Inhibition of Fungal Growth and Aflatoxin B1 Production by Some *Lactobacillus* Strains

Nanis H. Gomah * , W.S. Ragab **and L. B. Bullerman***
*Dairy Department , Fac of Agric. Assiut Univ, Assiut, Egypt.
**Food Science & Technology Dept. Fac of Agric, Assiut Univ
***Food Science & Technology Dept,Univ, Nebraska-Lincoln,USA

Key words: Lactobacilli, inhibition, aflatoxins, mold growth.

Abstract: The antifungal effect of five *Lactobacillus* strains namely *L. plantarum* ATCC 4008, *L. plantarum* 12006, *Lactobacillus plantarum 299V, L. paracasei* subsp. *paracasei* LMG 13552 and *L.rhamnosus VT1* was examind against some of common cheese-contaminant fungi (*A. parasiticus* NRRL2999, *A. flavus* , *A. versicolor*, *Penicillium roqueforti* and *P. communi*). The ability of *L. plantarum 299V*, *L. paracasei* subsp. *paracasei* LMG 13552 and *L .rhamnosus VT1* to inhibit aflatoxin B_1 production by *Aspergillus flavus and A. parasiticus* NRRL-2999 was also studied. All the studied *Lactobacillus* strains exhibited various degrees of growth inhibition against some but not all the studied molds. The three strains of *L. plantarum* showed an antimycotic activity against *A. versicolor* and *A. parasiticus* but not *against A. flavus*. *A. versicolor* showed the highest sensitivity toward *L* .rhamnosus and all of *L. plantarum* strains. This was followed by *A. parasiticus* which affected at lower extent by *L. plantarum* strains. Growth of *A. flavus* was slightly inhibited with the presence of *L. paracasei* subsp. *paracasei* and *L.rhamnosus* only. On the other hand, none of the studied *Lactobacillus* strains were found to inhibit growth of *P.roquefoti* or *P. commune*. Production of aflatoxin B_1 by *A. parasiticus* NRRL2999 was almost completely inhibited (98.8-99.99%) by all the investigated lactobacilli. However, the antiaflatoxigenic potential of *L. paracasei* subsp. *paracasei* was lower than that of *L. plantarum* and *L. rhamnosus*. The amounts of aflatoxin B_1 produced by *Aspergillus flavus* in the presence of these Lacobacilli were reduced by about 85-92% and 96.3-98.3% compared with control after 10 and20 days of incubation, respectively.

Received on: 26/12/2009 **Accepted for publication on**: 5/1/2010
Referees: Prof.Dr. Fathy E. ELgazar Prof.Dr. Mohamed E.Hassan

Introduction

During the last few years there has been a growing interest in biopreservation, i.e., the use of microorganisms and / or their metabolites to prevent spoilage and to extent the shelf-life of foods (Stiels, 1999). Lactic acid bacteria (L A B) are of particular interest as biopreservative organisms. Their preserving effect mainly relates to the formation of lactic acid, acetic acid, and hydrogen peroxide; competition for nutrients; and the production of bacteriocins (Lindgren and Dobrogosz, 1990).

Some species of molds are intentionally used for ripening purposes in cheese production such as, Roquefort, Stilton, Camembert and Gorgonzola. Nonetheless, many of these molds are considered undesirable contaminants of cheese during storage, even at refrigeration temperatures (Cabo *et al.*, 2002). Several studies on the distribution of fungal species on cheese have shown that *Penicillium* is one of the most predominant genera in the fungal mycoflora of cheese (Coallier-Ascah and Idziak, 1985 and Nielson *et al.*, 1998). Lund, *et al.* (1995) found that *P. commune* was the fungus most frequently observed in sime-hard and semi-soft cheeses collected from different European countries. Also, a high incidence of *Aspergillus versicolor* was found in Dutch cheese warehouses between 1975 and 1980, even though this species is not a frequent cheese contaminant (Scott, 1989). The contamination of dairy products with undesirable molds, such as the genera *Aspergillus* and *Penicillium* is a serious and frequently disturbing problem because their growth can cause alteration, with the production of mycotoxins which present a potential health hazard (Filtenborg et al., 1996). Aflatoxins are both acutely and chronically toxic to animals, including human, causing acute liver damage, liver cirrhosis, induction of tumors and teratogenic effects (Stoloff, 1977). They are produced in nature by *A. flavus, A. parasiticus* and a recently described species *A. nomius* (Klich and Pitt, 1988).

