to inhibit the growth of *S. Typhi*. Based on the findings of this experiment, ½ MIC inhibited bacterial growth (Figure 10).

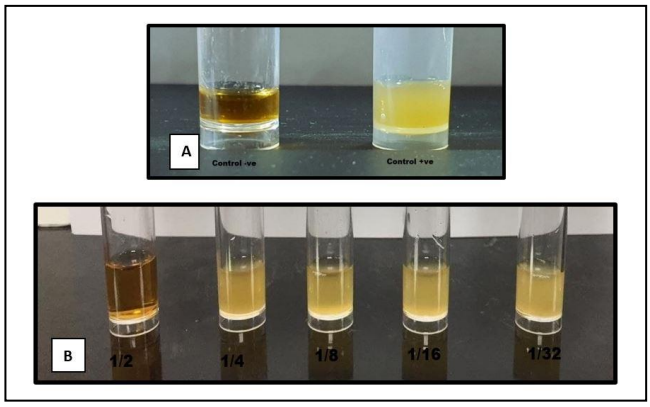

Figure 10. (A) Positive and negative control, (B) MIC for *Lactobacillus* Bacteriocins against *S. typhi*

Regarding the antibacterial activity of bacteriocins, the total and diluted concentrations of MIC 1, MIC 2, MIC 3, MIC 4, and MIC 5 for bacteriocins in *Lactobacillus* yogurt are illustrated in table 5.

3.1. Agar Well-Diffusion Method

Regarding the inhibition zone of bacteriocins of *Lactobacillus* from yogurt against *S. typhi* in plate, significant differences were identified regarding the inhibition zone values of *Lactobacillus* from yogurt against bacteria for those with or without prebiotic addition (Figures 11 and 12). Accordingly, the value of the inhibition zone for those without prebiotic addition (13.18±7.403) was significantly lower than that of cutoff values of 20 with a significant difference of -6.820 (t=-6.514, df: 49, P=0.000). Moreover, the inhibition effect of prebiotics (inulin and starch) against *S. typhi* at 37 °C for 24 h in part dish glucose as control, only the mean value of inulin was found to be significantly lower than that of the cutoff value of 18 with the mean difference of -3.900 (t=-4.115, df: 49, P=0.000). Other prebiotics of glucose and starch in 24 h showed negative inhibition (Table 6).

3.2. Identification of metabolites produced by the probiotic *Lactobacillus* spp. by the addition of prebiotics (inulin and starch)

Furthermore, the effects of prebiotics against *Lactobacillus* (inulin and starch) were identified at different hours in a microplate, as the OD values progressively increased from 1 h to 24 h among control glucose. The OD values were 1.063, 1.068, 1.092, and 1.266 for bacteriocins isolated from yogurt and 1.066, 1.069 1.093, and 1.228 for bacteriocins in the treatment group at 1 h, 2 h, 4 h, and 24 h in microplate, respectively. After the addition of the insulin prebiotic to yogurt, the OD values were 1.217, 1.273, 1.381, and 1.629 at 1 h, 2 h, 4 h, and 24 h in a microplate, respectively. Moreover, the OD values were 1.219, 1.277,1.386, and 1.632 after the addition of the insulin prebiotic to the treatment group at 1 h, 2 h, 4 h, and 24 h in a microplate, respectively. Furthermore, after the addition of starch to yogurt, the OD values were 1.963, 2.001, 2.028, and 2.053 at 1 h, 2 h, 4 h, and 24 h in a microplate, respectively. Besides, after the addition of starch to the treatment group, the OD values were 1.982, 2.005, 2.031, and 2.055 at 1 h, 2 h, 4 h, and 24 h ia n microplate, respectively (Figure 13).

3.3. Determination of the Minimal Inhibitory Concentration for *Lactobacillus* Supernatant to Add Prebiotics (inulin and Starch) and Glucose that Controls against *Salmonella typhi*

Unfourentely, no significant differences were found regarding the OD values at 1 and 2 h, compared to their cutoff values of 0.719 and 0.776, respectively (Figure 14 and Tables 7-10).

