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Antioxidant role of *Lactobacillus* sp isolated from honey bee against histological effects of Ochratoxin in vivo.

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Abstract

In this study One hundred honey bee worker were collected from two sites in AL-Diwanyia Governorate include Eastern Hamza and AL-Daghara city during April 2017. to detect antioxidant role of some bacteria isolated from honey bee gut workers against Ochratoxin.

Based on biochemical tests, the results show that out of one hundred samples of honey bee workers were screened, only Seventy isolates were isolated ,and the genera lactic acid bacteria (*Lactobacillus kunkeei ,lactobacillus plantrum and lactococus lactis)* were highly abundant in honey bee gut . we examined protective role of *Lactobacillus kunkeei* in reduce histological effects of Ochratoxin A, the results showed treated animals with *L. kunkeei*. mixed with OTA showing not found any histopathological changes in liver and kidney.while treated animals with OTA only appear several pathological changes ,in kidney tissue this changes include :vascular congestion , dystrophy and the disappearance of the glomerulus in addition to cell necrosis of glomerulus.and cell necrosis, vascular congestion and loss of normal structure of liver. *Lactobacillus kunkeei* isolated from honey bee appear good protective role against histological effect of OTA and may safely improve immune responsiveness**.** This is the first report for detection of protective role of honey bee bacteria against OTA .and this bacteria appear good role in degradation of mycotoxin.therfore we recommended for detection of LD50 and LD90 for this bacteria and use as supplement drugs for mycotoxicosis

Key word **:Ochratoxin A, Lactic acid bacteria, *Lactobacillus kunkeei,* Antioxidants.**

Introduction

Mycotoxins are secondary metabolites of some fungi belonging to *Aspergillus* , *Penicillium spp* and *Fusarium* spp and are common contaminants of human foodstuffs , such as wine, coffee beans, nuts and animal feed. Mycotoxins can cause many health problem in animals and humans known as mycotoxicosis (**Hussien and Brasel ,2001).** The major problem associated with animal feed contaminated with mycotoxins is not acute disease episodes, but rather, the ingestion of low levels of toxins, which may cause an array of metabolic, Physiologic and immunologic disturbances. **(Gianni *et al*.,2010)**. The most extensively investigated mycotoxins are Aflatoxins (AF), Deoxynivalenol (DON), Zearalenone (ZEN), Fumonisin B1 (FB1) Ochratoxin A (OTA) and T2 toxin .((**Bennett and Klich, 2003).**

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced by *Aspergillus ochraceaus* soluble in organic solvents, in aqueous solution of sodium bicarbonate and slightly soluble in water . OTA is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood, mainly to the kidney with lower concentrations found in liver, muscle and fat. Specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney where it accumulates. (**Benford *et al.*, 2001)**.

OTA has been found toinduce an increase in ROS levels and oxidative damage in some immortalized renalcell and cancer cell lines, Such as Human renal proximal tubularepithelial cells (HK-2), primary rat proximal tubular cells, proximal tubular cells (L.L.C-PK1), human hepatoma-derived cells (HepG2) and human colonic adenocarcinoma cells (CaCo-2**) .(Dörrenhaus and Föllmann ,1997).**

Assaf *et al*.(2004). showed that OTA could induce immunosuppression via marked apoptosis in Human lymphocytes , It has been generally accepted that the induction of cell cycle arrest and apoptosis was an important bioeffect of many carcinogenic mycotoxins.

Recent studies have led to a interest in the probiotics, which are claimed to have health benefits. Probiotics refer to live nonpathogenic microorganisms, which, when administered in adequate amounts, confer microbial balance, particularly in the gastrointestinal tract (Shen *etal*. ,2011). Evidence has showed that probiotic bacteria has significant antioxidant abilities both in vivo and in vitro (**Lin and Yen ,1999; Wang *etal*.,2016).**HoweverLactic acid bacteria (LAB) are ,the most versatile species extensively used in the food industry both as microbial starters and probiotic microorganisms. **(We etal.,2006).** In recent decades. selection of microbial molecules and/or bacterial strains able to produce antagonistic molecules to be used as antimicrobials and preservatives has been attracting scientific interest, in order to, eliminate or reduce chemical additives, because of the growing attention of consumers for healthy and natural food products.