Interactions between molds and different genera of LAB have been extensively described. However, the results of these studies are contradictory. While inhibition of mold growth in the presence of LAB has been reported by some authors (Karunaratne *et al.*, 1990 and Cabo *et al.*, 2002), a stimulatory effect of several LAB on mold growth and mycotoxin production has also been described (Suzuki *et al.*,1991 and Zaki *et al.*,1992).

The purpose of this study was to investigate (a) the ability three individual *Lactobacillus* species to inhibit growth of some commonly cheese-contaminant

molds. (b) The inhibitory effect of these bacterial cultures on aflatoxin B_1production by the highly aflatoxin- producing strains, *A.flavus* and *A. parasiticus* NRRL2999.

Materials and Methods

Five *Lactobacillus* strains namely: *L. plantarum* ATCC 4008, *L. plantarum* 12006, *Lactobacillus plantarum 299V, L. paracasei Subsp. paracasei* LMG 13552 and *L.rhamnosus VTI*) as will as, five fungal species (*A. parasiticus* NRRL2999, *A. flavus* , *A. versicolor, Penicillium roqueforti* and *P. communi)* were obtained from the culture collection of the Department of Food Science and Technology at the University of Nebraska-Lincoln, USA.

Inoculation:

Frozen stock cultures of Lactic acid bacteria were prepared by thawing under optimum temperature, activated in litmus milk. Then, cultured in deMan Rogosa Sharpe (MRS) broth and incubated at 37℃ for 24 h before use. The mold spore suspensions were prepared by growing the studied fungi on malt extract agar (Oxoid, Hampshire, England) slants at 25℃ for 5-7 days until sporulation was observed. Spores were harvested by washing the medium surface with sterilized aqueous solution of Tween 80 (0.05% v/v). Ten ml of this solution was added to the agar tube, and the spores were loosened by gentle brushing with sterile spatula. Mycelia debris was removed by filtration through sterile cheese cloth. Spore counts in the resulting suspension were determined by microscopy with a hemocytometer chamber. Spore counts were finally adjusted to 10^7spores of each fungus per ml and stored at 4°C until use.,

Antifungal activity assay:

This assay was a modification of the overlay technique described by Cabo *et al.* (2002). Three 10-µl drops from an active culture of each bacterial strains tested were spotted into agar plates and incubated until well-grown colonies could be observed (Ca. 48h).The plates then were overlaid with 10 ml of glucose yeast extract agar medium (GYA), (glucose 20g / L, yeast extract 5 g / L), on which 0.1 ml of a mold spore suspension (10^7 spores per ml) was finally spread out. After incubation for up to 5 days, the plates were examined for a halo zone formation around the bacterial colonies. These experiments were performed in triplicate.

Antiaflatoxigenic activity assay

The ability of three individual *Lactobacillus* species to inhibit aflatoxin B_1 production by *A. flavus* and *A. parasiticus* NRRL2999 was investigated by the simultaneous antagonism assay as described by Munimbazi and Bullerman (1998). Hundred

ml portions of glucose yeast extract broth medium were sterilized at 121°C for 15 min. in 250 ml Erlenmeyer flasks. Each flask was inoculated with 1 ml of fungal spores suspension containing 10^7 spores/ ml, and 1 ml containing 10^7 Cells of bacterial culture grown at 30° C for 24h in MRS medium without acetate. All flasks were incubated at 28° C and analyzed for aflatoxin B_1 production after 10 and 20 days of incubation.

Determination of aflatoxin B_1:

At the end of the incubation period, aflatoxin B_1 was extracted by adding of 25 ml chloroform to each culture flask which was then shaken for 15 minutes on a wrist-action shaker. After phase separation the chloroform layer was removed and the extraction repeated with additional 25 ml chloroform. Combined extracts were dehydrated over granular anhydrous sodium sulfate and evaporated to dryness at 60° C in a water bath. Residues were dissolved in 1 ml of chloroform for analysis. Aflatoxin B_1 concentration was determined by enzyme-linked immunosorbent assay (ELISA) as described by Sekhon, et al., 1996.