With respect to the effects of prebiotics of inulin against *S. typhi*, inulin showed no significant differences from its cutoff values at other time points (P>0.05) (Tables 11).

However, regarding the effects of prebiotic starch against *S. typhi*, a significant difference was found only in 1 h, as the mean OD value of 1 h (1.86426±0.085461) was significantly lower than that of the cutoff value of 1.958 with a mean difference of -0.093740 (t=-7.756, df: 49, P=0.000). Starch showed no significant difference from its cutoff values at other time points from 2 to 24 h (P>0.05) (Table 12).

Table 5. The OD and concentration of Bacteriocins in lactobacillus yogurt, lactobacillus treatment the concentration of stander protein 8 g/dl, OD of stander protein 0.546

No. of tube	OD Bacteriocins from yogurt	The concentration of Bacteriocins from Yogurt g/dl	OD Bacteriocins from treatment	The concentration of Bacteriocins g/dl
Total	0.450	6.6	0.375	5.5
MIC1	0.322	4.8	0.247	3.6
MIC2	0.264	3.8	0.192	2.8
MIC3	0.245	3.6	0.172	2.5
MIC4	0.212	3.1	0.153	2.4
MIC5	0.162	2.4	0.121	1.7

Biological effects of crude Bacteriocins with prebiotic against *Sal typhi*

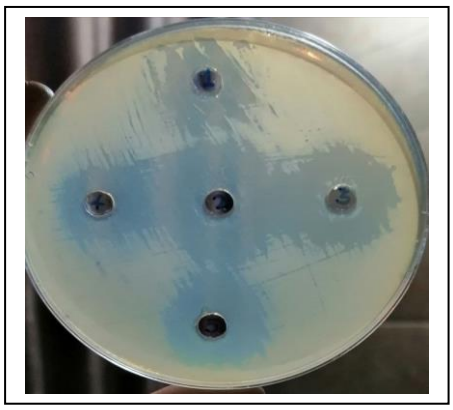

Figure 11. (1) glucose added to lactobacillus yogurt, (2) inulin added to *lactobacillus* yogurt, (3) inulin prebiotic, (4) inulin added to *lactobacillus* treatment, (5) starch added to *lactobacillus* yogurt on Muller Hinton plate

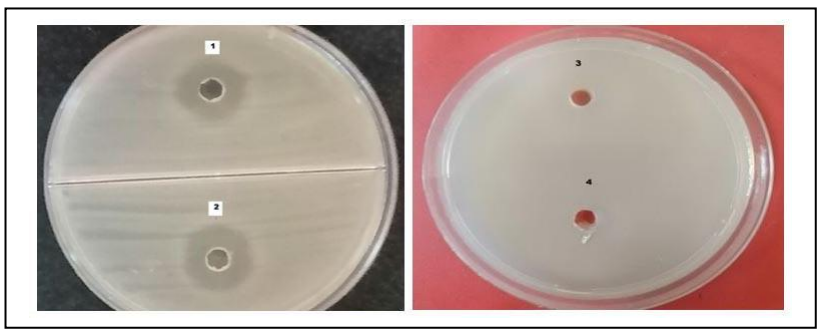

Figure 12. (1) starch added to *lactobacillus* treatment (2) glucose added to *lactobacillus* treatment (3) starch prebiotic (4) glucose control on Muller Hinton plate

Table 6. The inhibition value against *Salmonella typhi* using inulin in 24 hours within the plate (per dish)

Time	OD Values using Inulin			
	No.	Mean ± SD	Mean difference [a]	Significance
24 hours	50	14.10 ± 6.701	-3.900	*t*= -4.115, df: 49, *P*= 0.000

