Studies on Aflatoxins in Milk and Milk Products
MATERIALS AND METHODS

Examination of milk and milk products for presence of aflatoxins:

Collection of samples:

One hundred and forty eight raw milk samples were examined. Out of these, 86 were from the Institute's Experimental Dairy, 36 were from individual animals in the Cattle Yard and the remaining 26 were from the local market.

Among the 47 khoa samples examined, 18 were from the Institute's Experimental Dairy and the remaining 29 were collected from the local halwai shops. Ten samples of dahi collected from local halwai shops were also examined.

In addition to raw milk, khoa and dahi, 10 samples of dried milk, 3 of sweetened condensed milk and 17 of Cheddar cheese, all collected from the Institute's Experimental Dairy, were included in this study.

Isolation and identification of mold cultures:

Mold cultures were isolated from samples of raw milk, dahi, khoa, milk powder, cheese, cattle feed and soil. Some cultures isolated as laboratory contaminants were also screened for aflatoxin production.
Ten fold dilution of each sample was plated on Potato Dextrose Agar (IS - 3507 - 1960) having the following composition:

Potato extract (from 200 grams of peeled potatoes) 1000 ml
Dextrose 20 g
Agar 15 g

The medium was autoclaved at 121°C for 15 minutes.

Just before plating, the molten medium was adjusted to pH 3.5 using 10% tartaric acid. The plates were incubated at 28°C for 72 hours. Individual isolates were streaked on potato dextrose agar slants, and after growth, the slants were stored in the refrigerator. Subcultures were done at monthly intervals.

The mold cultures were identified according to Smith (1960). Czapek Dox Agar of the following composition was used for the purpose.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>2.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>AgNO₃·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>30.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 g</td>
</tr>
</tbody>
</table>

The medium was autoclaved at 121°C for 15 minutes.
Extraction of aflatoxin from milk:

Aflatoxin was extracted from milk according to the method of Jacobson et al. (1971) but modified later by McKinney (1972). Seventy-five milliliters of raw milk were thoroughly blended with 300 ml methanol for 2 to 3 minutes in a Waring blender. Twenty grams of Celite were added and the mixture was again blended for 30 seconds and filtered through Celite 545 under vacuum. The precipitated protein and the Celite bed were then pressed and washed with 100 ml methanol. The pooled filtrate and washings were transferred to a separating funnel and the concentration of methanol was adjusted to 50% by addition of appropriate quantity of 4% sodium chloride solution. The methanolic extract was extracted twice with 100 ml hexane, followed by extraction with 100 ml chloroform so as to extract out the aflatoxin $M_1$ completely. The combined chloroform extracts were washed with 4% sodium chloride solution and passed through a column of anhydrous granular sodium sulphate to remove the residues of moisture in the chloroform extract. The latter was then evaporated on a steam bath to dryness. The dried material was quantitatively transferred to a small glass vial and again dried. The dried residue was dissolved in an appropriate quantity of chloroform and used for analysis by thin layer chromatography (TLC).
Extraction of aflatoxin from milk powder

The method of Masri et al. (1968) was used for extraction of aflatoxin from dried milk. Ten grams of milk powder were defatted with petroleum ether, filtered and dried. The sample was then extracted with 80% methanol using a Waring blender. After filtration, the filtrate was extracted twice with chloroform. The remaining steps of the procedure were same as that followed for milk.

Extraction of aflatoxin from khoa and cheese:

The method described by Shih and Marth (1971) was followed for extraction of aflatoxin from cheese and khoa.

One hundred gram sample of cheese or khoa was minced and blended for several minutes in a Waring blender, using a mixture of chloroform-methanol-water (100:200:80 v/v/v). One hundred milliliters each of chloroform and distilled water were added to the mixture and blended again for one minute. The mixture was then filtered through a Buchner funnel (Celite was added as filter aid) to remove the residue. The filtrate was transferred to a separating funnel. The chloroform layer which contained the aflatoxins, was separated. In order to recover all the aflatoxin in the sample, the blender was again rinsed with chloroform. The washings were
filtered to remove any particulate matter. The methanol-water fraction was separated in a separating funnel by adding sufficient quantity of chloroform. The pooled chloroform extracts were passed through a column containing 5 g sodium sulphate to remove traces of moisture. The sodium sulphate column was rinsed with chloroform to recover traces of aflatoxin.

