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DETOXIFICATION OF AFLATOXIN M1 on LACTIC ACID BACTERIA IN CONTAMINATED WITH IT MILK.

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ABSTRACT

Commercial skim milk, naturally and artificially contaminated with aflatoxin M_1 (AFM_1), was incubated with 4 lactic acid bacteria; *L. casei sub sp. casei* (ATCC15008), *L. acidophilus* (ATCC 11975), *L. sp. GG* (ATCC 53103) and *L.rhamnosus* (ATCC 10863). In samples "spiked" with AFM_1 (0.8ng/ml) the coagulation time of all 4 isolates increased while the pH of the treatments reduced compared to control (contaminated skim milk before starter addition and incubation). All 4 bacteria also caused a reduction in AFM1 level ranging from 26.2- 34.0% depending upon the bacterial isolate, during the coagulation period, whereas AFM1 levels were reduced to only trace levels after storage at room temperature for about 48 hours. Thus, the bacteria used in the conversion of milk to milk products such as yogurt appeared to be very effective in reducing and even elimination of AFM_1in milk

Key words: Aflatoxin M_1 - Milk – *L actobacillus casei - Lactobacillus acidophilus - Lactobacillus rhamnosus.*

INTRODUCTION

Aflatoxins, a group of secondary metabolites produced by the fungus *Aspergillus flavus* Link and A*aspergillu parasiticus* Spear. Food and Agriculture Organization (FAO) estimates that 25%of the world food crops are affected by mycotoxins each year (FAO 1996). There are four major naturally occurring aflatoxins, the most hepatotoxic being B_1 (AFB_1), and three structurally similar compounds namely B_2, G_1 and G_2. , One of the principal aflatoxin B_1 biotransformation products is aflatoxin M_1 (Van Egmond, 1989). Human exposure to high levels of aflatoxin from the diet is an important risk factor for the development of liver cancer (Yeh, et al, 1989 and Wogan 1991).

Aflatoxin M_1 (AFM_1) which is a major metabolite of aflatoxin B_1,is produced in the liver of animals that have ingested feed contaminated with aflatoxin B_1 (Campbell and Hayes, 1976). Food and Drug Administration (FDA), established an action level of 0.5 μg/kg for AF M_1 in fluid milk and milk products (FDA, 1977). This level was selected as based upon the level of analytical capabilities, the need to minimize human exposure, and the finding, in transmission studies, that feed containing 20μg/kg of B_1 would result in 0.5 μg/kg of aflatoxin M_1 in the milk. (Wood and Trucksess, 1998). In Arizona, (1978), 909,442lb of milk was destroyed with AFM_1 levels as high as 10 μl/kg. (Park, 1993). Oliveira *et al.* (1996) found that AFM_1 was detected in 11%of milk powder samples to be consumed by infants in Sao Paulo at levels of 0.1-1.0 μg/L. The mean daily intake of AFM_1 for 4 months old children was 3.7ng/kg body weight/day. Galvano, *et al*. (1996) reported that majority of

countries in the world experience high contamination with AFM_1 in fluid milk, human milk or milk products. Studies have been done to reduce the level of aflatoxin in foods; Ciegler et al (1966) screened several microorganisms (including molds, yeast, bacteria, acetinomcetes, algae and fungal spores) for their activity to degrade and / or modify aflatoxin. They noted that AFB_1 may be detoxified in acidic media. El-Gendy and Marth (1981) mixed *Lactobacillus casie* with *Aspergillus parasiticus* in the same culture and found that *L.casei* can grow, decrease pH, and degrade aflatoxin B_1 and G_1, with maximum degradation during the first 3days (93.6%). Line and Brackett (1994) determined the factors that affected AFB_1 removal by *Flavobacterium auranntiacum,* which included; culture age, concentration, and viability of the bacteria. Some studies attempted to detoxify AFB_I in contaminated acidified milk and yogurt.

