Original Article

The effect of supernatant of Lactobacillus plantarum against Salmonella Typhi biofilm formation

Ulaa A. Kadhom[1], Dr. Ali Jaffar Saleem[2], Dr. Amal A. Kareem[3]
[1,2]Al-Iraqia University/Collage of Education for Pure Science/ University of Diyala
[3]Middle Technical University /College of Health& Medical Techniques /Baghdad/ Research Unite
Email: bioh.olla.kadhom@uodiyala.edu.iq
DOI: 10.47750/pnr.2022.13.S01.88

Abstract

Lactic acid bacteria are a safe and healthy alternative to antibiotics that have been attracted in many recent studies Methods: Salmonella. Typhi was diagnosed with the VETIC system, biofilm formation was detected by Congo red method and the microtiterplate method. L. plantarum capsule from (Swanson) company ATCC 53103 was used to obtain L.plantarum life cells. Results: The diagnostic results showed that 11 bacterial isolates from feces with a percentage 1.27% and 37(61%) bacterial isolates from blood represented S. Typhi bacteria with a total of 48 isolates and various levels of biofilm productivity, which were categorized as having strong production by 87%, average production by 6.25%, and weak production of membrane bioavailability by 2.1%. The inhibiting effect of L. Plantarum potential as a probiotic was investigated using the bacterial Cell Free Supernatant (CFS) of the same bacteria, which gave the highest inhibition area for S. Typhi and for three bacterial isolates, it was (16,18,17) mm compared to the inhibition of suspension which gave the lowest inhibition areas (16,15,15)mm. The results also showed that the CFS produced by L. plantarum had the ability to inhibit biofilm formation by 20 isolates of S.T. bacteria, in comparison with the positive control reading. The highest inhibition rate was 84.5% towards isolate S.T.4 and the lowest 70.8% inhibition of the S.t.35 isolate trend. Therefore, L. plantarum suspension can be presented as a viable healthy and therapeutic probiotic agent.

Keywords: Biofilm, Salmonella Typhi, Probiotic, anti-biofilm, Lactobacillus plantarum, supernatant.

INTRODUCTION

Salmonella Typhi causes typhoid fever, which is a common disease in society (1), and it is one of the main problems in the world, especially in developing countries where the disease is endemic, and Iraq is one of the countries that suffer from this disease annually (2), this bacterium is a parasite on humans in particular and is the only host for it (3), and infects multiple organs of it, as it invades the lymphatic tissues of the small intestine, liver, spleen and bloodstream of the infected person, resulting in the emergence of multiple strains that are resistant to antibiotics because they take A strategy of biofilm formation that helps them persist inside the host by aggregating with each other (4).

Biofilm forms in response to a number of factors such as high cellular density and unfavorable environmental conditions such as nutrient deficiencies or during antibiotic treatment (5), and biofilm-forming bacteria are responsible for chronic and incurable infections because they contribute to increased pathogenesis and (6) proved in his study that the biofilm has a protective role similar to the capsule in S. Typhi bacteria that is not surrounded by the capsule in giving it the ability to cause infection, as well as it has a key role as a shield for bacteria to stay for a longer period in the body, such as colonizing S. Typhi bacteria in chronic and asymptomatic gallbladder, This allows it to be transmitted to uninfected individuals (7), so treatment alternatives such as a probiotic are resorted to, which is characterized by its inhibitory effect on biofilm production and has no side effects in addition to its presence inside the body has multiple benefits (8).

Lactic acid bacteria is a safe and healthy alternative to antibiotics, which attracted a lot of recent studies. In this study, it was found that Lactobacillus plantarum has an antibacterial activity against S. Typhi bacteria. This study also proved that its floating CFS without cells has an effect in removing and preventing biofilm formation by S. Typhi .The study was carried out according to scientific methods described in some international sources and research (9 ,10). L.plantarum bacterium tolerates different stress conditions, resists acidic stomach, small intestine environment and pancreatic juice when passing through the part .The upper part of the alimentary canal (11), tends to stick to the cells lining the intestine. This binding prevents the invasion of pathogenic bacteria (12), due to the inhibitory property of L. plantarum against S. Typhi, because it produces antibiotics

Microbes, including lactic acid, acetic acid, hydrogen peroxide and bacteriocins, and scientific studies have shown that the L. plantarum′s CFS has an antagonistic action towards Escherichia coli and Vibrio cholera (13).

Materials and methods:

- Samples Collection

One hundred S. Typhi isolates (blood and feces) were collected from educational laboratories in Baghdad / Iraq, these samples (60 blood and 40 feces) were collected from age groups (1-58) years, from patients They are expected to have typhoid infection ,the samples cultivating in Brain heart infusion broth bottles for blood culture ,SS agar and XLD agar. The fully grown colonies on the surface of the agar plate were identified as S. Typhi as reported by (14), the phenotypic characteristics of the isolated bacteria and biochemical tests were studied then the diagnosis was confirmed using the VITEK® 2 GN Gram-negative bacteria diagnostic kit (BioMerieux).

- Biofilm formation methods

Congo-red method

S. Typhi was inoculated into Congo red medium and incubated for 18 h at 37°C. and the plates were incubated at 37°C for (48-24) h. The appearance of black colonies is evidence of a positive test, while the pink colonies refer to negative result (15).

Micro-titer plate method (MTP)

The susceptibility of all S. Typhi isolates to biofilm formation as described by (16) with an adjustment in the incubation time, which extended to 24 h according to (9) by activating these isolates on nutrient agar plates and incubating for 24 h at 37 °C, and the test was carried out by adding 200 µl Tryptic soy broth(TSB) to each pit of microtiter plate and inoculating with the isolates by 50 µl (three repetitions in the vertical rows of the titration dish for each isolate separately and in succession for isolates from (1 to 48) , in addition to a negative control by adding 200 µl of the same sterile culture medium not inoculated with bacteria for three replicates in the last vertical row. Then the wells were dyed with crystal violet (0.1%) for 15 min., then the dye was removed by washing them three times with physiological salt and left to dry at room temperature, then 200 µl of glacial acetic acid were added at a concentration (33%), and then the optical density was read with an ELISA reader for all contents at a wavelength of 630 nm to determine the efficiency of the isolates in biofilm production. The calculations were done according to Table (1-1) (9).The experiment was carried out with three replications and the average value was adopted.

