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Multi-locus sequences Technique for Identified *Lactobacillus salivarius* from Greek yogurt anti-biofilm agents

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Abstract
Although the idea of micro–organism such as bacteria in standard is dangerous to humans, A quantity of its species are taken into consideration to be of superb cost and safety, especially in organic therapy, including the institution Bacteria Probiotics. Lactobacillus salivarius is one of the maximum fundamental forms of bacteria which can be innocent to people and which may also play a main position in their bioremediation potential in general. Moreover, Lactobacillus salivarius was identified by traditional methods using MRS agar selective medium, then by biochemical methods using the VITEK 2 method. Finally, Lactobacillus salivarius was confirmed using Multi Sequencing (MLST) and Biofilm technique. The main objective of this research is to isolate and characterize the novel bacterium salivarius Lacto from Greek yogurt and their ability for biological assay and for study to determine the effect of bacteriocin, which is secreted by these bacteria, and to study the extent to which these by-products to reduce environmental pollution by removing heavy metals from various environmental media by these products.

Keywords: Lactobacillus, Yogurt, MLST, VITEK2 and Bio-film technique.

Introduction.

Lactobacillus is the most important genus of the own circle of relatives lactobacillaceae. Lactobacillus is one of the maximum fundamental forms of bacteria which can be innocent to people and which may also play a main position in their bioremediation potential in general. Lactic acid bacteria have been recognized at the start of the 19th century and have been described as "organisms that cause acidity of milk. Henneberg In 1904, observed that the bacteria which reason acidity in milk are just like the sorts of lactic acid bacteria observed with inside the soil. In 1905, Grigoroff was isolated this bacteria from milk that differ in their microscopic form, including rods, including spheres (Corsetti et al., 2011). It is known that lactic acid bacteria possess the characteristics of beneficial bacteria (probiotic), as they have the ability to produce inhibitory compounds such as Diacetyl, Lactic acid, Hydrogen peroxide, Acetaldehyde, Bacitracin, and these compounds are able to inhibit the growth of harmful microbes (Allameh, et al, 2012). Beneficial microorganisms are defined by the Food and Agriculture Organization/WHO as organisms that, when added in sufficient quantities, confer a health benefit on the host (Sieladie et al.,2011).

From May 2018 , it include 196 validly posted species, and those species are normally isolated now no longer best from environments related to fermented food, consisting of fruits, meat, sourdough, vegetables, and wine, but additionally from the gastrointestinal and vaginal tracts of people and animals (Tamang et al; 2015). Currently, lactobacilli are broadly implemented in fields associated with food ,feed, prescribed drugs and biotechnology; for example, they are used as dairy starters, probiotics, vaccine vendors and silage inoculants (Giraffa et al,2010),that are among the maximum economically thrilling packages of lactic acid bacteria(LAB). Lactobacilli are gram-positive, rod-shaped , facultative anaerobic or microaerophilic, non-spore-forming, acid-tolerant, and catalase-bad micro-organism with DNA G+C content material this is typically much less than 50 mol%. Lactic acid bacteria are fermented carbohydrates to produce (Abed,2013).

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Milk and fermented milk merchandise are healthful ingredients and incorporate lots of wherein calcium and nutrition D for vital elements of bone fitness in addition to protein or different nutriments (Anon,2011).

Materials And Methods

Isolation and Identification of bacterial

Samples have been gathered from Greek yogurt crafted from goat's milk originally made in Turkey called Siris of the KRI KRI business enterprise and transported to the laboratory via way of means of sterilized sealed glass packing containers and packing containers. The sample was grown on MRS agar medium and incubated at 37°C for 48 hours in aerobic conditions. The bacterial was stained by Gram stain kit to confirm that it is gram positive.

Biochemistry Diagnosis

The micro-organism had been identified by using the VITEK2 tool provided of using the bioMérieux company, which incorporates sixty four biochemical checks which are utilized in diagnosing micro-organism, in order that the accuracy of the analysis with this tool reaches 98%. Notably, the experiment has done according to manufacturer protocol.