The pooled chloroform extracts were evaporated to dryness on a steam bath. The concentrated residue was extracted with a mixture of methanol-water-hexane (50:45:129 v/v/v). The resulting mixture was transferred to a separating funnel and the lower layer of methanol-water was recovered and extracted twice with 100 ml chloroform to recover the aflatoxin. The hexane layer was also extracted once with methanol water (50:45 v/v). The methanol-water layer was again extracted twice with chloroform to recover traces of aflatoxin. The chloroform extracts were pooled and passed through a column of sodium sulphate. The column was washed with extra quantity of chloroform. The chloroform extracts were then evaporated to dryness. The residue was dissolved in an appropriate volume of chloroform and used for analysis by HPLC.

**Extraction of aflatoxin from dairy and sweetened condensed milk**

The procedure followed for the extraction of
aflatoxin from milk and sweetened condensed milk was the same as that for milk.

**Examination of mold isolates for aflatoxin production:**

All the mold cultures used in this study were grown on potato dextrose agar slants for a period of 1 to 3 weeks. Spore suspensions were prepared by adding 3 ml of 0.005% Triton x 100 to the agar slant cultures.

**Medium:**

The medium used for aflatoxin production had the following composition (Davis et al., 1966).

- Sucrose: 200 g
- Yeast extract: 20 g
- Distilled water: 1000 ml

The medium was dispensed in 100 ml aliquots in 500 ml Erlenmeyer flasks and autoclaved at 121°C for 15 minutes.

**Incubation:**

The medium was inoculated with the spore suspension prepared from 1 to 3 week old mold culture. The culture flasks were incubated for 6 to 8 days at 28°C.

**Extraction of aflatoxin from culture medium:**

At the end of incubation period, the contents of
the culture flask were steamed for 10 minutes to kill the organism and then filtered. The filtrate was used for extraction of aflatoxin. Appropriate aliquots of the filtrate were extracted thrice with chloroform in a separating funnel. The lower chloroform layer was separated and passed through a column filled with sodium sulphate to remove the residual moisture. The chloroform extracts were pooled and evaporated to dryness. The residue was dissolved in a suitable quantity of chloroform and used for TLC analysis.

Detection of aflatoxin producing molds by agar plate method:

Mold isolates were also screened by the method described by Bera et al (1974) for aflatoxin production.

Medium:

The composition of medium is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>10.0 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Fe₂(SO₄)₃·7H₂O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30.0 g</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>5x10⁻³ M</td>
</tr>
<tr>
<td>Corn steep liquor</td>
<td>0.5 g</td>
</tr>
</tbody>
</table>
Agar 15.0 g
Distilled water 1000 ml

pH was adjusted to 5.5 with 1 N NaOH before addition of agar. The medium was autoclaved at 121°C for 15 minutes.

**Cultivation of mold strains:**

The test mold cultures were inoculated at the centre of solidified agar medium on petri plates and incubated at 28°C in the dark for 6 to 10 days.

**Observation of fluorescence:**

The petri plates were examined under ultraviolet light from 6th up to 10th day of incubation for presence or absence of fluorescence in the agar surrounding the mold colonies. The intensity of fluorescence was found to be proportionate to the amount of toxin produced by the mold (Plate 1).

**Determination and confirmation of aflatoxin production by mold cultures:**

Thirty grams of agar from the portion exhibiting fluorescence, were blended with 75 ml distilled water for 5 minutes. The aqueous slurry was again mixed with suitable quantities of chloroform. After centrifugation, the chloroform layer was decanted and retained. The process was repeated by extracting the
Plate 1

A. Aflatoxin negative
Aspergillus flavus
colony showing no
fluorescence under uv.

B. Aflatoxin positive
Aspergillus flavus
colony showing
fluorescence under uv.
slurry with chloroform. The chloroform fractions were pooled, passed through the sodium sulphate column and concentrated to dryness. The residue was dissolved in a suitable quantity of chloroform and used for TLC analysis.

Production of aflatoxin by a standard culture:

*Aspergillus parasiticus* var. 2999, known to be a potent producer of aflatoxin (Hesseltine et al., 1966) was used to produce aflatoxin in the laboratory. The mold strain was obtained from the culture collection of the Department of Microbiology, Punjab University, Chandigarh. Spore suspension was prepared from 7 day-old potato dextrose agar slant cultures as described earlier.

