Original Article

Nutritional safety of Basillus subtilus. That isolated from Iraqi soil

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Abstract

This study demonstrates the effectiveness of biologics made from Bacillus, as research proves that these bacteria are food safe if they enter organisms(humans, animals) because they have no negative effects on biological systems. Bacillus Subtilis. Based on animal testing, it is considered food safe. No changes in normal parameters were observed when examining tissue sections compared to control treatments. In addition, no clinical symptoms were observed when examining the natural indicators of experimental animals during administration. They are in normal condition. This proves that their use in biological control is harmless.

Keywords: biological systems, Bacillus Subtilis, animal testing.

INTRODUCTION

Biological control is one of the modern research trends that has attracted the attention of researchers in recent decades and a "scientific" and honest solution to combat many plant diseases and pests, especially after realizing the dangers resulting from the use of chemical pesticides (Al-Zubaidi, 1992). Biological resistance is defined as the use of a natural microorganism. Or the hub in genes or gene products to reduce the effect of unwanted organisms so that no harm occurs to agricultural crops (Abu Arqoub, 2000).Biocontrol can be defined as products that contain cells from different microorganisms. They can be described as commercial preparations that contain living organisms or cells growing from microorganisms (Khanna et al., 2019).The development of biological control methods in modern ways enhances the preservation of the most important food crops for sustainable global food, as traditional chemical and physical control methods to meet future demands are uneconomic and environmentally unfeasible (Tyagi et al., 2019).The methods of using microorganisms in combating many plant pathogens are based mainly on the property of antagonism, as their use increased for development in the field of biological control, and the general concern over the use of chemicals, as well as their success in combating some diseases that cannot be controlled using traditional methods. Many studies have been conducted on the possibility of employing microorganisms in combating fungi that infect grains of important crops such as wheat, corn, rice and others in stores, especially when such grains are stored under poor storage conditions.One of the studies showed that the use of the antibiotic Itarin A (a lipoprotein isolated from Bacillus subtilis) at a concentration of 50 ppm, led to a limitation of the growth of A. parasiticus as well as a complete inhibition of aflatoxins production (Ono and Kimura, 1991). In another study, it was proved that the biological preparation made from the B. subtilis vaccine has a high effectiveness in protecting the maize seeds from the harmful effects of the fungi A.flavus and A.niger in the store (Ali and Muhammad, 2009). Another study carried out by (Al-Asady, 2013) proved that fumigation of maize and wheat seeds with Bacillus subtillus bacteria loaded with calcium bicarbonate was effective in preserving the studied grains from infection with A.flavus and A.parasitacus fungi at a concentration of 1 g/kg. The study also proved the health safety of the animal. Or the human if it is used in food preservation.

General and taxonomic characteristics of Bacillus subtilis

It is one of the common species of the genus Bacillus, also called Hay Bacillus or grass Bacillus. The cells of this bacterium are positively gram-positive for aerobic Gram test, obligatory and sometimes facultative anaerobic forming spores. This trait gives it the ability to withstand harsh conditions such as high temperatures and drought, with a sticky texture. It is found in water, soil and the hollow of ruminants and in humans it is a producer of enzymes, it grows at mesophilic temperatures, the optimum temperature for its growth is 30-25 C (Bandow et al., 2002).Brown, (2001) states that Bacillus spp., is a Gram-positive bacterium that is common in common soil microorganisms. Its species are heterotrophic, moderately heat-needed, and often

aerobic, endospores producing, heat-resistant, and flagellated, and this helps them to move. Wavy (Jamil, 2007).They are isolated from the soil by pasteurization processes and then poured into food media. Typical colonies are white to sticky in appearance (white and dry or past looking), mucousy in texture and shiny, with rectangular shapes often formed in the form of chains or pairs, appearing under the microscope in a dotted manner because of the blackboards the interior that they make up (Nester, 2001).Affect plant growth directly or indirectly by supporting the plant with special substances secreted by these bacteria such as plant hormones and plant growth regulators, facilitating the absorption of necessary substances from the environment, improving soil composition and biological treatment of contaminated soils by isolating toxic species and stimulating resistance to pathogens (Refish. et al.,2016).As for its industrial importance, it is represented in the production of a large number of enzymes with metabolic, hydrolyzing, oxidizing and reducing functions, but the quantities of these enzymes vary between species and even between strains of the same type. Amylase, lipase, protease and cellulase enzymes are among the most important enzymes used in industries Food, pharmaceutical, textile, leather, and other industries, where the enzymes produced by this genus constitute about 50% of the total enzymes present in the world market, such as the use of B. subtilis in Japan to ferment soybeans (Lyngwi and Joshi, 2014).