Molecular Diagnosis

A- DNA Extraction

Extraction of DNA was carried out in the central laboratory of the College of Agriculture and Forestry / University of Mosul. It was extracted by a special kit produced by (Sigma) and according to the kit's protocol.

B- PCR Primer

Polymerase chain reaction was conducted by using thermo-cycler equipment for eight housekeeping genes by designed sixteen primers that were selected from the whole bacterial genome sequences for the NCBI website. The sequences of the used primer showed in **Table1**.

Table 1. The Primers sequences for housekeeping genes for *Lactobacillus salivarius* that used in this study

	The name of primer	Sequencing
1	*clpX/F*	CGCACGGAAGCAGAAAC
2	*clpX/R*	GAGTCGGTCCCAAACCC
3	*dnaK/F*	GACAACGGTCCGCTCCACT
4	*dnaK/R*	TCGGCTTCTTCCTTCTTCTTCT
5	*groEL/F*	CCGACAACGACAAGATGG
6	*groEL/R*	CCAAGGCAGGGATAACG
7	*murC/F*	TTTGAAGCCGACGAATACC
8	*murC/R*	CGATGTCCTCGCTACCC
9	*pepX/F*	AAAGAAGACGAGCAACCAACC
10	*pepX/R*	CGGAGTCCTTAGTCCCGATT
11	*pyrG/F*	TCATTGGGTCGGCTGTT
12	*PyrG/R*	GGTCCATCCCTTGCTTTTG
13	*rpoB/F*	GAAGTTCCGCCGCTCTA
14	*rpoB/R*	GGTCCCATCTGGCATGTAC
15	*uvrC/F*	TCGTCACCTCCTCCAATAA
16	*uvrC/R*	TGGTTCGGTAATCCCTCC

C- PCR Amplification

Standard PCR reactions, which start with one preparatory thermal cycle, were carried out at a temperature of 96 °C for two minutes for the initial denaturation of the DNA tape as a preparatory step at the beginning of using the PCR device, then followed by 35 thermal cycles, one cycle includes three steps and each step has a specific temperature for a specific purpose as follows: The first step was carried out at 96 °C for 2 minutes to denature the double strip. The second step was done at 95 °C for 30 seconds to bind the primers to the complementary site on the DNA strand. The third step it was done at 45 °C for 30 seconds to start the

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elongation process of the initiator. Then last step heat cycle at 72°C for 1.5 minutes to complete the elongation of the starter. After done all PCR process, the DNA fragments determine by electrophoresis at 120 voltage,50 minutes and 400 mv, within 1% agarose gel (Albanna, 2017 and Alomari, 2017).

1. Extraction DNA

The Genome of bacteria of lactobacillus in general have been extracted depending on the company (sigma), Catalog Nos(NA2100)

2. Run to the PCR, according to manufacture (Promega).

The polymerase chain reaction it contains details as follows:

1.Includes:- (Nuclease Free water , Primers(forword,refers), DNA extracted , Green *Master mix*2.The cycle of PCR was 30 cycle starting of :- denaturation 95 and Annealing 45 , Extension 72

Notably:- The PCR fragments Agarose gel electro phoresis using to agarose gel 2% ,safe stain 5ML , voltage 100 , ampere 400.

Preventing the formation of biofilms (the rate of biofilm inhibition).

Bacillus subtilis, which usually form the biofilm, became used to research the capacity of bacteriocin to inhibit the formation of the biofilm. (two hundred microliters in the direction of the formation of stay membranes through pathogenic bacteria the use of the micro titer plate 96-wells method), as (20) microliters of the diluted bacterial suspension have been transferred to every hollow containing (two hundred) microliter of the nutrient broth medium, and (10) microliters of every awareness of dry filtrate of bacteria used in the examine have been introduced to (4) pits for every sort of pathogenic bacteria with the usage of manage pits containing the nutrient medium handiest and different pits containing inoculated nutrient medium The M.T.P. plates have been then incubated for (24) hours at (37) ranges Celsius, and after the incubation period, the contents of the pits have been eliminated with the aid of a micro pipette, the pits have been washed (3) instances with Alfo buffer solution. Sfat, then ethanol alcohol became used at a awareness of (95%) to restoration the cells connected to the partitions of the holes, the plates have been left for (10) minutes, the alcohol became eliminated and left to air dry, then the holes have been dyed with crystal violet dye at (1%) with inside the mild of adding (100). Microliter of dye for every hollow, and the dyeing procedure took (15) minutes, then the holes have been washed with sterile distilled water to cast off the hint of extra dye, in addition to cast off the weakly connected cells. The clean crystal pink dye because of treating pathogenic bacteria with exclusive concentrations of the bacterial infiltrate, the dye adhered to the partitions of the holes became extracted through adding (100) microliters of glacial acetic acid at (33%) to every hollow, after which the optical density of the biofilms became measured the use of a stretchable device. The wavelength is (450) nm, the test became repeated with (3) replications for every pathological bacterial isolate and for every awareness of the bacterial filtrate (Mathur et al., 2006; Abbas and Ahmed, 2014)