Table (1-1) Classification of adhesion susceptibility of isolates

Mean of OD value at 630nm	Biofilm formation
(OD ≤ ODc)	Non-biofilm
(ODc < OD ≤ 2× ODc)	Weakly biofilm
(2× ODc < OD ≤ 4× ODc)	Moderately biofilm
(4× ODc < OD)	Strong biofilm

- Activation and preparation of the probiotic L. plantarum

The freeze-dried bean of the commercial probiotic L.plantarum from (Swanson) company ATCC 53103 was crushed individually by light pressure using a ceramic mortar sterilized by alcoholic flame (17), then transferred to 9 ml of prepared and sterilized MRS broth and incubated at 37°C. For a period of 24 h with the activation process repeated three times (18), the logarithm of the number of living suspended cells in addition to the reading of the Optical density was calculated according to the MacFarland (0.5).

-Calculating the logarithm of the viable cells of the biostimulants used in the study

The logarithm of of L. plantarum bacteria was calculated according to the method described by (19). The necessary serial dilution were performed using sterile peptone water tubes, by transferring 1 ml of MRS broth inoculated with the L. plantarum and making the necessary dilutions, then transferring 1 ml From the dilutions to the sterilized plates , the plates were poured using sterile MRS agar and the medium was mixed with the sampel. after solidification of the medium, the plates were transferred and using anaerobic jars, emptied from the air and CO2 gas was pumped, then the container was placed in the incubator at 37°C for 48-72°C. An h (20), then the plates were taken out and the colonies formed were counted using a colony counter and the number of bacteria per ml was calculated by multiplying the colony rate * the reciprocal of the dilution and for three replications (21), and the numbers were converted to the logarithmic formula and through For its preparation / (CFU) Colony Forming Unit /(ml).

Preparation of probiotic bacteria CFS cell-free extract

The Cell Free Supernatant (CFS) filter for probiotic bacteria was prepared based on what was mentioned in (22), from isolates of L. plantarum, grown on MRS broth, and incubated at 37°C for 72 h under anaerobic conditions. Then 18,000 xg was centrifuged for 25 min. at 4°C to obtain the bacteria-free CFS. Then the suspension was filtered through millipore filters with a diameter of 0.22 µl. The CFS was kept refrigerated in sterile glass bottles to study the effect of its inhibitory activity by holes method against isolates of S. Typhi.

- Detection of the inhibitory activity of suspended bacteria L. plantarum

The well diffusion method was used to detect the inhibitory activity of the suspension of L. plantarum, which prepared by growing the bacteria on MRS broth, while the suspension of S. Typhi was spread on the medium of Muller Hinton(MH) compared with the McFarland tube (0.5). By means of a cotton swab, using a sterile cork poorer, made holes with a diameter of 5 mm on the surface of the medium and then filled it with 50 µl of the prepared suspension. The occlusion was incubated at a temperature of 37°C for 18 h, then the areas of inhibition around the holes were measured and the results were recorded and compared with the control treatment containing MRS broth without any bacterial inoculum (23).

- Detection of the inhibitory activity of L. plantarum CFS

The well diffusion method was used as mentioned by (10) to detect the inhibitory activity of the prepared L. plantarum CFS, while the suspension of S. Typhi was spread on MH medium after comparison with McFarland tube (0.5). By means of a cotton swab, using a sterile cork Poorer ,wells were made with a diameter of 5 mm on the surface of the medium in each plate, then filled with 50 µl of the prepared CFS. It was compared with the control treatment containing MRS broth medium without any bacteria (24).

Characterization of antimicrobial substances produced by L. plantarum

The CFS was divided into five tubes as reported by (25):

1. The first tube was treated with 1 mg/mL Trypsin to determine bacteriocin production.

2. The second tube was changing to pH 6.5 ± 0.1 by adding NaOH.

3. The third tube was treated with 0.5 mg/ml catalase for 30 min at 25°C to determine hydrogen peroxide production.

4. The fourth tube was set to pH 6.5 ± 0.1 and treated with catalase and trypsin.

5. The fifth tube was used as a positive control (untreated).

The experiment was completed by testing the damping efficacy according to the method of diffusion in the holes.

- Detection of the effect of CFS produced by L. plantarum in inhibiting the production of biofilm produced by S. Typhi:

The experiment was conducted for each of the CFS and the suspended one to reveal their effectiveness in inhibiting or preventing the formation of the biofilm individually and at the same time by the method of microtiterate plates. Amodified method was used based on the method (9):

The most biofilm-producing isolates of S. Typhi were selected and activated on TBS medium and incubated for 24 h at 37 °C. The culture medium containing the activated isolates was added at a rate of 100 µl to each hole of the microtiterplate (in the amount of three wells of the vertical rows). From the titration plate for each isolate separately and in succession), then 100 µl of the extracted CFS were added to the pits designated to study the effect of the CFS in inhibiting biofilm adhesion, and 100 µl of the suspension were added to the pits containing the bacterial culture and designated to study the effect of the suspension in inhibiting biofilm adhesion, in addition To (negative control) by adding 200 µl of sterile and uninoculated culture media to three holes in the last vertical row, and to (positive control) by adding 200 µl of bacterial culture without additions to three holes in the first vertical row, and completed the experiment according to (9) The percentage of adhesion inhibition of pathogenic bacteria was calculated by applying the equation given by (26,27).

$$\%\ of\ biofilm\ inhibition = 1 - \frac{Optical\ density\ (OD)\ of\ the\ inhibitor\ protein}{Optical\ density\ (OD)\ for\ positive\ control\ treatment}$$

100 samples were collected for age groups (1-58) years of patients with symptoms of typhoid fever, 40 faeces samples and 60 blood samples, the results showed that 11 faeces samples (27.5%) and 37 blood samples (61.6%) were of the genus S. Typhi, with a total number of 48 samples (48%), of the total sample as shown in Table (1-4). This result agreed with the result of the study of (28), which collected samples during the epidemiological detection during the outbreak of the disease from the center In Al-Obaidi, for the period from 2002-2003, 58 isolates were out of a total of 144 isolates belonging to the cultivar S. Typhi, as it agreed with the results of (29), which appeared from 47 positive blood samples, 32 isolates belonging to S. Typhi, and to indicate the infection status in The whole country was resorted to the study of (30,31)which took place in the cities of Babylon

