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Effect of certain Factors on surface binding of aflatoxin B_1 by some probiotic strains

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ABSTRACT

In this study ,increasing the aflatoxin B1 more than 30 ug/L in the medium decreased the count of the lexamined strains especially *Lactobacillus casido* which decreased from $9.4x10^8$ to $8.4x10^2$ cell/ml at concentration *225ug/L*. Although the amount of bound toxin increased from 25.2 to 57.75 *ug/L* , the percent of bounding decreased from 84% to 77%. Almost the same trend was observed in case of *Lactobacillus acidophilus* and *Bifidobacterium* .On other hand increasing the bacterial count from 10^6 to 10^9 cell/ml increased the percentage of binding toxin from 55,32, and 54 to 81,60 and 90% for *Lactobacillus acidophilus* and *Bifidobacterium Bifidum* respectively. The percentage of. Binding remaining almost the same by increasing the incubation time more than 30 min . At initial pH 4 and 7 the count of the tested bacteria slightly decreased after 3 hours of incubation while at initial pH 9 decreased to about 5 after the incubation time the results indicated that the initial pH between 2 to 4 slightly affected the bacterial count and the pH of the media after 24 hours of incubation while pH 5 or more till 9 sharply increased the count of the tested lactic acid bacteria while the final pH decreased to about 4 after the end of the incubation time . The results also revealed the increasing the initial pH more than 5 affected the percentage of binding aflatoxin B_1 while the percentage of media decreased at pH less than 5.

INTRODUCTION

Propbiotics are defined as living organisms which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition. One of the most significant groups of probiotic organisms are lactic acid bacteria (LAB). LAB might play in preventing or slowing the growth of colon cancer[1]; lowering cholesterol level[2]; preventing urogenital infections, alleviating constipation and treating food allergy[3]. Intestinal bacteria can produce from dilatory component that have genotoxic, carcinogenic, and tumor promoting activities[4]. It is clear that some groups of intestinal bacteria eg, Lactobacilli and bifidobacteria have much lower activities of enzymes that can generate carcinogens components than other gut microflora components such as *clostridium* and bacterioicids. This suggests that balance of microbial types in gut is important in terms of colorectal cancer risk4.LAB useful in preventing and shortening the duration of several types of diarria and act on the immune system[5]

Functional foods contain significant levels of biologically active components that provide health and basic nutrition. For example yogurt and other curdled milk products contain lactic acid bacteria and enhance gastrointestinal system function. *Lactobacillus acidophilus* (from a commercially available yogurt), *Lactobacillus gasseri (P79), Lactobacillus confusus* (DSM20196), *Streptococcus thermophilus* (NCIM 50083), *Bifidobacterium breve* and *Bifidobacterium longum* (from human infant stool) could strongly inhibit genotoxicity in the gastrointestinal tract of rats[6].

LAB distribute in human and animal gastrointestinal tract *Lactobacillus acidophilus* exist in the upper part of small intestine to the lower part of the small Intestine. *Biofdobacterium* exists from the lower part of the small intestine to the large intestine but is especially important to prevalent in high numbers in breastfed infants. *B. bifidum* is a common resident in the mucus membranes lining the distal part of the small intestine, the large intestine, and the vaginal tract, where it attaches to the luminal walls. Digestive system pH is not static but changes over time in the different parts of the system. Stomach pH varies between 1, 2-2 until 4 while duodenum pH range from 8-8.9[7].

Various food commodities including dairy products may be contaminated with aflatoxin, which, even in small quantities, have detrimental effects on

human and animal health[8].Food contaminates interning the body through the oral route is directly exposed to the action of gut micro flora. Normal healthy intestinal micro flora contains many strains of lactic acid bacteria (LAB) which consider a good binder for aflatoxins. When aflatoxin B_1 and B_2 contaminated food or feed is consumed, the toxins are metabolized to aflatoxins M1 andM2 and secreted in to the tissues, biological fluids, and milk of lactating animals; including breast milk[10]. *Lactobacillus plantarum* removed aflatoxin B1 corn crop[11]. *L. casei* and *L. Acidophilus* eleminat AFB1 from liquid media [9]. Binding of aflatoxin by intestinal bacteria should be fast in order to prevent toxin adsobtion in animal or human digestive tract.