Megalla and Hafez, (1982) found complete transformation of AFB_1 to its hydroxy derivative AFB_2 by the acids present in yogurt. Rasic, et al. (1991) found that, yogurt reduces AFB_1 concentration better than acidified milk, and reported that this could be a result of metabolic activity of yogurt bacteria which produce lactic acid, plus small amounts of volatile fatty acids, amino acids, peptides and other acidic compounds. However, several authors (Stoloff 1980, Wiseman and Marth 1983, Ismail et al. 1989and Blanco et al. 1993) reported no influence of yogurt manufacture on AFM_1content. Meanwhile, El Deeb et al. (1992) observed that enzymic, microbial and particularly acid coagulation caused degradation of AFM_1 in buffalo milk. El-Deeb (1989) observed some negative effects of AFM_1 on *Lactobacillus bulgaricus* (cell wall thickening and shortening of cell chain length) and *Staphylococcus thermopiles* (cell wall thickening and shape changing from coccid to oval). Karunaratne et al. (1990) noticed that *Lactobacillus acidophilus, Lactobacillus bulgaricus,* and *Lactobacillus planetarium* can reduce aflatoxin level because the bacteria were effective in preventing growth of the mold, and bacterial metabolites were effective in reducing the amount of aflatoxin produced. El- Nezami et al. (1997) studied the ability of some dairy strains of lactic acid bacteria, *Lactobacillus rhamnosus* (GG-ATCC53103), and *Lactobacillus rhamnosus* (LC-705); to remove AFB1 from contaminated media and they suggested the use of acid -treated *Lactobacillus rhamnosus* (GG or LC705) to remove aflatoxins in contaminated foods and feeds.

The present study is an investigation of the use of *Lactobacillus rhamnosus* (ATCC10863) and *Lactobacillus sp.* (GG-ATCC 53103) plus other two strains of dairy lactic acid bacteria (*Lactobacillus casei* sub *sp casei* (ATCC15008) and *Lactobacillus acidophilus* (ATCC11975) when added to yogurt or some dairy products, to improve the quality of these products, and detoxify or removal AFM_1 present in milk.

MATERIALS AND METHODS

Materials

Standard aflatoxins M_1: Pure aflatoxin M_1 from *Aspergillus flavus* (SIGMA Co., production of Israel MSDS). Aflatoxin concentration was prepared according to AOAC, procedures 49.2.03(1995).

Bacterial strains. The lactic acid bacteria (LAB), obtained from the American Type Culture Collection (ATCC) USA, were: *Lactobacillus casei* sub *sp.casei* (ATCC 15008) *Lactobacillus acidophilus* (ATCC 11975), *Lactobacillus sp.* And (ATCC 53103) *Lactobacillus rhamnosus* (ATCC 10863).

Skim milk powder: Commercial skim milk powder (Rich Food Co, Richmond, VA23261, USA) was used.

Preparation of samples

The skim milk was added to warm distilled water (1: 9). The mixture was stirred for 5 min. and sterilized in the autoclave for 20 min at 121°C.The strains of lactic acid bacteria were activated in suitable media (Lactobacilli MRS Broth "Difeco 0881") at 37°C and 5% CO_2. Lactic acid bacteria (LAB), were transferred to sterilized rehydration skim milk, incubated at 40°C until the milk coagulated and then stored at refrigeration temperature. Milk was contaminated with AFM_1 (o.8 ng/ml) and the contaminated milk was fermented with 5% of coagulated milk containing: *L. casie sub casie sp*. *L acidophilus*, *L. sp. GG* and *L. rhamnosus*. The treatments were incubated at 40°C until milk coagulated and then stored at room temperature (25-30°C) for 48 hours. One contaminated, sample (control) was acidified with solution of lactic acid (pH 4.0). Coagulation time (CT) were record and the samples analyzed for pH change and the aflatoxin concentration after coagulation and 48 hrs storage. Two control samples were prepared using skim milk only and milk contaminated with 0.8ngAFM_1/mL.