LAB were reported to have positive effects on the treatment and maintenance of ulcerative colitis (UC).**(Zocco *etal.*,2006).** They were also associated with the improvement of metabolic diseases **(Rad *etal*.,2007**). In addition to the beneficial effects mentioned above, in recent decades, many findings have shed new light on the understanding of the antioxidant capacity of probiotics. The culture supernatant intact cells and intracellular cell-free extracts of Bifid bacterium animals were found to scavenge hydroxyl radicals and superoxide anion in vitro while enhancing the antioxidase activities of mice in vivo **(Shen and Shang ,2011).** Further, the oxidative stress in patients with type 2 diabetes can be ameliorated by multispecies probiotics, LAB strains have been studied widely both in animals and the human body . It is revealed that LAB can resist ROS, including peroxide radicals **(Asemi *etal.*,2013),** superoxide anions, and hydroxyl radicals **(Kullisaar *etal.*,2002). (Bao *etal*.,2012).**

In this study we attempt to use some bacteria isolated from the honey bee worker gut as biological strategies to reduce OTA effect on biological systems.

Material and Methods

1-Ochratoxin A

One gram purified and crystalline OTA (Sigma chemical CO. Louis ,USA) was diluted in 1ml PBS and keep until using.

2-Samples of honey bee worker collection

One hundred honey bee worker(*Apis Mellifera*) were collected from two Apples Honey sites in Al-Diwanyia Governorate include Eastern Hamza and Al-Daghara .All collections were carried out in April , 2017 and samples were stored on ice before bacterial isolation.

3-Isolation and cultivation of bacteria

Fresh honey bee samples were surface-sterilized with 7% sodium hypochlorite in sterile plates, to dissect the worker bees, sterile dissecting scissors were used to decapitate the bee and remove the cuticle before dissecting with a sterile blade. The midgut of the bee was removed with a sterile blade and homogenized in 1 ml of phosphate-buffered saline (PBS). The gut homogenate (100 µl) was spread using the standard dilution method onto three different media: Nutrient Agar (NA); Brain Heart Infusion Agar (BHI); and MacConkey Agar (MA). The plates were incubated aerobically at 37°C for 24 h. The bacterial colonies growing on the plates were selected and purified on sub culture media for identification. .**(Disayathanoowal *etal*.,2012)**.

4-Bacterial identification

Bacteria isolated were identified by using morphological and biochemical characterizations as follow:

A-Gram stain *:*

Smear was taken from bacteria and applied on them the steps of Gram stain (Collece *et al*., 1996.

B-**Oxidase test**

This test was Conducted by using oxidase reagent which consists of a tetra methyle-p phenylene diamine dihydorchloride as it developed a bacterial colony by a wooden stick on a sterile filter paper and put a drop of reagent on the colony where appeared purple color within 30 seconds **(Benson, 2002)**

C-Catalase test

Part of the bacterial growth was transferred and put on a glass slide sterile by sterile wooden stick and add a drop of reagent of hydrogen peroxide (H_2O_2) in concentration of 3%, which gave air bubbles **(Macffadin, 2000).**

D-Urease test

Bacterial colonies were cultured in urea agar in a stabbing method on the sloping surface and incubated 37 ^{0}C, and then it observed color change of the media from yellow to pink (Macffadin , 2000).

E-Citrate utilization test

Simmon's citrate slopes were prepared in Flask as recommended by the manufacturer (stored at 2-8oC). And the slopes were then stabbed and incubated at 37oC aerobically for 48 hours. bacteria are citrate negative as such Simmon's citrate agar slopes remained as green in color. And blue color indicates a positive reaction (**Benson, 2002**).