The objectives of this study are (1) determine the amount of aflatoxin B1 that bound by *Lactobacillus acidophilus*, *Lacto bacillus casido* and *Bifidobacterium* in vivo experiments in physiological buffer for different time .2) determine effect of different pHs on toxin binding and (3) examine the stability of complex formed between AFB_1 and bacterial cells.

MATERIAL AND METHODS

Bacterial strains and growth conditions

The strains *Lactobacillus acidophilus*, *Lacto bacillus casei* and *Bifidobacterium bifidum* were obtained from Chr. Hansen's Lab. Inc., Denemark. All the strains were cultured for 24h in de Man-Rogosa-Sharpe (MRS) (Oxoid, Hampshire, united Kingdom) Under aerobic condition except *Bifidobactreium bifidum* which grown under anaerobic condition at 30 ^{0}C. Bacterial counts were determined by pour plat method.

Surface binding experiments

AFB1 binding assay

Solid AFB_1 (Sigma, St. Louis, MO) was suspended in benzene-acetonitrile (97:3; vol/vol) the actual concentration of this stock solution was calculated from the Lambert- Beer equation spectrophtometrically at 365 nm and E_{365}=20.767. Different concentrations of AFB_1 were prepared in buffer (pH7.3) and benzene acetonitrile was evaporated by heating in water bath (70°C, 5 to10 min).

Bacterial strains were grown up in MRS for 24 h then were centrifuged for 15 mints at 3000 rpm .The bacterial cells were washed with 5 ml of buffer (pH 7.3) to avoid the removal AFB_1[12].Bacterial pellets were suspended in 1.5 ml of AFB_1 solution (the concentrations depend on the experiments) for 1 hour. The bacterial cells were recentrifuged. Samples of the supernatant fluid contained AFB_1were estimated by thin layer chromatography.

Effect of incubation time on AFB_1 binding

The tested strains (10^8CFU/ml) were suspended in buffer (pH 7.3) and incubated with 10 *ug* /ml of AFB_1 for 0, 30, 45, 60, and 75 mints at 37°C. Each sample was centrifuged and the reminder toxin in supernatant was determined.

Effect of different pH on AFB_1 release and binding

Binding of viable cells –AFB was evaluated under various physiological buffers at pHs 3, 5, and 7. Bacterial pellets for each strain (10^8 CFU) was suspended in 1.5 ml buffer solution contain 10µg of AFB_1, the suspension was incubated for 1h at 37°C. After incubation all bacterial suspensions were centrifuged and the toxin reminder in supernatants was determined. Bacterial pellets were washed by buffer solution pH 5 five times then washed an other five times by buffer pH 7, released toxin for each pH was quantified by thin layer chromatography (TLC).

RESULTS AND DISCUSSION

Effect of AFB_1 concentration and viability of tested bacterial strains

Relationship among bacterial viability and AFB_1 concentrations represented in Fig. 1. By increasing AFB_1 concentration to 30 µg, the viability decreased approximately 2.5 log cycles for all examined strains. Meanwhile when AFB_1 concentration increased 75 µg, the counts decreased approximately 2 log cycles for all the tested strains. The small amount of AFB_1 may penetrate the cell wall of LAB casing death[9].

Effect of AFB_1 concentationsand capability of tested bacterial strain for binding the toxin

Figure 2 showed that by increasing AFB_1 concentration, there was remarkable decrease in binding rate by microbial cells. *B. bifidum* removed 90% of 30 µg/ml of AFB_1 from buffer solution while it remove 84.3 %, 79.7%

and 67.4% of 45,60 and 75 µg/l AFB_1 concentrations, respectively. Meanwhile, *L. casei* removed 84%, 80.9%, 79% and 77%. *L.acidophilus* bined 85%, 77.7%, 62.75% and 42% of the same previously toxin concentrations, respectively (Fig. 2).