3. Results

Isolation and identification of lactic acid bacteria.

The observe blanketed choice of an isolate of Lactobacillus salivarius developing on MRS agar medium taken from samples of dairy products (Greek yogurt). The growing of colonies was developing in this medium and gave white and yellow color, and the relevance of those isolates to lactic acid micro -organism turned into showed via way of means of subjecting them to some of examinations and tests. Afterward, microscopic examinations had been carried out, wherein the cells seemed with inside the microscopic exam of the organized slides from colonies of micro-organism with inside the shape of small quick bacilli regularly organized in pairs (chains) or can be single, because it became out to be Gram-nice and immobile. As proven with inside the **figure (1)**. Where our outcomes had been in settlement with preceding studies (Bin Masalam et al., 2018) (Shida et al., 1997).

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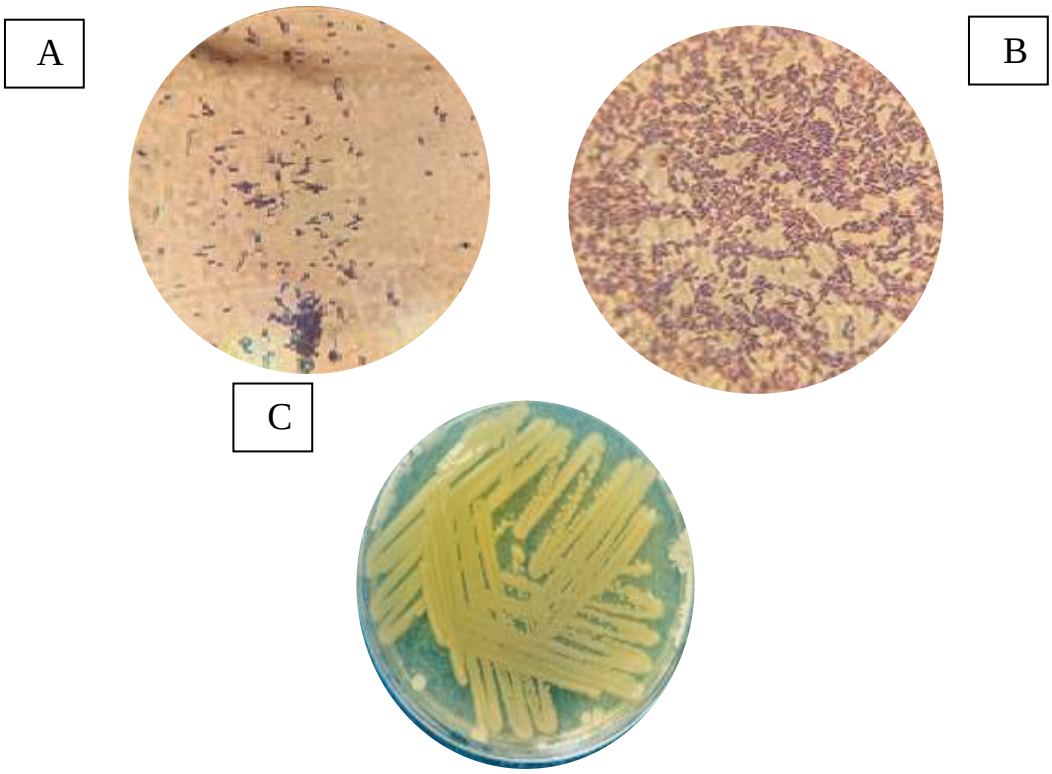

Figure (1): Shows the cultivation traits and microscopic exam of *Lactobacillus spp..*
(A+B) is Lactobacillus cells after staining with gram stain and examined under a light microscope using a 100x oil lens.
(C) is refers to Lactobacillus colonies growing on MRS agar solid medium.