Results and Discussion

Inhibition of fungal growth:

Results of the antimycotic activity test of three *L. plantarum* strains, as well as, one strain of each of *L. paracasei* subsp. *paracasei* and *L.rhamnosus* against five species of *Aspergillus* and *Penicillium* are summarized in Table (1). All the studied *Lactobacillus* strains exhibited various degrees of growth inhibition against Aspergillus species. All strains of *L. plantarum* showed an antimycotic activity against *A. versicolor* and *A. parasiticus* but not against *A. flavus*. *A.* versicolor showed the highest sensitivity toward *L* .rhamnosus and all of *L. plantarum* strains. This was followed by *A. parasiticus* which affected at lower extent by *L. plantarum* strains. Growth of *A. flavus* was slightly inhibited with the presence of *L. paracasei* subsp. *paracasei* and *L.rhamnosus* only. On the other hand, none of the studied *Lactobacillus* strains were found to inhibit growth of *P.roquefoti* or *P. commune.* The typical aspect of the inhibition halos is shown in Fig.1.

The inhibitory action of LAB have been demonstrated by several investigators (Corsetti *et al.*, 1998; Coloretti, et al., 2007). Lavermicocca, *et al.*, (2000) reported on the fungal inhibitory effects of LAB isolated from sour dough. They found that a mixture of organic acids to be synergistically responsible for the inhibitory effect of these bacteria. . All these identified substances are low molecular weight compounds, but there are also reports of unidentified proteinaceous compounds with broad antifungal activity (Gourama & Bullerman, 1995, 1997, Magnusson & Schnürer, 2001).

Table 1: Inhibitory effect of five strain of *Lactobacillus* on various species of *Aspergillus* and *Penicillium*.

	L. plantarum 12006	*L. plantarum* ATCC 4008	*L. plantarum* 299V	*L. paracasei* subsp. *Paracasei* LMG 13552	*L.rhamnosus* VT 1
A. parasiticus	+	++	++	-	-
A. flavus	-	-	-	+	+
A. versicolor	+	+++	+++	-	++
P.roquefoti	-	-	-	-	-
P. commune	-	-	-	-	-

+ Weak inhibition , ++ Moderate inhibition, +++ Strong Inhibition, - No inhibition.