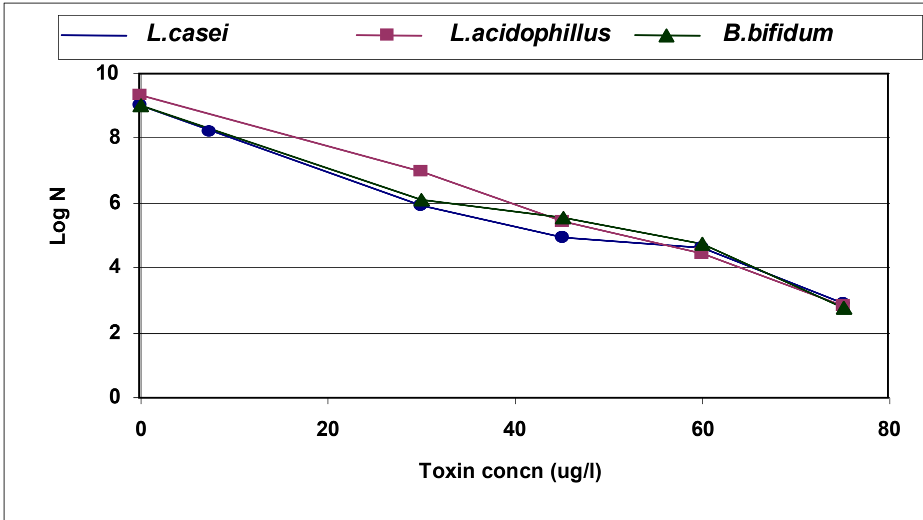

Fig. 1. Effect of different concentration of AFB_1(10 µg) on *L.casei L.acidophilus and B.bifidum* growth

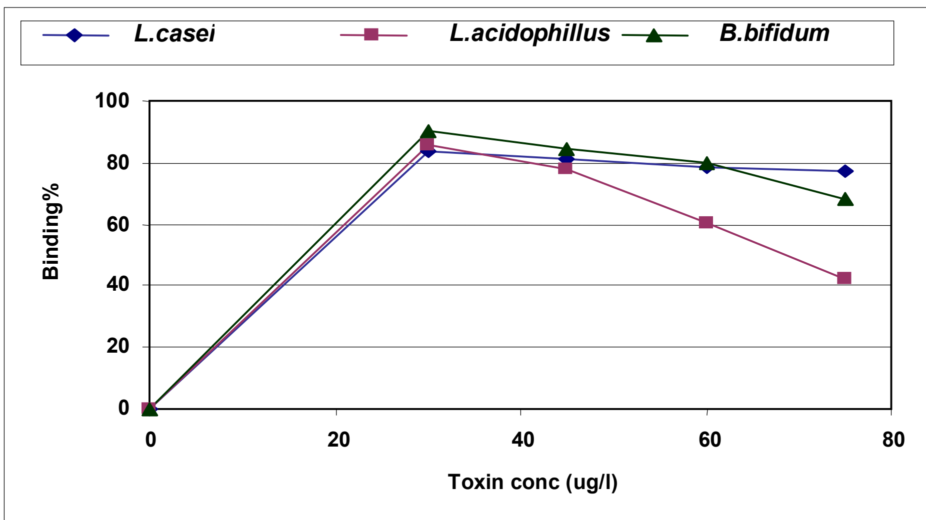

Fig. 2. Effect of different concentration of AFB_1 (10 µg) on the capability of *L. casei L.acidophilus and Bifidobacteria bifidum* for binding toxin.