Biochemical Examinations.

After getting ready natural cultures of Lactobacillus spp. Laboratory diagnostic biochemical exams have been performed. **Table (2)** and **figures (2 and 3**) display the biochemical outcomes, via way of means of adopting sixty four biochemical analyzes the usage of VITEK2 technology, in which the biochemical evaluation turned into matched and the very last end result of Lactobacillus salivarius turned into given consistent with the outcomes of bioMérieux Company, the proportion of isolates identified turned into 100% **Table (2)** biochemical checks for sixty four assays the usage of VITEK2 technique.

Table (2) refers to the biochemical examinations for 64 assays via using VITEK2 technique.
(+) Positive test result (the ability of bacteria to grow).
(-) Negative test result (disability of bacteria to grow).

2	AMY	-	4	PIPLC	-	5	dXYL	+
13	APPA	-	14	CDEX	-	15	AspA	-
20	LeuA	+	23	ProA	-	24	BGURr	-
28	AlaA	+	29	TyrA	-	30	Dsor	-
38	dRIB	-	39	ILATk	-	42	LAC	-
47	NOVO	+	50	NC6.5	+	52	dMAN	-
57	dRAF	-	58	O129R	-	59	SAL	+
8	ADH1	+	9	BGAL	-	11	AGLU	-

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16	BGAR	-	17	AMAN	-	19	PHOS	-
25	AGAL	+	26	PyrA	-	27	BGUR	-
31	URE	-	32	POLYB	-	37	dGAL	-
44	NAG	+	45	dMAL	+	46	BACL	-
53	dMINE	-	54	MBdG	-	56	PUL	-
60	SAC	-	62	dTRE	+	63	ADH2s	+
64	OPTO	+						

figures (2 and 3) show the Identification of Lactobacillus salivarius by Vitek 2 technique, which has sixty-four biochemical analyzes.

Molecular Diagnosis.

When the bacterial isolates have been recognized the usage of tradition and biochemical tests, DNA became extracted from the received isolates, in which the extracted DNA became left on agarose gel the usage of electrophoresis technique. A wavelength of 320 nm to hit upon the DNA bundles of the samples and to make sure their presence, purity and comparable molecular weight to all isolates, because it turned into observed that the DNA of the isolates is near 18000 Kb, and **figure (4)** suggests the consequences of the electric switch of genetic material at the gel with a capacity distinction of (100) volts and (400)) mill amperes for an hour.

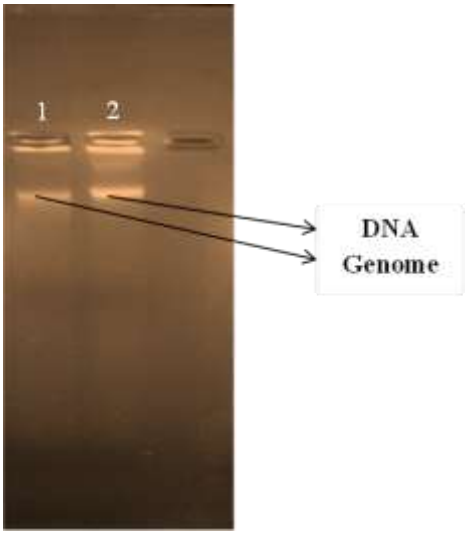

Figure (4) shows the Migration of the DNA genome extracted from Lactobacillus salivarius onto agarose gel the use of the diagnostic kit. The numbers (1-2) refer to the samples used with inside the migration.