Extraction of aflatoxin M_1

Samples were extracted for aflatoxin analysis by the method adoped by Chang and De Vries (1983) and modified by Stubblefield and Kwolek (1986). A milk sample (25-mL) was added to separatory funnel with 5ml saturated aqueous NaCl and 60 mL chloroform. The mixture was gently rolled for 3 min. the chloroform layer was drained into flasks containing 5g anhydrous Na_2SO_4, stirred for 3mins. and filtered through fluted filter paper into a measuring cylinder . The chloroform extract was evaporated to dryness at 60°C under vacuum. The residue was transferred to another separator funnel containing 25ml acetonitrile and the solution was extracted with two 25ml portions of petroleum ether. The acetonitrile layer was evaporated to dryness. The residue was taken up with a minimum amount of methylene chloride and equal portions was transferred to two vials and re-evaporated to dryness under nitrogen steam.

HPLC analysis:

The content of one vial was dissolved in 450 µl injection solvent mobile phase (H2O+acetonitrile +MeO 700:150:150). The content of the second vial were derivatized according to Trucksess, et al. (1994). Vial contents were mixed well with 100 ml acetonitril; 350µl derivatization solution (10mL trifluroacetic acid + 5mLglacial acetic acid +35mL H_2O), was added and the vial heated for 9 minutes at 65 °C then cooled to room temperature before opening.

GLC:

The GLC system (Waters Co. Model 440 USA) was run 10-20 minutes to stabilize and adjust sensitivity control of fluorescence detector to give a

reasonable integrator response for lowest concentration of standard working solution. Samples (50 μl) were injected onto Waters μBonda Pak 086684 - 4.6mm 25cm, 5mm C18 ultras phere cat#23533 Beckman; at flow a rate 1.0 mL/min., Fluorescence detector-operating conditions (nm); were: excitation 360, emission440. Shimadzu Model RF-535 and the run time was 25 minutes. To limit the retention time (RT) of AFM_1 before injection samples; two standard samples of aflatoxin M_1 (1μg/1mL) were injected; one derivative (50μL) and other sample was non-derivative (50μL). The retention time (RT) of AFM_1 was at 4.7 minutes for derivative samples while it was at 16.7 minutes for non-derivative samples as shown in Figs. (1- a,b, c and d).

Fig. (1-a) Standard Derivative

Fig. (1-b) Controlled Derivative

Fig. (1-c) Standard Non-Derivative

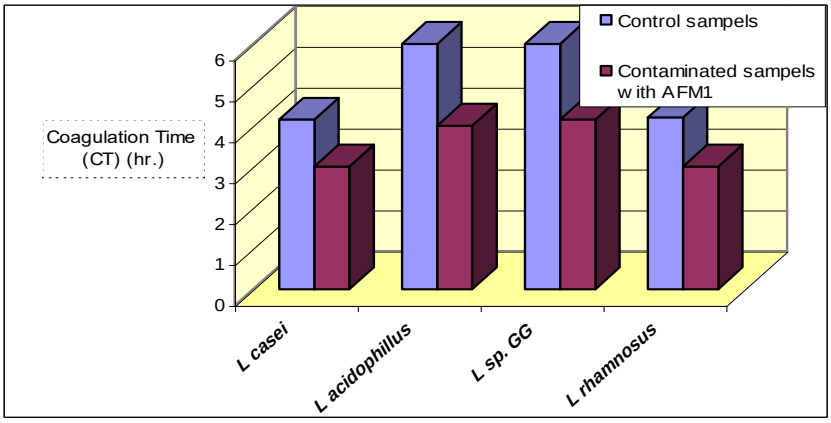
Fig. (1-d) Controlled Non-Derivative

Fig.(1) The Retention Time (RT) for Derivative and Non-Derivative Standard and Control Samples.