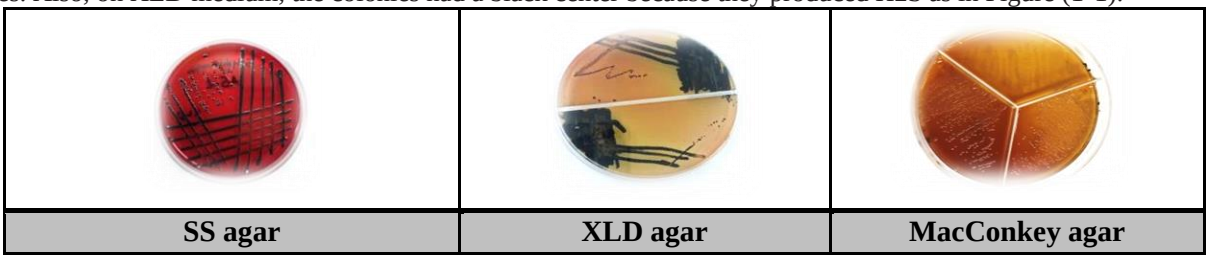
and Thi Qar, and the percentages were close to 24(68.57)%, 26 (59%) respectively. This study is close correlated with (28,32), where the serotype S. Typhi recorded the highest isolation rate of 61 (12.7%), 58 (47.1%), respectively. The agreement of the results with the different time periods and place of infection. This is due to the increase in health awareness and the lack of sufficient symptoms and old diagnostic methods, and on the other hand, neglect of health conditions by restaurants and street vendors, deterioration of food preservation in markets and shops, damage to drinking water networks and their inclusion in many areas of leaching. This leads to the entry of groundwater contaminated with sewage water and pollution Drinking water (33).

Table (1-2) shows the number of isolates, infection rate and their sources

source of isolation	No.	Number and percentage of *S.* Typhi isolates
The blood	**60**	**(3777)%**
Faeces	**40**	**(%22.9) 11**
The total	**100**	**(% 48) 48**

A study in India showed that the percentage of S.Typhi isolates was (35%), while in the United States of America the percentage of isolates was (25%) (34). The closeness of the isolation rates of S.Typhi in local and international studies with the different geographical location and environmental conditions of the source of isolation, may be due to several reasons, the most important of which is the misuse of antibiotics, whether the patient's use of drugs that prevent the growth of bacteria or those that are resistant to bacteria contributed greatly to their spread, Therefore, the tendency to conduct more studies on the S.Typhi that causes typhoid fever remains one of the important trends because it is one of the serious public health problems in many countries and developing ones in particular, as typhoid disease is considered a frightening disease because of the long duration and complications associated with it if it is not detected and treated early)33).

The microscopic examination of S. Typhi stained with Gram stain showed that they are Gram-negative rod-shaped cells, not forming spores. It is lactose-fermented and has an unpleasant odor on MacConkey. On S.S agar medium, it appeared as black colonies. Also, on XLD medium, the colonies had a black center because they produced H2S as in Figure (1-1).



SS agar	XLD agar	MacConkey agar

Figure (1-2) shows the growth of S. Typhi colonies on differential media

The diagnosis of the isolates was confirmed by VITEK 2 compact system using the negative card (GN ID card). The results were in agreement with the results of the biochemical tests. 48 isolates belong to the serogroup S. Typhi. Using the card for Gram-negative bacteria gives greater diagnostic accuracy through the 43 test and through within 5-8 h, the isolates with a high accuracy of 99% (35).

Results of qualitative and quantitative detection of biofilm formation of S. Typhi

1) Qualitative methods: The Congo red method is considered a qualitative method because it shows isolates, productive or non-productive (36) and as shown in Figure (1-3).

Figure (1-3): The Congo Red Method

The study showed that 3 isolates were producing biofilm using the Congo red method at a rate of (6.25%) and as mentioned in Table (1-3). According to (37) study findings, this approach revealed that 29 out of a total of 82 isolates were highly bio-

producing.The micro-titer plate method is more accurate and sensitive in studying biofilm production than the Congo Akar method, this was confirmed by(38), because the Congo Akar method is affected by oxygen and incubation conditions.

Quantitative method (Micro-titer plate):This test is a quantitative assay to detect the ability of biofilm formation, as it gives a digital value of the absorbance at a wavelength (630) nm using an ELISA reader to determine the amount of live films formed by adhesion to the surfaces of the microtiterplate. The absorbance represents the thickness of the live films formed by the isolates.Crystal violet dye is also used in this method, which is a basic dye that has the advantage of attaching it to negatively charged molecules on the cell surface as well as nucleic acids and polysaccharides, and thus it gives acomprehensive measure of the entire bio-membrane and gives the result of whether the bacteria are motile or non-motile (39), and from it is worth noting that the biofilm composed of Salmonella on the walls of the wells because the Salmonella bacteria have motile (fimbria, polar flagella) compared to non-motile bacteria such as Staphylococcus aureus that form the biofilm at the bottom (40).

The results of the study showed that 46 (95.83%) produced biofilm by titration method with different production rates from strong 42 (87.5%) ,moderate 3 (6.25%) to weak biofilm formation (2.1%) as shown in Table (1-4)) The difference in thickness of biofilms is due to various reasons such as differences in the ability of biofilms; perhaps the initial number of bacteria that succeeded in adhesion, the qualitative and quantitative differences induced QS quorum sensing signaling molecules produced from each isolate (41).

The results of the study agreed with the study of researcher (32), as the production rate of his isolates by this method reached (93.75%) with 15 isolates out of a total of 16 isolates, while it differed with the study of (42) in Malaysia, where the production of his isolates in this way was (28.33). %) 29 out of 60 isolates from the gallbladder, in which it was mentioned that the presence of S. Typhi frequently is associated with the presence of (GSD) gallstone disease and without symptoms in the case of the human carrier and is generally localized in the gallbladder, as it is not believed in this case that it is necessary for its development as it attracts bacteria that invade and then spread intracellular, colonize and persist as a biofilm on gallstone disease (GSD). The spread of bacteria through urine and feces, especially in the host human chronic infection with S. Typhi, epidemiological studies conducted in areas endemic with S. Typhi, such as Chile, Bolivia, Ecuador as well as some areas of India, Pakistan and Japan and Korea, nearly 90% of carriers with chronic infection have also been shown to be gallstone carriers, and this association is termed as the major predisposing factors for the development of GC (gallbladder cancer) (6, 43).