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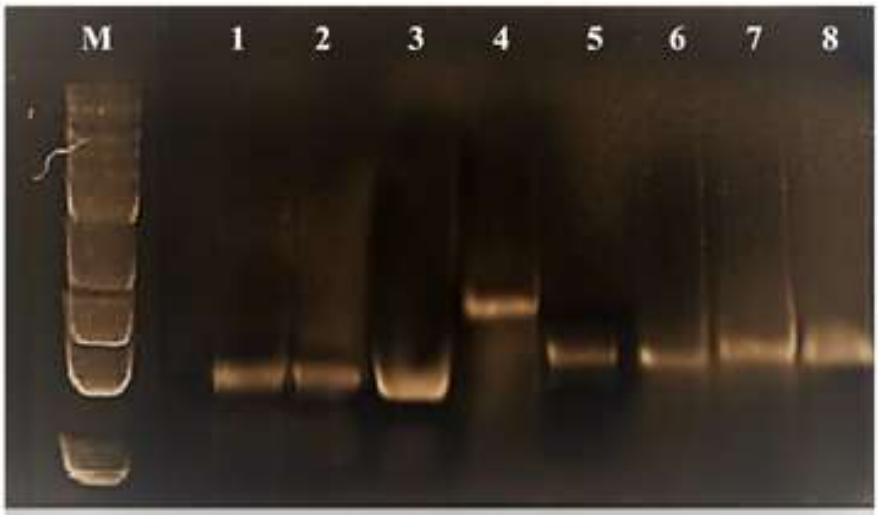
The selection Method for Multi_Loccus Sequence typing (MLST).

A phenotyping test concerned the identity of 8 eight house- keeping genes which there are (*ClpX,DnaK,GroEL,MurC,PepX,PyrG,RpoB,UvrC*), and to identfy and verify Lactobacillus salivarius remoted from Greek yogurt and recognized morphologically and biochemically.

Polymeres Chains Reaction (PCR).

Diagnosis of micro-organism (bacteria) through biochemical assessments is generally unreliable, because of the necessities of bacteria inclusive of nutrients and different comparable matters for distinct species of the identical genera. So, researchers have followed molecular diagnostic techniques, inclusive of analysis with the aid of using sequencing the nitrogenous bases of genes, as a supplement to phenotypic analysis and now no longer as an alternative for it. The genetic sequence of bacteria become trusted to diagnose the micro-organism belonging to the genus Lactobacillus salivarius after the genes of the genetic cloth had been amplified through PCR method and a manage price of one KB become used for comparison. Lactobacillus salivarius, wherein nearly all of the DNA packets seemed on the molecular length of 800bp as proven in the **figure (5)**. The phenotypic analysis coincided with the genetic analysis of bacterial isolates. It is really well worth noting, that the 8 DNA cuts with home tasks genes regarded comparable in comparison to the current study (Xu et al., 2022). It became emphasized in our effects that using the MLST approach become used in the detection of bacteria due to its significance in the accuracy and pace in figuring out the sort of micro bacteria further to be diagnosed.



1 Kb

Figure (5) is shown the DNA PCR cuts for eight housekeeping genes (house -keeping genes) for Lactobacillus salivarius

Examine the ability of Lactobacillus salivarius to inhibit biofilm formation.

The look at protected the detection of the sensitivity of pathogenic bacteria to biofilm manufacturing the usage of 9 special concentrations, beginning from (1, 5, 10, 20, 40, 60, 80, 100, 200) microliters of Lactobacillus salivarius filtrate, which have been isolated within side the look at with the aid of using the usage of the plate technique. For biofilm formation, the samples have been incubated for forty eight hours with the aid of using following the technique cited in (Mathur et al., 2006, Abbas and Ahmed, 2014). The consequences confirmed the capacity of the bacterial infiltrate to inhibit the capacity of pathogenic bacteria to shape biofilms mediated with the aid of using Bacteriocin TXJ, that's extracted from Bacteria Milk (Lactobacillus salivarius), and this become located thru the clear color gradation of the dye (C.V) used in the test and the **figures (5-6)** illustrates the impact of Lactobacillus salivarius filtrate at the increase of pathogenic bacteria and inhibiting their capacity to supply biofilm, thru By recording the absorbance cost of the dye used in the test the usage of a

