EVALUATION OF PROBIOTIC *LACTOBACILLUS* SPP. FOR REDUCTION OF AFLATOXIN M_1 BIOACCESSIBILITY AND TOXICITY

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENT

FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

DAIRY MICROBIOLOGY

BY

ROHIT PANWAR

(M.Sc. Dairy Microbiology)

DIVISION OF DAIRY MICROBIOLOGY

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

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Approved by

27.08.2021

(Dr. Birbal Singh)
EXTERNAL EXAMINER

27.08.2021

(Dr. Chand Ram Grover)
MAJOR ADVISOR & CHAIRMAN

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5. **Dr. Suman Kapila** .....
   (Director Nominee)
   Principal Scientist, Animal Biochemistry Division

Dr. Chand Ram Grover
Principal Scientist (DM Division)

CERTIFICATE

This is to certify that the thesis entitled, **"Evaluation of probiotic *Lactobacillus* spp. for reduction of aflatoxin M_1 bioaccessibility and toxicity"** submitted by **Mr. Rohit Panwar** towards the partial fulfilment of the award of the degree of **DOCTOR OF PHILOSOPHY IN DAIRY MICROBIOLOGY** of the **ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana), India,** is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 27.08, **2021**

(Chand Ram Grover)
Major Advisor

DEDICATED TO MY RESPECTED GUIDE..

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Date: **(Rohit Panwar)**

EVALUATION OF PROBIOTIC *LACTOBACILLUS* SPP. FOR REDUCTION OF AFLATOXIN M_1 BIOACCESSIBILITY AND TOXICITY

ABSTRACT

Present study was aimed to evaluate probiotic *Lactobacillus* spp. for reduction of aflatoxin M_1 (AFM_1) bioaccessibility *in-vitro* and *in-vivo* to avert its toxic effect. Thirty probiotic lactobacilli strains were screened for AFM_1 binding/bioadsorption potential *in-vitro.* Aflatoxin M_1 bioadsorption ranged from 2.46±2.31 - 64.16±5.60% after incubation of 6, 12 and 24 hrs. Five probiotic strains exhibiting >50% AFM_1 binding ability were selected. *Lactobacillus plantarum* CRD7 exhibiting highest AFM_1 binding of 64.14±5.60% was selected for detailed investigations. Selected probiotic *Lactobacillus* strains were also assessed for their probiotic lactobacilli-AFM_1 complex stability. Result on lactobacilli-AFM_1 complex stability demonstrated highest percentage of AFM_1 release during first wash (7.40±0.23 - 20.45±2.10%) as compared to second wash (0.98±0.14 - 18.20±1.24%) however, no $AFM_1$was released during third wash. Results of chloroform treatment showed release of 88.57±0.16 - 92.30±2.23% of bound AFM_1 from the bacterial cells indicates AFM_1 binding to bacterial cell wall instead of absorption or metabolic degradation. Observations on reduction of AFM_1 bioaccessibility *in-vitro* digestion model showed significant ($p < 0.05$) reduction in AFM_1 bioaccessibility by selected probiotic strains, which ranged from 21.56±1.66 - 52.84±3.34%. Investigations on AFM_1 binding and bioaccessibility reduction by heat killed cells also demonstrated that selected probiotic *Lactobacillus* strains exhibited AFM_1 binding (49.01±3.18 - 60.06±3.08%) and reduction in AFM_1 bioaccessibility (32.23±2.73 - 50.7±1.90%). *In-vivo* efficacy of probiotic *L. plantarum* CRD7 was conducted in mice model for its protective effect against AFM_1 intoxication alongwith *L. rhamnosus* GG as reference. The mice were randomly divided into six major groups *i.e.* normal control group (NCG), AFM_1 group (PCG), LGG group, AFM_1-LGG group, CRD7 group and AFM_1-CRD7 group fed with respective experimental diets. *In-vivo* efficacy data on AFM_1 bioaccessibility reduction ranged from 3.14±0.12 - 36.21±0.72%. Significantly ($p<0.05$) higher AFM_1 bioaccessibility reduction (36.21±0.14%) and protective effect *w.r.t.* immune cell counts (TLC and DLC) as well as viability of splenocytes and thymocytes was also noticed in AFM_1-CRD7 fed group as compared to other treatment groups. Levels of liver function enzymes (aspartate transaminase, alanine transaminase and γ-glutamyl transferase) were also found significantly ($p<0.05$) reduced in AFM_1-CRD7 fed group as compared to PCG indicative of its ameliorative effect. Both probiotic-AFM_1 fed groups (AFM_1-CRD7 and AFM_1-LGG) also exhibited significantly ($p<0.05$) lower levels of oxidative stress markers (superoxide dismutase, glutathione peroxidase, catalase) in liver as compared to PCG. Apparent protective effect of *L. plantarum* CRD7 was also observed against DNA damage of liver cells as compared to PCG and AFM_1-LGG fed groups. The *L. plantarum* CRD7 has proved remarkable AFM_1 binding ability both *in-vivo* and *in-vitro,* thus it could be delivered through daily diet alongwith milk and dairy products as a complementary bio-therapeutic agent to discharge AFM_1 from human body through excretion as well as amelioration of oxidative stress.

PhD Scholar	Major Advisor	Head of Division
(Rohit Panwar)	(Chand Ram Grover)	(Dairy Microbiology)

एफ्लाटॉक्सिन एम$_1$ बायोसिसेसिबिलिटी और विषाक्तता में कमी के लिए प्रोबायोटिक लैक्टोबैसिलस जाति का मूल्यांकन

सारांश

वर्तमान अध्ययन का उद्देश्य प्रोबायोटिक लैक्टोबैसिलस जाति का मूल्यांकन करना था। इस विषैले प्रभाव को रोकने के लिए एफ्लाटॉक्सिन एम$_1$ (एएफएम$_1$) की इन-विट्रो और इन-विवो की कमी के लिए। इन-विट्रो एएफएम$_1$ बाइंडिंग पोटेंशियल के लिए, तीस प्रोबायोटिक लैक्टोबैसिली उपभेदों का **मूल्यांकन किया गया**। एफ्लाटॉक्सिन एम$_1$ के जैवसंरक्षण की **क्षमता** 2.46 ±2.31 - 64.16±5.60% से 6, 12 और 24 घंटे के ऊष्मायन के बाद **पाई** गयी। पांच प्रोबायोटिक उपभेदों का प्रदर्शन> 50% एएफएम$_1$ बाध्यकारी क्षमता का चयन किया गया। एल. प्लांटरम सीआरडी7 ने 64.14±5.60% की उच्चतम एएफएम$_1$ बाइंडिंग का प्रदर्शन करते हुए विस्तृत जांच के लिए चुना गया था। चयनित प्रोबायोटिक लैक्टोबैसिलस उपभेदों का मूल्यांकन उनके प्रोबायोटिक लैक्टोबैसिली- एएफएम$_1$ जटिल स्थिरता के लिए भी किया गया। दूसरे वॉश (0.98±0.14 - 18.20±1.24%) की तुलना में लैक्टोबैसिली- एएफएम$_1$ जटिल स्थिरता के परिणाम ने पहले वॉश (7.40±0.23 - 20.45±2.10%) के दौरान एएफएम$_1$ रिलीज का उच्चतम प्रतिशत प्रदर्शित किया (तीसरे चरण के दौरान कोई एएफएम$_1$ रिलीज़ नहीं किया गया)। क्लोरोफॉर्म उपचार के परिणामों ने 88.57±0.16 - 92.30±2.23% की रिहाई को दिखाया। बैक्टीरिया कोशिकाओं से बाध्य एएफएम$_1$ का अवशोषण या चयापचय में गिरावट के बजाय एएफएम$_1$ को बैक्टीरिया की कोशिका की दीवार से बांधना दर्शाता है। एएफएम$_1$ जैवसक्रियता इन-विट्रो पाचन मॉडल की कमी पर टिप्पणियों ने चुनिंदा प्रोबायोटिक उपभेदों द्वारा एएफएम$_1$ जैवसक्रियता में महत्वपूर्ण (पी <0.05) कमी दिखाई, जो 21.56±1.66 - 52.84±3.34% से लेकर थी। एएफएम$_1$ बाइंडिंग और बायोसिसेसिबिलिटी रिडक्शन निष्क्रिय सेल्स की जांच में यह भी सामने आया है कि चुनिंदा प्रोबायोटिक लैक्टोबैसिलस स्ट्रेन ने एएफएम$_1$ बाइंडिंग (49.01±3.18 - 60.06±3.08%) और एएफएम$_1$ बायोकैसेबिलिटी में कमी (32.23±2.73 - 50.7±1.90%) का प्रदर्शन किया। संदर्भ के रूप में एएफएम$_1$ के साथ इसके सुरक्षात्मक प्रभाव के लिए चूहों के मॉडल में प्रोबायोटिक एल प्लांटरम सीआरडी7 की इन-विवो प्रभावकारिता का आयोजन किया गया। चूहों को छह प्रमुख समूहों यानी सामान्य नियंत्रण समूह (NCG), एएफएम$_1$ समूह (पीसीजी), LGG समूह, एएफएम$_1$-LGG समूह, सीआरडी7 समूह और एएफएम$_1$- सीआरडी7 समूह में विभाजित किया गया। एएफएम$_1$ जैव-क्षमता में कमी पर विवो प्रभावकारिता डेटा 3.14±0.12 - 36.21±0.72% से लेकर था। गौरतलब है (पी <0.05) उच्च एएफएम$_1$ बायोकेसेबिलिटी में कमी (36.21±0.14%) और साथ ही सुरक्षात्मक प्रभाव w.r.t. प्रतिरक्षा सेल मायने रखता है (टीएलसी और डीएलसी) के साथ-साथ अन्य उपचार समूहों की तुलना में एएफएम$_1$-सीआरडी7 खिलाया समूह में स्प्लेनोसाइट्स और थायमोसाइट्स की व्यवहार्यता भी देखी गई। लीवर फंक्शन एंजाइमों (एस्पार्टेट ट्रांसएमिनेस, एलेनिन ट्रांसएमिनेस और गामा-ग्लूटामाइल ट्रांसफ़रेज़) के स्तर भी एएफएम$_1$-सीआरडी7 फीड किए गए समूह में काफी कम (पी <0.05) मात्रा में पाए गए, जो कि इसके अम्लीरेटिव प्रभाव के पीसीजी सूचक की तुलना में बहुत कम था। दोनों प्रोबायोटिक फेड ग्रुप (एएफएम$_1$-सीआरडी7 और एएफएम$_1$-LGG) ने भी पीसीजी की तुलना में लीवर में ऑक्सीडेटिव स्ट्रेस मार्कर (सुपरऑक्साइड डिसम्यूटेस, ग्लूटाथिओन पेरोक्सीडेज, केटेरेज) के निचले स्तर का काफी प्रदर्शन किया। एल. प्लांटरम सीआरडी7 के स्पष्ट सुरक्षात्मक प्रभाव को पीसीजी और एएफएम$_1$-LGG खिलाए गए समूहों की तुलना में यकृत कोशिकाओं की डीएनए क्षति के लिये भी देखा गया। एल. प्लांटरम सीआरडी7 ने इन-विवो और इन-विट्रो दोनों में उल्लेखनीय एएफएम$_1$ बाइंडिंग क्षमता साबित की है, इस प्रकार इसे दैनिक आहार के माध्यम से दूध और डेयरी उत्पादों के साथ पूरक बायो-चिकित्सीय एजेंट के रूप में और साथ ही उत्सर्जन के माध्यम से मानव शरीर में एएफएम$_1$ के निर्वहन के लिए दिया जा सकता है।