In this study and many other studies,(39) in Ethiopia revealed the substances that inhibit or kill alternative antibiotics and substances that affect biofilm construction were found. Therefore, this study came to probiotic bacteria and their products as a safe, effective and cost-effective alternative.

Table (1-4) shows the biofilm productivity by the two methods of micro titration plate and Congo red

No.	biofilm formation	Micro titer plate method	Congo red method
1	strong	(87.5 %) 42	2 (% 4.17)
2	moderate	(6.25 %) 3	0
3	weak	(2.1 %) 1	1 (% 2.1)
Total		**(95.83 %) 46**	**3 (%** 6.**25**)

The inhibit activity of the probiotic L. plantarum against S.Typhi .

The impact of suspended and CFS together for L. plantarum probiotic against S. Typhi bacteria were studied. Three highly biofilm-forming isolates were selected, which were named S.t1, S.t.3, and S.t.2 and the statistical analysis showed significant differences. Clear with a significant level of significance (P > 0.05) between the inhibitory effects of the studied components of both types of lactobacilli on the isolates of S. Typhi as in Figure (1-5).

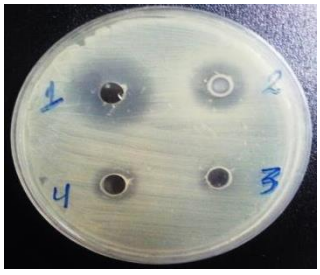


Figure (1-5): The inhibition zone of L. plantarum CFS

1: L. plantarum suspended ,2: L. plantarum CFS ,3:C- (MRS broth),4: Chloramphenicol

The inhibition zone of L. plantarum suspension with a diameter of 17 and 18 mm against isolates S.t.1 and S.t.2,

respectively, while the lowest inhibition zone was with a diameter of 15 mm for the two isolates S.t.2 and S.t.3 caused by the effect of suspended bacteria L.plantarum as in Table (1- 5), and as a result of the results shown, the L. plantarum CFS was approved in subsequent experiments to inhibit and kill S. Typhi and prevent it from forming a biofilm.

Table (1-5) Inhibitory activity of L. plantarum against S.Typhi

Isolates	*L.plantarum*	
	CFS	**suspension**
S.t.1	17mm	16mm
S.t.2	18	15
S.t.3	16	15

The results showed that L. plantarum has a clear inhibitory effect towards S.Typhi, but there is a discrepancy in this effect according to the type of isolate, as the diameters of the highest inhibition were for the CFS, which includes many inhibitory and lethal substances for pathogens, due to its production of secondary metabolic products such as microbial antibiotics,such as bacteriocins (11). It contains lactic acid and Biosurfectant (BS), in addition to its fermentation products (44).as for the inhibitory activity of the suspension against S. Typhi bacteria, because it contains live cells that have a competitive role with pathogenic bacteria by competitive exclusion against pathogens on the adhesion site and prevent their assembly (45), in addition to the components of the CFS that kills pathogenic bacteria that work together in a synergistic way to kill and prevent the growth or accumulation and adhesion of pathogenic bacteria (46), which also confirmed that there is a clear effect of the extract of lactobacilli on the DNA of the albicans by destroying the DNA, and that such an effect does not go away with the disappearance of the effect. Antifungals used in the treatment of these candida.

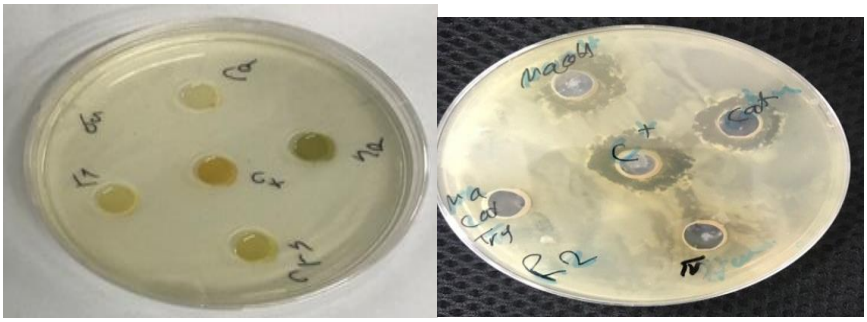
The results were in agreement with what was obtained (47) when using quinoa at a rate of 4% synbiotically with the probiotics L. rhamnosus (GG) and L. plantarum separately against some types of pathogenic bacteria, including Gram-negative pathogens. S. enterici serovir typhimurium The diameter of the inhibition halos was (16) mm for each of the two species.

L. plantarum produces an antimicrobial (dipeptide bacteriocins) Plantarici (48) which is a heat-resistant and antimicrobial protein for a number of food spoilage-causing Gram-positive and Gram-negative pathogens such as Listeria spp. L. plantarum also produces an antimicrobial role. In its competition with pathogenic bacteria for the place of attachment in the body (49), L. plantarum produces another antimicrobial, Bacteriocin SLG10, with properties that resist heat, pH, and the digestive enzymes Trypsin and Pepsin (50).

The use of lactobacilli and its filters was better than the use of the antibiotic itself in the study (51), explaining the reason that the use of these bacilli is safe and far from many of the side effects associated with antibiotics, and on the other hand, the emergence of resistant strains of these antibiotics in addition to their deadly effect on bacteria Antibiotic-resistant disease is a successful alternative to antibiotics, and this is consistent with what was mentioned (52).

Characterization of the antigens produced by L.plantarum

Based on what was shown in the results of the experiments in this study that Lactobacillus is characterized by antimicrobial activity due to their ability to produce antimicrobial substances, such as organic acids, hydrogen peroxide, bacteriocins and the rest of the produced substances that have a similar inhibitory spectrum, the results showed that the inhibitory effect against S.Typhi bacteria was mainly due to bacteriocin. When Trypsin was added to the crude leaf, the action against the microbial was inactivated (no inhibition aura appeared), but in the absence of Trypsin, the result was the appearance of an inhibitory action, while organic acids and Hydrogen peroxide has a secondary role, not a primary one, which is to enhance the role of bacteriocin, because the inhibition halos persisted even when the pH was titrated to (0.6 ± 0.1) using NaOH and treated with Catalase as shown in Figure (1-6).