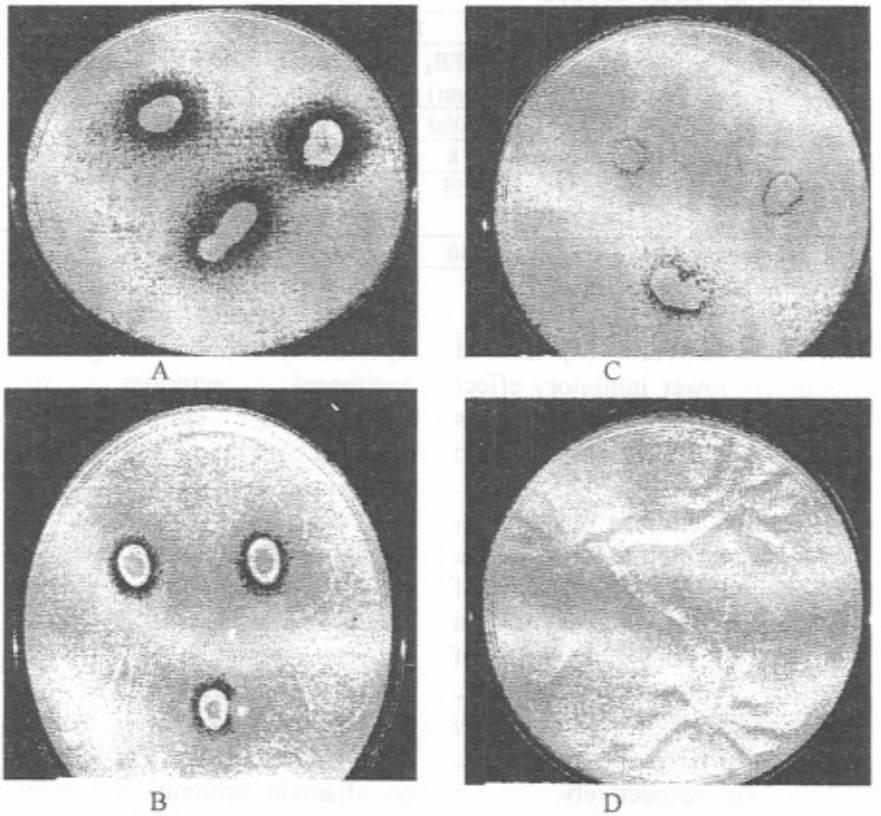

Figure 1: Antifungal effect of *L. plantarum* against: (A) *A. versicolor* " Strong inhibition". (B) *A. versicolor* " moderate inhibition". (C) *A. parasiticus* NRRL2999 "weak inhibition" (D) *P. roqueforti* "no inhibition".

Inhibition of aflatoxin B_1 production:-

Aflatoxin B_1 production was greatly affected by the presence of all the investigated *Lactobacillus* species. Data presented in table (2) indicated that the inhibitory effect on aflatoxin B_1 production by *A. parasiticus* NRRL2999 ranged from 98.8% in the presence of *L. paracasei* subsp.*paracasei* to 99.9% in the presence of *L. plantarum* or *L. rhamnosus* after 10 days of incubation. The toxin production was almost totally suppressed with extension of the incubation period to 20 days. The percentage of inhibition reached to 99.9 % when any of the tested bacterial culture was simultaneously grown with *A. parasiticus NRRL2999.*

Table 2: The inhibitory effect of *L. plantarum, L. paracasei* subsp. *paracasei* and *L. rhamnosus* on aflatoxin B_1 produced by *A. parasiticus* NRRL – 2999

Lactobacillus species	10 days		20 days	
	*AFB_1 (ppm)	Inhibition (%)	*AFB_1 (ppm)	Inhibition (%)
Control	70000	--	77500	--
L. plantarum 299V	28	99.96	20	99.98
L.paracasei subsp.*paracasei*LMG 13552	855	98.80	105	99.86
L. rhamnosus VTI	40	99.94	12	99.99

* AFB_1 = Aflatoxin B_1

Results in Table (3) showed relatively lower inhibitory effect of all the three lactobacillus species against the aflatoxigenic fungus *A. flavus*.

The production of aflatoxin B_1 by *A. flavus* was reduced by ca. 90, 85 and 92% after 10 days of incubation and by about 97, 96 and 98% after 20 days of incubation in the presence of *L. Plantarum* , *L. paracasei* subsp.*paracasei* and *L. rhamnosus*, respectively.

Similar results have been reported by other investigators. Antifungal activities by *Lactobacillus* species that inhibited both the growth and the aflatoxins production of *A. parasiticus* has been reported by Vanne, *et al.* (2000) and Onilud, *et al.* (2005). Also, Coallier-Ascah and Idziak (1985) found that the inoculation of *A. flavus* spores into a culture of *Streptococcus lactis* in synthetic broth medium resulted in little or no aflatoxin accumulation even though the growth of the fungus was not hindered.

Table 3: The inhibitory effect of *L. plantarum, L. paracasei* subsp. *Paracasei* and *L. rhamnosus* on aflatoxin B_1 produced by *A. flavus*.

Lactobacillus species	10 days		20 days	
	*AFB_1 (ppm)	Inhibition (%)	*AFB_1 (ppm)	Inhibition (%)
Control	10000	--	30000	--
L. plantarum 299V	1000	90.00	1000	96.77
L. paracasei subsp. *paracasei* LMG 13552	1500	85.00	1100	96.33
L. rhamnosus VT1	800	92.00	500	98.33

* AFB_1 = Aflatoxin B_1

In conclusion, results of the present study suggest that the use of LAB offers a potential alternative as a natural food-grade biocontrol agent of growth of cheese- contaminant molds and aflatoxin production in different food commodities. Further work is needed to identify and characterize the active metabolites involved in the inhibition of the toxigenic Aspergilli by LAB species.

References:

Cabo, M.L.; Braber, A.F. and Koenraad, P.M.F.J. 2002. Apparent antifungal activity of several Lactic acid bacteria against Penicillium discolor is due to acetic acid in the medium. J. Food Prot. 65: 1309-1316.