विद्यार्थी	**मार्गदर्शक**	विभागाध्यक्ष
(रोहित पंवार)	(चांद राम ग्रोवर)	(डेयरी माइक्रोबायोलॉजी)

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LIST OF ABBREVIATIONS

AFM_1	Aflatoxin M_1
AFB_1	Aflatoxin B_1
AOE	Ant oxidative enzyme
IARC	International Agency for Research on Cancer
GIT	Gastrointestinal Tract
ELISA	Enzyme-linked Immunosorbent Assay
HPLC	High performance liquid chromatography
YGLM	Yeast Glucose Litmus Chalk Milk
IAEC	Institutional Animal Ethics Committee
CPCSEA	Committee for the Purpose of Control & Supervision of Experiments on Animal
NCG	Negative Control Group
PCG	Positive Control Group
Lr-LGG	*Lactobacillus rhamnosus GG*
AFM_1-Lr-LGG	Aflatoxin M_1- *Lactobacillus rhamnosus GG*
Lp-CRD7	*Lactobacillus plantarum* CRD7
AFM_1-Lp-CRD7	Aflatoxin M_1-*Lactobacillus plantarum* CRD7
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma-glutamyl transferase
SGOT	Serum Glutamic Oxaloacetate Transferase
PLP	Pyridoxal Phosphate
ALAT	Alanine aminotransferase
ALT	Alanine transferase
LDH	Lactate dehydrogenase
IFCC	International Federation of Clinical Chemistry

CAT	Catalase
SOD	Superoxide Dismutase
GPx	Glutathione Peroxidase
BHT	Butylated hydroxytoluene
TBA	Thiobarbituric Acid
TLC	Total Leucocytes Count
DLC	Different Leucocytes Count
PE	Phycoerythrin
ROS	Reactive Oxygen Species
WHO	World Health Organization
EU	European Union
RIA	Radioimmunoassay
SPFS	Enhanced Fluorescence Spectroscopy
SPs	Surface Plasmons
UHT	Ultra High Temperature
CYP	Cytochrome P450 enzymes
ATP	Adenosine tri-phoshate
MDA	Melondialdehyde
MRS	deMan, Rogosa and Sharpe
NCDC	National Collection of Dairy Cultures
NDRI	National Dairy Research Institute
OD	Optical density
TBAR	Thiobarbituric acid reactive
TBARS	Thiobarbituric acid reactive substances
BW	Body weight

Units

ºC	Degree centigrade
µg	Microgram(s)
µl	Microlter(s)
CFU	Colony forming units(s)
EU	Enzyme unit
G	Gram(s)
H	Hour(s)
Kg	Kilogram
L	Liter(s)
M	Molar
Mg	Milligram
Min	Minute(s)
MI	Milliliter(s)
mM	Milli molar
N	Normal
Nm	Nanometer
pH	-Log10 $[H^+]$
Psi	Pressure in standard international units
Rpm	Revolution per minute
Sec	second(s)
kDa	Kilo Dalton

1.0 INTRODUCTION

Food safety has become a public health priority worldwide and consumption of foods has been acknowledged as the major route for human exposure to certain contaminants. Milk has been recognized as the best form of nutrition and obligatory for growth, development and maintenance of human health. Although, it is more prone to contamination by variety of physical and biological contaminants from farm to consumer levels. Consumption of contaminated milk and milk products results in food borne illnesses, especially in infants, who are more susceptible than adults due to their weak immunity. Among several sources, milk and milk products is one of the main source responsible for consumer exposure to aflatoxin M_1 (AFM_1) (Iqbal *et al.,* 2015; de Freitas *et al.,* 2018; Min *et al.,* 2020; Goncalves *et al.,* 2021b). Various scientific studies have reported that the presence of AFM_1 in dairy foods is a serious health issue because in many countries, every age group regularly consumed these products in their daily diet (Fallah *et al.,* 2009; Michlig *et al.,* 2016; Goncalves *et al.,* 2018; Ahmadi, 2020). Increased amounts of dietary AFM_1 may contribute to the development of mutagenic, carcinogenic, teratogenic and immunosuppressive effects in humans, so it is necessary to monitor AFM_1 in human diet (Jebali *et al.,* 2015). Cytochrome P450 enzymes (CYP) including CYP1A2, CYP 3A4 and CYP2A6 expressed in the liver and other tissues are responsible for conversion of aflatoxin B_1 (AFB_1) to epoxides such as AFB_1-8,9-exo-epoxide, AFB_1-8,9-endo-epoxide, AFM_1, AFP_1, and AFQ_1 (Kamdem *et al.,* 2006; Marchese *et al.,* 2018). The AFM_1 is major hydroxylated metabolite of AFB_1, which are excreted in animal or human milk, of those who have consumed some quantities of contaminated food burdened with AFB_1. About 0.3-6.2 % of AFB_1, is metabolized into AFM_1 by animal and their transformation depends on various factors such as genetic background of the animal, its health, diet, digestion rate, milking process, lactation stage, season, and environmental conditions (Unusan, 2006; Michlig *et al.,* 2016; Muaz *et al.,* 2021).

Therefore, AFM_1 is not strictly a detoxified product of AFB_1 in biological responses in which genotoxicity and immunotoxicity plays a significant role (Heinonen *et al.,* 1996; Bianco *et al.,* 2012). The acute toxicity caused by aflatoxins in 1-day-old ducklings suggests that both AFM_1 and AFB_1 have similar mechanism to cause acute toxicity and subcellular alterations, such as changes in liver parenchymal cells, dissociation of ribosomes from the rough endoplasmic reticulum (van Egmond, 1989). Recently, Marchese *et al.,* (2018) evaluated the effects of AFM_1

on cell viability, apoptosis, cell cycle, and metabolomic and cytokinomic profile of HepG2 cells after treatment showed decrease in cell viability by blocking cell cycle in the G0/G1 phase, decrease of formiate levels and increased level of some amino acids and metabolites as well as increase in concentration of three pro-inflammatory cytokines, IL-6, IL-8, and TNF-α, and decrease in anti-inflammatory interleukin, IL-4. Thus, AFM_1 inhibit growth of HepG2 cells, inducing both a modulation of lipidic, glycolytic, and amino acid metabolism and an increase of the inflammatory status of these cells.

Initially AFM_1 was categorized as group 2B human carcinogen but later on the basis of its toxicity and prevalence levels in milk, it was reclassified as group 1 human carcinogen (IARC, 2002). Several countries have established regulatory limits for AFM_1 in milk and dairy products, which vary from 0.05 ppb (European Community, Codex Alimentarius Commission) to 0.5 ppb in United States Food & Drug Administration and in Food Safety and Standards Authority of India (Berg, 2003; Commission of the European Communities, 2006).

The maximum prevalence of AFM_1 has been reported in milk and milk products specifically, in the developing African and Asian countries *viz.* Indonesia, Japan, Thailand, Korea, China and India. However, AFM_1 in milk and milk products have been found at alarming state. According to EU limits for AFM_1 about 100 % of the samples of milk and milk products have been reported to contain exceptionally higher levels of AFM_1 than the stated norms in Nigeria and Sudan (Darsanaki *et al.,* 2019; Moghaddam *et al.,* 2019). The levels of AFM_1 are comparatively high in milk and other dairy products in South African countries and thus it is major concern a serious health hazard in consumers. The wide variations in AFM_1 levels in milk and milk products over the globe not only related to climatic and geographic differences but also due to differences in feeding systems, farm management practices, and analytical methods (Alborzi *et al.,* 2006; Cherkani-Hassani *et al.,* 2020; Suman, 2021).

The AFM_1 present in milk and milk products is highly heat stable. Heat processing such as pasteurization, boiling, UHT treatment and sterilization do not reduce an appreciable amount of AFM_1 in milk and milk products (Omeiza *et al.,* 2018; Daou *et al.,* 2020; Gavahian *et al.,* 2021). Although, several chemical and physical methods are available to eliminate or reduce AFM_1, however both of the treatments can not readily applicable to dairy industry directly due to scanty research data available about their biological safety and nutritional quality of treated

products. Moreover, the cost of existing processes in considerably prohibitive for large-scale application. Till date, there is no effective detoxification method available, which can help dairy industry to mitigate or remediate the problem of AFM_1 from milk and milk products. This has led to search for alternative strategies such as use of biological agents. There has been increasing interest in concept that inhabitate microorganisms of gastrointestinal tract (GIT) can reduce the absorption of AFM_1 in consumed foods. Selected strains of lactic acid bacteria and bifidobacteria have been used as bioremediant for AFM_1 (Hashemi *et al.,* 2018; Raduly *et al.,* 2020; da Cruz *et al.,* 2021). This strategy is considered the most promising due to its effectiveness, specificity and environment friendly nature to reduce or eliminate possible AFM_1 contamination in milk and milk products. The binding of AFM_1 with microbial cells has been reported as a rapid decontamination process but the exact mechanism of binding is still has to be fully established.