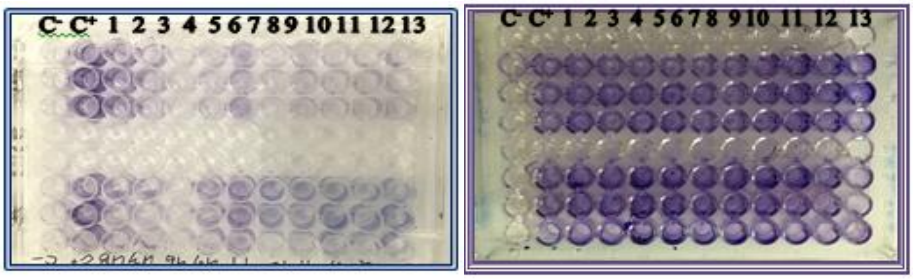
Befor incubation After incubation

Figure (1-6): shows the inhibitory effect of supernatent on S. Typhi.

The results of the study agreed with (46) that bacteriocin produced by L.acidophilus has a strong inhibitory action against Psudomonas aerogenosa, S.aureus and E.coli, In another study, it was found that bacteriocin extracted from L.acidophilus has a deadly activity against multiple antibiotic-resistant bacteria isolated from the throats of patients suffering from upper respiratory tract infections (53). It also agreed with the study of (54), which confirmed that the inhibitory effect of Lactobacillus on multi-antibiotic resistant E.coli isolated from children with diarrhea was the effect of bacteriocin only. It also agree with (47) study on L. rhamnosus (GG) and L. plantarum which had an inhibitory effect on a group of pathogenic bacteria resistant to Gram-positive and Gram-negative antibiotics, like S.aureus, B.cereus, E.coli O157:H7, and S.enterici serovir typhimurium. While it disagree with (55), represented inhibitory effect of bacteriocin produced by L. acidophilus had very little effect on P. aeruginosa.

Inhibition of biofilm formation by L.plantarum (CFS)

CFS filter L. plantarum was used to inhibit the adhesion and formation of biofilm formed by S. Typhi bacteria by micro titer plate method as in Figure (1-7).



Biofilm formation without suspension Biofilm formation with suspension

Figure (1-7): Inhibition of the biofilm formed by S. Typhi bacteria with CFS produced by L.plantarum bacteria

Figure (1-7) showed that L. plantarum CFS had the ability to remove the biofilm formed by S. Typhi bacteria on the inner surface of the micro titration plate, which is the most accurate and best method in detecting the formation or inhibition of the biofilm and bacteria adhesion (56), the results in Table (1-6) showed that CFS CFS produced by L. plantarum had the ability to inhibit biofilm formation by 20 S. Typhi isolates in comparison with the positive control reading (S. Typhi cultured on TSB media). Without the CFS, the highest inhibition was 98.0% towards isolate S.T.6 and the lowest was 96.2% towards isolate S.T.15.

Table (1-6): shows Inhibitory activity of of L. plantarum′s suspension

NO	AV of OD	Percentage of biofilm inhibition
C+	**2.979**	**-----**
S.T.1	**0.075**	**96.8%**
S.T.2	**0.082**	**96.5%**
S.T.3	**0.088**	**96.3%**
S.T.4	**0.081**	**96.6%**
S.T.5	**0.088**	**96.3%**
S.T.6	**0.048**	**98.0%**
S.T.7	**0.077**	**96.8%**
S.T.8	**0.075**	**96.8%**
S.T.9	**0.063**	**97.3%**
S.T.10	**0.068**	**97.1%**
S.T.11	**0.089**	**96.2%**
S.T.12	**0.066**	**97.2%**
S.T.13	**0.069**	**97.1%**
S.T.14	**0.086**	**96.4%**
S.T.15	**0.089**	**96.2%**

LSD value	---	**12.063 ****
* (P≤0.05).		

The results of the study agree with(57), who showed that CFS components of L. plantarum comprising bacteriocin and biosurfactant have anti-adhesion activity, thus preventing biofilm formation when treating a group of multi-antibiotic-resistant bacteria,(26) that it has anti-microbial activity, the highest inhibition rate was 70% against S.aureas. It also agreed with the study of (58), which confirmed that L. plantarum works to reduce biofilm formation in two types of pathogenic bacteria S.typhimurium and E.coli to a significant extent and also works to dismantle membrane components and instability by removing the membrane components. Its polarization thus dismantled it. While it differed with the study (59), which stated that the lactobacilli inCFS is not sufficient to inhibit the adhesion of some pathological bacteria in the digestive system, including E.coli.

Crude bacteriocin depends on a special inhibitory mechanism, as it can bind to the surfaces of most types of pathological cells as a result of having a large amount of affinity towards the components of the walls of many pathogenic bacteria causing damage by more than one mechanism. The membrane, which leads to a loss of ions and energy, as evidenced by the influx of potassium ions K+ out of the target cell, and after 30 seconds of exposure of the target cell to bacteriocin, this leads to the flow of other cytoplasmic contents and then the death of the cell. As a result of its final destruction and death (60), it is also effective at pH 4.2-8.0, and it has an inhibitory and deadly spectrum for a number of pathogenic bacteria and food pollutants, including (S. aureus, S. typhimurium, B. cereus, and E. coli , B. subtilis and Listeria innocua), and is not affected by the enzymes Lysozyme and Catalase, but they are destroyed by protease enzymes such as Pepsin and Trypsin and Papin, used to preserve and extend the shelf life of foodstuffs (Shelf-life) . (61) also mentioned that CFS L. plantarum CFS contains active substances called biosurfactant (BS). BS can be defined as glycoproteins (with a high content of proteins), which produce biologically active surface factors in the stabilization phase as in BS produced from L. plantarum bacteria, its protein content is the important part in the process of inhibiting surface binding and has a major role in reducing surface tension when adding this substance to surfaces or solutions associated with other factors, including pathogens, as these proteins compete with the receptors of other factors to bind to surfaces and replace them. As mentioned by(6), the highest activity of BS at pH ranges between 3 - 7.4 and at a temperature of 25 °C, and its effectiveness is not specialized and has a wide spectrum in influencing the adhesion of pathogens to surfaces (62), that L. plantarum′s CFS capable of raising the level of active oxygen and depolarizing the biofilm and destroying the wall of the pathogenic bacteria S. Typhi, thus its death and destruction of the biofilm and the release of cellular materials, in addition to its impact on the ability of adhesion, movement and ability to form biofilm with a significant removal rate, for this it can Presentation of CFS CFS of L. plantarum as a health and therapeutic probiotic factor applicable in multiple uses in industrial applications and biological activities in addition to its uses in other fields (26).