Rice was used as the substrate for production of aflatoxin. Fifty grams of rice and 25 ml of tap water were transferred to each Erlenmeyer flask. After soaking for 2 hours, the flasks were autoclaved at 121°C for 15 minutes and cooled. The rice clumps were broken with a sterile glass rod and inoculated with one ml spore suspension. The culture flasks were incubated at 28°C. Small quantities of sterile water were added at 24 and 48 hour intervals to each culture flask and examined to ensure that individual rice kernels did not adhere to one another. The contents of each flask were shaken
vigorously at least three to four times a day. At the end of 5-7 days incubation, 10 ml of water were added to each flask and steam sterilized to destroy the organism.

Extraction of aflatoxin

Twenty five grams of moldy rice were blended for 5 minutes with 250 ml water in a Sorvall Omni-Mix. Chloroform (250 ml) was added and the contents were again blended for another 5 minutes. The mixture was centrifuged at 3000×g for 15 minutes and filtered through cheese cloth into a separating funnel. The chloroform layer was treated with 20 g anhydrous sodium sulphate and washed with 50 ml of chloroform. The pooled chloroform extracts were evaporated to dryness and the residue was dissolved in a suitable quantity of chloroform and used for TLC analysis.

Isolation of aflatoxin

The chloroform extracts collected after extraction of the contents of 8 Erlenmeyer flasks were pooled together and concentrated. Ten volumes of hexane were added to the concentrate to precipitate out the crude aflatoxin. The precipitate was then dried.
Fractionation of aflatoxins by column chromatography:

Chromatographic columns were prepared by packing silicic acid (100 mesh) made as a slurry in 1% ethyl alcohol in chloroform (v/v). Positive pressure was applied while packing the columns and also to elute the toxin fractions. Crude aflatoxin isolated from moldy rice was dissolved in a minimal volume of 1% ethyl alcohol in chloroform, transferred on to the chromatographic column and washed into it with small quantities of the same solvent. One percent ethyl alcohol in chloroform was used for elution (Wijdan van der A 1969). While the eluting solvent was added, 20 ml fractions were collected and chromatographed on TLC plates. Appropriate fractions were then combined, concentrated and used in the present study.

Separation and identification of aflatoxin by Thin Layer Chromatography:

Preparation of TLC plates:

Glass plates of 20 x 10 cm and 20 x 20 cm dimensions were used. Silica gel 6 was shaken with two parts by weight of distilled water in a stoppered conical flask for half a minute. The slurry was poured into an applicator and plates were coated with the slurry to 250 μm and 500 μm thickness. The coated
plates were air dried for 2 hours. After heating at 110°C for 2 hours, the plates were cooled to room temperature and stored in a desiccator until use.

For visual comparison of aflatoxin, the plates coated to 250 μ thickness were used. For spectrophotometric analysis, plates coated to 500 μ thickness, were employed.

Aflatoxin standards:

Pure standard mixtures of aflatoxin containing the four components B₁, B₂, G₁ and G₂ were prepared and used in the present study. Aflatoxin reference standards for B₁, B₂, G₁ and G₂ as well as M₁ and M₂ were gifted by Dr. R.D. Stubblefield and Dr. C.L. Shotwell of NRRL, Peoria, Illinois, U.S.A. Standard aflatoxins were also procured from Biochemical Unit, V.P. Chest Institute, University of Delhi, India. All these standard aflatoxins were used in the present study.

Separation and detection of aflatoxins on TLC plates:

The following solvent systems were used in the development of TLC plates for detection of aflatoxin in different dairy products and in the culture extracts of molds.
Raw milk, Milk powder, Dahi and Sweetened condensed milk:

Isopropyl alcohol-acetone-chloroform 
\(5:10:85\ \text{v/v/v}\)  
(McKinney, 1972)

Methanol-chloroform 
\(3:97\ \text{v/v}\)  
(Masri et al., 1966)

Cheese:

Water-methanol-chloroform 
\(1:1:98\ \text{v/v/v}\)  
(Shih and Marth, 1971)

Culture extracts:

The plates were first developed by diethyl ether 
(Habney and Nesbitt, 1965), followed by

Methanol-chloroform 
\(2.5:97.5\ \text{v/v}\)

Hexane-petroleum ether-benzene-chloroform-acetone 
\(1:1:1:6:1\ \text{v/v}\)  
(Strzelecki and Jadura, 1972)

Toluene-amyl alcohol-methanol 
\(90:32:3\ \text{v/v/v}\)

Quantitation of aflatoxins:

Visual comparison:

The amount of toxin present in the experimental samples and in the culture extracts was determined by
comparing the Rf value and the fluorescence with the reference standards of aflatoxins (Plate 2 and 3).