Effect of bacterial strains count and percentages of AFB_1

Relationship between bacterial load and binding percentages were examined (Fig. 3). When the total count were increase the binding percentage were also increase. When the bacterial counts increased from log 6 to log 7 the differences between binding percentages increased by 1%, for *L.casei.* and *1.3%* for both *L acidophilus*, *B. bifidum,* respectively. By increasing the microbial load from log 8 to log 9 the difference decreased to 1 % for *L . casei* and to 0.6% and 0.5% for *L acidophilus* and *B. bifidum* respectively. The bacterial concentration influences the AFB1 removal. Different minimum concentrations have been reported such as $5x10^9$ CFU/ml of either *L. acidophilus* or *B. longum* to remove only 13% of the AFB1 within one hour [16]or 2 x 10^9 CFU/ml of *Lactobacilli* and *Propionibacterium* to remove 50% of free AFB1 but higher binding occurred at 10^{10} CFU/ml[12]

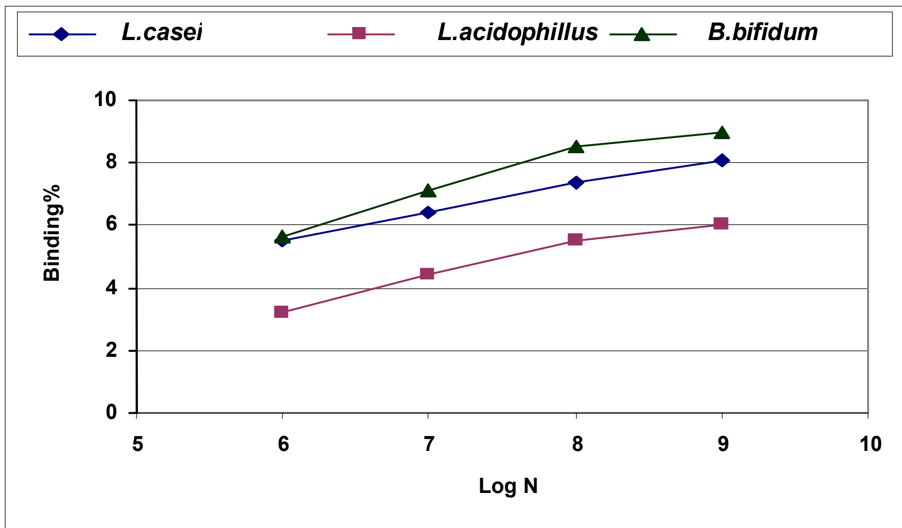

Fig. 3. Relationship between different bacterial counts of *L. casei*, *L acidophilus*, *Bifidobacterim bifidum* and binding percentage of AFB1.

Effect of incubation time end binding percentages of AFB_1 by tested strains

Figure 4 represent the effect of incubation time on AFB_1 binding on bacterial cells. Binding percentage increase at first 15 and 30 mints. After 60 mints the binding percentage become constant until the end of the

experiments(80 h) .The bacterial count was slightly decreased due to the AFB_1 toxicity.