RESULTS AND DISCUSSION

Coagulation times were recorded and compared with control samples (skim milk + 5%starter only) to determine the effect of aflatoxin M1 on coagulation time. The obtained results as shown in Fig. (2) indicated that coagulation times for all treatments increased compared to the control samples. Aflatoxin M_1 retarded milk coagulation by 75 - 120 minutes, depending on the bacteria species. *Lactobacillus casie sub sp. Casie and l. rhamnosus* had the shortest coagulation time in both AFM_1 treated and non-treated samples. *Lactobacillus acidophilus* and *L. sp. GG* had the longest times for coagulation (4: 00 & 4: 15 h.) respectively. They were also susceptible to M_1 toxin in the milk having a delay in coagulation of 120 and 105mins, respectively. These results were similar to those obtained by Sutic and Banina, 1979 &1990 and EL-Deeb, 1989. Increasing coagulation time during manufacturing of yogurt or other dairy products was the reason that some authors suggested of examining milk for the presence of aflatoxin M_1 before manufacturing (Sutic and Banina 1990).

Fig. (2) Effect of Aflatoxin M1 (AFM1) on Coagulation Time for Fermented Milk by Some Lactic Acid Bacteria



In this study pH values were determined to evaluate bacterial growth in milk during coagulation and storage times and to detect the effect of aflatoxin M_1 on growth of bacteria. Fig. (3) shows pH values of samples after coagulation time and storage at room temperature (25-30 °C). The pH of control samples (contaminated skim milk before starter added and incubation) was 6.32, and afteradding starter and incubating samples at 40°C until complete coagulation, pH value were; 4.28, 4.55, 5.25 and 4.80 in *L.csei, L. acidophilus, L. sp. GG* and *L. rhamnosus* respectively. The ability of the 4 bacteria to decrease pH during storage at room temperature (25-30°C) for 48 hours slightly different, *L acidophilus* had the lowest pH (3.41), *L. csei* was 3.45, *L.rhamnosus* was 3.50 where *L. sp. GG* had the highest pH (3.92).

These results were similar to those obtained by EL-Gendy and Marth (1981) and EL- Nezamy et al. (1996).

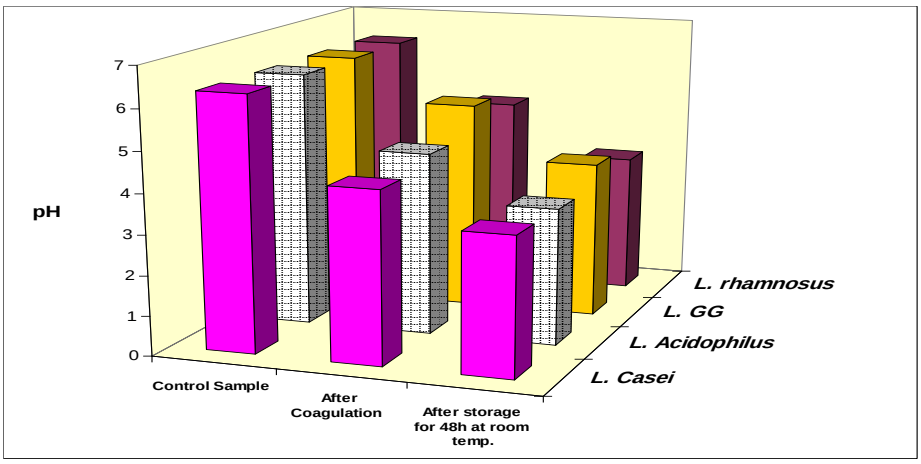


Fig. (3) Effect of Aflatoxin M1 (AFM1) on pH of Fermented Milk by Some Lactic Acid Bacteria after Coagulation Time (CT) and Storage for 48 hrs at Room Temperature (25~30°c)