Spectrophotometric estimation of aflatoxins

The method described by Nabney and Nesbitt (1965) was used for spectrophotometric estimation of aflatoxins.

Known quantities of aflatoxin-containing sample extracts were spotted and developed on the TLC plates. The spots of aflatoxin B<sub>1</sub> were located under UV light and scraped out. The scrapings were extracted with methanol and filtered. After washing the silica gel with methanol, the filtrate was made up to 5 ml. The optical density of the methanolic extract was recorded using Beckman 20-2 spectrophotometer at 363 mμ and 420 mμ. Pure methanol was used as a blank. The amount of aflatoxin B<sub>1</sub> present in the test sample extracts was calculated as follows:

\[
\text{Aflatoxin B}_1 \text{ concentration} = \frac{\mu \times 200 \times 10^6}{\xi \times 200 \times 2} \mu g / 5 \text{ ml}
\]

where \( \mu \) = the corrected density (density at 363 mμ-density at 420 mμ)

\( M \) = molecular weight of aflatoxin B<sub>1</sub>

\( \xi \) = the molar extinction coefficient of B<sub>1</sub> in methanol.
Chromatogram showing comparison of standard aflatoxins with culture extracts of two mold isolates.

\[ S = \text{Standard} \]

\[ C_1 = \text{Culture extract from a mold isolate containing all four fractions of aflatoxin (indicated by arrows).} \]

\[ C_2 = \text{Culture extract from another mold isolate containing only aflatoxin B_1 (indicated by an arrow).} \]
Plate 3

Chromatogram showing comparison of standard aflatoxins with culture extracts from eight different individual mold isolates.

S = Standard

C₁ to C₄ = Culture extracts of four mold isolates showing aflatoxins B₁ and G₁.

C₅ to C₈ = Culture extracts of four other mold isolates showing aflatoxin B₁ only.
The \( R_f \) and fluorescence values of the four types of aflatoxins are as follows:

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>( R_f )</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>312</td>
<td>22,000</td>
</tr>
<tr>
<td>Aflatoxin B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>314</td>
<td>23,400</td>
</tr>
<tr>
<td>Aflatoxin G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>328</td>
<td>18,700</td>
</tr>
<tr>
<td>Aflatoxin G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>330</td>
<td>21,000</td>
</tr>
</tbody>
</table>

Chemical characterization of aflatoxin B<sub>1</sub> and G<sub>1</sub>:

After comparing the \( R_f \) value and fluorescence of the test spots with standard aflatoxin samples, chemical characterization of the different aflatoxins was done by the method of Stack and Pohland (1975).

A thick vertical line marking was drawn in the centre of the silica gel plate. One half of the plate was covered with a clean glass plate. Known quantities of experimental extract and a mixture of standard aflatoxins B<sub>1</sub> and G<sub>1</sub> were separately spotted on the open half of the plate. In both the spots, two microliters of trifluoroacetic acid (TFA) were spotted and allowed to react for 5 minutes. Warm air (35 - 40°C) was blown over the plate for a period of 10 minutes. The glass plate which had been covering the other half of the plate was removed. Spotting was done as above.
except that TPA was not spotted. All the plates were developed in a mixture of chloroform-acetone (85:15 v/v) and examined under UV light. Untreated aflatoxin appeared near the top of the plate. Blue fluorescent derivatives ($a_{2a}$ and $G_{2a}$) appeared at an Rf approximately 1/4 of that of $B_1$ and $G_1$. Additional confirmation was done by spraying the plates with 25% sulphuric acid and observing the change in fluorescence from blue or blue green to yellow fluorescence (Plate 4 and 5).

**Confirmation of the presence of aflatoxin by microbiological assay:**

A strain of *Aspergillus niger* maintained in the culture collection of Dairy Bacteriology Division was selected after preliminary trials using aflatoxin $B_1$ as the test standard. The above organism produced clear zones around the filter paper discs impregnated with 1, 2 and 5 μg of aflatoxin $B_1$, thereby indicating inhibition of growth around the discs. The size of the zone was found to be proportionate to the concentration of aflatoxin.