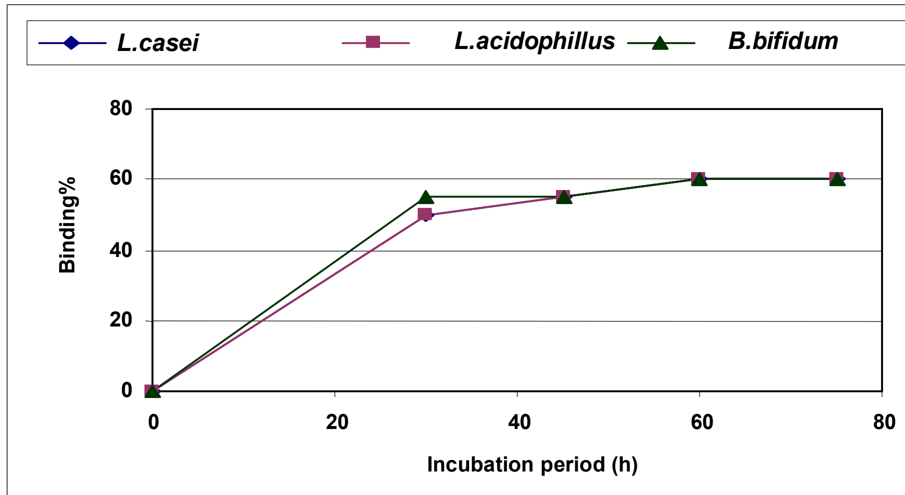

Fig. 4. Effect of incubation time on AFB_1 binding percentage on bacterial cells

Effect of the pH of the growing medium and the counts of the tested strains

From the resent study (Table 1), all the examined strains could not grow under pH 3 but they were still life in artificial media combined by changing in pH. *L.casei.,* *L. acidophilus* and *B. bifidum* changed pH 2 to 2.35, 2.24 and 2.3, respectively and from pH 3 to 3.6, 3.29 and 3.36 for the same organism's after first 3 hours .while counts were equal to the inoculums count (10^4cuf/ml) in liquid media. From pH 4 to pH 9 the microbial counts were approximately constant (10^6cuf/ml) for *B. bifidum* while they were (10^8cuf/ml) for *L. casei and L.acidophilus*. The tested strains lowered the initial pHs 4, 7, and 9 to about 4 indicating the unabilitity of the these strains to grow under pH 4 after 24 hrs. Lactic acid bacteria lowers the pH due to lactic acid production[17]. Marteau *et al.* A static experments showed that *Bifidobacterium* ssp and *L.acidophilus* are acidic resistant[18]; after 120 min. more than 40% of injected *L. acidophilus* and B. *bifidum* remained viable in gastric dynamic model via the stomic and small intestine. A other static experments have shown that *Bifidobacterium* sp, *L. acidophilus* are more acidic resistant than *S. thermphulus*[19].

Table 1. Effect of incubation time on bacterial count and pH of growing media.

Bacterial strains	**pH before inoculation**	**Incubation time in hours**							
		1 hr		**2 hrs**		**3 hrs**		**24hrs**	
		Final pH	**BCA (cfu m^{-1})**	**Final pH**	**BCA (cfu m^{-1})**	**Final pH**	**BCA (cfu m^{-1})**	**Final pH**	**BCA (cfu m^{-1})**
	3	3.0	$3x10^{4}$	3.6	$3x10^{4}$	3.6	$3x10^{4}$	3.6	$5x10^{4}$
L. casei	4	3.8	$3x10^{5}$	3.8	$3x10^{5}$	3.8	$2x10^{5}$	3.7	$5x10^{5}$
	7	6.4	$3x10^{5}$	6.3	$5x10^{5}$	6	$5x10^{5}$	3.79	$3.3x10^{8}$
	9	7.6	$3x10^{5}$	5.5	$5x10^{5}$	5	$5x10^{5}$	4.2	$3.5x10^{8}$
L. acidophilus	3	3.2	$6.7x10^{4}$	3.2	$6.7x10^{4}$	3.29	$6.7x10^{4}$	3.29	$1.5x10^{4}$
	4	3.8	$6.7x10^{5}$	3.8	$6.7x10^{5}$	3.8	$6.9x10^{5}$	3.5	$8x10^{4}$
	7	6.6	$6.7x10^{5}$	6.5	$6.7x10^{5}$	6.4	$7.5x10^{5}$	4.02	$3x10^{8}$
	9	6.6.	$6.7x10^{5}$	5.5	$6.7x10^{5}$	5	$7.0x10^{5}$	4.4	$3.8x10^{8}$
B. bifidum	3	3.0	$4.4x10^{4}$	3.3	$4.4x10^{4}$	3.36	$4.4x10^{4}$	3.36	$4.4x10^{4}$
	4	3.5	$4.4x10^{5}$	3.5	$4.4x10^{5}$	3.4	$4.4x10^{5}$	3.39	$4X10^{3}$
	7	6.5	$4.4x10^{5}$	6.3	$4.5x10^{5}$	6	$4.5x10^{5}$	3.62	$2.9X10^{6}$
	9	6.3	$4.4x10^{5}$	5.2	$4.4x10^{5}$	4.7	$4.4x10^{5}$	3.8	$3.5X10^{6}$

