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Detoxification of Aflatoxin by *Streptococcus lactis* and Lactic Acid Bacteria in Commercial Yoghurt

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ABSTRACT

The inoculation of *S. lactis* in a mixed culture with *Aspergillus parasiticus* and into a 3-day-old *Aspergillus parasiticus* culture reduced aflatoxin accumulation from 108.33 to 94.18 and 31.01 µg/ml,respectively, after two days of incubation. When *S. lactis* was cultured for 3 days before the inoculation of *A. parasiticus* spores, it was found that *A. parasiticus* could produce only 58.01 µg/ml of aflatoxin on day 2. *S. lactis* was further examined for the ability to detoxify aflatoxin B1 in yoghurt in comparison with lactic acid bacteria from commercial yoghurt. The result showed that *S. lactis* could detoxify aflatoxin B1 from 50 to 33.70 µg/ml whereas bacteria from commercial yoghurt detoxified aflatoxin B1 from 50 to 37.25 µg/ml after 7 days of storage.

Key words : aflatoxin, detoxification, *Aspergillus parasiticus*, *Streptococcus lactis*

INTRODUCTION

Aflatoxins are secondary metabolites produced by some certain strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Aflatoxin B1 is shown to be the most potent hepatocarcinogen causing cholangiocarcinoma and hepatocellular carcinoma in the livers of many animal species including human (Chu, 1977). A variety of food and animal feed are often contaminated with aflatoxins. Frank (1966) found that condensed milk showed mould growth and aflatoxin production in experimental condition. Jordano *et al.* (1989) detected aflatoxin producing strains of *A. flavus* in 7 out of 20 samples of yoghurt whereas Mateos-Garcia and Suarez-Fernandez (1983) also found that 5 strains of *A. parasiticus* isolated from yoghurt were aflatoxin-producing fungi. *Aspergillus* strains isolated from contaminated cheese were shown to produce aflatoxinin culturemedia (Bullerman and Olivigni, 1974). Lie and Marth (1967) found that *A. flavus* and *A. parasiticus* could produce aflatoxin in cheddar cheese whereas Park and Bullerman (1983) reportedthat yoghurt and summer sausage were poor substrates for *A. parasiticus* but favourable substrates for *A. flavus* for aflatoxin production at 15 and 25°C.

Detoxification of aflatoxin contaminated food has been continuing challenge for the food industry. Aflatoxin production can be influenced by various factors including pH, temperature, available nutrients, A_w and competitive growth. Wiseman and Marth (1981) found the *Streptococcus lactis* (chesse starter) inhibited aflatoxin produced

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by *A. parasiticus*. Coallier-Ascah and Idziak (1985) also found that little or no aflatoxin accumulation was observed when *A. flavus* was grown in a broth culture of *S. lactis*. Megalla and Hafez (1982) demonstrated that aflatoxin B1 could be detoxified by acidogenous yoghurt.

This experiment was performed in order to study the interaction between *S. lactis* and *A. parasiticus* on production of aflatoxin in liquid medium and to compare the ability of *S. lactis* with lactic acid bacteria in commercial yoghurt for the detoxification of aflatoxin B1.

MATERIALS AND METHODS

Cultures and cultivation methods

Aspergillus parasiticus IMI 102566 obtained from International Mycological Institute, UK was grown on potato dextrose agar (PDA) slants for 7 days at 30°C. Stock culture of *Streptococcus lactis* TISTR 457 obtained from MIRCEN, Thailand was transferred to nutrient agar slants and incubated at 30°C for 48 h. Spores of *A. parasiticus* and cells of *S. lactis* were then collected in sterile distilled water containing 0.1% Tween 80. The total spore and cell counts of the suspension (10^8 per ml) were determined using an improved Neubrauer Haemacytometer.

Medium

Lablemco tryptone broth (LTB) was used in this study. The medium consisted of1% glucose, 1% yeast extract [Difco], 1% Lablemco beef extract [Oxoid], 1% tryptone[Difco], 0.5% NaCland 0.2% $NaHPO_4$ (Hurst, 1966). The pH of the medium was adjusted aseptically with HCl (5N) to 4.3 after sterilization.