Classify this genus (Promon et al, 2015) according to the following **table (1)**

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*

Material and methods

Isolation and identification of Bacillus subtilis

Three areas were selected in Karbala governorate, as a sample of soil was taken from each area, with a size of 250 gm, and at different depths (5 cm, 10 cm) and for each of the specified sites. Heat of 80 ° C for ten minutes to get rid of the vegetative cells. Then, an amount of 1 g was taken from each soil sample and a series of dilutions was made from it 10-1-10-12. 1 ml of the last dilution was drawn and distributed on three Petri dishes containing 20 ml of Nutrient agar (sterilized at a temperature of 121 °C and a pressure of 1 atmosphere for a period of time15 minutes) and spread by the planning method by a glass diffuser, and then the occlusion was incubated in the incubator at a temperature of - +37 °C for a period of 24 hours. This process was repeated for each soil sample and for each site, and the following were studied.

Study of the phenotypic characteristics of Bacillus bacteria

The phenotypic characteristics of Bacillus colonies were examined and studied, the colony size and color were determined, and their strength was studied after making sure that those colonies were similar to Bacillus colonies.

Study of the microscopic properties of Bacillus bacteria

A sample was selected from colonies similar to the characteristics of Bacillus bacteria by taking a smear by the culture lube and spreading it on a sterile glass slide and fixing it, then staining it with gram dye and examining the shape of the bacterial cell and the location of the spore in it. Gram, either the bacteria cells that take the red dye are Gram-negative (Al-Jubouri, 1990: Collee and his group, 1996).

Diagnosis of Bacillus isolate

After sifting the bacterial isolates belonging to the genus Bacillus and selecting the most efficient isolate among them, the isolate was diagnosed laboratory using the Vitec device.

The stages of manufacturing a biological preparation from B. subtilis.

Determining the appropriate fermentation medium for the development of B.subtilis.

The efficiency of three types of extracts of wheat, barley and yellow maize to be used as fermentation media was tested to grow B. subtilis inoculum.

1- He took 250 g of grains of yellow corn, barley and wheat, moistened with sterile water, then crushed in a metal container, and a liter of distilled water was added to it and left for 24 hours at room temperature.

2- Filter the mixture and take the filtrate and add to it (20) g of sucrose, then sterilize the mixture with an autoclave at a temperature of 121 H and a pressure of (1) atmosphere for 15 minutes and leave the media to cool

3.Each flask was inoculated with five colonies of B. subtilis bacteria grown on Nutrient agar medium and 24 hours old for each of these fermenting media and each separately. Then the fermenting media were incubated for 24 hours at a temperature of 37°C. Then a series of dilutions (1-10 - 10-10) were made for each of these mediums, 0.1 ml of the last dilution was taken for each fermenting medium and planted on Nutrient agar medium with three replications for each medium, and then incubated at a temperature of 37 °C for 24 hours. The average number of bacteria in each dish was calculated and then the final concentration of bacteria in one milliliter was extracted according to Clark's (1965) equation.

Number of bacteria cells (ml) = average number of colonies in each dish × inverted dilution

The medium that gave the largest number of bacterial cells was chosen as the fermentation medium for the production of the biological preparation, and it was the yellow corn extract medium.