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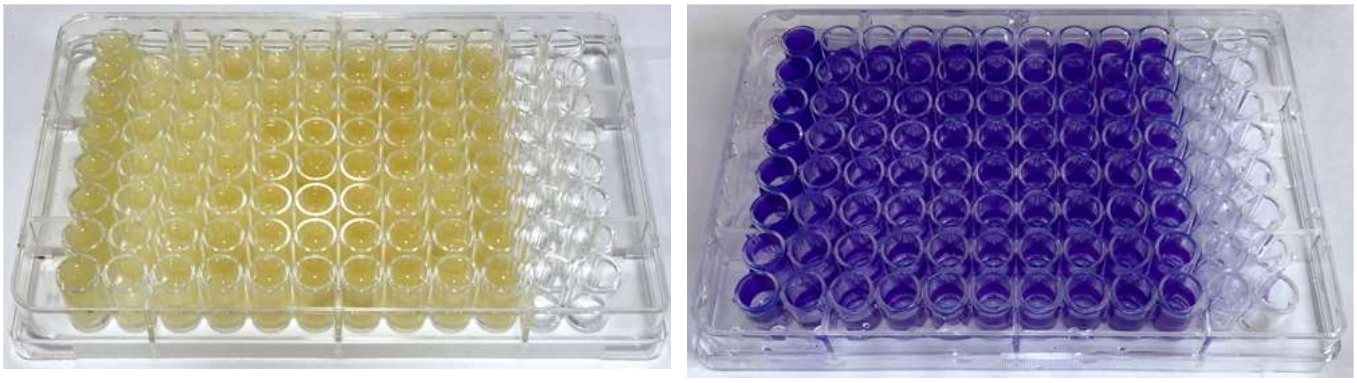
spectrophotometer at a wavelength of 450 nm, the absorbance depth is without delay associated with the formation of the biofilm



Figures (5-6) are showing an assay to detect biofilm formation and its inhibition by bacteriocin and extracted from Lactobacillus salivarius. Nine concentrations starting at (1, 5, 10, 20, 40, 60, 80, 100, 200) microliters were used and positive (bacteria only) and negative (distilled water) hard solutions were compared.

The consequences confirmed as proven in the **figures (7-8)**, wherein it confirmed that the inhibition of Bacillus subtitles filtrate had an excessive inhibitory impact in stopping biofilm formation at concentrations eighty to two hundred µl, even as the inhibition at concentrations forty to 60 µl become fairly intense, even as it seemed at concentrations 1 to twenty µl is low and is non-existent while in comparison to high quality scales and at a wavelength of 450 nm; The motive is because of the mechanism of formation with inside the bacterial mobileular wall, specially in the peptidoglycan layer.

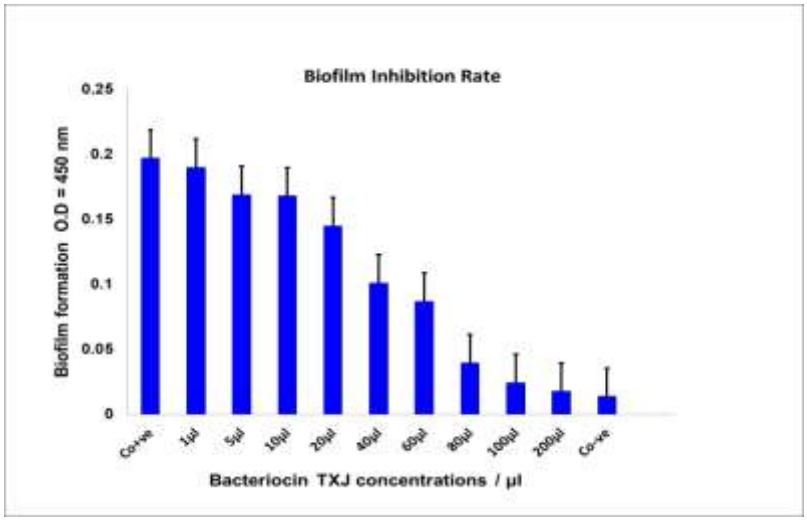


Figure (7) above shows the comparing the ability of bacteriocin TXJ to inhibit the action of the biofilm, where the inhibition was high at concentrations 80 to 200 µl, while the inhibition was moderate at concentrations 40-60 µl. But at concentrations 1 to 20 µl, the inhibition was somewhat low and non-existent when compared to positive measures.