Method

1) Aflatoxin production of *A. parasiticus*

100 ml of LTB medium were inoculated with spores of *A. parasiticus* and incubated for 7 days at room temperature on a Gallenkamp orbital shaker at 200 rpm.

2) Effects of *S. lactis* on aflatoxin production of *A. parasiticus*

In the first experiment, spores of *A. parasiticus* and cells of *S. lactis* were simultaneously inoculated into 100 mlof LTB medium. In the second experiment, *A. parasiticus* was cultivated in the medium for 3 days before the inoculation of *S. lactis*. In the last experiment, *S. lactis* was cultivated for 3 days before the inoculation of *A. parasiticus*. All cultures were then incubated at room temperature for 7 days on a Gallenkamp orbital shaker at 200 rpm.

3) Detoxification of aflatoxin by *S. lactis* and lactic acid bacteria in yoghurt.

Yoghurt was prepared by mixing 100 ml of fresh milk with 5 g of skimmed milk powder and heated at 80-90°C for 15 min before cooling at 48° C. Either *S. lactis* or lactic acid bacteria from commercial yoghurt was then added into the milk. Standard aflatoxin B1 with the concentration of 50 µg/ml was also added. Two batches of milk were then incubated at 45°C for 2-5 h and yoghurts with optimum acidity were obtained. Yoghurts were then stored at 5-10°C and taken for further analysis on day 1, 2 and 7 respectively.

Determination of pH and acidity

Liquid cultures were determined for pH values by pH meter whereas acidity of yoghurt was examined by titration with 0.1 N NaOH and percentage of lactic acid was calculated.

Aflatoxin extraction

1) Extraction from liquid medium

Cultures were filtered and filtrates were extracted for aflatoxin analysis by the modified method of Shih and Marth (1974). 20ml of filtrates was extracted with 20 ml of chloroform in a

separatory funnel. The chloroform layer was then removed and the sample was washed twice with 100 ml of chloroform. The chloroform extracts were pooled together and evaporated to dryness and kept in a small vial for further determination.

2) Extraction from yoghurt

The sample was extracted for aflatoxin by modification from Sep Pak method (Matsubara, 1985). 25g of sample was extracted with 150 ml of chloroform and 25 ml of distilled water in a shaker at 250 rpm for 30 min. The extract was then filtered through Whatman no. 1 filter paper with the aid of Celite and the chloroform layer was separated from the filtrate. The filtrate was washed twice with 50 ml of chloroform and the chloroform extracts were pooled together and evaporated to dryness. The extract was dissolved with 10 ml of chloroform-hexane (3:7) and then added to the Sep Pak silica gel cartridge column. The column was drained and eluted with 10 ml of hexane followed by 10 ml of benzene- acetic acid (95.5:4.5) and 10 ml of ethyl ether-hexane (60:40). The eluate was discarded and aflatoxin was eluted with 15 ml of methylene chloride-acetone (9:1), evaporated to dryness and kept for further analysis.

Aflatoxin quantification

The aflatoxin extracts were dissolved with 1 ml of methanol, filtered through 0.45 µm membrane filter (Millipore) and 10 µl were injected to the HPLC (Shimadzu) using the following condition : flow rate of 1 ml/min, mobile phase solvent (methanol: water: acetic acid = 2:1:1), reverse phase column C18 and UV spectrophotometric detector at 365 nm. Aflatoxin concentrations were read against standard curve.

Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA) and Least Significant Difference (LSD) to indicate the effect of *S. lactis* on aflatoxin production at significant level of 0.05. The statistical methods used were those described by Montgomery (1991). All experiments were done in triplicate.