Coallier-Ascah J. and Idziak, E.S. 1985. Interaction between Streptococcus lactis and Aspergillus flavus on production of aflatoxin. Appl. Environ. Microbiol. 49: 163-167.

Coloretti, F.S.; Armaforte, E.; Chivari, E.; Grazia, L. and Zambonelli, C. 2007. Antifungal activity of Lactobacilli isolated from salami. FEMS Microbial Lett. 271: 245-250.

Corsetti, A.; Gobbetti, M.; Rossi, J. and Damiani, P. 1998. Antimold activity of sour dough Lactic acid bacteria: identification of a mixture of organic acids produced by Lactobacillus sanfrancisco CBI. Appl. Microbiol. Biotechnol. 50: 253-256.

Filtenborg, O.; Frisvad, J.C. and Tharni, U. 1996. Molds in food spoilage. Int. J. Food Microbiol. 33: 85 – 102.

Gourama, H. and Bullerman, L.B. 1995. Inhibition of growth and aflatoxin production of Aspergillus flavus by Lactobacillus species. . J. Food prot. 58:1249-1256.

Gourama, H. and Bullerman, L.B. 1997. Antimycotic and antiaflatoxigenic effect of lactic acid bacteria. J. Food Prot. 58: 1275-1280.

Karunaratne A., Wezenberg E. and Bullerman, L.B. 1990. Inhibition of mold growth and

aflatoxin production by Lactobacillus spp. J. Food Prot. 53: 230-236.

Klich, M. A. and Pitt, J.I. 1988. Differentiation of Aspergillus flavus from A. parasiticus and other closely-related species. Trans. Brit. Mycol. Soc. 91: 99-108.

Lavermicocca, P.; Valerio, F.; Evidente, A.; Lassaroni, S.; Corsetti, A. and Gobbetti M. 2000. Purification and characterization of novel antifungal compounds from the sour dough Lactobacillus plantarum strain 21 B. Appl. Environ. Microbiol. 66: 4084 – 4090.

Lindgren, S.E. and Dobrogosz, W. 1990. Antagonistic activities of lactic acid bacteria in feed and food fermentations. FEMS Microbiol. Rev. 87: 149-164.

Lund, F.; Filtenborg O. and Frisvad, J.C. 1995. Associated mycoflora of cheese. Food Microbiol. 12. 173-180.

Magnusson J. and Schnurer J. 2001. Lactobacillus coryniformis subsp. coryniformis strain si3 produces a broad-spectrum proteinaceous antifungal compound. Appl. Environ. Microbiol. 67: 1-5.

Munimbazi C. and Bullerman, L.B. 1998. Inhibition of aflatoxin production of A. parasiticus NRRL2999 by Bacillus pumilus. Mycopathologia 140: 163-169.

Nielson M.S.; Frisvad, J.C. and Nilson, P.V. 1998. Protection of fungal starters against growth and secondary metabolite production of fungal spoilers of cheese. Int. J. Food Microbiol.42: 92-99.

Onilude, A.A., Fagade, O.E.; Bello, M.M. and Fadahuns, I.F. 2005. Screening of the antifungal effects of Lactic Acid bacteria against toxigenic Penicillium and Aspergillus strains. In http://www.vtt./bel/2000micro biology/ antifungal attributes of lactic acid bacteria.

Scott, P.M. 1989. Mycotoxigenic fungal contaminants of cheese and other dairy products.p.193-259. In H.P. Van Edmond (ed.), Mycotoxin in Dairy products. Elsevier Applied Science. N.Y.

Sekhon, C.P.S. Jyotik; Kapur, A.; Sodhi, S.S. and Jand, S.K. 1996.Qualitative and quantitative detection of aflatoxin B1 in poultry sera by enzyme-linked immunosorbent assay J. Biosci., 21(4): 471-476.

Stiels, J. 1999. Antifungal activity of Lactobacilli. Ph. D. Thesis. Institute of Chemical Technology. Prague, Czech Republic.

Stollof, L. 1977. Aflatoxin – an overview. In: Rodrigues, J.V., Hesseltine,C. W. , Mehlman, M. (eds) Mycotoxins in Human and Animal Health. Park Forest Sout, IL. Pathotox Publishers. pp. 7-28.