Effect of pH on removing the binding toxins by thested bacterial strains

The binding ability of the examined strains to remove AFB_1 from buffer solution at different pHs were tested and the stability of the bound aflatoxin B_1 was examined (Fig. 5) From results changining in pH has little effect on AFB_1-*L.casido* binding. The binding precentage at pH 3 and 4 was 46% then increased to 56% at pH 5 and pH6 and up the binding % became constent(57%). *L. acidophilus* -AFB1 binding were 50% and 59% at pH 3and 5 respectivily .After pH5 the binding precsentages became constent. While *bifidobacteria* – AFB1 binding persentages were 55% and 57% at pH 3 and 4 and increased to 62% after pH 6. The sharp increase in binding at pH 6 may be due to the increasing in bacterial counts from10^4 t0 10^8 .

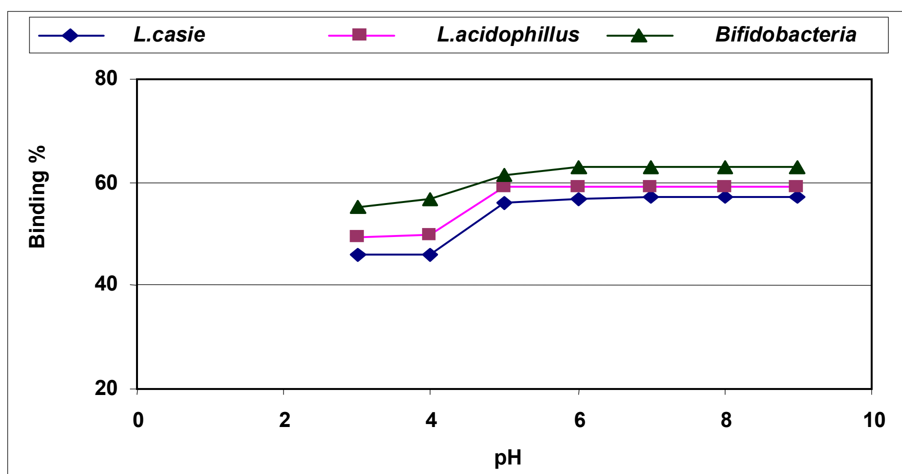

Fig. 5. Effect of pH on toxin binding by bacterial isolates.

pH 5.5 increase the binding ability of lactic acid bacteria to bind AFB1 more than pH 3 or 4.5[20]. These results suggest that a relationship between reduction of AFB1, pH of the medium and temperature of incubation typical of an enzymatic reaction could exist. Other investigations reported the transformation of AFB1 by lactic acid bacteria into the nontoxic aflatoxins B_2a in acidogenous yogurt and showed also that the fermentation of yogurt(pH 4) and acidified milk contaminated with AFB1 reduced the amount of the toxin [11] . Even though the mechanism of AFB_1 removal by Lactic acid bacteria is still unkown it has been suggested that aflatoxins molecules are bound to the bacterial cell wall components of bacteria.. Nine potential proprietary sequestering agents were compared in a novel *In vitro* assay for aflatoxin B1 (AFB_1) binding.The results suggested that these questering agents tested here had sufficient potential to bind AFB_1 at pH values commonly found in the gastrointestinal tracts of ruminants and other animals. All nine agents bound more than 95% of the 5 µg of AFB_1 in solution, regardless of pH[21]. Binding was not affected by pH[22]. Lactic acid bacteria and Bifidobacteria removed the AFB1 from contaminated solution[8]. The extract exopoly saccharides and cell wall content of *L. Rhmnosus* GG and AFB1 binding properties has been tested[23]. Their was no evidence for exopolysaccharidess proteins,Ca^{2+} or Mg^{+} being involved in AFB1 bindings wall isolate indincates that AFB1 binds to the cell wall peptidoglycanof LGG tightly associated with thenpeptidoglycan.