However, most widely accepted hypothesis regarding the mechanism involved in microbial removal of AFM_1 and other mycotoxins, is the adhesion of toxin molecules with cell wall components such as proteins, polysaccharides and peptidoglycan particularly teichoic acid present (Serrano-Nino *et al.,* 2013; Assaf *et al.,* 2018; Muaz *et al.,* 2021). Recently, researchers are trying to understand whether the absorption of aflatoxins in consumed food is reduced by microorganisms or they make it less bioaccessible to GIT. Bioaccessibility of various mycotoxins such as zearalenone, deoxynivalenol, nivalenol, AFB_1, fusarium and AFM_1 in different food matrices have been investigated (Raiola *et al.,* 2012; Meca *et al.,* 2012). However, there is diminutive published data regarding the influence of probiotic lactic acid bacteria on the bioaccessibility of AFM_1 under gastrointestinal conditions. Further, research investigations could not be retrieved on search engine regarding use of indigenous probiotic lactobacilli for their ability to bind and reduce AFM_1 bioaccessibility. Therefore, keeping in view of the above facts the proposed research program was designed with following objectives:

1. Screening of probiotic *Lactobacillus* strain for aflatoxin M_1 binding ability.
2. Evaluation of selected probiotic *Lactobacillus* strain for reduction of aflatoxin M_1 bioaccessibility (*in vitro* and *in vivo*) and its preventive role against aflatoxin M_1 immunotoxicity and genotoxicity.

2.0 REVIEW OF LITERATURE

2.1 MYCOTOXINS

Mycotoxins are poisonous chemical compounds produced by certain fungi. There are many such compounds, but only a few of them are regularly found in food and animal feedstuffs such as grains and seeds. Nevertheless, those that do occur in food have great significance in the health of humans and livestock. In the 1960s more than 1,00000 young turkeys on poultry farms in the United Kingdom died in a period of a few months from an unidentified disease, which was named "turkey × disease"(Hedayati, 2016; Moretti *et al.,* 2018; Shi *et al.,* 2018; Taheur *et al.,* 2021). Ducklings and other poultry animals were also affected, and high mortalities were observed. A careful survey of the inputs and environment of the affected farms indicated that the disease was associated with feeds and specifically with peanut meal imported from Brazil. A disease with symptoms typical of turkey × disease was reproduced when animals were fed the same peanut meal. Intensive investigations were then carried out on the suspected ingredient to identify the nature of the toxin, which was soon found to be of fungal origin. The chemical structures of some of the important mycotoxins are presented in the **Fig 2.1**.

The toxin-producing fungus was identified as *Aspergillus flavus* and the toxin was accordingly called aflatoxin/mycotoxin. While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins (Nesbitt *et al.,* 1962; Hassan *et al.,* 2014; Wochner *et al.,* 2018; Gbashi and Madala, 2019; Kebede *et al*., 2020). Soon, the mycotoxin rubric was extended to include a number of previously known fungal toxins (e.g., the ergot alkaloids), some compounds that had originally been isolated as antibiotics (e.g., patulin), and a number of new secondary metabolites revealed in screens targeted at mycotoxin discovery (e.g., ochratoxin A). The period between 1960 and 1975 has been termed the mycotoxin gold rush (Manabe, 2001; Markaki and Melissari, 1997) because so many scientists joined the well-funded search for these toxigenic agents. Depending on the definition used, and recognizing that most fungal toxins occur in families of chemically related metabolites. Some 300 to 400 compounds are now recognized as mycotoxins, of which approximately a dozen groups regularly receive attention as threats to human and animal health (Cole *et al.,* 1995; Adebiyi *et al*., 2019; Kebede *et al*., 2020; Adebo *et al.,* 2021; Suman, 2021). Mycotoxicoses are the animal diseases

caused by mycotoxins; mycotoxicology is the study of mycotoxins (Martin and Gilman, 1976). The term mycotoxin is derived from the Greek word 'mycos' meaning fungus (mould), and the Latin word 'toxicum', which means poison. Mycotoxins are produced by fungi through their secondary metabolism. Mycotoxin concentration can therefore be independent of the growth of the fungi, which is associated with the primary metabolism. The diversity of the compounds formed and the specificity of the fungal strain for mycotoxin production result from the secondary metabolism, which is usually activated by signals from the environment (cold, heat, dryness etc.). Among the numerous mycotoxins, several groups have been identified, produced by three major fungus genera *Aspergillus, Penicillium* and *Fusarium* (Jouany *et al.,* 2009; Cheraghali *et al.,* 2007; Jalili and Scotter, 2015; Xiong *et al.,* 2018; Zhao *et al*., 2019; Celik, 2020). Mycotoxins present in food products and animal feeds are an important problem concerning food and feed safety as significant economic losses are associated with their impact on human and animal health (Shundo *et al.,* 2009; Vila-Dinat *et al*., 2018).

In addition to being acutely toxic, some mycotoxins are now linked with the incidence of certain types of cancer and this aspect has evoked global concern over feed and food safety, especially for milk and milk products.

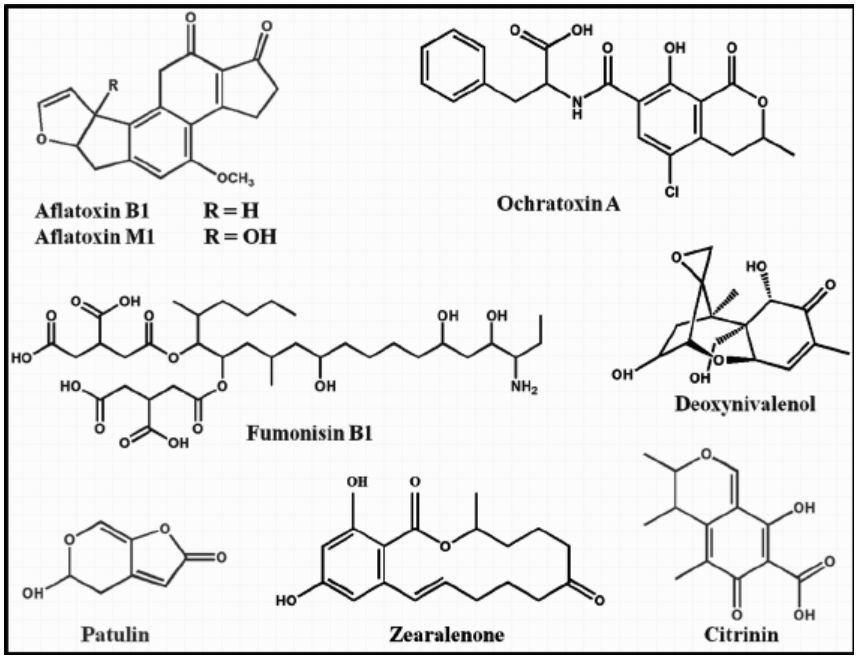
2.2 AFLATOXINS

The aflatoxins are a group of chemically similar toxic fungal metabolites (mycotoxins) produced by certain moulds of the *Aspergillus* species growing on a number of agriculture raw food commodities.

These are highly toxic compounds responsible for both acute and chronic toxicity in humans and animals. Different types of aflatoxin are produced in nature. The aflatoxins consist of about 20 similar compounds belonging to a group called difuranocoumarins, but only four named AFB_1, B_2, G_1 and G_2 are naturally found in foods (**Fig 2.2**). AFB_1 is the most commonly found in food and also the most toxic (Bandyopadhyay *et al.,* 2016; Jiang *et al.,* 2018; Yang *et al.,* 2020). Aflatoxin B_1 is considered to be the most toxic metabolites produced by *A. flavus* and *A. parasiticus*. AFG_1 and G_2 are produced exclusively by *A. parasiticus*. When lactating cattle and other animals ingest AFB_1 in contaminated feed, toxic metabolites can be formed and may be present in milk. These metabolites, AFM_1 and M_2, are potentially important

contaminants in dairy products. There are four major aflatoxins: B_1, B_2, G_1, G_2 and two additional metabolic products, M_1 and M_2 that are of significance as direct contaminants of foods and feeds (Hathout *et al.*, 2014; Alshannaq and Yu, 2017; Slizewska *et al.*, 2019; Huang *et al.*, 2020). The physicochemical properties of some major aflatoxins are presented in the **Table 2.1**.

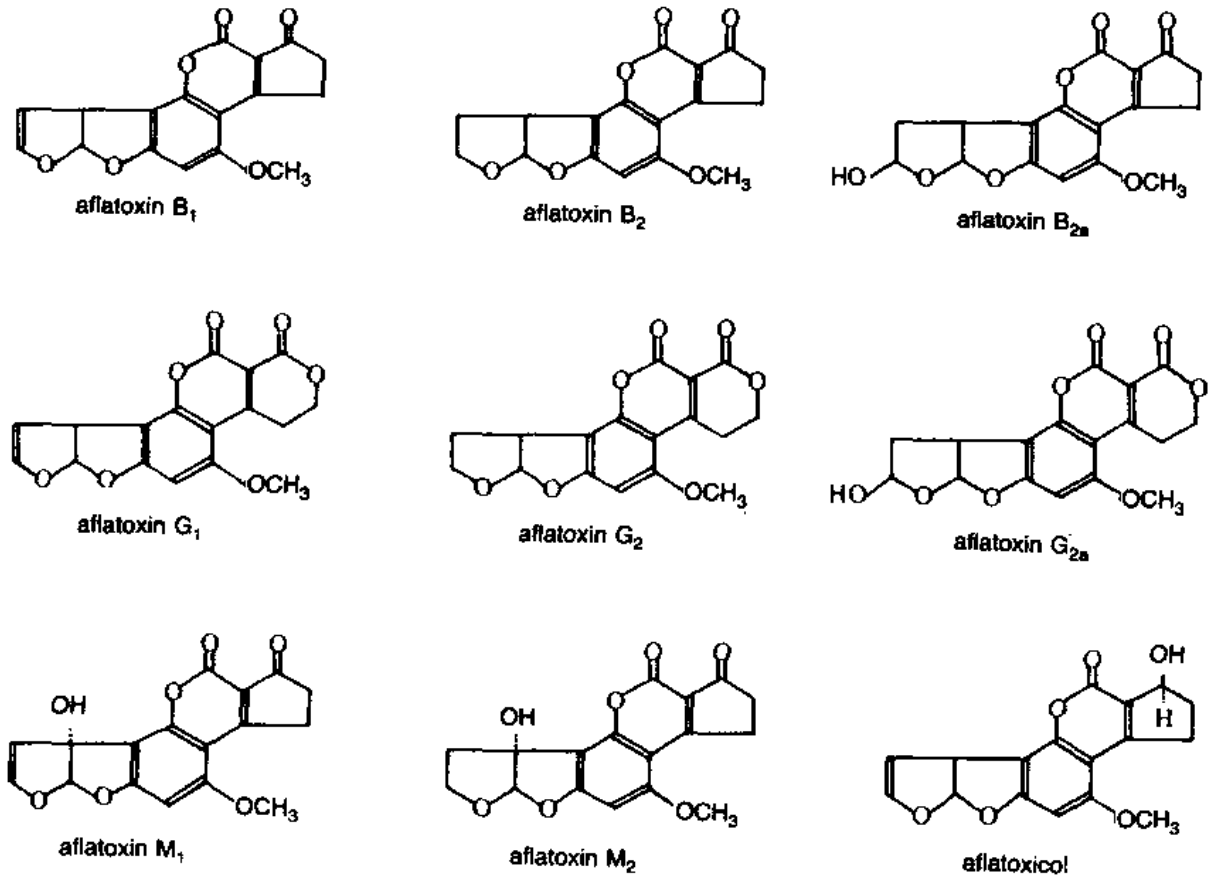
Fig. 2.1 Chemical structures of some of the important mycotoxins



