AFLATOXIN PRODUCTION IN A MEAT MIX MODEL SYSTEM IN THE PRESENCE OF *PEDIOCOCCUS* AND *LACTOBACILLUS*: LEVELS OF GLUCOSE NECESSARY TO INDUCE PRODUCTION.

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SUMMARY

Aflatoxin production has been related to the presence of readily utilizable sugars. This study was conducted in an axenic cultural system in APT (modified) and LTB medium. Irradiated meat was also employed in the formulation of a meat medium. The medium composition and incubation temperatures were sumulations of Brazilian salami processing conditions. Eight individual strains of *Pediococcus* and *Lactobacillus* were used. Toxin and resudual sugars were determined by ELISA and HPLC, respectively.

All single cultures of *Aspergillus parasiticus* supported aflatoxin production. Aflatoxin was not detected when *A. parasiticus* was grown with lactic acid bacteria, altough visible mould growth was observed in all such cultures. There was a correlation between the levels of toxin produced and the concentration of glucose to which the mould had been exposed in a pre-incubation period. The combination of lower levels of sugar, low temperature of incubation and the presence of an active competitor, probably had an effect on the levels of sugar that were taken up by mould, thus compromising aflatoxin production.

INTRODUCTION

Salami is a fermented dry sausage made from coarsely ground pork and beef, combined in variable proportions with salt, sugar, spices and nitrate and / or nitrite. Recent practices of production have introduced the use of starter cultures, usually lactic acid bacteria. Mold growth develops during the salami ripening. Since a number of investigators have reported findings of toxigenic strains, the potential risk of aflatoxin being produced may be present.

The preservation of a great number of important fermented foods such as salami is due mainly to the consumption of carbohydrates with formation of lactic acid as a main product. This fact prompt us to study the effect of glucose concentration on aflatoxin production. Aflatoxin production has been conditioned to the presence of a readily metabolizable sugar source. ABDOLLAHI AND BUCHANAN (1981) demonstrated that *A. parasiticus* does not produce aflatoxins when cultured on a peptone mineral salts medium, but will commence synthesis when transferred to a glucose mineral salts medium. They further demonstrated that the proacess could be blocked if either a protein synthesis or a RNA synthesis inhibitor were incorporated into the glucose mineral salts medium. The authors concluded that the sugar acts not only as a metabolizable carbon source, but also as an inducer of aflatoxin biosynthesis.

The objectives of the study were: (a) To determine whether aflatoxin can be produced in a meat mix model system inoculated with *A. parasiticus* and under incubation temperatures simulating Brazilian salami processing conditions. (b) To determine the effect of medium composition and pH on the minimal concentration of glucose needed to induce aflatoxin production in *A. parasiticus*.

MATERIALS AND METHODS

Microorganisms.

Mold. *Aspergillus parasiticus* CBS 921.70 was obtained from Centraalbureau voor Schimmelcultures (Delft, Netherlands). An inoculum was prepared as described previously (LUCHESE AND HARRIGAN, 1990).

Lactic acid bacteria (LAB). These bacteria were obtained from National Collection of Food Bacteria (Shinfield, Reading UK) and from commercial source (Chr. Hansen's Lab.). Working cultures were prepared by subculturing three times in APT broth.

Salami based meat medium.

The liquid medium, which was devised by one of the authors (Martins), was prepared using irradiated lean pork and beef, sucrose, glucose, salts, phosphates, manganese and water and simulated salami mix in pH, water activity and buffer capacity.

Media for determining the glucose effect on aflatoxin production.

The media employed on the determination of minimal levels of glucose needed to induce aflatoxin production were Peptone mineral salts medium (PMS) which was prepared as described previously (BUCHANAN AND LEWIS, 1984) and was used for the initial culturing of *A. parasiticus*. The replacement media were Lab-Lemco Tryptone broth (LTB) (HURST, 1966) and a modified All Purpose Tween Broth (APT). Modified APT broth was prepared following Difco commercial formulae, except that glucose was omitted and yeast extract was substituted by Lab-Lemco beef extract (Oxoid) for the reason that the first was shown to contain some small residues of glucose. Filter sterilized glucose solutions were used for supplementation of the replacement media to final concentrations of 0, 5, 10 and 100 mM.