5-Confirm identification

Polymerase Chain Reaction

PCR assay was performed for detection of *Lactobacillus kunkeei* based on 16S rRNA gene and this assay was carried out according to method described by **(Lidia and El-bieta ,2005)** Genomic DNA was prepared from all *Lactobacillus kunkeei* bacterial isolates. PCR was performed using the universal primers F (5'-GAGAGT TTG ATC CTG GCT CAG-3') and R (5'-CTA CGC CTA CCTTGT TAC GA-3') which covered almost full length 16S RNA gene. as following steps:

1. Genomic DNA extraction

Genomic DNA was extracted from *Lactobacillus kunkeei* bacterial isolates by using Genomic DNA Mini Bacteria Kit, as following steps:

1- 1 ml of (18 hours) incubated cultured bacterial cells was transferred to a 1.5 ml microcentrifuge tube then centrifuged in high speed centrifuge at 15000 rpm for 1 minute then the supernatant discarded.
2- Lysozyme buffer(200µl) were added to the tube and re-suspended the cell pellet by shaking vigorously by vortex, then incubated at room temperature for 10 minutes, and the tubes inverted every 3 minutes through incubation periods.
3- GB buffer (200 µl) were added to each tube and mixed by shaking vigorously for 5 seconds. Then the tubes were incubated at 60ºC for 10 minutes and inverted every 3 minutes through incubation periods.
4- Absolute ethanol (200 µl) were added to the clear lysate and immediately mixed by shaking vigorously, and then precipitates broke it up by pipetting.
5- A GD column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to the GD column. Then centrifuged at

15,000 rpm for 2 minutes. And the 2 ml collection tube containing the flow-through was discarded and placed the GD column in a new 2 ml collection tube.

6- W1 buffer (400µl) was added to the GD column, then centrifuge at 15,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.

7-Wash Buffer 600µl was added to the GD column. Then centrifuged at 15,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube and the tubes were centrifuged again for 3 minutes at 15,000 rpm to dry the column matrix.

9- The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of pre-heated elution buffer were added to the center of the column matrix.

10- The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 15,000 rpm for 30 seconds to elute the purified DNA.

PCR master mix preparation

PCR master mix was prepared by using (AccuPower® PCR PreMix Kit) and this master mix done according to company instructions as following table (1):

Table(1): PCR master mix contents according to company instractions

PCR Master mix	Volume
DNA template	5µL
16S rRNA gene Forward primer (10pmol)	1.5µL
16S rRNA gene Reveres primer (10pmol)	1.5µL
PCR water	12µL
Total volume	20 µL

After that, these PCR master mix components that mentioned in table above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,and loading dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (MyGene, Bioneer. Korea).

PCR Thermocycler Conditions

PCR thermocycler conditions were done by using convential PCR thermocycler system for each gene as following table (2):

Table (2) **PCR Thermocycler Conditions**

PCR step	Temp.	Time	Repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	30 cycle
Annealing	60.3	30sec	
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	-

PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1- 1% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5-520bp PCR products were visualized by using UV Transilluminator

6-**Experimental Design**

In present study albino male rats were used, weight (200-220)g ,and obtained from animals house in Science Faculty /Al-Kufa University .The animals kept in room with temperature (25±2 C^0) and light/dark cycle (12/12h)and provide with standard laboratory diet. Atotal of Twenty four animals were divided into 4 groups (6 animls for each group) as follow:

Group 1: Treated with OTA ,injection intraperitoneal at dose 100µg/kg body weight for seven day.

Group 2: Treated with $1x10^7$ cell/ml from *L. kunkeei* ,injection intraperitoneal at dose 1 ml/kg body weight for seven day

Group 3: Treated with OTA ,injection intraperitoneal at dose 100µg/kg body weight and $1x10^7$ cell/ml from *L. kunkeei* at dose l ml/kg body weight for seven day.

Group 4: treated with normal saline , injection intraperitoneal at dose 1ml/kg body weight for seven day.

7.General histopathological preparations

The specimens of livers and kidneys were fixed by 10% buffered formalin solution still the preparation of histological sections. Tissues were embedded in paraffin and several tissue sections were prepared for histopathological sections were stained with Hematoxylin-Eosin (H and E) according to **Bancroft and Stevens (1982).**

8.Statistical analysis

ANOVA and T student test were used to analyze the data .Value of $P \leq 0.05$ were regarded as significant.so Mean and SEM was used for analyze data.(

Results and Discussion

Based on biochemical tests, the results show that out of one hundred samples of honey bee workers were screened, only seventy isolates were isolated and these distributed as 20 (28.57%) identified as *Lactobacillus kunkeei* , 11(15.71%) for *Lactobacillus plantarum* and(14.28) for *Lactococcus lactis* ,9 (12.85%) identified as *Escherichia coli* ,8(11.42%) identified as *Klebsiella oxytoca*,6 (8.57%) for *Enterobacter cloacae* so *Enterobacter aerogenes* and *Citrobacter freundii* represent 4.28% for each one. These results summarized in table (3).