RESULTS AND DISCUSSION

Effect of *S. lactis* on aflatoxin production

The production of aflatoxin by *A. parasiticus* in LTB medium during 7 days of incubation was shown in Table 1. The result showed that the highest yield of total aflatoxin was produced on day 2 with the amount of 108.33 µg/ml. Table 2 showed the production of aflatoxin when *A. parasiticus* was grown in a mixed culture with *S. lactis*. The total production of aflatoxin was reduced from 108.33 to 94.18 µg/ml on day 2. When *A. parasiticus* was cultivated for 3 days before the addition of *S. lactis*, it was found that the total aflatoxin production was even further reduced from 108.33 to 31.01 µg/ml on day 2 (Table 3). Total aflatoxin production of *A. parasiticus* in a 3-day-old culture of *S. lactis* was also reduced from 108.33 to 58.01 µg/ml (Table 4).

Comparison of total aflatoxin production of *A. parasiticus* in various conditions with *S. lactis* was also presented in Figure1. It could be concluded that in the presence of *S. lactis*, the production of total aflatoxin B1+G1 of *A. parasiticus* was reduced in all treatments. The addition of *S. lactis* into a 3-day-old culture of *A. parasiticus* was shown to be the best condition for detoxifying aflatoxins.

The difference between the control and the treatments were found to be significant. However, the difference in the amounts of aflatoxin B1 on day 5 and aflatoxin G1 on day 4 between the control and the mixed culture was found to be statistically insignificant. In addition, the amounts of aflatoxin G1 on day 1 comparing between the control and *A. parasiticus* plus a 3-day-old *S. lactis* culture were also insignificant.

Table 1 Aflatoxin production of *Aspergillus parasiticus* in liquid medium.

Day	pH of medium	Aflatoxin (µg/ml)		
		B1	G1	Total B1 + G1
1	4.55	11.67	87.04	98.71
2	6.33	18.93	89.40	108.33
3	8.17	17.41	85.96	103.37
4	8.26	16.39	49.30	65.69
5	8.45	12.82	35.01	47.83
6	8.73	22.57	33.55	56.12
7	8.80	8.73	34.18	42.91

Table 2 Aflatoxin production of *Aspergillus parasiticus* in a mixed culture with *Streptococcus lactis*.

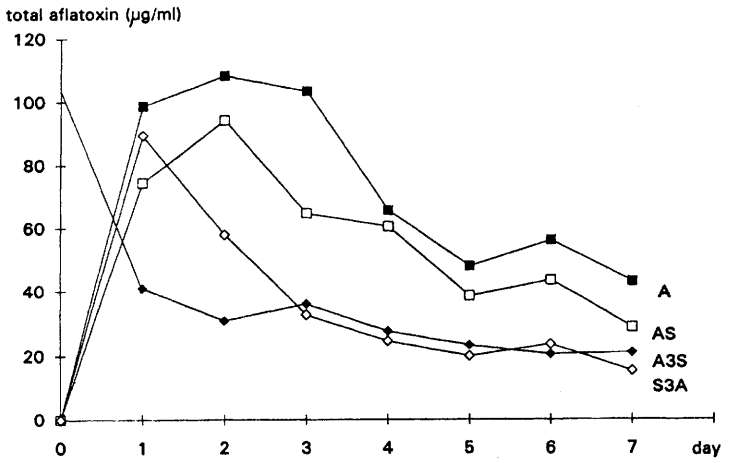
Day	pH of medium	Aflatoxin (µg/ml)		
		B1	G1	Total B1 + G1
1	4.44	9.55	64.80	74.35
2	6.10	14.49	79.69	94.18
3	7.98	12.84	51.92	64.76
4	8.09	14.02	46.45	60.47
5	8.43	11.68	26.74	38.42
6	8.49	19.09	24.28	43.37
7	8.80	5.79	22.90	28.69

Table 3 Aflatoxin production of *Aspergillus parasiticus* when cultured for 3 days before the inoculation of *Streptococcus lactis*.