Aflatoxins may be present in a wide range of food commodities, particularly cereals, oilseeds, spices and nuts. Maize, groundnuts (peanuts), pistachios, chillies, black pepper, dried fruit and figs are all known to be high risk foods for aflatoxin contamination, but the toxins have also been detected in many other commodities. Milk, cheese and other dairy products are at risk of contamination by AFM_1 and M_2 (Mohajeri *et al.*, 2013; Mohammadian *et al.*, 2010; Benkerroum, 2020; Gavahian *et al.*, 2021). The highest levels are usually found in commodities from warmer regions of the world where there is a great deal of climatic variation. It is important to recognize that, although primary food commodities usually get contaminated with aflatoxins by mould growth, and toxins produced by them are very stable and may remain stable through quite severe processes and thus due to this reason they can be a problem in processed foods (Ahlberg *et al.*, 2018; Dada *et al.*, 2020; Muaz *et al.*, 2021).

The AFM_1 and M_2 were first isolated from milk of lactating animals fed with aflatoxin preparations; hence, they were designated as M (Talebi and Abedi, 2015).Whereas, B designation of AFB_1 and B_2 resulted from exhibition of blue fluorescence under UV-light, while G refers to yellow-green fluorescence of the relevant structures under UV-light. The AFB_1 has been classified as group 1A carcinogenic agent by the International Agency for Research on Cancer (IARC). High level exposure, to aflatoxins can cause acute toxicity and potentially death, in mammals, birds, fish and humans. The liver is the principal organ affected, however high levels of these aflatoxins have also been found in the lungs, kidneys, brains and hearts of individuals dying of acute aflatoxicosis (Caloni *et al.,* 2012; Kazemi *et al.,* 2008; Gavahian *et al.,* 2021; Plaz Torres *et al.,* 2020; Namulawa *et al.,* 2020).

Fig. 2.2 Chemical structure of aflatoxins



Milk is an important basic food commodity particularly for infants that can get contaminated with AFM_1, a highly toxic substance, has been a matter of considerable concern

and thus is a subject of legislative regulation in many countries. It is necessary to prevent its presence in milk and milk products intented for human consumption or younger ones in particular (Europian commission, 2006; Darsanaki *et al.,* 2019; Moghaddam *et al.,* 2019; Cherkani-Hassani *et al.,* 2020; da Cruz *et al.,* 2021).

2.3 BIOSYNTHESIS OF AFLATOXIN M_1

Aflatoxin M_1 is the principal hydroxylated metabolite of AFB_1.The metabolism process take place at the hepatic level by microsomal cytochrome P450 and can be secreted in the milk of mammals (Caloni *et al.,* 2012; Guven *et al.,* 2020; Muaz *et al.,* 2021, Knipstein *et al.,* 2015).

Table: 2.1 Physicochemical properties of aflatoxins

Types of aflatoxin	Melting point (°C)	Mol. Wt. (Da)	Mol. formula
B_1	268-269	312.3	$C_{17}H_{12}O_6$
B_2	286-289	314.3	$C_{17}H_{14}O_6$
G_1	244-286	328.3	$C_{17}H_{12}O_7$
G_2	237-240	330.3	$C_{17}H_{14}O_7$
M_1	299	328.3	$C_{17}H_{12}O_7$
M_2	293	330	$C_{17}H_{14}O_7$

The conversion rate of ingested AFB_1 to AFM_1 present in the milk is in the range of 0.5-5 % for many species, including dairy cattle and humans (Patterson and Anderson, 1982; Kim and Lee, 2021). Human exposure to AFM_1 is partly from consumption of contaminated milk and dairy products and partly from endogenous production through AFB_1 metabolism in the liver (Neal *et al.,* 1998). Aflatoxin B_1 is a common food contaminant and exposure in humans and animals mainly occurs trough the oral route. The ingestion of AFB_1 results in efficient absorbtion mainly in duodenum (Hsieh and Wong, 1994). Being low molecular weight its passive diffusion into the enterocyte has been suggested as a mechanism of absorption (Kumagai, 1989; Hsieh and

Wong, 1994; Fernandez *et al.*, 1997). Once ingested inside the body, it is absorbed by the intestine and carried over to the liver, the main site of metabolism.

Wherein, AFB_1 is activated and metabolized by cytochromes p450 (CYP) of hepatocytes to AFB_1-8, 9-exo-epoxide, AFB_1-8, 9-endo-epoxide aflatoxin Q_1, M_1 and P_1. The CYP3A4, 3A5, 3A7 and 1A2 are the enzymes involved in its metabolism. Aflatoxin undergoes enzymatic conversion by the microsomal mixed function oxidase (MFO) primarily present in the liver, and probably may also present in the lungs, kidneys and elsewhere. Aflatoxin B_1-8, 9-exo-epoxide is highly unstable when join to nitrogen of guanine, which binds to DNA to form predominant 8, 9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB_1 (AFB_1– N7-Gua) adduct. Aflatoxin B_1-N7-Gua adducts confers the mutagenic properties of the compound (Groopman *et al*., 1985; Madali and Ayaz, 2017; Moon *et al.*, 2018; Celik, 2020). In presence of water, epoxide will be rapidly and nonenzymatically hydrolyzed to AFB_1-8, 9 dihydrodiole, which is able to form Schiff bases with primary amino groups in lysine residues (Sabbioni *et al.*, 1987). One of the proteins, readily available for AFB_1 adduct formation is serum albumin that forms a stable adduct which persist in blood circulation for several days in rats and several weeks for humans. The major CYP enzymes involved in human aflatoxin metabolism are CYP3A4 and 1A2 (Gallagher *et al*., 1996; Ueng *et al*., 1995). The CYP3A4 results in the formation of the *exo*-epoxide and AFQ_1, while CYP1A2 can lead to formation of some *exo*-epoxide but also a high proportion of *endo*-epoxide and AFM_1.

2.4 AFLATOXIN M_1

The health of human population is often reflecting the condition of their food-producing ecosystems. Moreover, implementation of food regulations may be directly linked with the quantity and quality of available food. Therefore, consumers from developing countries, especially from rural areas, face issues related to food security and food safety because they depend on locally produced foods (Marroquin-Cardona *et al.*, 2009; Michlig *et al.*, 2016; Goncalves *et al.*, 2018). The presence of AFM_1 in milk and dairy products is an important issue, especially for developing countries (Prandini *et al.*, 2009; Ismaiel *et al.*, 2020; de Souza *et al.*, 2021).

Milk is a valuable source of nutrients and widely considered a complete nutritional meal. It can also be considered as a source of toxic compounds such as mycotoxins (Ghazani, 2009;

Fallah, 2010; Yaroglu *et al.,* 2005). Aflatoxin M_1 and M_2 are the hydroxylated metabolites of AFB_1 and B_2 and can be found in milk or milk products obtained from animals fed with contaminated feed. In mammals, after 12-24 h of AFB_1 ingestion, AFM_1 can be detected in milk and usually disappears within 24-72 h after stopping the consumption of contaminated feed. The main sources of aflatoxins in feeds are peanut meal, maize and cottonseed meal (Gul *et al.*, (2014; Golge, 2014; Daou *et al.,* 2020). Aflatoxin M_1 has a relative molecular mass of 328 Da and its molecular formula is $C_{17}H_{12}O_7$.

With an intake of AFB_1 for 2-60 mg/cow/day, AFM_1 residues in milk could range between 1 and 50 ppb. Their differences have been reported in amounts of AFM_1 produced by different bovine species. The distribution of AFM_1 in dairy products prepared from contaminated milk is approximately 40-60 % in cheese, 10 % in butterfat and <2 % in buttermilk (Creppy, 2002). The quantity of AFM_1 in milk depends on the concentration of AFB_1 in the contaminated feed. It has been reported that milk is one of the main risk factors of human exposure to AFM_1. Infants are the foremost milk consumers, which make them more susceptible to the adverse effects of mycotoxins (Shundo *et al.,* 2009; Kullmann *et al.,* 2019). The lactating animal could be regarded as intermediate host also due to the biological transformation of AFB_1 to AFM_1 inside the animal body. Consequently, the farm animals may be considered as a reservoir for AFM_1 (Kazemi *et al.,* 2008; Kim *et al.,* 2021).

The information on possible adverse health effects of AFM_1 on human's is scarce. The limited experimental animal studies carried out to determine toxicity and carcinogenicity of AFM_1 indicate it's hepatotoxic and hepatocarcinogenic potential. The acute toxicity of AFM_1 seems to be similar or slightly less than that of AFB_1 but its carcinogenic potency is probably one or even two orders of magnitude lower than that of AFB_1 (Frisvad *et al.,* 2019; Caceres *et al.,* 2020). It should be noted that AFM_1 is not only present in dairy milk, but also in breast milk of nursing mothers. Using AFM_1 as possible marker of exposure to AFB_1, El-Sayed *et al.,* (2002) reported from Egypt a mean level of 0.3+0.5 µg/L in breast milk of nursing mothers, and a corresponding mean blood level of 1.2 µg/L.