Table(3) Diversity of bacteria isolated from the gut of honey bee workers

Bacterial species	**Number of isolates**	**Percentage (%)**
Lactobacillus kunkeei	20	28.57
Lactobacillus plantarum	11	15.71
Lactococcus lactis	10	14.28
Escherichia coli	9	12.85
Klebsiella oxytoca	8	11.42
Enterobacter cloacae	6	8.57
Enterobacter aerogenes	3	4.28
Citrobacter freundii	3	4.28
Total	70	

This results agreement with many previous culture-dependent studies have been carried out to investigate the bacteria of honey bee gut (Gilliam et al., 1997; Inglis et al., 1998; Mohr and Tebbe, 2006; Yoshiyama and Kimura, 2009). It is interesting to note that the genera lactic acid bacteria (*Lactobacillus kunkeei ,lactobacillus plantrum and lactococus lactis)* were highly abundant in honey bee gut .

The members of other bacteria have been isolated from honey bee workers may be found in environment of honey bee such as in water , living trees , plant products , vegetation and soil (**Brisse *et al.*, 2006).** They have been reported to have a symbiotic relationship with various insects **(Dillon *et al.*, 2002).** These bacteria may play important roles in food digestion such as pollen grains for the honey bee, where many studies referred to these bacteria had ability to produce some enzyme such as protease and lipase that play important role in food ingestion **(Zhang and Guan., 2009; Feng *et al.*, 2011).**

From above results its found that *L. kunkeei* was isolated in highly percentage comparative with other bacteria therefore we focused on it to investigate their role in reduced histological effect of OTA in *vivo* and confirm it identification by PCR technique by 16s RNA ,and results showed that all isolates was *L. kunkeei*. (figure 1 and 2).

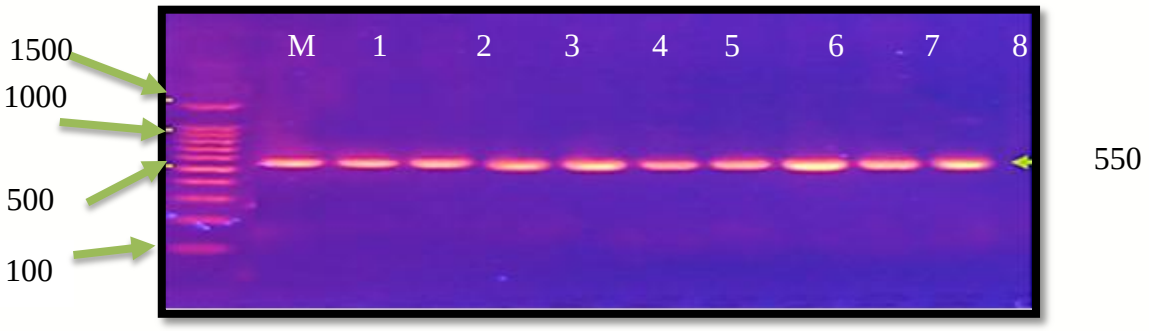


Figure (1) PCR product for confirm diagnosis of *L.kunkee* ,M=Leader ,(lane 1-10) *16sRNA* of bacteria