Test suitability of calcium carbonate as carrier

The efficiency of calcium carbonate as a carrier of B. subtilis. vaccine was tested by placing 100 g of calcium carbonate in a clean, sterile container and placing it in the electric oven at a temperature of 160 m for an hour and left to cool. Then (100) ml of the liquid fermentation medium of the pre-prepared yellow corn crop, on which the bacteria were grown, was added to it.B.s ubtilis at 24 hours of age, which proved highly efficient in developing the inoculum of this bacteria. Then the pots were transferred to an electric dryer at a temperature of 40 mC for 5 days until they dried well. Then the powder loaded with bacteria was ground in a sterile room (Hood), after which a series of dilutions of the prepared sample was prepared. (10-10-10-12) Then (0.5) ml of the last dilution of the bacterial suspension was transferred to sterile dishes containing Nutrient agar medium, with three replicates, and the dishes were incubated at a temperature of 37 ° C for 24 hours. (Hamid, 2001). Then the number of bacteria per gram of the carrier material was estimated according to Clark's (1965) equation.

Determination of the ratio of the fermentation medium to the carrier

After calcium carbonate proved its efficacy as a carrier for B.subtilis. vaccine, a test was conducted to determine the ratio of the fermentation medium to the carrier by taking 5 ml of the yellow corn medium in which the bacteria grew at 24 hours old and taking 5 ml of it and mixing it with 5 g of calcium carbonate, i.e. in a ratio of 1:1 He also took 5 ml of bacteria and mixed it with 10 g of calcium carbonate, i.e. in a ratio of (2_1), and took 10 ml of bacteria and mixed it with 5 g of calcium carbonate, so the ratio was (2:1). These treatments were mixed separately and placed in the oven at a temperature of 40°C until drying. After drying, 1 gm of each treatment was taken and a series of dilutions were made from it (10-15-10-1) and 0.5 ml of the last dilution was taken and spread on the middle of the previously prepared nutritious broth. Replicates from each dilution and the number of colonies developing after that was calculated according to the concentration using Clark's (1965) equation.

Production of the final biological preparation from the isolation of B. subtilis bacteria

In light of the previous tests, the fermentation medium prepared from the yellow corn crop was adopted for the development of B. subtilis bacteria. Calcium carbonate was used as a carrier for the same bacteria vaccine in a ratio of 1:2 (fermentation medium: carrier substance) for the final preparation of the manufactured biological preparation.

Biopharmaceutical safety test

For the purpose of identifying the safety of the biological preparation on the vital tissues of laboratory animals as an indication of its safety for humans, an experiment was carried out in which white rat animals were used, as follows:

1- Preparing laboratory animals

Take (12) old male albino rats were prepared and divided into four groups, each group included 3 animals, and they were treated by oral administration.

2- Treatment of animals Animals were treated according to what is shown below

Table (2) Table of laboratory animal treatment

Transaction Description	Transactions
Not treating animals	control treatment
The animals were dosed with the loaded bacteria only, at a dose of 0.5 g/kg animal weight	Biopharmaceutical treatment
Animals were inoculated with *B. subtilis* only inoculated with NB nutrient medium at 1 ml/kg (1.5×10^{10} CFU/ml).	B. *subtilis* vaccine treatment
Animals were dosed with the nutrient medium only, at an amount of 1 ml / kg animal weight	Treating the nutrient medium only

These treatments were repeated three times over a two-week period, after which the animals were left for seven days. During this period, the clinical symptoms that could appear on the treated animals were followed up. After the end of the experiment period, the animals were sacrificed after being anesthetized with chloroform and explained by opening the abdominal cavity and drawing blood by heart stab method (Heart Pincture), and the drawn blood was placed in test tubes containing EDTA to conduct physiological blood tests, then samples were taken from the liver, kidneys and intestines and preserved in formalin at a concentration of 10% to study histological changes (Al-Asady, 2013 modified).

histological study

Histological Section Preparation

Histological sections were prepared in Al-Sadr Teaching Hospital in Al-Najaf Governorate, and they followed the method of (1982) and Stevens Bancroft, which included the following:

1- Dehydration: The samples were passed in ascending concentrations of ethyl alcohol (70, 80, 90, 95, 100 and 100%) for 2_1.5 hours in each concentration to remove water from them.

2- Clearing:The samples were washed with xylene twice for 1.5-1 hour each time to remove the oxalate solution from the tissues.