The milk could be established as a major carrier of AFM_1 which affects the human health. Generally, presence of aflatoxins in animal or human bodies cause a disease named aflatoxicosis, so the presence of AFM_1 may be specified as aflatoxicosis M_1. The main target

organ in mammals is the liver thus aflatoxicosis is primarily a hepatic disease (Hjartaker *et al.*, 2002; Felicia *et al.*, 2011; Gavahian *et al.*, 2021; Raduly *et al.*, 2020). Aflatoxins also cause decreased milk yield. The occurrence of AFM_1 in milk and milk products is a serious problem of food hygiene. Aflatoxin contamination in milk and products is produced in two ways either toxins pass to milk with ingestion of feeds contaminated with aflatoxin, or it results as subsequent contamination of milk and milk products with fungi. Aflatoxin M_1 has been reported to cause certain hygiene difficulties in milk and milk products used for food (Veldman *et al.*, 1992; Khlangwiset and Wu, 2010; Serraino *et al.*, 2019; Costamagna *et al.*, 2019).

2.5 MECHANISM OF INTESTINAL ABSORPTION OF AFLATOXIN M_1

Toxins absorption is the process whereby any toxins gain access into the body. Ingested materials considered outside the body until they cross the cellular barriers of the gastrointestinal tract. Absorption varies with specific chemicals and route of exposure. The gastrointestinal tract is an organ system responsible for consuming and digesting foodstuffs, absorbing nutrients, and expelling waste (Akande *et al.*, 2006; Kumar *et al.*, 2016; Hassan *et al.*, 2021). Any foreign substances once get enter into the body; it must have to pass through gastrointestinal mucosa, crossing several membranes before entering the blood stream. It was found that absorption of aflatoxins is less within the mouth and esophagus. This may be due very short residential period of substance within these portions of the gastrointestinal tract.

The maximum absorption of chemicals and nutrients takes place in the intestine, particularly in the small intestine; however, very little absorption takes place in the colon and rectum (Battilani *et al.*, 2016). The surface of the proximal intestinal tract is the optimal site of absorption of chemicals or xenobiotics (Jiujiang, 2012). Two major modes of absorption are described for uptake of chemicals from the lumen into the systemic circulation: (a) passive permeability down a concentration gradient (which is most common for xenobiotic absorption) or (b) carrier mediated uptake which can happen either facilitatively (not energy requiring) or actively (energy consuming) (Sahoo *et al.*, 2014; Peles *et al.*, 2021). A third mode of absorption is paracellular passive permeability. Once a chemical has entered the enterocytes, it will be subject to metabolism by the several enzymes, and basically for xenobiotics it is carried out in two steps *i.e.* phase-I and phase-II reactions, both aiming at making components more water soluble and easier to excrete (Chhabra and Tredger, 1978).

Aflatoxin B_1 efficiently absorbed into the duodenum region of small intestine by the mechanism of passive diffusion (Hsieh and Wong, 1994). The AFB_1 enters in the blood stream from the site of absorption and is transported to major site of its metabolism *i.e.* liver. One of the most prominent phase I enzyme families is the cytochrome p450 enzymes (CYPs), a superfamily of membrane associated haemoproteins, concentrated in the endoplasmatic reticulum of liver and intestinal cells (Chhabra and Tredger, 1978; Ding *et al.,* 2012, Wochner *et al* ., 2018; Frisvad *et al.,* 2019; Caceres *et al.,* 2020). Among these, CYP 3A4 is the most important of all human drug metabolizing enzymes, and plays a major role in the intestine, since it is strategically located in high concentrations at the tip of the villus, and is always positioned in close vicinity to the P glycoprotein within the enterocytes (Lindell *et al.,* 2003; Moore *et al.,* 2018). It also plays a role in bioactivation of xenobiotics such as aflatoxin. Furthermore, phase II conjugation enzymes are also found in enterocytes in the gut epithelium (Tejada-Castaneda *et al.,* 2008).

2.6 TOXICITY OF AFLATOXIN M_1

Aflatoxin M_1 has been reported in many parts of the world both in raw milk and many dairy products, caused huged economic losses and human disease. Aflatoxin B_1 is considered to be the most potent hepatocarcinogen, teratogen and mutagen of this group of mycotoxins. The AFM_1, is hydroxylated metabolite of AFB_1, may be found in the dairy products and meat of dairy cattle and mammals that have ingested feedstuffs contaminated with AFB_1. It is therefore not strictly a detoxication product of AFB_1, it can cause serious human disease, especially primary liver cancer, DNA damage and acute toxicity and carcinogenicity comparable with that of the parent molecule *i.e.* AFB_1 (Heinonen *et al.,* 1996; Jalili and Scotter, 2015, Ahlberg *et al.,* 2018; Marchese *et al.,* 2018; Mahuku *et al.,* 2019; Singh, 2021). The acute toxicity of aflatoxins in 1-day-old ducklings suggested that AFM_1 and AFB_1 act by a similar mechanism in causing acute toxicity and subcellular alterations, such as changes in liver parenchymal cells, dissociation of ribosomes from the rough endoplasmic reticulum (van Egmond, 1989; Cai *et al.,* 2020).

Shibahara *et al.,* (1995) studied the potency of AFB_1 and AFM_1 in inducing DNA damage and genotoxicity in *Drosophila melanogaster*. They demonstrated that AFM_1 was a DNA-damaging agent, with an activity about one-third that of AFB_1 however with similar genotoxicity as that of AFB_1. Most of the studies indicated that heat processing, such as pasteurization; UHT treatment and sterilization do not cause an appreciable change in the

amount of AFM_1 in these products (Vagef and Mahmoudi, 2013; Unusan *et al.*, 2006; Ghazani, 2009; Alborzi *et al.*, 2007; Prandini *et al.*, 2009; Daou *et al.*, 2020; Muaz *et al.*, 2021; Wacoo *et al.*, 2020).

Studies on the stability of AFM_1 in milk during cool or frozen storage showed variable results. The storage of frozen contaminated milk and other dairy products for a few months did not appear to affect AFM_1 content (Motawee, 1989). Several possibilities for eliminating or inactivating AFM_1 in milk, involving chemical (sulfites, bisulfites, and hydrogen peroxide) and physical (adsorption and radiation) treatment, have been investigated. Both of described treatments are not readily applicable in the dairy industry, at least at present, as little is known about the biological safety, or the nutritional value of the treated products. Moreover, the costs of the processes may be considerably higher and thus prohibitive for large-scale application.

According to Neal *et al.*, (1998), the human liver microsome has very limited capacity to catalyse the epoxidation of AFM_1. Furthermore, this epoxide also has a lower capacity to bind microsomal protein in comparison to AFB_1. It has been concluded that AFM_1 is a genotoxic carcinogen and less toxic than AFB_1 (Creppy, 2002) with a classification as a group 2B carcinogen, with possible causative effects of cancer to human. It has been observed that AFM_1 has a lower mutagenic potency than its parent compound in one-day-old ducklings, much less carcinogenic than AFB_1 in male Fischer rats and less hepatocarcinogenic in trout (Hsieh *et al.*, 1984; Bailey *et al.* 1996). Aflatoxin M_1 has the same acute toxicity as that of AFB_1 in rats (Sinnhuber *et al.*, 1970; Pong and Noga, 1971).

Caloni *et al.*, (2012) investigated the absorption profile of AFM_1 and possible damage to tight Junction of Caco-2/TC7 cells. It can cause different events including: (i) increase in paracellular permeability to ions; (ii) changes in transcellular ion flux through altered plasma membrane channels or pumps; or (iii) uncontrolled cell death within the monolayer. Aflatoxin M_1 was poorly absorbed in Caco-2/TC7 cells.

Abbes *et al.*, (2013) studied the ability of *L. rhamnosus* GAF01 to remove AFM_1 *in vitro* and to counteract AFM_1 immunotoxicity *in vivo.* Immunotoxicity was determined to measure the number of blood cells and subtype analyses using 2-color flow cytometry, heparinized blood was incubated for 20 min at room temperature together with 0.05 ml of a suspension containing monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)

against CD4, CD8, CD54, or CD56 surface markers. Erythrocytes were lysed with 0.02 ml FACS lyses solution for 10 min at 4°C. The samples were then analyzed on a FACScan flow cytometer. Results induced that damage of immune system reflected as significant decreases in the relative levels of CD4, CD8+, CD54+, CD56+ and total WBC and RBC. Bianco *et al.,* (2012) investigated the immune-effects of AFB_1, AFB_2, AFM_1 and AFM_2, alone and differently combined, in J774A1 murine macrophages.

MTT assay showed that AFB_1, alone and combined with AFB_2, possess anti-proliferative activity only at the highest concentration; such effect was not shown by their hydroxylated metabolites, AFM_1 and AFM_2, respectively. Furthermore, the mixtures of AFB_1+AFB_2 and AFM_1+AFM_2 significantly affected nitrous oxide release both at the concentration of 15 and 30 μM. These data suggest that the co-exposure to AFB_1, AFB_2, AFM_1 and AFM_2, frequently present as food contaminants, may adversely affect immune system. Salah-Abbes *et al.,* (2015) studied the potential preventive role of lactic acid bacteria against AFM_1 immunotoxicity and genotoxicity in mice. The results showed that, compared to in control mice, AFM_1 treatment led to significantly decreased body weight gains; total WBC levels in the blood were significantly increased in the AFM_1-treated hosts; suppressive effect in thymus and spleen cellularity was observed in mice treated with AFM_1 and caused cytotoxic/genotoxic effects as indicated by increases in frequencies of polychromatic erythrocytes, as well as those with micronucleation (PCEMN) and chromosomal aberrations, among bone marrow cells.

Mice receiving AFM_1+*Lactobacillus plantarum* MON03 co-treatment displayed no significant differences in the assayed parameters as compared to the control mice. Similarly, Jebali *et al.,* (2015) investigated the immunotoxic effect of AFB_1 and AFM_1 in mice. They found suppressive effect on thymic and splenic cellularity; in mice treated with AFB_1 and AFM_1. Spleens of mice treated with AFB_1 and AFM_1 showed focal necrosis, vascular dilatation, and lymphoid infiltration. Thymus swelling in cortical cells of the proximal lobules, granular degeneration, shrunken dendritic cells, a presence of eosinophilic casts, and blood vessel dilatation was observed with both of aflatoxins. Significant decreas in mRNA expression of IFNγ and TNFα and increased expression level of IL-4 in the spleen.