Conclusions:

The results showed that the CFS of L.plantarum was able to inhibit biofilm formation by microtiter plate method, as a result of the effectiveness of the CFS components produced by L.plantarum bacteria, which are able to raise the level of active oxygen, depolarize the biofilm and destroy the wall of S. Typhi, thus its death and destroying the biofilm by releasing cellular materials, in addition to affecting the ability of adhesion, movement and the ability to form the biofilm with a significant removal rate, for this reason, the suspension of L. plantarum bacteria can be presented as a viable healthy and therapeutic probiotic antibiotic.

REFERENCES

1. Booijink CC, Zoetendal EG, Kleerebezem M, de Vos WM (2007) Microbial communities in the human small intestine: coupling diversity to metagenomics. Future Microbiol 2(3):285–295. https://doi.org/10.2217/174 60913.2.3.285.
2. Rada, Donia Jawaid,1999,‘ Stady of efficieny diagnostic tests for Salmonella Typhi bacteria and resistance to antibiotics by molecule methods ’,Thesis, College of Science /AL-Mustansiriyah University.
3. Chia, T.W.R., Goulter, R.M., Mcmeekin, T., Dykes, G.A. & Fegan, N., 2009, ‘Attachment of different Salmonella serovars to materials commonly used in a poultry processing plant’, Food Microbiology 26, 853–859. http://dx.doi.org/10.1016/j. fm.2009.05.012.
4. Chauhan, N., Ma, K., & Farooq, U. (2016). Biofilm: an Important Factor in the Prevalence of Mdr Salmonella Typhi. 5(10), 2464–2473.
5. Stokniene, J., Varache, M., Rye, P. D., Hill, K. E., Thomas, D. W., & Ferguson, E. L. (2022). Alginate oligosaccharides enhance diffusion and activity of colistin in a mucin-rich environment. Scientific Reports, 12(1), 1–12. https://doi.org/10.1038/s41598-022-08927-1
6. Raza, A., Sarwar, Y., Ali, A., Jamil, A., Haque, A., & Haque, A. (2011). Effect of biofilm formation on the excretion of Salmonella enterica serovar Typhi in feces. International Journal of Infectious Diseases, 15(11), e747–e752. https://doi.org/10.1016/j.ijid.2011.06.003
7. Goer, A., Blanchard, L. S., Van Belkum, A., Loftus, K. J., Armstrong, T. P., Gatermann, S. G., Shortridge, D., Olson, B. J., Meece, J. K., Fritsche, T. R., Pompilio, M., Halimi, D., & Franceschi, C. (2022). Multicenter Evaluation of the Novel ETEST Fosfomycin for Antimicrobial Susceptibility Testing of Enterobacterales, Enterococcus faecalis, and Staphylococcus Species. Journal of Clinical Microbiology. https://doi.org/10.1128/jcm.00021-22
8. DaSilva PE, Von Groll A, Martin A, Palomino JC. (2011). Efflux as a mechanism for drug resistance in Mycobacterium tuberculosis. FEMS Immunol.