**Medium:**

Tryptone dextrose agar of the following composition was used:
Plate 4

Chromatogram showing changes in Rf values of aflatoxin B₁ (standard as well as a culture extract from a mold) to B₂₅ after the treatment of the former with TFA.

S = Standard
C = Culture extract from a mold isolate.
Plate 5

Chromatogram showing changes in Rf values of aflatoxins B₁ and G₁ (Standard as well as culture extracts from three molds) to B₂a and G₂a; after treatment of the former with TFA.

S = Standard

C₁ to C₃ = Individual culture extracts from three mold isolates.
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Dist. water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH adjusted to</td>
<td>6.4 to 6.6</td>
</tr>
</tbody>
</table>

Petri plates were poured with tryptone dextrose agar medium seeded with 1.5 ml spore suspension of *A. niger*. In each plate, one filter paper disc impregnated with 1 μg aflatoxin B₁ as positive control and other filter paper discs impregnated with aflatoxin from culture extracts of molds or from other samples, were placed. A filter paper disc impregnated with pure chloroform was used as a negative control. The petri plates were incubated at 37°C for 16-18 hours and were examined for inhibitory zones according to the procedure of Clements (1968 a, b).

**Production of aflatoxin M₁:**

Aflatoxin M₁ was produced by biological transformation. Aflatoxin B₁ was administered to a sheep.

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*The organism was grown on tryptone dextrose agar in Roux bottles at 37°C for about 20 days or until 99% sporulation was achieved. The spores were harvested and washed with sterile phosphate buffer (pH 7.2) (Clements, 1968 a, b). The suspension was centrifuged at 5000 x g for half an hour. The centrifugation was repeated until a clear supernatant was obtained. The growth was adjusted to 40% transmittance at 665 nm using a photoelectric colorimeter (Spectronic – 20). The spore suspension was stored in the refrigerator until use. A fresh spore suspension was prepared once in 3 months.*
as a single oral dose of 400 μg/kg body weight. Aflatoxin \( M_1 \) was recovered from urine of the animal by extracting it with chloroform and by purifying the toxin by thin layer chromatography using chloroform-methanol (97:3 v/v) as the solvent system (Grant and Carlson, 1971). The identification and estimation of aflatoxin \( M_1 \) was carried out by visual comparison of \( R_f \) value and by fluorescence using standard aflatoxin \( M_1 \) (Roberts and Allcroft, 1968).

**Production of aflatoxin in khoa**

Fresh khoa samples prepared from cow's milk were used in these trials. Blocks of freshly made khoa were inoculated on the top portion with the spore suspension of \( A. \) parasiticus NRRL 2999. In another set of trials, \( A. \) flavus \( K_3 \) (an isolate from khoa) was used. The inoculated blocks of khoa were incubated at 5 ± 1°C, 28 ± 1°C, and 37 ± 1°C, for one and two weeks. Thereafter, each block was cut horizontally into a series of one cm layers. The top most layer contained the mold growth. All the layers were stored in the refrigerator until used. Aflatoxins were extracted and quantitated from the samples as described earlier.

**Partitioning of aflatoxin \( M_1 \) in milk after inoculation with different lactic cultures**

Two hundred milliliter lots of pasteurized milk
containing 1 μg aflatoxin M₁ were inoculated with the test lactic cultures and incubated for 24 hours at 30 / 37°C. The culture medium was filtered to separate the protein fraction. The concentration of aflatoxin in the protein fraction as well as in whey and washing were determined.

**Partitioning of aflatoxin M₁ in Paneer**

**Paneer** was prepared according to the procedure described by Srinivasan and Ananthakrishnan (1964).

Milk containing 1 μg of aflatoxin M₁ was brought to boiling temperature and then coagulated by 1% citric or lactic acid. The whey was filtered and collected for analysis. The concentration of aflatoxin M₁ in paneer (the coagulated protein fraction) as well as in whey and in washing was estimated.

**Effect of addition of milk clotting enzymes from different sources to milk on the partitioning of aflatoxin M₁**

Coagulation of 500 ml pasteurized cow's milk containing 2 μg of aflatoxin M₁ was carried out according to the procedure described by Kosikovski (1970) for commercial cheese, except that no starter culture was used. The coagulating agents used in different trials were animal rennet, 'Meito' rennet and 'Absidia'
rennet. The milk was allowed to set after adding the rennet. The firm coagulum was cut into small cubes and whey was drained.