This indicated that AFB_1 and AFM_1 could have a direct impact on inflammatory cytokine expression. Cytotoxic effect on Caco-2 cells, especially the differentiated one that resemble

mature small intestinal enterocytes. Both undifferentiated (UC) and differentiated (DC) cells were treated with AFB_1 and AFM_1 at various concentrations (0, 0.01, 0.05, 0.1, 0.5 and 1 mg/mL AFB_1 and AFM_1) for up to 72 hours. Cell viability, lactate dehydrogenase (LDH) release, reactive oxygen species (ROS) production and DNA damage were determined. Results showed that AFB_1 and AFM_1 significantly inhibited UC and DC cell growth, increased LDH and caused genetic damage. In comparison, AFB_1 was found to be more toxic than AFM_1 on both UC and DC. All these cytotoxic outcomes might be associated with intracellular ROS generation, leading to membrane damage and DNA strand break.

Additionally, DC was found to be more sensitive to aflatoxins, which might be due to the alteration of enzymes during cell differentiation (Zhang *et al.*, 2015). Earlier taking into consideration the toxicity of AFM_1, it was initially classified by IARC as an agent in Group 2B with possible carcinogenic effect on humans, however it has been recently reclassified as Group 1 carcinogenic agent (IARC, 2002). Codex Alimentarius have fixed the limit to a maximum of 0.5 ppb. When AFM_1 is present at concentration of 0.5 ppb or greater, the milk is discarded because it cannot be used for product preparation that goes into the human food supply chain. Milk is a major commodity for introducing aflatoxin M_1 in human diet. Evidence of hazardous human exposure to AFM_1 through dairy products has been reported (Kamkar *et al.*, 2005; Rahimi *et al.*, 2010).

Recently, Jebali *et al.*, (2015) studied disturbances in intestinal genes expression and DNA fragmentation in mice treated orally with aflatoxin B_1 (AFB_1) or aflatoxin M_1 (AFM_1) and the protective activity of *Lactobacillus plantarum* (LP). Male Balb/c mice were divided into 6 groups including the control group, the group treated with 2 mg/kg BW of LP (2×10^9 cfu/mL), the groups treated with AFB_1 or AFM_1 (100 μg/kg BW), and the groups treated with AFB_1 or AFM_1 during, after or before LP. Small intestines were collected for the determination of DNA fragmentation, gene expression and target protein content. They were found that AFB_1 or AFM_1 increased DNA fragmentation, down regulated the expressions of caspase-3, caspase-9, CYP3A13, Bax and p53 as well as up-regulated the expression of TNF-α and Bcl-2 and their target proteins. LP succeeded to alleviate the disturbances in DNA fragmentation and the expression of these genes. The improvement was more pronounced in the group co-administered with the toxins plus LP. It could be concluded that AFB_1 and AFM_1 induced disturbances

in intestinal function via the disturbances in DNA fragmentation and genes expression. LP induced a potential protective effect and is considered a promising agent against the genotoxicity induced by these mycotoxins.

Marchese *et al.,* (2018) evaluated the effects of AFM_1 on the cell viability, apoptosis, cell cycle, and metabolomic and cytokinomic profile of HepG2 cells after treatment and they were found AFM_1 induced: (1) a decrease of HepG2 cell viability, reaching IC_{50} at 9 µM; (2) the blocking of the cell cycle in the G0/G1 phase; (3) the decrease of formiate levels and incremented level of some amino acids and metabolites in HepG2 cells after treatment; and (4) the increase of the concentration of three pro-inflammatory cytokines, IL-6, IL-8, and TNF-α, and the decrease of the anti-inflammatory interleukin, IL-4. Our results show that AFM_1 inhibited the growth of HepG2 cells, inducing both a modulation of the lipidic, glycolytic, and amino acid metabolism and an increase of the inflammatory status of these cells.

In the recent study Shirani *et al.,* (2021) investigated the effects of AFM_1 on immune system and its modulation by MicroRNA (miR)-155. They were found that the spleen weight was reduced in mice exposed to AFM_1 compared to negative control. Proliferation of splenocytes in response to phytohemagglutinin-A was reduced in mice exposed to AFM_1. IFN-γ was decreased in mice exposed to AFM_1, whereas IL-10 was increased. Concentration of IL-4 did not change different in mice exposed to AFM_1 compared to negative control. Exposure to AFM_1 reduced the expression of miR-155. Significant upregulation of phosphatidylinositol-3, 4, 5-trisphosphate 5-phosphatase 1 (Ship1) and suppressor of cytokine signaling 1 (Socs1) was observed in isolated T cells from spleens of mice treated with AFM_1, but the transcription factor Maf (c-MAF) was not affected. These results suggest that miR-155 and targeted proteins might be involved in the immunotoxicity observed in mice exposed to AFM_1.

2.7 LEGISLATION OF AFLATOXIN M_1

In today's changing world, safety and security have generally remained basic human needs. Ensuring the safety of food has been a major focus of international and national action over the last years. Both microbiological and chemical hazards are of concern. Among chemical hazards, the contamination of food and feed by mycotoxins, fishery products by phycotoxins (toxins produced by algae) and edible plant species by their plant toxins have been characterized

by the World Health Organization (WHO) as significant sources of food-borne illnesses. Amongest three categories of natural toxins, most attention have been directed to mycotoxins particularly aflatoxins until now. In several parts of the world, aflatoxins currently represent a major food safety issue. The knowledge that aflatoxins can have serious effects on humans and animals has led many countries to establish regulations on aflatoxins in food and feed in the last decades to safeguard the health of humans, as well as economical interests of producers and traders.

Due to the grave health impacts of aflatoxins, they are highly being observed in food imports and exports. Within the European Union (EU), diverse detailed and harmonized regulations for aflatoxins in food exist, and maximum levels have been set for AFB_1 and for total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) in cereals, grains, groundnuts, dried fruit and spices, as well as for AFM_1 in milk and milk-products (Commission Regulation 2003/2174/EC amending Commission Regulation 2001/466/EC). According to the FAO (2011) approximately 60 countries have set specific limits for AFM_1. Since declaration of AFM_1 as human carcinogen by IARC (2002), it is particularly monitored by regulatory bodies and the agencies have imposed stringent limiting standards in comparison with the 20 ppb specified limit of other mycotoxins in foods (Serrano-Nino *et al.*, 2013).

The maximum permissible limit for AFM_1 varies greatly from country to country **(Table 2.2)** depending on the developmental and economic condition of a country. The EU limits the total aflatoxin's level to 20 ppb in lactating dairy feeds and 0.05 ppb in milk. A number of factors are involved in setting legal limits for aflatoxins such as AFM_1 including availability of survey data for the toxic compounds, data regarding the level of toxin contamination in various commodities and methods of analysis. More or less all the developed countries have set maximum permissible limits for AFM_1, while most of the developing countries are relying on the legal limits set by international agencies like European Union (European Commission, 2006). The legal limit for AFM_1 in milk ranges from 0.05 ppb as adopted by EU and Codex Alimentarius Commission and as high as 0.5 ppb adopted by Brazil and Serbia. The lenient standard limits for AFM_1 adopted by many of the developing countries could be linked with the higher prevalence rate of liver cancer in developing countries of Asia and Africa and vice versa for the developed countries of Europe and America (Jemal *et al.*, 2010).

The maximum limit of AFM_1 for mother's milk established by European Community is 0.025 ppb (Adejumo *et al.,* 2013). However, many of the developing countries have more flexible permissible limits for AFM_1 than that of the EU possibly due to economic constraints and lack of scientific and consumer based knowledge.

2.8 PREVALENCE OF AFLATOXIN M_1 IN MILK AND MILK PRODUCTS

Worldwide occurrence of AFM_1 in milk and milk products has been summarized in **Table 2. 3**. The maximum prevalence of AFM_1 has been reported in the developing economies, specifically the African and Asian countries where levels of toxin in milk and milk products were found alarming. Considering the EU limits for AFM_1 as standards, nearly 100 % of the samples of milk and milk products studied in Nigeria and Sudan were reported to contain exceptionally higher levels of AFM_1 than the stated norms (Suliman and Abdalla, 2013; Susan *et al.,* 2012; Venâncio *et al.,* 2018). Several studies have been conducted in South Asia for the presence of AFM_1 in milk and dairy products. Among them, most of the reports are from Iran and Pakistan, and some of the studies presented very high mean concentrations of AFM_1 in milk.

In Iran, 98 samples were positive for AFM_1 with an overall mean level of 0.053 ppb. Levels of the toxin were also higher in winter and spring than in summer and autumn Bovo *et al.,* (2012), while in Sarab city of Iran, 77 % (total 111 raw milk) samples were found contaminated with AFM_1 levels exceeded the tolerance limit of 0.050 μg/L (Kamkar *et al.,* 2005).

Table: 2.2 Maximum permissible limits of AFM_1 for milk and milk-products followed in various countries

Country	Maximum permissible limit (ppb)	Type of product	References
European Union	0.05	Milk	European Union, (2006)
	0.25	Cheese	Elkak *et al.,* (2012)
USA	0.50	Milk	Ertas *et al.,* (2011)
Switzerland	0.05	Milk	Dashti *et al.,* (2009)
	0.25	Cheese	Dashti *et al.,* (2009)
Australia	0.05	Milk	Fallah, (2010)

Germany	0.05	Milk	Fallah, (2010)
Belgium	0.05	Milk	Fallah, (2010)
Sweden	0.05	Milk	Fallah, (2010)
France	0.05	Milk	Dashti *et al.,* (2009)
Iran	0.05	Milk	Sani *et al.,* (2010)
	0.20	Cheese	Mohajeri *et al.,* (2013)
	0.05	Yoghurt	Fallah, (2010)
	0.05	Ice cream	Fallah, (2010)
China	0.50	Milk	Xiong *et al.,* (2013)
Turkey	0.05	Milk	Bakirci, (2001)
	0.025	Cheese	Sarimehmetoglu and Kuplulu, (2004)
	0.05	Yoghurt	Atasever *et al.,* (2011)
Netherlands	0.02	Butter	Fallah, (2010)
	0.20	Cheese	Fallah, (2010)
Brazil	0.50	Milk	Dashti *et al.,* (2009)
Japan	0.50	Milk	Han *et al.,* (2014)
India	0.50	Milk	Siddappa *et al.,*(2012)
Morocco	0.05	Milk	Zinedine *et al.,* (2007)
	0.50	Milk Powdered	Zinedine *et al.* (2007)

Fallah *et al.,* (2009) also conducted a study to determine the prevalence of AFM_1 in milk and milk products from Iran. They reported mean levels of AFM_1 in milk (0.323 ppb), cheese (0.085 ppb) and yogurt (0.016 ppb).