[a]: Mean difference from cutoff values, [b]: One-sample T-Test

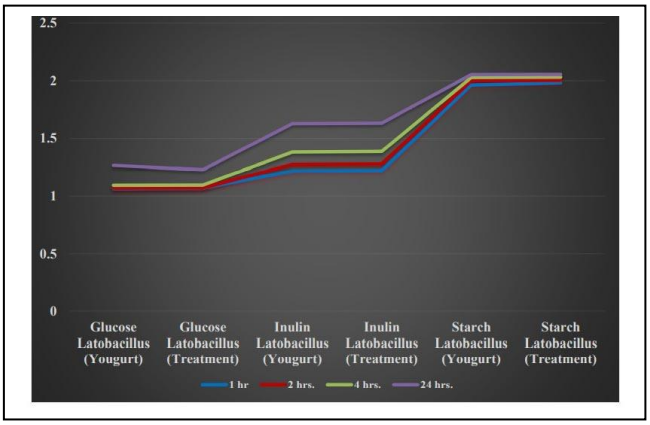

Figure 13. The OD values of prebiotic add to *Lactobacillus* against *S. typhi* (glucose, inulin, and Starch) in 1 hr, 2 hr, 4 hr, and 24 hr within a microplate

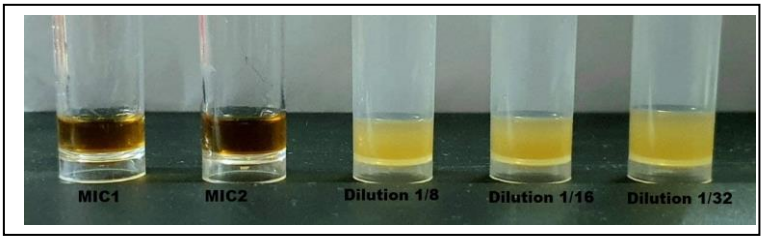

Figure 14. MIC for *Lactobacillus* Bacteriocins adds to prebiotic against *S. typhi*

Table 7. The OD and concentration of Bacteriocins in lactobacillus yogurt add inulin, lactobacillus treatment added inulin

No. of tube	OD Bacteriocins from yogurt add inulin	The concentration of Bacteriocins from Yogurt add Inulin	OD Bacteriocins from treatment add inulin	The concentration of Bacteriocins add inulin
Total	0.778	11.4	0.737	10.8
MIC1	0.658	9.6	0.617	9.8
MIC2	0.592	8.7	0.551	8.00
MIC3	0.573	8.4	0.532	7.8
MIC4	0.541	8.00	0.500	7.3
MIC5	0.528	7.7	0.487	7.1

Note: The concentration of stander protein 8 g/dl, OD of stander protein 0.546

Table 8. The OD and concentration of Bacteriocins in lactobacillus yogurt add glucose (control), lactobacillus treatment adds glucose (control), the concentration of stander protein 8 g/dl, OD of stander protein 0.546

No. of tube	OD Bacteriocins from Yogurt adds glucose	The concentration of Bacteriocins from Yogurt add Glucose	OD Bacteriocins from treatment, add glucose	The concentration of Bacteriocins add glucose
Total	0.696	10.2	0.443	6.5
MIC1	0.576	8.4	0.323	4.7
MIC2	0.510	7.5	0.257	3.8
MIC3	0.478	7.00	0.228	3.3
MIC4	0.465	6.8	0.206	3.0
MIC5	0.452	6.6	0.193	2.8

Table 9. The OD and concentration of Bacteriocins in lactobacillus yogurt added Starch; lactobacillus treatment added Starch

No. of tube	OD Bacteriocins from yogurt add Starch	The concentration of Bacteriocins from yogurt add Starch	OD Bacteriocins from treatment add Starch	The concentration of Bacteriocins add starch
Total	0.743	10.9	0.490	7.2
MIC1	0.623	9.1	0.378	5.5
MIC2	0.557	8.2	0.312	4.6
MIC3	0.538	7.9	0.293	4.3
MIC4	0.506	7.4	0.280	4.1
MIC5	0.493	7.2	0.267	3.9

Note: the concentration of stander protein is 8 g/dl, OD of stander protein 0.546

Table 10. The OD value against *Salmonella typhi* using glucose in different hours within microplate