The experimental designs.

Competitive studies in the meat system: The freshly prepared salami based meat medium suspension was distributed into conical flasks and inoculated with lactic acid bacteria. The flasks were incubated standing for 24 h at 20 °C, followed by another 24 h at 25 °C. At the end of the third day, the flasks were transferred to a 15 °C incubator.

At the fifth day a spore suspension of *A. parasiticus* was inoculated into each flask to a final concentration of 5×10^3 spores ml^{-1}.

All flasks were incubated for a further 30 d at 15 °C with continuous shaking at 60 cycles per minute.

Studies on glucose effect: The technique employed was a modification of the sequential culturing described by ABDOLLAHI AND BUCHANAN (1981) which allows the separation of glucose action as both inducer and substrate for synthesis.

Analysis

Aflatoxin: Aflatoxin was quantified by enzyme immunoassay using Total aflatoxin assay kit (Biokits, Deeside, Clwyd, UK).

Some positive and negative ELISA filtrates were subjected to a confirmatory thin layer chromatography (TLC) anlysis. The presence of aflatoxins were detected by their fluorescènce under long-wave UV light.

Residual carbohydrates. The glucose and sucrose consumption by single cultures of lactic acid bacteria at the 5th d (moment of mold inoculation) and after 15 d, were determined by high-performance liquid chromatography (HPLC).

RESULTS AND DISCUSSION

The results shown on Tables 1 to 3 represents the mean of duplicate determination, and each toxin determination was obtained as a mean value of the readings from duplicate wells.

All samples of single cultures of *A. parasiticus* supported aflatoxin production (Table 1). More aflatoxin was produced in flasks treated by the addition of lactic acid than in non treated ones. Enough lactic acid was added to obtain a pH of 5.0 which is that normally found in salami after fermentation. It is not known how LAB causes a total suppression of aflatoxin production by *A. parasiticus*. However, since all LAB including different species were capable of eliciting the same response, this suggests that the effect was due to failure of *A. parasiticus* in establishing effective competition for the nutrients necessary for aflatoxin production, when growing at a low temperature,

TABLE 1: Aflatoxin content and pH changes in a meat mix medium in the presence or absence of lactic acid bacteria.

Treatment	5 day* pH	35 day pH	35 day Aflatoxin (ng/ml)
Lactic acid	5.00	6.63	150
Not treated**	5.90	6.62	30
P. pentosaceus NCFB 1220	4.50	6.35	nd
P. pentosaceus NCFB 559	4.47	6.50	nd
P. pentosaceus CH	4.28	6.28	nd
L. plantarum CH	4.05	5.35	nd
L. alimentarius CH 33	4.10	5.92	nd
L. sake CH 10	5.06	6.41	nd
L. sake CH 17	5.09	5.85	nd
P. pentosaceus CH (control)	4.28	4.31	nd

* moment of *A. parasiticus* inoculation at a level of 5×10^3 spores/ml.
** not inoculated with lactic acid bacteria and not treated with lactic acid
control - not inoculated with *A. parasiticus*

TABLE 2: Residual sucrose (mmol/l) left in the meat medium by individual strains of lactic acid bacteria at 5 th day (moment of mould inoculation) and after growing as single culture.

CULTURE	DAYS 0	5	15
P. pentosaceus NCFB 1220	47.5	26.6	22.2
P. pentosaceus NCFB 559	48.5	33.8	23
P. pentosaceus CH	48	29.4	19.6
L. plantarum CH	48.5	18.9	3.3
L. alimentarius CH 33	48.6	32.2	13.9
L. sake CH 10	48.6	37.1	28.6
L.sake CH 17	49.4	41	31.6

nd - not detected

rather than production of antiaflatoxigenic compounds by LAB.

One important nutrient for aflatoxin production for which the mold will have to compete with LAB is carbohydrate. Previous studies have established that a readily metabolizable sugar source is necessary not only to serve as carbon source for aflatoxin production, but also is involved in the induction of aflatoxin synthesis (ABDOLLAHI AND BUCHANAN, 1981).