The ability of lactic acid bacteria to detoxify or remove AFM1 from contaminated milk was the major aim of this study. When the level of AFM1in control samples (0.8 ng/ml AFM_1) was compared to spiked standard before and after extraction, the efficiency of extraction was about 96%. The control samples (rehydrated skim milk) was contained 4.84 ng/L AFM_1, which is slightly lower than the FDA action level for AFM_1 which is 0.5µl/kg (FDA, 1977). When levels of AFM_1in bacteria treated samples were determined (Fig. 4). *L. casie sp. casie* after AFM_1 28.2% after 4.25 hours (CT), it had eliminated all of the AFM_1 after during 48hours storage at 25: 30°C. *L. acidophilus* after 6.0 hr reduced AFM_1, 26.2% but after 48 hours storage at 25:30°C only trace levels of AFM_1 remained. *L. sp. GG* after 6.o hr reduce AFM_1 29.5% likewise after storage at 25:30°C for 48 hr only trace concentrates of AFM1, were detected . The highest reduction of AFM1 (34.0%) resulted from L. rhamnosus after only 4.33 hr (CT), also when storage at 25-30°C, AFM_1 was reduced to trace levels. On other hand samples acidified with water solution of lactic acid (pH 4.0) reduced AFM_1 12.4% only, both during incubation and storage at 25-30°C.

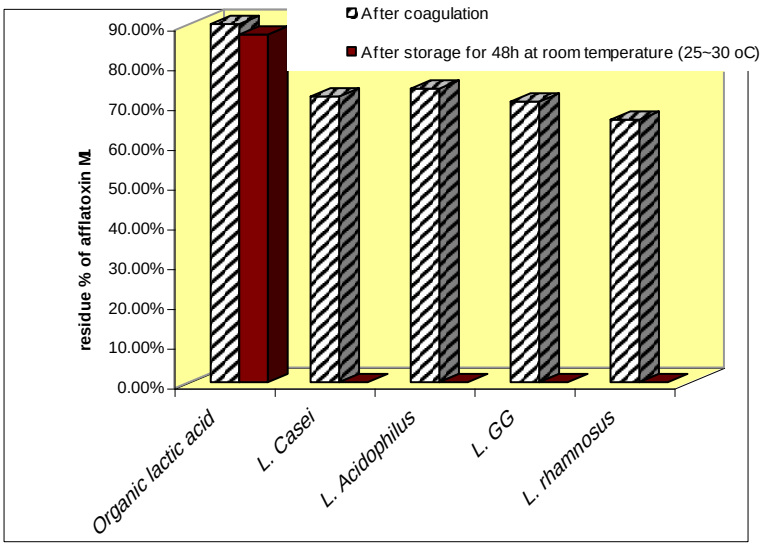


Fig. (4) Effect of Some Lactic Acid Bacteria on Aflatoxin M1 (AFM1) Concentrations

We conclude that, the lactic acid bacteria used in the production of yogurt and other coagulated milk products are effective means of reducing the levels of aflatoxin M_1 when present in milk prior to milk fermentation. Break down of AFM_1 occurs soon after the bacteria are added to milk and after 4.5 hrs we could detect only trace levels of AFM_1.

Thus it appear that the normal processing including through of milk in products such as yogurt essentially eliminate the health threat of aflatoxin M1 contaminated in lactic acid bacteria treated milk products. *Lactobacillus casie, lactobacillus acidophilus, Lactobacillus sp. GG* and *Lactobacillus rhamnosus* can be used for these purposes.

REFERENCES

Blanco, J.L., Carrion, B. A., Liria, N., Diaz, S., Garica, M. E., Domingues, L. And Suarez, G. 1993. Behavior of aflatoxin during manufacture and storage of yogurt. *Milichwissenschaft 48:385-387*.