3- Infiltration: The samples were impregnated with molten paraffin wax at a temperature of 56 °C, by placing the samples in it twice, for 1.5-1 hour each time.

4- Embedding: The samples were embedded in special molds containing molten paraffin wax and left to solidify

5- Sectioning: Serial tissue sections with a thickness of 5 µm were prepared using a rotary microtome, and the samples were fixed on glass slides using s albumine'Meyer adhesive. Then the slices were placed in the oven at 56 °C vertically for 20 minutes to remove the excess wax.

6- staining: The sections were stained with hematoxylin dye, as they passed the slides in (Xylene - 100% ethyl alcohol - 95% alcohol - 90% - 70% - hematoxylin dye) for two minutes each, then the sections were washed with tap water, and dipped in eosin dye for two minutes. Then it was washed with tap water and then passed with increasing concentrations in each of ethyl alcohol (70%-90%-95%-100%) and xylene for two minutes for each concentration.

7- Mounting: Put the slide cover using Canada balm. Then the integrity of the tissue sections was diagnosed

8. Histological sections were photographed using a high-resolution electron microscope

Result and Discussion

Isolation and identification of Bacillus

Five Bacillus isolates were obtained in three areas from Karbala Governorate. The isolates were diagnosed based on:

Phenotypic characteristics of the isolates on the culture medium

Colonies of bacteria isolates appeared on the medium of the solid nutrient broth in the form of round, white, opaque colonies tilted to little brown with the advancing age of the colony. The adjectives match what Al-Ashour (2009) indicated.

Microscopic characteristics of bacterial isolate cells

The results of the microscopic examination of the colonies of bacterial isolates fixed on glass slides and stained with Gram stain showed that they are short to medium-length rod cells, some are thick and swollen, and the other are relatively thin rods with equal ends positive for Gram stain forming spores. It does not have a wallet, and some sites of the spores were central (central) and to the other semi-terminal (sub-terminal). These characteristics correspond to what was indicated by Collee and Al-Ashour (2009) by collee and Al-Ashour (2009) regarding the shapes of Bacillus cells and the nature of their regularity.

Phenotypic characteristics of B. subtilis isolate colonies

Through laboratory examinations of bacterial growth on the nutrient agar medium N.B, its colonies appeared in a circular shape and relatively large, smooth with a rounded edge that quickly turns into a lobed edge with the progress of growth and its color is white to pale brown and tends to a dark brown color with the aging of growth. The diameter of the colony ranges between 1.5 mm - 3.5 mm This is consistent with what was stated by McFadden (2002) and Al-Ashour (2009).

Microscopic description of B. subtilis isolate cells

The results of microscopic examination of the Gram-stained Bacillus colonies showed that they are a relatively short gram-positive bacillus and form sporophytes. These spores are clearly formed after a 24-hour incubation period and are in the middle

of the vegetative cells of the bacteria. When the growth continues for 48 hours, most of the bacterial cells are decomposed and the spores are liberated. The attributes are consistent with what Al-Ashour (2009) brought.

Biochemical tests for the isolation of B. subtilis bacteria

The results of the biochemical tests shown in the figure (1) showed that they apply to the type of bacteria Bacillus subtilis. In which the diagnosis was relied on the viteks device

bioMérieux Customer: Microbiology Chart Report Printed March 16, 2022 10:02:02 AM CDT

Patient Name: Patient ID: 153202210
Location: Physician:
Lab ID: 153202210 Isolate Number: 1

Organism Quantity:
Selected Organism : Bacillus subtilis

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	13.92 hours	**Status:**	Final
Selected Organism	91% Probability **Bionumber:**	**Bacillus subtilis** 1373060615557671		
ID Analysis Messages				

Biochemical Details

1	BXYL	+	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	-	12	AlaA	+	13	TyrA	+	14	BNAG	(-)
15	APPA	-	18	CDEX	-	19	dGAL	-	21	GLYG	-	22	INO	+	24	MdG	+
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	-	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	+	44	PHC	(-)	45	PVATE	+	46	AGLU	+	47	dTAG	-	48	dTRE	+
50	INU	+	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_I	+						