Day	pH of medium	Aflatoxin (µg/ml)		
		B1	G1	Total B1 + G1
0	8.17	17.41	85.96	103.37
1	8.35	9.52	31.51	41.03
2	8.33	4.68	26.33	31.01
3	8.35	6.97	29.07	36.04
4	8.35	6.15	21.47	37.62
5	8.34	4.92	18.29	23.21
6	8.37	4.32	16.08	20.40
7	8.36	4.69	16.21	20.90

Table 4 Aflatoxin production of *Aspergillus parasiticus* in a 3-day-old culture of *Streptococcus lactis*.

Day	pH of medium	Aflatoxin (μg/ml)		
		B1	G1	Total B1 + G1
0	4.43	0	0	0
1	4.43	8.50	80.87	89.37
2	3.38	8.82	49.19	58.01
3	7.24	6.14	26.59	32.73
4	7.63	4.14	20.36	24.50
5	7.96	3.62	16.26	19.88
6	8.03	3.73	19.75	23.48
7	8.15	3.48	11.76	15.24



A = culture of *A. parasiticus*
AS = mixed culture of *A. parasiticus* and *S. lactis*
A3S = *S. lactis* in a 3-day-old culture of *A. parasiticus*
S3A = *A. parasiticus* in a 3-day-old culture of *S. lactis*

Figure 1 Production of total aflatoxin B1+G1 in LTB medium.

Various investigators reported that a number of microorganisms affected the production of aflatoxin in a competitive environment. Gourama and Buller-man (1995) reported that a mixture of *Lactobacillus* species reduced mould growth and inhibited aflatoxin production by *A. flavus* subsp. *parasiticus*. Coallier-Ascah and Idziak (1985) found that the inoculation of *A. flavus* into a culture of *S. lactis* in LTB medium resulted in little or no aflatoxin accumulation even though the growth of *A. flavus* was not hindered.

According to Wiseman and Marth (1981) and El-Gazzar and colleagues (1987), a potential inhibitory effect on aflatoxin production was lactic

Table 5 Detoxification of aflatoxin B1 in yoghurt.

	S. lactis			Commercial lactic acid bacteria		
Storage day	Aflatoxin (ug/ml)	Detoxification (%)	Acidity (%)	Aflatoxin (ug/ml)	Detoxification (%)	Acidity (%)
0	50	-	0.70	50	-	0.70
1	44	12	0.98	46.85	6.3	0.95
2	39.65	20.7	1.16	43.25	13.5	1.08
7	33.70	32.6	1.5	37.25	25.5	1.4

acid. This suggestion was discounted by Coallier-Ascah and Idziak (1985), when they cultivated *A. flavus* in LTB supplemented with lactic acid at pH 4.3 and found that aflatoxin produced in the medium was similar to the control (LTB without lactid acid). Buchanan and Ayres (1975) reported that lactate enhanced the production of more aflatoxin when added to synthetic and semisynthetic media containing glucose.

Nisin, a product of *S. lactis* might be another potential inhibitor (Coallier- Ascah and Idziak, 1985). However, Yousef *et al.* (1980) reported that 200 to 5000 units of nisin per ml partially delayed the growth of *A. parasiticus* but had no effect on aflatoxin production.

Another possibility was proposed by other investigator (Ciegler *et al.*,1966; Masimango *et al.*,1978) in which aflatoxins were adsorbed to the bacterial cell wall as the mechanism of aflatoxin degradation by lactic acid bacteria. Coallier-Ascahand Idziak (1985) also explained that *S. lactis* in someway, might interfere with the synthesis of aflatoxin. Furthermore, it is generally known that lower pH favours the production of aflatoxin (Cotty, 1988; Gourama and Bullerman, 1995) whereas in the present experiment, pH of the medium was raised from 4.3 to around 8 and the content of aflatoxin B1 and G1 was also reduced (Tables 1-4) when *S. lactis* was grown with *A. parasiticus* in all conditions.