Time interval	OD Values using Glucose			
	No.	Mean±SD	Mean difference [a]	Significance [b]
1 hours	50	0.7168±0.072621	-0.00282	t= -0.275, df: 49, *P=0.785*
2 hours	50	0.79172±0.069093	0.015720	t= 1.609, df: 49, *P=0.114*
4 hours	50	0.82134±0.070117	0.029340	t= 2.959, df: 49, *P=0.005*
24 hours	50	0.93848±0.132656	0.054480	t= 2.904, df: 49, *P=0.006*

Table 11. The OD value against *S. typhi* using inulin in different hours in microplate

Time interval	OD Values using Inulin			
	No.	Mean ± SD	Mean difference a	Significance [b]
1 hour	50	0.72458 ± 0.078168	0.007580	*t*= 0.686, df: 49, *P*= 0.496
2 hours	50	0.77278 ± 0.072771	0.000780	*t*= 0.076, df: 49, *P*= 0.940
4 hours	50	0.81904 ± 0.067017	0.031040	*t*= 3.275, df: 49, *P*= 0.002
24 hours	50	0.88969 ± 0.103263	0.021688	*t*= 1.485, df: 49, *P*= 0.144

[a]: Mean difference from cutoff values, [b]: One-sample T-Test

Table 12. The OD value against *S. typhi* using Starch in different hours within microplate

Time interval	OD Values using Starch			
	No.	Mean ± SD	Mean difference A	Significance
1 hour	50	1.86426 ± 0.085461	-0.093740	*t*= -7.756, df: 49, *P*= 0.000
2 hours	50	1.89846 ± 0.085985	0.013460	*t*= 1.107, df: 49, *P*= 0.274
4 hours	50	1.92182 ± 0.115868	-0.010180	*t*= -0.621, df: 49, *P*= 0.537
24 hours	50	1.95570 ± 0.122541	-0.010300	*t*= -0.594, df: 49, *P*= 0.555

[a]: Mean difference from cutoff values, [b]: One-sample T-Test

4. Discussion

The results shown in figure 2 were compatible with those of a study conducted by Ramadan (19), who collected samples from Baghdad Teaching Hospital in Baghdad, Iraq between 2003 and 2004 and reported the incidence rate of *S. typhi* at 27.67%. Probably this incidence rate was due to a lack of health awareness, while Bhatta, Bangtrakulnonth (20) revealed that the prevalence rate of *Salmonella* infection was 8.9655% in Nepal. *The salmonella* infection rate in Iraq is 44.25%, which is a very high percentage, compared to other countries, especially developed countries, like the United States (0.015%) (21). This could be the result of continuous exposure when traveling throughout the world.

In developing countries with poor sanitation, *Salmonella*, which causes typhoid fever, is widespread. *Salmonella* bacterium can be found and transmitted to humans through contact with contaminated food, water, or the environment of animals, such as birds or reptiles, that are kept as pets (22, 23). Natural resistance was a common trait observed during our research. This was consistent with the findings of a study performed by Klemm, Shakoor (24) which proved that the multi-antibiotic resistance of *S. typhi* strains have become a significant problem in Asia Klemm, Shakoor (24).

Man Rogosa Sharpeagar which contains complex nutritional requirements was used as a selective medium promoting the growth of *Lactobacillus* as shown in figures 4-12 while suppressing other bacterial species. *Lactobacillus* species have probiotic properties, i.e., they can tolerate specific environmental stress present in the gastrointestinal tract, such as acidity and bile conditions, that are non-pathogenic. All these factors are considered for the proper selection of probiotics. This makes *Lactobacillus* an interesting bacteria to microbiologists due to their high potential health and nutritional benefits (25).

Natural resources, specifically *Lactobacillus* spp., were utilized to overcome the problem of MDR. Present and isolated in ordinary yogurt, this medicine was deemed a standard that had already been produced to cope with various issues that required interaction with *Lactobacillus* extraction of bacteriocins and inhibition of *S. typhi*. The difference between phases was quite clear when using the well diffusion method with and without prebiotics. The largest inhibitory zone was about 17-20 mm in diameter; this value comes from the isolate bacteriocins versus *S. typhi* (1, 3, 4, 6, 7, 9, 19, 20, 27, 28, 29, 30, 31, 32, 36, 37, 39, 40, 41, 47, 48, 49, 50). This isolate had already developed MDR and was close to exceeding the ATCC minimal inhibition zone of 20. The responses of the other stains were within the range of 0-16. The present research supports the findings of a study conducted by Zacharof and Lovitt (26).