Med. Microbiol. 63(1), 1–9.
9. Dheepa, M.; Rashme,L.; & Appalaraju, F. (2011). Comparision of biofilm production and multiple drug resistance in clinical isolates of Acinetobacter baumanii from atertiary care hospital in south India :Int J. Pharm. Biomed. Sci. 2011, 2(4), 103 – 107 ISSN No:0976-5263.
10. Razzak, M., AlCharrakh, A. and Al-Greitty, B. (2011) Relationship between lactobacilli and opportunistic bacterial pathogens associated with vaginitis . North Am.J.Med .Sci. .3:185-192.
11. Oh, N. S., Joung, J. Y., Lee, J. Y., & Kim, Y. (2018). Probiotic and anti-inflammatory potential of Lactobacillus rhamnosus 4B15 and Lactobacillus gasseri 4M13 isolated from infant feces. PLoS ONE, 13(2), 1–15. https://doi.org/10.1371/journal.pone.0192021
12. Marras, L., Caputo, M., Bisicchia, S., Soato, M., Bertolino, G., Vaccaro, S., & Inturri, R. (2021). The role of bifidobacteria in predictive and preventive medicine: A focus on eczema and hypercholesterolemia. Microorganisms, 9(4), 1–21. https://doi.org/10.3390/microorganisms9040836
13. Matejčeková, Z., Liptáková, D., Spodniaková, S., & Valík, Ľ. (2016). Characterization of the growth of Lactobacillus plantarum in milk in dependence on temperature . Acta Chimica Slovaca, 9(2), 104–108. https://doi.org/10.1515/acs-2016-0018
14. MACFADDIN, J. F. Individual biochemical tests. Biochemical tests for identification of medical bacteria, 2000, 3: 27-439.
15. Freeman , D. J. ; Falkiner , F. R. and Keane , C. T (1989) . New method for detecting slime production by coagulase negative staphylococci . Journal of Clinical Pathology . 42(8) : 872-874pp.
16. Christensen,G.D.:Simson W.A.:Bisno,A.L. and Beachey ,E.H. (1982). Adherence of slime-producing strains of Staphylococcus epidermidis to smoth surface. Infect.immun. 37:318-326 .
17. Myers, D. N. and Sylvester, M. A. (2022). "7.1.1. Equipment and Equipment Sterilization Procedures" (PDF). National field manual for the collection of water-quality data. Techniques of Water-Resources Investigations, book 9. USGS.Page last reviewed: February 2, 2022.Content source: Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases (DFWED)
18. Savini, M.; Cecchini, C.; Verdenelli, M. C.; Silvi, S.; Orpianesi, C. and Cresci, A. (2010). Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. Nutrients, 2(3), 330-339.
19. Mathialagan, M.; Edward, Y. J. T.; David, P. M. M.; Senthilkumar, M.; Srinivasan, M. R. and Mohankumar, S. (2018). Isolation, Characterization and Identification of Probiotic Lactic Acid Bacteria (LAB) from Honey Bees. International Journal of Current Microbiology and Applied Sciences, 7, 894-906.
20. Buck, L. and Gilliland, S. (1995). Comparisons of freshly isolated strains of Lactobacillus acidophilus of human intestinal origin for ability to assimilate cholesterol during growth. J. Dairy Sci. 77:2925-293
21. Weber, A. and Hekmat, S. (2013). The Effect of Stevia rebaudiana on the growth and survival of Lactobacillus rhamnosus GR-1 and sensory properties of probiotic yogurt. J. dairy technology, 2(2):136-142.
22. Gupta, U.; Radramma; Rati, E.R. and Joseph, R.(1998). Nutritional quality of lactic acid fermented bitter gourd and fenugreek leaves. International Journal of Food Sciences and Nutrition, 49(2): 101-108.
23. Lin, C.K.; Tsai, H.C.; Lin, P.P.; Tsen, H. Y. and Tsai, C.C. (2008). Lactobacillus acidophilus LAP5 able to inhibit the salmonella choleraesuis invasion to the human Caco -2 epithelial cell Anaerobe., 14:251-255.
24. Ivanova, I.; Kabadjova, P.; Pantev, A.;Danova, S. and Dousset, X. (2000) Detection , Purification and partial characterization of a noval bacteriocine substance production by Lactobacillus lactis subsp. Lactis b14 isolated from Boza-Bulgarian traditional cereal beverage. Biocatalysis, 41(6):47-53.
25. Lim, E. S. (2016). Inhibitory effect of bacteriocin-producing lactic acid bacteria against histamine-forming bacteria isolated from Myeolchi-jeot. Fisheries and Aquatic Sciences, 19(1), 42.
26. Gudiña EJ, Teixeira JA, Rodrigues LR (2010) Isolation and functional characterization of a biosurfactant produced by Lactobacillus paracasei. Colloids Surf B Biointerfaces 76:298–304. doi:10.1016/j.colsurfb.2009.11.008
27. Heinemann C., Hylckama Vlieg J.E. Van, Janssen D.B., Busscher H.J., Van der Mei H.C., Reid G., FEMS Microbiol. Lett. 190 (2000) 177.
28. Al-Maadidi, Asif Hassan Abdel-Razzaq (2004). Epidemiological and Molecular Study of Antibiotic-Resistance of Some Salmonella Species Isolated from Clinical and Non-Clinical Specimens. Master Thesis, Department of Life Sciences/Microbiology, College of Science/University of Baghdad.
29. Abood, Ali musa kadhim.(2020). Immunogen Preparation of Salmonella Typhivirulence Factors Isolated from Typhoid fever Patients. Msc thesis college of Science, University of Babylon.
30. Albanwawy, J. N. A., & Abdul-lateef, L. A. (2021). Molecular Detection of Some of the Salmonella Typhi Virulence Genes Isolated in the Province of Babylon / Iraq. 25(2), 675–685.
31. AL-Oqaili, N. A. D. (2019). Detection of some virulence factors of Salmonella Typhiisolated from patients' blood by PCR and phylogenetic tree. Periodicals of Engineering and Natural Sciences, 7(4), 1915–1923. https://doi.org/10.21533/pen.v7i4.941
32. AL Jobouri, Abdul Aziz Thamer Abdul Aziz Farhan,(2010). Molecular Characterization of Some Virulence Genes of Salmonella enterica in Babylon province. Thesis ,B.Sc.in Microbiology\ College of Science \ Babylon University-2010.
33. World Health Organization.(2007).Communicable Disease Profile for Iraq.
34. Dutta, S., Das, S., Mitra, U., Jain, P., Roy, I., Ganguly, S. S., Ray, U., Dutta, P., & Paul, D. K. (2014). Antimicrobial resistance, virulence profiles and molecular subtypes of Salmonella enterica serovars Typhi and paratyphi A blood isolates from Kolkata, India during 2009-2013. PLoS ONE, 9(8), 1–13. https://doi.org/10.1371/journal.pone.0101347
35. Forbes, B.A., Saham, D.F.& Weissfed, A.S.(2007).Baily and scott's diagnostic microbiology .12th ed. Mosby .Inc.St. Louis. USA.
36. Sallami, I., Turki, Y., Werheni Ammeri, R. et al. Effects of heavy metals on growth and biofilm-producing abilities of Salmonella enterica isolated from Tunisia. Arch Microbiol 204, 225 (2022). https://doi.org/10.1007/s00203-022-02836-7.
37. Al-Sa′adi, Amal Azeez Kareem,(2014). The relationship between collagenase production and biofilm formation by Pseudomonas aeuroginosa. Doctorate dissertation/ College of Science/ University of Baghdad.
38. Krysenko, S., Okoniewski, N., Nentwich, M., Matthews, A., Bäuerle, M., Zinser, A., Busche, T., Kulik, A., Gursch, S., Kemeny, A., Bera, A., & Wohlleben, W. (2022). A Second Gamma-Glutamylpolyamine Synthetase, GlnA2, Is Involved in Polyamine Catabolism in Streptomyces coelicolor. International Journal of Molecular Sciences, 23(7), 3752. https://doi.org/10.3390/ijms23073752
39. Hajri, A., Zmantar, T., Altayb, H. N., Kouidhi, B., & Chaieb, K. (2022). Synthesis, antibacterial, antibiofilm evaluation and molecular docking studies of 3-methyl- 2-propyl-2H-[1,2,4]triazolo[4,3b] [1,2,4,6]thiatriazine-1,1-dioxide. Bulletin of the Chemical Society of Ethiopia, 36(1), 109–117. https://doi.org/10.4314/bcse.v36i1.10
40. Duffus, J. H., Nordberg, M., & Templeton, D. M. (2007). Glossary of terms used in toxicology, 2nd edition (IUPAC recommendations 2007). Pure and Applied Chemistry, 79(7), 1153–1344. https://doi.org/10.1351/pac200779071153
41. Kerr, E. J., Stafford, R., Rathnayake, I. U., Graham, R. M. A., Fearnley, E., Gregory, J., Glasgow, K., Wright, R., Sintchenko, V., Wang, Q., Howard, P., Leong, L. E. X., Valcanis, M., Pitchers, W., Lambert, S. B., & Jennison, A. V. (2022). Multistate Outbreak of Salmonella enterica Serovar Heidelberg with Unidentifi ed Source, Australia, 2018-2019. Emerging Infectious Diseases, 28(1), 236–241. https://doi.org/10.3201/eid2801.211462.