Campbell, T. C. and Hayes, J. R.1976. The role of aflatoxin metabolism in its toxic lesion. *Toxical Appl. pharmacol. 1976 (35):199.*

Chang, H. L. and De Vries, J. W. 1983. Rapid high-pressure liquid chromatographic determination of aflatoxin M_1 in milk. *Journal of the Association of Official Analytical Chemists. 66. 913-917. [In Food Addit. Contam.1995. (6): 255-261].*

Ciegler, A., Lillehoj, B., Peterson, R. E.and Hall, H. H. 1966 Microbial detoxification of aflatoxin. *Appl. Microbiol 14: 934-939*.

EL Deeb, S. A. 1989. Interaction of aflatoxin M_1 on the physiological and morphological properties of lactobacillus bulgaricus and streptococcus thermophilus. *Alexandria Journal of Agricultural Research. 34(3) 61-72*

EL Deeb, S. A., Zaki, N., Shoukry, Y. M. R. and Kheadr, E. E. 1992. Effect of some technological processes on stability and distribution of aflatoxin M_1 in milk. *Egypt J. of Food Sci.(suppl.) 20: 29-42*

EL-Gendy, S. M. and Marth, E. H. 1981.Growth and aflatoxin production by *Aspergillus parasiticus* in the presence of l*actobacillus casei*. *J. Food Prot., 44, 211-212.*

El-Nezami, H. S., Kankaanpaaa, P. E., Salminen, S. and Ahokas, J. T. 1997. Physicochemical alterations enhance ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media*. J. of Food Prot. 61(4) 1998: 466-468.* FAO.1996. Basic facts of the world cereal situation. Food out look 5/6, 1996. FDA, 1977. Aflatoxin contamination of milk establishment of action level*. Fed Reg 61630,1977.*

Galvano, F., Galofaro, V. and Galvano, G. 1996. Occurrence and stability of aflatoxin M_1 in milk and milk products*. A worldwide review journal of Food Protection, 59 (10): 1079-1090.*

Galvano, F., Galofaro, V., Angelis, A. D., Galvano, M., Bognanno, M. and Galvano, G. 1998. Survey of the occurrence of aflatoxin M_1 in dairy products marketed in Italy*. Journal of Food Protection, 61(6) 738-741*.

Ismail, A. A., Tawfek, N. F., Abd Alla, A. M., El-Dairouty, R. K. and Sharaf, O. M. 1989. Fate of aflatoxin M_1 during kefir processing and its effect on the microflora and the chemical structure. *Dtsch Lebenesm. rundschair* 85: 76-78.

Karunaratne, A., Wezenberg, E. and Bullerman, L. B.1990 . Inhibition of mold growth and aflatoxin production by *lactobacillus spp. J.Food Protection., 53, 230-236.*

Line, J. E. and Brackett, R. E. 1994. Factors affecting aflatoxin B_1 removal by *Flavobacterium aurantiacum*. *J. Food Protection, 58(1) 91-94*.

Magella, S. E. and Hafez, A. H. 1982. Detoxification of aflatoxin B_1 by acidogenous yogurt. *Mycopathologia 77:89-91*.

Oliveria, C. A. F., Germano, P. M. L. ,Bird, C. and Pinto,C. A. 1977. Immunochemical assessment of aflatoxin M_1 in milk powder consumed by infants in Sao Paulo Brazil*. Food Additives and Contaminants 14(1) 7-10*.

Park, D. L. 1993. Controlling aflatoxin in food and feed*. Food Technology 47(10): 92 .*

Rasic, J. L. Skrinjar, M. and Markov, S. 1991. Decrease of aflatoxin M1 in yogurt and acidified milks. *Mycopathologia 113: 117-119.*

Stoloff, L. 1980. Aflatoxin M_1 in perspective. *J. Food Prot. 43: 226-230*

Stubblefield, R. D. and Kwolek, W. F. 1986. Rapid liquid chromatographic determination of aflatoxins $M_1$and M_2 in artificially contaminated fluid milks ANAL. CHEM. 69(5) 880-885. [In *Food Addit. Contam.1995. (6):255-261].*

Sutic, M. and Banina, A. (1979). Variability of lactic acid bacteria caused by aflatoxin $B_1$and its importance to manufacture. *Mljekarstove v.29(5) 106 –111*

Sutic, M. and Banina, A. (1990). Influence of aflatoxin Bı on gas production by lactic acid bacteria*. J. Environ. Pathol. Toxicol. Oncol., 10, 149-153*.