Figure (1) biochemical tests in viteks test

Biopharmaceutical manufacturing process

Determining the appropriate fermentation medium for the growth of B. subtilis

The results of this test showed that the yellow corn extract was suitable for the growth of Bacillus isolate compared to wheat and barley extract. The bacteria preparation for the isolate in the first medium (maize) was 5.5 x 1010 colony-forming units, while it was 1010 x 2 colony-forming units in the barley extract or the wheat extract. The preparation of bacterial isolate was slightly lower than the preparation found in barley extract

The reason for the superiority of the yellow corn extract medium may be due to the fact that it contains nutrients suitable for bacterial growth such as sugars, amino acids and vitamins such as vitamin A, B, E and others, as well as containing major and minor mineral elements such as potassium, iron, sodium and others, which stimulate the speed of division of bacterial vegetative cells and thus increase the biomass of these bacteria (Al Ashour,2009)

Evaluation of the efficiency of calcium carbonate as a carrier for Bacillus subtilis vaccine

The results of the test showed that there were no negative effects of calcium carbonate on the growth of B. subtillus bacteria, and this was manifested by the presence of large numbers of live bacterial cells per gram of this substance if it reached 1010 x 5.5 colony-forming units / gram and this result is consistent with what Al-Ashour mentioned (2005) It is suitable for this substance to carry Bacillus cereus cells. Calcium carbonate plays an important role in increasing the effectiveness of the biological preparations included in their manufacture through its ability to inhibit the growth of pathogenic fungi with an indirect effect such as changing the nature of the food medium and changing the pH as it changes the medium The culture is basal when it is hydrolyzed by giving it negative hydroxyl ions (Awad, 1986), Since the fungi generally prefer neutral or slightly acidic medium in their growth (Enclaud, 1980), the change in the PH value of the medium affects the readiness of the nutrients needed by the fungi and then inhibiting their growth rates (Balkarami and Ferma, 1988), in addition to being a locally available and cheap material Which reduces the cost of mass production.

Determination of the ratio of the fermentation medium of the yellow corn kernel extract to the carrier substance caco3

Among this test, the best ratio of adding the fermentation medium to the carrier (caco3) is 1:2, as the bacteria preparation

per gram reached 1010 x 95 colony-forming units/gm, while the preparation decreased at the ratio 1:1 in the fermentation medium: carrier substance to 1010 x 76 colony-forming units. The same is the case with the ratio 2:1. Fermentation medium: carrier substance.

These results depend on some studies that indicated such results. Researcher Al-Ashour (2005) said that the ratio of 1:2 fermentation medium: a carrier substance is effective in increasing the number of bacteria per gram compared to other ratios, as it is similar to what was found by Al-Obaidi (2011), which indicated that The ratios 1:1 and 1:2 in fermenting medium: a carrier material gave an effective preparation of B. licheniformis per gram of the carrier, and there was no significant difference between these two ratios in the preparation of bacteria present in one gram.

Effect of the biological preparation on the histological parameters of laboratory animals

The results of the examination and laboratory diagnosis of the tissue sections of the organs (liver, kidney, and small intestine) showed that no pathological change occurred in those organs of the treated animals. It is completely similar to the tissue sections of the control treatment, which indicated the absence of pathological hanges, and these results converge with previous studies on biological preparations. For Bacillus spp. Which proved the health safety of these preparations. For example, a study conducted by Al-Asadi (2013) on the biological preparation B. subtilis loaded on calcium carbonate proved the health safety of the preparation through histological tests conducted on the tissue sections of male white rats. After conducting histological examinations, there were no signs of disease or danger to the health of the tested animals.