42. Kalai Chelvam, K., Chai, L. C., & Thong, K. L. (2014). Variations in motility and biofilm formation of Salmonella enterica serovar Typhi. Pathogens, 6(1), 1–10. https://doi.org/10.1186/1757-4749-6-2
43. Di Domenico, E. G., Cavallo, I., Pontone, M., Toma, L., & Ensoli, F. (2017). Biofilm producing Salmonella Typhi: Chronic colonization and development of gallbladder cancer. International Journal of Molecular Sciences, 18(9). https://doi.org/10.3390/ijms18091887.
44. Gut AM, Vasiljevic T, Yeager T, Donkor ON. Salmonella infection - prevention and treatment by antibiotics and probiotic yeasts: a review. Microbiology (Reading). 2018 Nov;164(11):1327-1344. doi: 10.1099/mic.0.000709. Epub 2018 Aug 23. PMID: 30136920.
45. Chenoll E. 2011. A novel probiotic Bifidobacterium bifidum CECT 7366 strain active against the pathogenic bacteria Helicobacter pylori. Appl. Environ. Microbiol. 77:1335–1343.
46. AL-Dulaimi, T.H., Aziz, H.W., Al-Marzoqi, A.H., Al-Aziz, S.A. and Mohsin, S.A.A.)2015(. Molecular characterization and antibiotic susceptibility of diarrheagenic Escherichia coli from Children. Medical Journal of Babylon, 12(2): 541-550
47. Al-Roumi, Fouad Farouk Youssef (2021). The use of quinoa seeds (Chenopodium quinoa Willd. Previously vital in therapeutic lactic ferments), College of Agricultural Engineering Sciences - University of Baghdad.
48. Seddik, H. A.; Bendali, F.; Gancel, F.; Fliss, I.; Spano, G. and Drider, D. .(2017). "Lactobacillus plantarum and its probiotic and food potentialities," Probiotics Antimicro Prot, vol. 9, No. 2, pp. 111–122.
49. Aguilar-Toalá, J. E.; Santiago-López, L.; Peres, C. M.; Peres, C.; Garcia, H. S.; Vallejo-Cordoba, B. and Hernández-Mendoza, A. (2017). Assessment of multifunctional activity of bioactive peptides derived from fermented milk by specific Lactobacillus plantarum stains. Journal of dairy science,100(1), 65-75.
50. Pei, J.; Jin, W.; Abd El-Aty, A.M.; Baranenko, D. A.; Gou, X.; Zhang, H.; Geng, J.; Jiang, L.; Chen, D. and Yue, T. (2020). Isolation, purification, and structural identification of a new bacteriocin made by Lactobacillus plantarum found in conventional kombucha. Food Control 110, 106923, 2020.
51. Muhammad, Bushra Jassem (2004). Studying the effect of different concentrations of alum, hydrogen peroxide and bitter melon extract on the growth and genetic content of Pseudomonas aeruginosa isolated from hospital contaminated. Higher Diploma thesis, Institute of Genetic Engineering and Biotechnology, University of Baghdad.
52. Sambanthamoorthy, K., Feng X., Patel R., Patel S. and Paranavitana C. (2014). Antimicrobial and antibiofilm potential of biosurfactants isolated from lactobacilli against multi-drug-resistant pathogens. BMC Microbiol. 14:197-206.
53. Zubair, F. and Nawaz, S .K. (2017). Antibacterial activity of bacteriocin isolated from Lactobacillus acidophilus against throat infections causing bacteria. Int. J. Biosci. 10(4): 372-379.
54. AL-Imam, Mokhtar Jawad Kadhim.(2020). Evaluation of Lactobacillus spp. supernatant effect on gene expression of some virulence determinants in Enteropathogenic and Enterohaemorrhagic Escherichia coli from diarrheal patients. College of Science/ University of Baghdad.
55. Kamel, Fouad Hussein. (2005). Purification and characterization of Surlactin produced by Lactobacillus acidophilus. PhD thesis, College of Science, Al-Mustansiriya University.
56. Al Shaikh, I. and Musaddiq M.(2010). Detection and analysis of Biofilm production and extracellular polymeric substance in Pseudomonas aeruginosa isolated from clinical specimens . Biosci. Biotech. Res. Comm.3(1):74-78.
57. Ashraf, A. Abd El-Tawab, Ahmed, A. A. Maarouf, Fatma, I. El Hofy, and Zeinab, A. M. Mahdy. Characterization and bioactivities of Lactobacillus plantarum and Pediococcus acidilactici isolated from meat and meat products. Nat Sci 2019;17(12):187-193]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 26. doi:10.7537/marsnsj171219.26.
58. Yan, X., Gu, S., Cui, X., Shi, Y., Wen, S., Chen, H., & Ge, J. (2019). Antimicrobial, anti-adhesive and anti-biofilm potential of biosurfactants isolated from Pediococcus acidilactici and Lactobacillus plantarum against Staphylococcus aureus CMCC26003. Microbial Pathogenesis, 127(October 2017), 12-20 . https://doi.org/10.1016/j.micpath.2018.11.039
59. Russo, P.; Spano, G. and Capozzi, V. (2017). Safety evaluation of starter cultures. Starter Cultures in Food Production, eds Speranza, B.; Bevilacqua, A.; Corbo, M.R. and Sinigaglia, M. (Hoboken, NY: John Wiley and Sons), pp. 101-128.
60. Adetoye, A., Pinloche, E., Adeniyi, B. A., & Ayeni, F. A. (2018). Characterization and anti-salmonella activities of lactic acid bacteria isolated from cattle faeces. BMC Microbiology, 18(1), 1–11.
61. Giordani, B., Parolin, C., & Vitali, B. (2021). Lactobacilli as Anti-biofilm Strategy in Oral Infectious Diseases: A Mini-Review. Frontiers in Medical Technology, 3(October), 1–8. https://doi.org/10.3389/fmedt.2021.769172
62. Abudoleh, S. M., & Mahasneh, A. M. (2017). Anti-quorum sensing activity of substances isolated from wild berry associated bacteria. Avicenna Journal of Medical Biotechnology, 9(1), 23–30.