Similarly, the incidence of AFM_1 in milk (0.252 ppb) was reported by Sadia *et al.* (2012) from Pakistan. The mean AFM_1 levels in milk (0.212 pbb), yogurt (0.147 ppb), cheese (0.189 ppb), and butter (0.156 ppb) have been also reported from Pakistan (Iqbal *et al.,* 2012). Moreover, in India 87.3 % of a total of 87 liquid milk samples, showed contamination of AFM_1 ranging from 28–164 μg/L. Almost 99 % of the contaminated samples exceeded the European Commission limit (Bovo *et al.,*2012). The analysis of one hundred and ten milk samples

collected from dairy farmers in Ethiopia. Results showed presence of AFM_1 in all milk samples, and contamination level ranged between 0.028 and 4.98 ppb. Overall, only nine (8.2 %) out of a total of 110 milk samples contained less than or equal to 0.05 μg/L of AFM_1. Furthermore, 29 (26.3 %) milk samples exceeded 0.5 μg/L of AFM_1 (Dashti *et al.,* 2009).

Bahrami *et al.,* (2016) analyzed 358 samples consisting of raw milk of cow (n = 64), goat (n = 56) and sheep (n = 52); traditional cheese (n = 40), yoghurt (n = 42), Kashk (n = 40), Doogh (n = 44) and Tarkhineh (n = 20) for AFM_1 by using an ELISA. They found AFM_1 contamination in 84.3, 44.6 and 65.3 % for cow, goat and sheep raw milks, respectively. Whereas it was 65.5, 23.8, 14, 13.6 and 35.0 % of cheese, yoghurt, Kashk, Doogh and Tarkhineh samples, respectively. Percentages of cow milk, goat milk, sheep milk and cheese samples exceeding the EU limit were 35.9, 11.1, 26.9 and 10, respectively. The levels of AFM_1 were comparatively higher in milk and other dairy products that might have been a serious health hazard for consumers.

The extensive variations in AFM_1 levels among different studies conducted in South Asia could not only be related to climatic and geographic differences but also due to differences in feeding systems, farm management practices, and analytical methods (Asi *et al.,* 2012). During summer, especially in South Asia, fresh animal feed such as pasture, grass, weeds and green fodder is available. However, due to the shortage of fresh green feed during the winter, more concentrated feeds consisting of wheat, corn and cotton seeds are used. Furthermore, green fodder and hay preserved as silage under inadequate storage conditions may be attacked by *Aspergillus* species, and subsequently, aflatoxins may be produced (Kamkar, 2005; Tajkarimi *et al.,* 2008).

Van Egmond, (1989) reported that excreted amount of AFM_1 in milk of dairy cow was 1-2 % of ingested AFB_1. However, the mean carry-over rates for mares were 0.04-0.05 %, for ewes they ranged from 0.60 to 0.72 % (with a maximum of 2.7 %), and for goats they ranged from 2.5 to 2.7 % (Shephard, 2008). Aflatoxin M_1 can be found in milk within 12–24 hours after the first ingestion of AFB_1 (Battacone *et al.,* 2003), and increased as soon as the first milking after animal ingestion with a pattern of increment up between 7^{th} and 12^{th} days of AFB_1 ingestion (Battacone *et al.,* 2003).

In a related study, dairy cows in the early lactation stage (2-4 weeks) and in late lactation weeks (34-36 weeks) were fed with AFB_1 contaminated feed. After 12 days, the carry-over of AFM_1 in the milk was 6.2 % in the early stage, but it declined to 1.8 % in the late lactation stage (Veldman *et al.,* 1992; Masoero *et al.,* (2007) has suggested that milk yield is one of the major factors affecting the total excretion of AFM_1. High yielding dairy cows with yield of up to 40 liters per day, showed a carry-over percentage as high as 6.2 % has been reported.

A health and food survey of 112 Nigerian children of whom 79 were experiencing protein energy malnutrition have been reported with the risk of AFM_1 toxicity through contaminated milk. Extremely dangerous levels of AFM_1 were reported in the milk samples fed to protein energy malnourished and healthy children of Nigeria (Onyemelukwe *et al.,* 2012). Similarly, earlier studies from Pakistan also reflect the same picture of food safety, indicating that 94 % of the milk possessed AFM_1 levels higher than EU tolerable limits (Hussain and Anwar, 2008). Almost parallel trends of AFM_1 prevalence in milk were suggested by Sefidgar *et al.,* (2011) and Panahi *et al.,* (2011) validating 100 % of raw and pasteurized milk samples in Iran carrying the contaminant at higher than prescribed EU standards.

A study conducted in India by Siddappa *et al.,* (2012) analyzed 45 sample of raw milk, 7 of pasteurized and 45 UHT milk for contamination of AFM_1 from Karnataka and Tamilnadu, they found that 100 % of raw milk samples were contamination at the level of 0.6-3.8, 42.9 % of pasteurized milk at 1.8-3.8 and 64.4 % of UHT milk at the range of 0.5-2.1 ppb. Out of total of 52 samples of raw and pasteurized milk samples received from the same states were showed 61.6 % were positive for AFM_1 within a range of 0.1 to 3.8 ppb. The content in 17.3 % of these samples was more than 0.5 ppb. In India, there have been reports on the prevalence of AFM_1 in raw milk. Choudhary *et al.,* (1997) have reported AFM_1 contamination of 94 % of raw and pasteurized milk ranging from 0.066 to 0.763 ppb in the 223 samples collected from Anand, Gujarat.

Rajan *et al.,* (1995) analyzed 504 raw milk samples from Thrissur, Kerala noted that 17.7 % were contaminated with AFM_1 at a range of 0.1–3.5 ppb. Kang and Lang, (2009) analyzed 81 raw milk samples from Ludhiana, and found 6.2 % positive with one sample having a high concentration of 13.3 ppb. Six hundred and thirteen milk samples were analyzed from four urban centers of Kenya for AFM_1 contamination using competitive enzyme immunoassay. They

reported 72 % of milk from dairy farmers, 84 % from large and medium scale farmers, 99 % of the pasteurized milk was positive for AFM_1. Twenty, 35 and 31 % of milk samples, from medium, large scale dairy farmers and market outlets respectively, exceeded WHO/FAO levels of 0.05 ppb.

However, in some East Asian countries such as Indonesia, Japan, Thailand, Korea, and China relatively high levels of AFM_1 have been documented. Stark, (2010) reported AFM_1 levels in milk (0.04-0.16 ppb) and milk powder (0.16-0.32 ppb) from China. Similarly, Xiong *et al.,* (2013) reported a range of 0.01-0.42 ppb in milk from China. Moreover, the incidence of AFM_1 in milk from Thailand is considerably high relative to other countries. Middle East, Africa, Latin America, AFM_1 in milk and dairy products has also been documented in Syria (Ghanem and Orfi, 2009), Egypt (Motawee *et al.,* 2009), Lebanon (El-Khoury *et al.,* 2011; Elkak *et al.,* 2012), Sudan , Kuwait (Dashti *et al.,* 2009), Morocco (El Marnissi *et al.,* 2012), Serbia (Kos *et al.,* 2014), and Brazil (Iha *et al.,* 2013; Oliveira and Ferraz, 2007; Shundo *et al.,* 2009). The highest concentration (2.07 ppb) and incidence (42/44) of milk samples contaminated with AFM_1 were noticed in Sudan. A total of 42 samples were above the recommended limit of 0.05 ppb.

In Morocco, Zinedine *et al.,* (2007) found that 89 % of milk samples were contaminated with AFM_1 at a mean level of 0.0186 ppb, and 3 samples were found above the EU recommended limit. Similarly, a high mean level (0.062 ppb) of AFM_1 in milk was reported by Oliveira *et al.,* (2007) in Brazil, and the levels ranged from 0.011 to 0.161 ppb. Mean levels of less than 0.018 to greater than 0.250 ppb were reported in milk from Egypt (Motawee *et al.,* 2009). These data show a high incidence of AFM_1 in milk and dairy products, especially from African countries such as Sudan. The lack of awareness and constraints in analytical facilities are major causes of the high incidence of this toxin in milk and milk products.

However, in some European countries, relatively low levels of AFM_1 were determined in milk samples (Trucksess, 2006). These differences may be related to stringent regulations of AFB_1 in complementary feedstuffs for dairy cattle in European countries (Kamkar *et al.,* 2011). The occurrence of AFM_1 in European milk and dairy products has been reported in Turkey, France, Italy, Spain, Croatia and from Greece. In Turkey, Ardic *et al.,* (2009) found a mean AFM_1 level of 0.284 ppb in white brined cheese with the concentration ranging from 0.052 to 0.860 ppb.

In another report, Tekinse *et al.,* (2008) analyzed 100 milk and 132 cheese samples and reported that 67 and 83 % of these milk and cheese samples, respectively, were found contaminated with AFM_1. The levels of AFM_1 in milk and cheese ranged from 0.010 to 0.630 ppb and from 0.05 to 0.690 ppb, respectively. The range of AFM_1 levels from Turkey, followed by Croatia, is considerably higher as compared to other countries. Eighty five pasteurised milk samples were analysed for AFM_1 with ELISA technique in Turkey. Seventy-five samples (88.23 %) were found to be contaminated with AFM_1, and 48 samples (64 %) exceeded the legals level of AFM_1 in milk according to the Turkish Food Codex and Codex Alimentarius limits 50 ppb (Celik *et al.,* 2005).