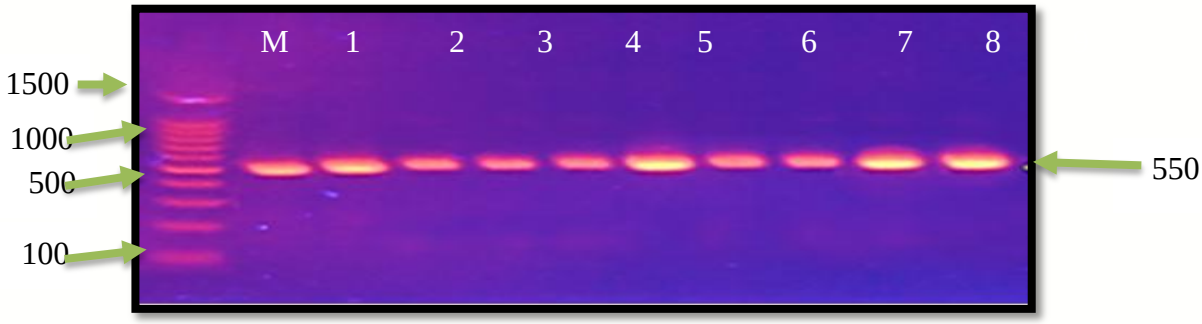


Figure (2) **PCR product for confirm diagnosis of *L.kunkee* ,M=Leader ,(lane 1-10) *16sRNA* of bacteria**

For detection protective role of this bacteria against histological effect of OTA we treated animal with OTA,the Microscopically results of kidney and Liver tissue sections male white rat that treated with OTA at dose100µg/kg body weight for seven day appeared several pathological changes ,in kidney tissue this changes include :vascular congestion , dystrophy and the disappearance of the glomerulus in addition to cell necrosis of glomerulus. (Picture2)

In liver tissue histopathological changes include: cell necrosis, vascular congestion and loss of normal structure of liver (Picture 5)**. Angela *etal*. (2005)** showed that treated rats with OTA lead to liver carcinomas, and cause of these effect may be belong to ability of OTA on starting a cancer by interfering with DNA molecule or via triggered abnormal growth of cells in this case called carcinogenic agents and perhaps cancer originates because of genetic mutations in DNA. Our results agreed with each **Hanif *et al.* (2008) and Peng *etal*. (2010)** whom referred to OTA lead to cause pathological effect in animals liver such as hepatocytes necrosis and vascular congestion, this may be because of OTA highly toxic compound have toxic effect on liver and kidney and that appear through Inhibition of enzymes like Glutathione reductase enzymes and AST, ALT, ALP and these enzymes are important in the expulsion of toxic free radicals in the body and inhibited it may lead to increased accumulation of free radicals and the inability of the liver to be removed . . it is yet unclear how OTA effects on the liver which is consider the largest detoxification

organ in the body. This effect may be due to effect of OTA on Hepatocytes that lead to release above enzymes into blood and increase it level.

The oxidative tension considered important mechanism for the effects that occur by Ochratoxin A, This toxin lead to release of ROS in addition. to those released by redox reactions That normally occur in different activity of cell . ROS if not controlled on it can offensive all active molecules in body for example Nucleic acid, Lipids, Enzymes and Proteins, therefore Reactive oxygen species are involved in many serious diseases e,g. diabetes , Alzheimer's , Parkinson's , cancer and other diseases **(Halliwell and Gutteridge ,1986**).

While treated animals with *L. kunkeei*.at dose l ml/kg body weight for seven day and saline at dose10ml/kg body weight for seven day didn't appeared any pathological changes (Picture1,4).So treated animals with *L. kunkeei*. mixed with OTA showing not found any histopathological changes in liver and kidney (Picture3,6) This confirms the safety role of *L. kunkeei*.and have capacity to OTA toxicity Reduction or decompose products have lost effectiveness toxic for biological systems in treated animals. **Asama *etal*. (2015**) referred in his study that Heat-killed. *L. kunkeei* isolates from bee pollen promoted. IgA production. in mouse Peyer's Patchcells and had little mutagenic activity or effect on IL-2 production in mouse spleen cells in comparison with *Listeria monocytogenes*, which does exhibit mitogen activity . A pilot study in 11 healthy adults showed that 4-week intake of 1000 mg day(-1) heat-killed *L. kunkeei* increased secretory IgA (SIgA) concentrations and secretion in saliva with no adverse effects.