On washining the toxin bound cells by buffer solution (pH 5) five times no remarcable AFB1 was detcted. The cells were rewashed an other 5 times by buffer solution pH 7 there no toxin released . The binding process of lactic acid bacteria and strains of *Bifidobacteria w*as reversible and AFB1 was released by repeated aqueous washes[8].

AFB_1 is bound to the bacteria by weak noncovalent interactions, such as associating with hydrophobic pocketson the bacterial surface[9].Thier study shows that small amounts of bound AFB1 are released from the bacterial surfaces in aqous slution between pH 2 and 10 at human body temprature. Lactic acid bacteria reduce tissue uptake of AFB1 from duouden of chicks. However ,full in vivo studies are required to assess the effects of these bacteria on the bioavailbility and mutagenicity of consumed aflatoxin[15].

CONCLUSIONS

- Aflatoxin decrease the viability of the examined strains.
- The binding rate of AFB_1 decreased by increasing it, s concentration.
- Binding percentage increased by increasing the bacterial counts.
- All the tested strains bound AFB1 at pHs ranged from 3(stomach pH) to (duodenum pH) at the human body temperature 37 ^{0}C.
- Lactic acid bacteria has the ability to neutralized the alkaline pH in duodenum.
- This study must be follows by several in vivo studies.

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مجلة البحوث الإشعاعية والعلوم التطبيقية

مجلد 1 عدد 2 ص ص 349 – 361 (2008)

تأثير بعض المعاملات على أدمصاص السم الفطرى (افلا توكسين بى بواسطة) بعض بكتريا الأمعاء

هالة أحمد حسين

قسم الميكروبيولوجى، المركز القومى لبحوث وتكنولوجيا الاشعاع، ص. ب. 29 مدينة نصر، القاهرة، مصر.

في هذة الدراسة وجد أن بزيادة تركيز الأفلا توكسين من 30 إلى 75 ميكرو جرام فى اللتر حدث أنخفاض حاد فى العد البكتيرى اللاكتوباسليس كاسيومن $9.4x10^{8}$ إلى $8.4x10^{2}$ فىالمليليتر ولوحظ أيضا برغم زيادة أدمصاص الأفلاتوكسين على السطح الخارجى للكائن من 25.2إلى57.75 ميليجرام فى اللتر إلا أن نسبة الأدمصاص أنخفضت من 84% إلى 77% على التوالى ولوحظ ذلك أيضا على كلا من اللاكتوباسيليس أسيدوفليس والبفيدوبكتريم بفيديوم وبدراسة تأثير العد الحيوى على نسبة الأدمصاص وجد أنه بزيادة العدد البكتيرى من 10^{6} الى 10^{9} فى الميلليتر زادت نسبة أدمصاص السم الفطرى من55,32 و54 إلى 81,60 و90% لكلاً من لاكتوباسيليس كاسيدو , لاكتوباسيليس أسيدوفيلاس و البفيدوبكتريم بفيديوم على التوالى. وعند تحضين السم الفطري مع الخلايا البكتيرية لفترات زمنية مختلفة وجد أن نسبة أدمصاص السم الفطرى ثبتت بعد ساعة من التحضين . وعند دراسة تأثير الرقم الهيدروجينى على الخلايا البكتيريا وجد أن العد البكتيرى زاد زيادة طفيفة خلال 24 ساعة من التحضين عند الأرقام الهيدروجينية 2,3,4 ولكن عند التحضين عند الأرقام الهيدروجينية أعلى من 5 تضاعف العدد البكتيرى لكل السلالات التى أجريت عليها الدراسة .أما نسبة الأدمصاص فقد أرتفعت من 46,50,55% عند الرقم الهيدروجينى3 إلى 57, 59 و63 % عند الأرقام الهيدروجينية الأعلى من 5 وعند غسيل الخلايا البكتيرية الحاملة للتوكسين بواسطة محاليل رقمها الهيدروجينى 5و7 لم يتحرر التوكسين من الخلايا البكتيريا.