Detoxification of aflatoxin B1 in yoghurt

When *S. lactis* was used to detoxify aflatoxin B1 during yoghurt preparation in comparison with commercial lactic acid bacteria fermentation, the result showed that *S. lactis* could detoxify more aflatoxin B1 than commercial lactic acid bacteria (Table 5). The statitical analysis also showed that there was a significant difference in aflatoxin detoxification between *S. lactis* and commercial lactic acid bacteria. Megalla and Hafez (1982) revealed that aflatoxin B1 added to yoghurt was completely transformed to a new fluorescing compound corresponding to aflatoxin B2a. The new compound has been referred to as hydroxydihydro-aflatoxin B1. Ciegler and Peterson (1968) found that this compound caused no bile duct hyperplasia and no deaths by duckling test.

In conclusion, *S. lactis* could reduce the amounts of aflatoxin produced by *A. parasiticus* under the conditions of this experiment and aflatoxin B1 present in the yoghurt could also be detoxified by *S. lactis* even though the level of detoxification was not satisfactory.

LITERATURE CITED

Buchanan, R.L. and J. C. Ayres. 1975. Effect of

initial pH on aflatoxin production. App. Microbiol. 30: 1050-1051.

Bullerman, L. B. and F. J. Olivigni. 1974. Mycotoxin producing potential of moulds isolated from cheddar cheese. J. Food Sci. 39:1166-1168.

Chu, F.S. 1977. Mode of action of mycotoxins and related compounds. Adv. Appl. Env. Microbiol. 49:163-167.

Ciegler, A. and R. E. Peterson.1968. Aflatoxin detoxification: hydroxydihydro- aflatoxin B1. App. Microbiol. 16:665-666.

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

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

Cotty, P. J. 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*: influence of pH. Phytopathol. 78: 1250-1253.

El-Gazzar, F. E.,G. Rusul, and E. H. Marth. 1987. Growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999 in the presence of lactic acid and different initial pH values. J. Food Prot. 50: 940-944.

Frank, H. K. 1966. Aflatoxine in Lebensmittelen. Arch. Lebensmittelhyg. 17: 237-242.

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

Jordano, R., M. Jodral, P. Martinez, J. Salmeron, and R. Pozo. 1989. Aflatoxin-producing strains of *Aspergillus flavus* in yogurt. J. Food Prot. 52:823-824.

Lie, J.L. and E. H. Marth. 1967. Formation of aflatoxin in cheddar cheese by *Aspergillus flavus* and *Aspergillus parasiticus*. J. Dairy Sci. 50:1708- 1710.

Masimango, N., J. Remacle, and J. L. Ramant. 1978. The role of adsorption in the elimination of aflatoxin B1 from contaminated media. Eur. J. App. Microbiol. Biotechnol. 6: 101-105.

Mateos-Garcia, A. and G. Suarez-Fernandez. 1983. Micoflora contaminante del yogur:aislamiento, comprobacion de toxigenicidal e inoculacion de especies aflatoxinogenicas. Alimentaria. 48: 39-40.

Matsubara, I. 1985. Some remarks on aflatoxin problems, pp. 1-14. *In* Seminar on Aflatoxin, Export Training Center, Department of Commercial Relations, Ministry of Commerce, Bangkok, March 8, 1985.

Megalla, S.E. and A. H. Hafez. 1982. Detoxification of aflatoxin B1 by acidogenous yoghurt. Mycopathol. 77:89-91.

Montgomery, D.C. 1991. Design and Analysis of Experiments. John Wiley and Sons, Inc., New York. 649 p.

Park, K. and L. B. Bullerman. 1983. Effects of substrate and temperature on aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus*. J. Food Prot. 46:178-184.

Shih,C.N.and E. H. Marth. 1974. Aflatoxin formation, lipid synthesis and glucose metabolism by *Aspergillus parasiticus* during incubation with and without agitation. Biochim. Biophys. ACTA 338:286-296.

Wiseman, D.W. and E. H. Marth. 1981. Growth and aflatoxin production by *Aspergillus parasiticus* when in the presence of *Streptococcus lactis*. Mycopathol. 73:49-56.

Yousef, A.E., S. M. El-Gendy, and E. H. Marth.1980. Growth and biosynthesis of aflatoxin by *Aspergillus parasiticus* in cultures containing nisin. Z. Lebensm. Unters. Forsch. 171: 341-343.