Trucksess, M. W., Stack, M. E.,Nesheim, S., Albert, R. H. and Romer, T. R. 1994. Multifunctional column coupled with liquid chromatography for determination of aflatoxin B_1, B_2, G_1 and G_2 in corn, Almonds, Brazil Nuts, and Pistachio Nuts : collaborative study. *Journal of AOAC International 77(6) 1512-1521.*

Van Egmond, H. P. 1989.Aflatoxin M_1: Occurrence, toxicity and regulation, p. 11-55 in H. P. Van Egmond (ed.), Mycotoxins in dairy products. *Elsvier Applied Science, London.*

Wiseman, D. W. and Marth, E. H. 1983. Behavior of aflatoxin M_1 in yogurt, buttermilk and kefir. *J. Food Prot. 46: 115-118*.

Wogon, G. N. 1991.Aflatoxins as risk factors for primary hepatocellular carcinoma in humans. In*; GA Bton Rouge; Louisiana state university press, 1991, p 18.*

Wood,G. E. and Turcksess, M. W. 1989. Regulatory control programs for mycotoxin – contaminated food. Ch. in *Mycotoxins in Agriculture and Food*

Safety. N. S. Sinha, K. K. and Bhatnagar (Ed),p. 459-481.D. Marcel and Dekker, Inc. Newyork

Yeh S., Yu M. C., Mo C. C., Wo S., Tong M. J. and Henderson, B. J. 1989. Hepatitis B virus, aflatoxins and hepatocellular carcinoma in southern Guangxi, China*. Cancer Res. 49: 2506, 1989*

القضاء على الأثر السام للأفلاتوكسين (AFM 1)فى اللبن الملوث باستخدام سلالات من بكتريا حمض اللاكتيك

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***** قسم الكيمياء الحيوية هيئة الرقابة على الأغذية والأدوية FDA واشنطن - الولايات المتحدة الأمريكية 0**

أجرى هذا البحث بتلويث بعض عينات اللبن التجارى بأفلاتوكسين (AFM1) بتركيز 80 نانوجرام /مل - وقد تم تلقيح هذه العينات بأربع سلالات من بكتريا حمض اللاكتيك هى

L. casei sub sp. casei (ATCC15008), *L. acidophilus* (ATCC 11975), *L. sp. GG* (ATCC 53103) and *L.rhamnosus* (ATCC 1086)

وقد تم الحصول على النتائج التالية :-

- فى جميع العينات الملقحة ببكتريا حمض اللاكتيك زاد زمن التجبن فى العينات الملوثة عنه فى العينات الغير ملوثة بالأفلاتوكسين (AFM1)
- استطاعت بكتريا حمض اللاكتيك خفض درجة ال pH لعينات اللبن الملوث بدرجات متفاوته 0
- أمكن للأربع سلالات تخفيض تركيز أفلاتوكسين (AFM1) بنسبة تراوحت ما بين 2 , 26 - 34 % معتمدة على نوع كل سلالة وذلك خلال زمن التجبن 0 بينما وصل تركيز أفلاتوكسين (AFM1) إلى مجرد آثار بعد تخزين جميع هذه العينات لمدة 48 ساعة على درجة حرارة الغرفة لمدة 48 ساعة 0
- وقد اتضح أن هذه السلالات والتى يمكن استخدامها فى صناعة الزبادى ذات تأثير واضح فى التخلص من الأفلاتوكسين (AFM 1) فى اللبن 0