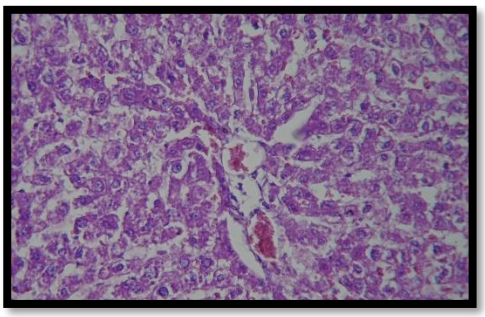

Figure (2) a cross-section of the rat liver Control treatment (magnification power 40X) liver

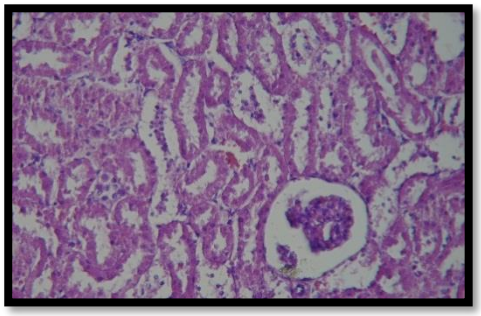

Figure (3) a cross-section of the rat kidny Control treatment (magnification power 40X) liver

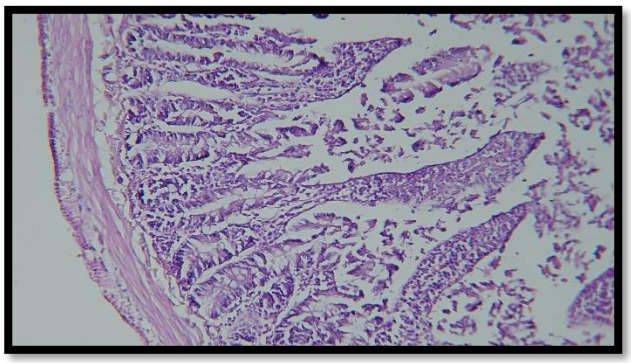

Figure (4) a cross-section of the rat intestinal Control treatment (magnification power 40X) liver

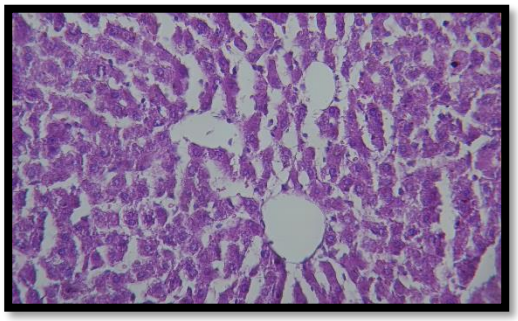

Figure (5) a cross-section of the rat liver caco3 (magnification power 40X) liver

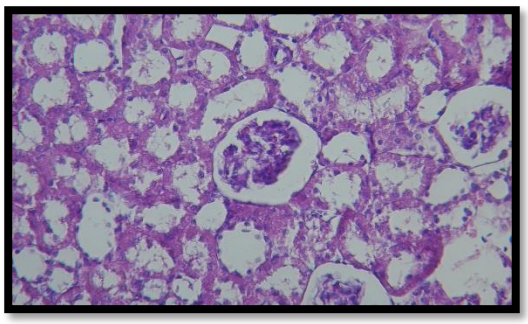

Figure (6) a cross-section of the rat kidny caco3 (magnification power 40X) liver

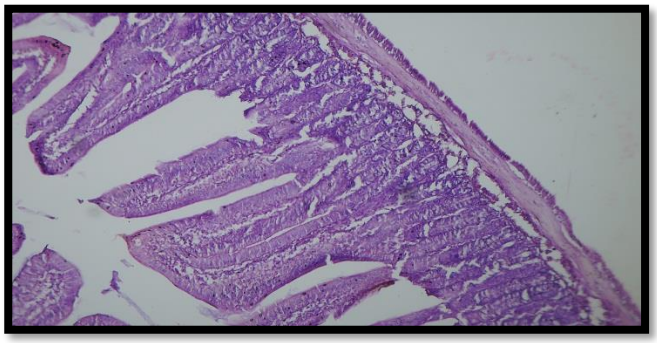

Figure (7) a cross-section of the rat intestinal caco3 (magnification power 40X) liver