In the present investigation we employed the technique described by ABDOLLAHI AND BUCHANAN (1981) but using two different replacement media supplemented with various glucose concentrations. The levels of aflatoxins were directly correlated with the glucose content to which the mycelia were exposed during the pre-incunbation period (Table 3).

However, this study also revealed that glicose alone is not a risk determinat. When pre-grown mycelia were transferred to LTB media, glucose was not a limiting factor for the synthesis of aflatoxin.. Aflatoxin was detected even in the absence of glucose (Table 3), and was dependent on the pH of the media rather than the presence of glucose.

In contrast to what happened in LTB medium, aflatoxin was not detected when mycelia were pre-incubated in modified APT containing <10 mM glucose either after 20 h pre-incubation or after 48 h post-transference to medium containing 100 mM glucose and cycloheximide (Table 3).

Although yeast extract was shown to contain trace amounts of glucose, it is unlikely that the levels of aflatoxin detected in LTB medium without addition of glucose were due to such a low glucose content as it is far less than the minimum amount required in mAPT medium which does not contain yeast extract. On the other hand, yeast extract is a reach source of zinc and of vitamin B-complex, components whose importance has been well demonstrated.

TABLE 3: Aflatoxin production by mycelia of *A. parasiticus preincubated for 20 h in a modified APT or LTB media (pH 6.9 and pH 4.2) supplemented with different levels of glucose and then transferred to modified APT or LTB media containing 100 mM glucose and 200 µg/ml of cycloheximide.*

PREINCUBATION MEDIUM			POST-TRANSFER INCUBATION MEDIUM CONTAINING 100 mM GLUCOSE AND 200 µg/ml CYCLOHEXIMIDE		
Medium Initial pH	pH after 20 h	Aflatoxin (ng/ml)	Medium Initial pH	pH after 48 h	Aflatoxin (ng/ml)
GLUCOSE	0 mM				
LTB 4.2	4.76	100	LTB 4.2	4.49	>100
LTB 6.9	7.40	nd	LTB 6.9	7.10	nd
mAPT 4.2	5.07	nd	mAPT 4.2	4.65	nd
mAPT 6.9	7.64	nd	mAPT 6.9	7.42	nd
GLUCOSE	5 mM				
LTB 4.2	4.62	100	LTB 4.2	4.45	190
LTB 6.9	7.19	nd	LTB 6.9	7.05	nd
mAPT 4.2	4.72	nd	mAPT 4.2	4.51	nd
mAPT 6.9	7.34	nd	mAPT 6.9	8.25	nd
GLUCOSE	10 mM				
LTB 4.2	4.40	30	LTB 4.2	4.37	500
LTB 6.9	7.29	3.5	LTB 6.9	6.85	12
mAPT 4.2	4.55	nd	mAPT 4.2	4.46	nd
mAPT 6.9	7.01	nd	mAPT 6.9	8.21	nd
GLUCOSE	100 mM				
LTB 4.2	4.30	>100	LTB 4.2	4.40	>500
LTB 6.9	6.20	5.0	LTB 6.9	6.40	150
mAPT 4.2	4.22	100	mAPT 4.2	4.39	125
mAPT 6.9	6.22	95	mAPT 6.9	6.53	40

nd - not detected

The different glucose requirements obtained here with different media clearly indicate that induction of aflatoxin synthesis is not related to just one factor, but an interrelation of factors.

On the other hand, the total sugar consumption (sucrose + glucose) in the meat system, by lactic acid bacteria, at 5 d (moment of mold inoculation) was in the range of 17 to 62 % of the initial content (Table 2). Even after 15 days total depletion of both sugars was not observed. *L. alimentarius* CH and *L. plantarum* CH were the most consuming strains. In any case, however, sugar exhaustion was not alone a risk determinant. However, the combination of lower levels of sugar, low temperature of incubation and the presence of an active competitor, probably had an effect on the levels of sugar that were taken up by mold, thus compromising aflatoxin production.

The findings with replacement cultures in LTB medium thus confirm those obtained with conidia initiated cultures in the meat system. Sugar alone does not determine the presence of aflatoxin, but an integration of factors such as temperature, competitive microflora, other nutrient components (nitrogen, vitamins and trace metals) and pH which will probably interfiere with the solubility and/or transport of nutrients necessary for aflatoxin synthesis.

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