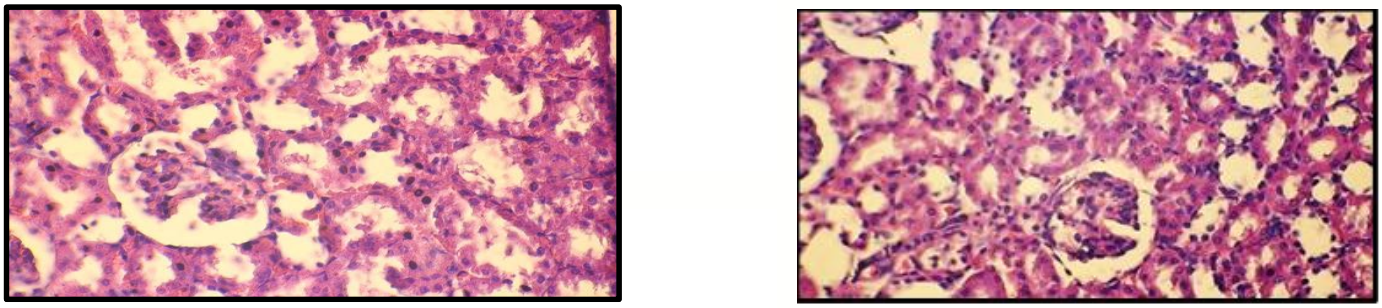
Previous reviews suggested that probiotics could lower the Frequency and duration of diarrhea ; stimulate humoral and cellular immunity ; prevent cancer.; and decrease unfavorable metabolites, including ammonium and procancerogenic Enzymes in the colon **(Mishra*etal*.2015).** In humans, *Lactobacillus rhamnosus* exerted strong antioxidant activity in situations of elevated physical stress. Athletes exposed to oxidative stress might benefit from the ability of *Lactobacillus rhamnosus* to increase antioxidant levels and neutralize the effects of reactive oxygen species **(Martarelli *etal*. 2011)**. Moreover, in recent years, a great number of studies have focused on the impacts of intestinal microbiota on an individual's health status. Probiotics, which are capable of colonizing the intestinal tract, are reported to improve metabolic diseases such as obesity and diabetes through modulating intestinal Microorganisms **(Wang *etal*015).**

For all above there were no study about protective effect of *L. kunkeei* for OTA-induced kidney and liver damage to compared our study with it.

CONCLUSION:

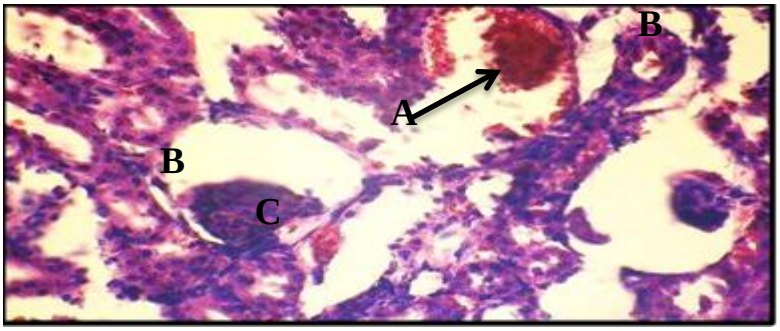
Lactobacillus kunkeei isolated from honey bee appear good Protective role against histological effect of OTA and may safely improve immune responsiveness.

This is the first report for detection of protective role of honey bee bacteria against OTA .and these bacteria appear good role in degradation of mycotoxin.

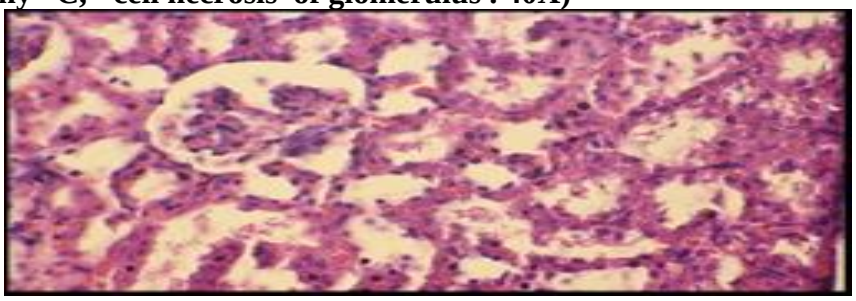


Picture (1) Histological section of control group Kidney. A=treated with *L.kunkee* at dose 1ml/kg b.w ($1x10^7$ cell/ml) B = treated with saline at dose 1ml/kg b.w(H and E stain 40X)