Suzuki, I.; Momura. M. and Morichi. T. 1991. Isolation of Lactic acid bacteria suppress mold growth and show antifungal action. Milchwissenschaft 46: 635-639.

Vanne, L., Kleemola, T. and Haikara, A. 2000. Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels African Journal of Biotechnology 4 , 12 : 1404-1408

Zaki, N.; Shoukry, Y.M.R.; Kheadr, E.E. and El –Deep, S.A. (1992). Effect of sodium chloride and some lactic acid bacteria on aflatoxin production and growth rate of Aspergillus flavus. Egyptian J. Dairy Sci.20: 359-369.

تثبيط نمو الفطريات وإنتاج الأفلاتوكسين B_1 بواسطة بعض سلالات اللاكتوباسيلاس

نانيس حسنين جمعه[1] ، وفيق سند رجب[2] ، لويد بوليرمان[3]

1- قسم الألبان ، كلية الزراعة – جامعة أسيوط

2- قسم علوم وتكنولوجيا الأغذية – كلية الزراعة – جامعة أسيوط

3- قسم علوم وتكنولوجيا الأغذية_ جامعة نبراسكا - لينكولن - الولايات المتحدة الأمريكية

تناول البحث دراسة تأثير خمس سلالات من بكتريا جنس الـــ *Lactobacillus* وهى :
L. plantarum ATCC4008, *L. plantarum* 12006, *L. plantarum* 299V, *L. paracasei* subsp. *paracasei* LMG13552 and *L. rhamnosus* VT1
على نمو بعض الفطريات الشائع وجودها كملوثات للجبن وهى :
Aspergillus parasiticus NRRL2999, *A. flavus*, *A. versicolor*, *Penicillium roqueforti* and *P. communi*
كما تناول البحث أيضاً دراسة مدى قدرة السلالات البكتيرية
L. plantarum 299V, *L. paracasei* subsp. *paracasei* LMG13552 and *L. rhamnosus*VTI
على تثبيط إنتاج أفلاتوكسين B_1 بواسطة فطرى *A. parasiticus* NRRL 2999 and *A. flavus* .

وكانت النتائج كما يلى :

أظهرت جميع سلالات بكتريا الـــ *Lactobacillus* المستخدمة درجات متفاوتة من تثبيط النمو لأنواع معينة من الفطريات دون الأخرى .وقد أعطت سلالات *L. plantarum* الثلاث تأثيرا مثبطا قويا لنمو نوعى الفطر *A. versicolor* and *A. parasiticus* ، بينما لم يتأثر بها نمو فطر *A. flavus* . كان نمو الفطر *A. versicolor* هو الأعلى تأثرا بتلك البكتريا وكذلك ببكتريا النوع *L. rhamnosus* يليه فى ذلك فطر *A. parasiticus* . أما فطر *A. flavus* فقد تأثر نموه فقط وبدرجة ضعيفة فى وجود نوعى البكتريا *L. rhamnosus* and *L. paracasei* subsp. *paracasei* .

ومن ناحية أخرى ، فلم يحدث أى تأثير مثبط لجميع سلالات بكتريا حمض اللاكتيك المستخدمة على نمو نوعي جنس البنسليوم *P. roqueforti* and *P. communi* .

وفيما يتعلق بقدرة الأنواع الثلاثة لبكتريا *Lactobacillus* على تثبيط إنتاج أفلاتوكسين B_1 بواسطة فطرى *A. parasiticus* and *A. flavus* فقد حدث تثبيطاً شبه كاملاً (بنسبة 98.8 – 99.99%) لإنتاج التوكسين بواسطة فطر *A. parasiticus* ، فى حين انخفضت فقط كمية التوكسين المفرزة بواسطة فطر *A. flavus* بنسبة 85-92% وبنسبة 96.3-98.3% بعد عشرة وعشرين يوماً من التحضين على التوالى . كانت فعالية بكتريا *L. paracasei* subsp. *paracasei* فى تثبيط إنتاج الأفلاتوكسين أقل من فعالية كلاً من بكتريا *L. rhamnosus* and *L. plantarum* .