The concentration of aflatoxin \( M_1 \) was estimated in coagulated protein fraction as well as in the whey and in the washing.

The procedure for extraction of aflatoxin from protein fraction was the same as described for khoa and cheese.

**Effect of heat treatment of milk on aflatoxin \( M_1 \)**

*Milk samples* containing known quantities of aflatoxin \( M_1 \) were heat treated at 63°, 80°, 90°, 100° and 121°C for 30 minutes. The residual concentration of aflatoxin \( M_1 \) in each sample was determined.

**Effect of aflatoxin \( M_1 \) on selected lactic cultures:**

The effect of aflatoxin \( M_1 \) on the growth of the following lactic cultures was studied:

1. *Streptococcus lactis* (C10)
2. *Streptococcus cremoris* (C7)
3. *Streptococcus cremoris* (496)
4. *Streptococcus thermophilus* (H1T)
5. *Streptococcus diacetilactis* (DRC1)
6. *Lactobacillus bulgaricus* (1373)
7. *Lactobacillus acidophilus*

The above cultures were obtained from the culture collection of Dairy Bacteriology Division. Cultures were maintained in litmus milk and subcultured at weekly intervals. The following media were used.

**Yeast Dextrose Broth:**

- Peptone: 20.0 g
- Dextrose: 10.0 g
- Sodium chloride: 5.0 g
- Yeast extract: 6.0 g
- Beef extract: 10.0 g
- Ammonium citrate: 5.0 g
- Dist. water: 1000 ml
- pH 6.5

**Lactic Broth:**

- Tryptone: 20.0 g
- Yeast extract: 5.0 g
- Dextrose: 5.0 g
- Gelatin: 2.5 g
- Lactose: 5.0 g
- Sucrose: 5.0 g
Tomato juice broth:

Tomato juice* 400 ml
Peptone 15.0 g
Dextrose 20.0 g
Sodium chloride 5.0 g
Yeast extract 5.0 g
Dist. water 600 ml

ph 6.4 to 6.6

*(Tomato juice - 200 grams of fresh tomatoes were cut into small pieces and steamed for 30 minutes and filtered. The filtrate was kept in the refrigerator overnight, filtered and used).

All the media were autoclaved at 121°C for 15 minutes after incorporating aflatoxin B1.

Effect of aflatoxin B1 on the growth of starter cultures:

Yeast dextrose broth was used for the cultivation of L. lactis, L. cremoris, L. diacetylactis, lactic broth for L. thermophilus and tomato juice broth for
L. bulgaricus and L. acidophilus cultures. The tubes of the test media containing 0 to 50 μg of aflatoxin B₁ were inoculated with growing cells of the above organisms and incubated at appropriate temperatures.

Assay procedures for measurement of growth:

The inhibitory/stimulatory properties of aflatoxin B₁ were determined by:

1) Visual comparison of turbidity,
2) Absorbance at 540 mμ ("Spectronic 20" Lausch and Lomn).

Aflatoxin B₁ binding by the intact cells of lactic acid bacteria:

Erlenmeyer flasks (250 ml) containing 50 ml aliquots of the test medium were inoculated with the test lactic culture and incubated at 30 / 37°C. Cells were harvested by centrifugation and washed twice in saline followed by centrifugation. The cell pellet was resuspended in saline. Specific number of cells were added to 50 ml medium containing known quantities of aflatoxin B₁ and incubated for one hour on a rotary shaker. At the end of the incubation period, the medium was centrifuged at 10,000 x g for 20 minutes. The supernatant medium was decanted and used for estimation of toxin. The sediment was resuspended in 25 ml
distilled water. The procedure was repeated 4 times using fresh distilled water and the washings were collected separately. The washed cells were resuspended in distilled water, sonicated at 25 KC for 30 minutes in an ultrasonic disintegrator (Model HT-20, Measuring Scientific Equipment Co., U.K.) and extracted with chloroform. Aflatoxin was estimated in the chloroform extracts of the medium, washings and disintegrated cells.

**Effect of aflatoxin B₁ on the lactic acid producing ability of the lactic acid bacteria**

Aliquots of sterile skim milk containing different levels of aflatoxin B₁ such as 5, 10, 20 and 50 μg/ml were inoculated with the test lactic culture and incubated at 30 / 37°C. Acidity was determined at intervals of 6, 8, 12 and 24 hours by using N/9 sodium hydroxide.