In order to evaluate the lost consideration that can hinder *S. typhi* while ignoring the inhibition zone of less than 20 mm, MIC dilution was performed to take a strain of *S. typhi* that has a response number of 20 mm (7, 15, 27, 30, 32, 37, 40, 47, 49, 50). The above dilution resulted in only the first dilution (1/2) giving inhibition, while the others showed no inhibition. Results of this research are in line with those of a previous study performed by Abdel-Daim, Hassouna (27). Due to the pharmaceutical characterization of preparation by freezing, which may interfere with medicinal purposes by lyophilizing the sedimented bacteria after extra centrifugation, *Lactobacillus* was employed as a medication that did not show any inhibition.

The results showed that the best option was inulin, giving a very high and positive response to inhibition by increasing the diameter zone. Even for samples that did not give any response before, for example, 13, 23, 24, 26, 38, 42, 43, 44, 45, and 46, the inhibition zone was about 10-25 mm. Findings of the current study are consistent with those of a previous study performed by Shoaib (28).

Inulin-type prebiotics contain inulin-type fructans, a category of nutritious substances that includes naturally-occurring plant oligo- and polysaccharides in

which one or more fructosyl-fructose linkages form the majority of glycosidic connections. Fructans must have a beta (2-1) fructosyl-fructose glycosidic linkage to be classified as "inulin-type", which gives inulin its distinctive structural and physiological qualities, including the ability to withstand enzymatic hydrolysis by human salivary and small intestinal digesting enzymes. Fructooligosaccharides (FOS) and oligo fructose are inulin-type prebiotics, which can have a variety of chemical compositions. Since inulin, oligofructose, and FOS are resistant to enzymatic digestion in the upper gastrointestinal tract, they reach the colon almost undamaged, fermented by bacteria. All inulin-type prebiotics stimulate the growth of *Lactobacillus* species. The effects on other intestinal microbes are less understood. A small amount of inulin-type prebiotic appears to be required to create a probiotic effect, an intraindividual response to the same inulin-type prebiotic at the same dose.

Regarding the antibacterial activity of bacteriocins, the total and diluted concentration of MIC 1, MIC 2, MIC 3, MIC 4, and MIC 5 for bacteriocins in *Lactobacillus* yogurt, with added inulin and starch for probiotic, and with prebiotic inulin were calculated in order to estimate the lost consideration that can inhibit *S. typhi*, considering the exclusion of the inhibition zones of less than 20-27 mm. Based on the results of the above-mentioned dilutions, only the first and second dilutions (½ and ¼) showed inhibition, while the others did not. Results of the present study were in agreement with those of a study carried out by Abdel-Daim, Hassouna (27).

Prebiotics inulin, starch, and glucose as standards have been investigated alone against *S. typhi*. Without further addition as disposing of diffusion plate agar methods, the result reading was obsolete. Inulin made very successful inhibition readings; accordingly, 50 samples were sensitive to inulin, except samples 1, 12, 13, 31, 32, and 50, which made no inhibition zones.

The microbial environment of the large intestine is necessary for physical conditions, and imbalances can lead to diseases. Inulin stimulates the development and metabolic action of a limited number of bacteria in the colon, *Lactobacilli*, and thus promotes their health. This is referred to as a prebiotic.

Probiotics are live microorganisms having beneficial host effects by enhancing microbial balance in the intestine, whereas prebiotics is indigestible food components having beneficial effects by enhancing the activity and growth of one or more colonic bacteria (29). *Lactobacillus* filtrates had considerable effects against the test *S. typhi* isolates.

Authors' Contribution

Study concept and design:
Acquisition of data:
Analysis and interpretation of data:
Drafting of the manuscript:
Critical revision of the manuscript for important intellectual content:
Statistical analysis:
Administrative, technical, and material support:

Conflict of Interest

The authors declare that they have no conflict of interest.

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