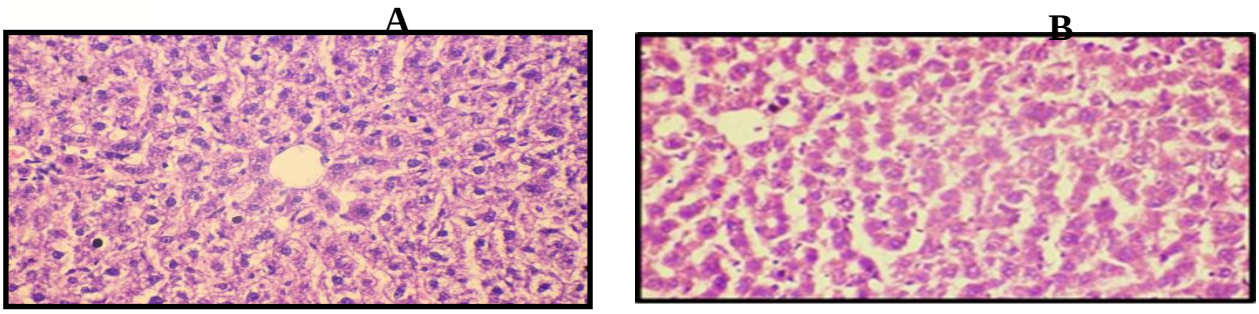
A



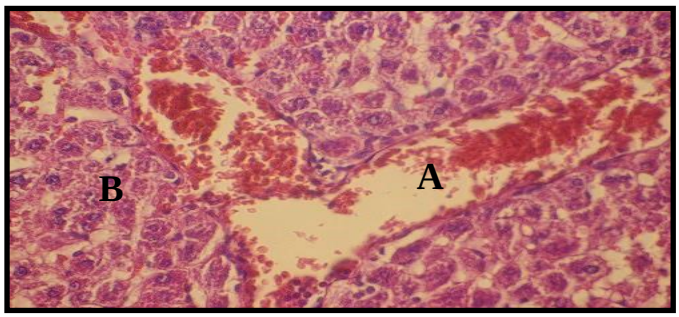
Picture (2): Histopathological section of Kidney for rat after 7 days of treatment with OTA at dose 100µg/kg b.w. shows A = vascular congestion, B =dystrophy C, =cell necrosis of glomerulus . 40X)



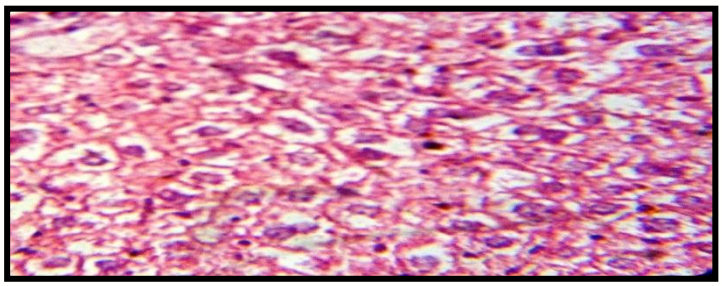
Picture (3): Histopathological section of Kidney for rat after 7 days of treatment with OTA at dose 100µg/kg b.w. + *L. kunkeei* at dose 1ml/kg b.w.($1x10^7$ cell/ml) shows normal structure for kideny. (H and E stain 40X).



Picture (4):Histological section of control group liver .A=treated with *L. kunkeei* at dose 1ml/kg b.w ($1x10^7$ cell/ml)B = treated with saline at dose 1ml/kg b.w (H and E stain 40X)



Picture (5): Histopathological section of liver for rat after 7 days of treatment with OTA at dose 100µg/kg b.w. shows **A** = vascular congestion, **B** =cell necrosis (H&E stain 40X)



Picture (6): Histopathological section of Liver of rat after 7 days of treatment with OTA at dose 100µg/kg b.w. + *L. kunkeei* at dose 1ml/kg b.w.(($1x10^7$ cell/ml)shows normal structure . (H and E stain 40X.

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