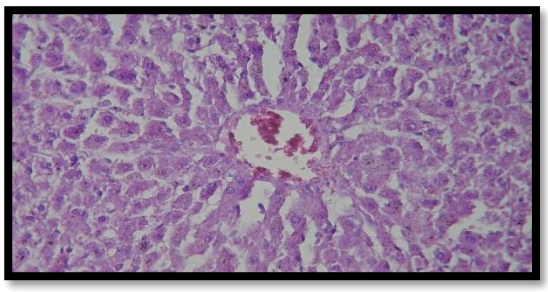

Figure (8) a cross-section of the rat liver nutrint broth (magnification power 40X) liver

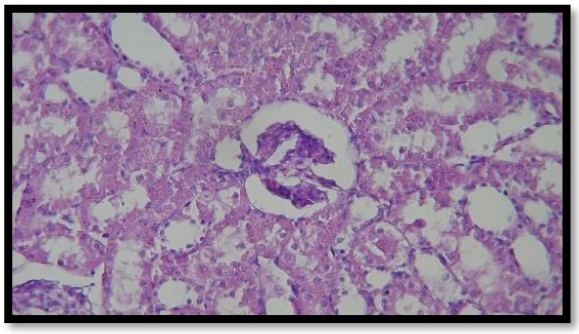

Figure (9) a cross-section of the rat kidny nutrint broth (magnification power 40X) liver

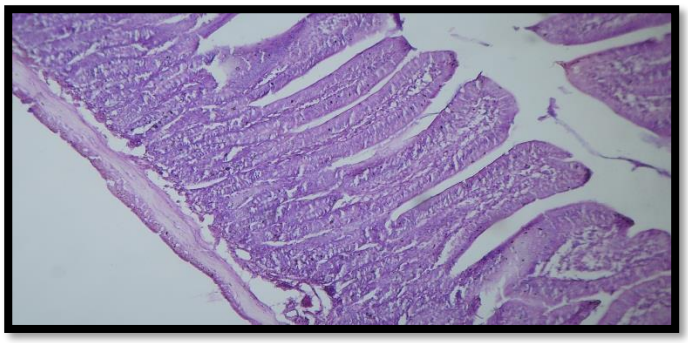

Figure (10) a cross-section of the rat intestinal nutrint broth (magnification power 40X) liver

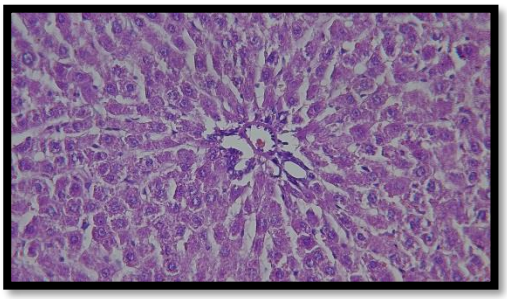

Figure (11) a cross-section of the rat liver Bacteria loaded with calcium carbonate (magnification power 40X) liver

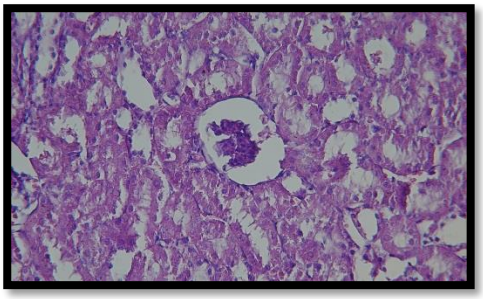

Figure (12) a cross-section of the rat kidny Bacteria loaded with calcium carbonate (magnification power 40X) liver

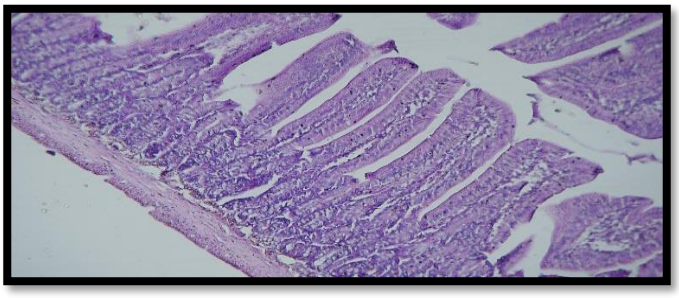

Figure (13) a cross-section of the rat intestinal Bacteria loaded with calcium carbonate (magnification power 40X) liver

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