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The outcomes results additionally confirmed the capacity of bacteriocin TXJ to inhibit the paintings of the biofilm, in which the very best discount fee changed into at a attention of 2 hundred microliters with the aid of using 91% and a attention of 80-one hundred microliters, 87.6-79.8%. While the discount percent at a attention of 40-60 µl changed into 48.7-55.8%, respectively, and at 5-20 µl, 26.4-14.7 -14.2%, respectively, and nearly non-existent at one µl, as proven in the figure (8).

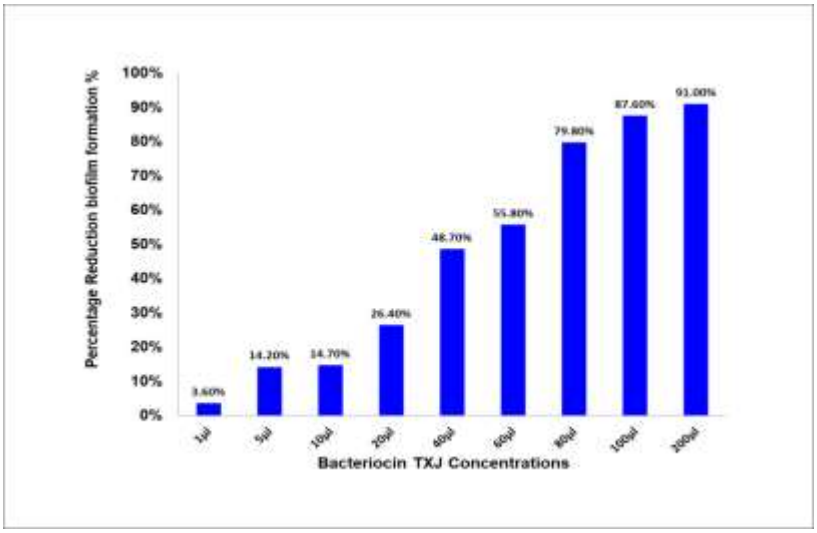

Figure (8) shows the proportion of biofilm reduction, in which the inhibition became at a awareness of two hundred µl it became very excessive via way of means of 91% and at one µl the inhibition became nearly non-existent.

Discussion

Here in this study involved the selection of an isolate of *Lactobacillus salivarius* growing in MRS agar medium taken from samples of dairy products (Greek milk) as it gives the colonies growing in this medium a white and yellow color and the relevance of these lactic acid bacterial isolates has been confirmed by subjecting them to a series of tests and number of tests. After that, microscopic examinations were carried out, whereby cells appeared on microscopic examination of slides prepared from bacterial colonies in the form of small short bacilli, which were often arranged in pairs (chains) or may be single, as it turns out to be gram-positive and turned out to be immobile. Where our results were consistent with previous studies (Bin Masalam et al. , 2018) (Shida et al., 1997). The media that used for isolation in standard must be appropriate and comprise many elements which have the cap potential to choose bacteria which have unique and complicated necessities along with lactic acid micro-organism (lactobacillus bacteria), wherein it ought to be introduced to the media used for isolation, the usage of MRS agar medium is complicated. In setting apart those bacteria, it is also specialized for them, as this medium is selective (Al-Khafaji, 1990). Many research have indicated in fashionable to Lactobacillus isolates acquired from more than one sources (milk, milk, soil, water, etc.) and their use in numerous research together with the bio-antagonism of pathogenic bacteria, as preservatives, Lactobacillus isolates 21 Various dairy products, (Erdogrul and Erbilir, 2006), in addition to thirteen isolates of the equal genus from fish and shrimp (Nair and Surendran, 2005). Ahmed and Kanwal (2004) have been capable of isolate L.acidophilus from camel milk and take a look at its susceptibility to the species. Bacteria convert lactose into lactic acid.

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