A total of 1668 milk samples were analyzed by ELISA which identified 36 (2.2 %) milk samples positive abd these were subsequently confirmed by HPLC (Alborzi *et al.,* 2016). The levels of AFM_1 in the positive samples ranged between 0.182 and 0.208 ppb. Of the total samples, only eight (0.5 %) were found non-compliant with the EU regulatory limit (0.50 ppb). The major reasons behind the high incidence of AFM_1 in developing countries could be farmer and consumer's ignorance, economic and technological constraints and suitable environment for optimum growth of toxin producing fungus. Better control over AFM_1 occurrence in milk and milk products developed nations might be associated with strong food and feed standards, stringent vigilance systems, law enforcement, standards, technological advancements, increased farmer and consumer awareness and unfavorable environmental conditions for toxin production (Cano-Sancho *et al.,* 2010; Ertas *et al.,* 2011, de Freitas *et al.,* 2018, Rahmani *et al.,* 2018; Bran *et al.,* 2020). A study was conducted to explore the incidence of AFM_1 in milk and dairy products marketed in Qatar. Milk ($n = 72$), yogurt ($n = 21$), cheese ($n = 46$), butter ($n = 18$) and laban ($n = 25$) samples were initially screened by competitive enzyme linked immunosorbent assay (ELISA) for the presence of AFM_1, followed by confirmation with ultra-high-performance liquid chromatography (UHPLC). AFM_1 was detected in 85 %, 76 %, 85 %, 67 % and 76 % of the milk, yogurt, cheese, butter and laban samples, respectively.

Ansari *et al.,* (2019) assess AFM_1 contamination in 100 samples of pasteurized milk which were conventionally gathered during spring, summer, autumn, and winter from supermarkets located in Maragheh city of northwestern Iran. Samples were evaluated for AFM_1 with a high-performance liquid chromatography (HPLC) method and with fluorimetric

detection. They were found that approximately 44 % (11.25) of samples in winter, 32 % (8.25) of samples in spring, 24 % (6.25) of samples in summer, and 20 % (5.25) of samples in autumn had AFM_1 concentrations that exceeded the limit (0.05 μg/L) set by the European, Codex Alimentarius Commission and Iran standards. According to the statistical analysis of the data, there was no significant variation between the mean content of AFM_1 during different seasons ($P = 0.076$). The results of this study suggest a high level of contamination of AFM_1 in pasteurized milk in all seasons which may be due to the fact that milk supply for dairy factories is provided from dairy farms that are low in livestock feed quality.

Ahmad *et al.,* (2018) was quantified AFM_1 in raw and processed milk from Lahore and Multan. The results indicated that approximately 90 % of the raw milk samples collected from Lahore city was contaminated with aflatoxin M_1. Similarly, around 92 % of the raw milk samples collected from Multan city was contaminated with aflatoxin M_1.

In this survey aflatoxin M_1 was quantified in raw and processed milk from various areas of two big cities of Punjab province, *i.e.* Lahore and Multan. The results indicated that approximately 90 % of the raw milk samples collected from Lahore city was contaminated with aflatoxin M_1. Similarly, around 92 % of the raw milk samples collected from Multan city was contaminated with aflatoxin M_1 (Ahmad *et al.,* 2019).

A total of 734 milk samples were examined for occurance of aflatoxin M_1 and among of them 590 milk samples were found aflatoxin M_1 contaminated (Xiong *et al.,* 2020). The 150 samples of milk from Hisar city of Haryana, India, were investigated for AFM_1 contamination. Out of these, 40 samples contained AFM_1 at a concentration below the limit of detection (LOD) of 0.052 μg/kg. Among the AFM_1 contaminated samples, 46 raw milk samples contained a concentration above the LOD but less than the limit of quantitation (LOQ), whereas 64 samples were above the LOQ. Of these samples, 31 exceeded the maximum limit of 0.5 μg/kg prescribed by FSSAI, India (Sharma *et al.,* 2020).

The total 67 liquid milk samples (46 pasteurized and 21 UHT) randomly collected during 2019 from supermarkets and dairy shops in four Moroccan cities and tested for AFM_1 contamination. Analytical results indicated that out of the 67 analyzed samples, 9 (13.4 %) were

considered as positively contaminated, while 58 samples (86.6 %) had AFM_1 under the detection limit (Mannani *et al.*, 2021).

Table: 2.3 World wide incidence of AFM_1 in milk and dairy products

Country	Type of product	Number of samples analysed	Number of positive samples	References
Argentina	Milk	56	6	Lopez *et al.*, (2003)
	Powdered milk	5	4	
	Pasteurized milk	16	8	
	Raw milk	160	160	Michlig *et al.*, (2016)
Algeria	Raw milk	47	5	Garrido *et al.*, (2003)
Brazil	UHT milk	60	53	
	Pasteurized milk	79	58	
	Pasteurized milk	43	17	Gonçalez *et al.*, (2005)
	Raw milk	36	19	Pereira *et al.*, (2005)
	Pasteurized milk	34	13	
	Raw milk	42	10	Sassahara *et al.*, (2005)
	UHT milk	40	40	Shundo *et al.*, (2009)
	Pasteurized milk	10	7	
	Powdered milk	75	72	
	UHT milk	75	23	Oliveira *et al.*, (2013)
	Minas frescal cheese	58	49	Iha *et al.*, (2011)
	Yoghurt	53	47	
	Dairy drink	12	10	
	Minas frescal cheese	24	6	Oliveira *et al.*, (2011)
	Minas padrao cheese	24	7	

	Raw milk	129	129	Picinin *et al.,* (2013)
	Milk Powder	112	50	Goncalves *et al.,* (2018)
China	Raw cow milk	200	65	Han *et al.,* (2014)
	UHT milk Pasteurized milk	153	84	Zheng *et al.,* (2013)
	Milk	233	112	Guo *et al.,* (2013)
	UHT Milk	111	32	Xiong *et al.,* (2018)
	Raw Milk	1207	56	Li *et al.,* (2018)
Croatia	Fresh milk	61	61	Bilandzi *et al.,* (2010)
	Bulk cow milk	337	59	Bilandzi *et al.,* (2014)
	Raw goat milk	32	2	
	Raw sheep milk	19	0	
	Raw donkey milk	14	0	
Egypt	Yoghur	25	3	Baranyi *et al.,* (2013)
	Processed cheese	25	11	
	Infant formula milk powder	15	2	
	UHT milk	15	7	
	Raw milk	50	10	Yaroglu *et al.,* (2005)
	Milk powder	50	19	
	Roomy cheese	50	24	
	Kariesh cheese	50	14	
Greece	Raw cow milk	30	22	Roussi *et al.,* (2002)
	Raw goat milk	10	4	
	Raw sheep milk	12	8	
	Pasteurized milk	82	70	
	UHT milk	17	14	
	Concentrated milk	15	14	
	Bulk cow milk	234	43	Malissiova *et al.,* (2013)

India	Infant milk food	17	17	Rastogi *et al.,* (2004)
	Infant formula	18	17	
	Milk based cereal weaning food	40	38	
	Liquid milk	12	4	
	UHT (plain Milk)	21	21	Siddappa *et al.,* (2012)
	UHT (flavored Milk)	24	9	
Iran	Raw milk	111	85	Kamkar, (2005)
	Pasteurized milk	624	624	Alborzi *et al.,* (2007)
	Pasteurized milk	128	128	Oveisi *et al.,* (2007)
	Infant formula	120	116	
	Raw milk	72	72	Tajik *et al.,* (2007)
	Pasteurized milk	72	72	
	Raw milk	98	98	Tajkarimi *et al.,* (2007)
	Raw milk	319	172	Tajkarimi *et al.,* (2008)
	Pasteurized milk	50	50	Ghazani, (2009)
	UHT milk	210	116	Heshmati and Milani, (2010)
	Pasteurized milk	116	83	Fallah, (2010)
	UHT milk	109	68	
	Pasteurized milk	91	66	Fallah, (2010)
	Raw milk	240	226	Mohammadian *et al.,* (2010)
	Pasteurized milk	32	31	
	Pasteurized milk	90	90	Nemati *et al.,* (2010)
	Raw milk	122	122	Kamkar *et al.,* (2011)
	Raw cow milk	88	74	Fallah *et al.,* (2011)
	Raw goat milk	65	28	
	Raw sheep milk	72	43	
	Pasteurized milk	42	41	Mohamadi *et al.,* (2012)
	Pasteurized milk	80	77	Moosavy *et al.,* (2013)

	Raw milk	90	56	Rokhi *et al.,* (2013)
	Pasteurized milk	45	45	Riahi-Zanjani and Balali-Mood, (2013)
	Feta cheese	80	66	Kamkar, (2006)
	Yoghurt	68	45	Fallah, (2010)
	White cheese	72	59	
	Ice cream	36	25	
	Butter	31	8	
	Lighvan cheese	75	49	Fallah *et al.,* (2011)
	Industrial yoghurt	61	30	
	Traditional yoghurt	60	14	
	Industrial kashk	64	34	
	Traditional kashk	61	19	
	Industrial doogh	71	16	
	Traditional doogh	65	9	
	Yoghurt	40	14	Nilchian and Rahimi, (2012)
	Cheese	40	16	
	Ice cream	40	12	
	White cheese	50	30	Tavakoli *et al.,* (2012)
	White cheese	45	29	Mohajeri *et al.,* (2013)
	Lighvan cheese	37	10	
	Cow milk	192	46	Fallah *et al.,* (2010)
	Ice-cream	90	62	Darsanaki *et al.,* (2019)
	Milk	518	515	Moghaddam *et al.,* (2019)
Italy	UHT milk	161	125	Galvano *et al.,* (2001)
	Raw milk	296	5	Decastelli *et al.,* (2007)
	Fresh pasteurized and UHT milk	316	2	Nachtmann *et al.,* (2007)

	Raw milk	51	27	Santini *et al.,* (2013)
	Bulk milk	10	3	
	UHT milk	12	5	
	Grana padano cheese	223	219	Peitri *et al.,* (2009)
	Yoghurt	120	73	Galvano *et al.,* (2001)
	Dry milk for infant formula	92	49	
	Sheep cheese	94	12	Montagna *et al.,* (2008)
	Cow cheese	92	25	
	Buffalo cheese	51	0	
	Sheep-goat cheese	16	5	
	Goat cheese	12	2	
	Cheese	102	85	Anfossi *et al.,* (2012)
	Cheese	17	7	Santini *et al.,* (2013)
	Milk cream	7	3	
Japan	UHT milk	208	207	Nakajima *et al.,* (2004)
Kuwait	Milk and milk products	54	14	Srivastava *et al.,* (2001)
	Fresh cow milk	177	176	Dashti *et al.,* (2009)
	UHT cow milk	105	-	
	Powdered milk	27	-	
	Cheese	40	32	
Lebanon	Cheese	111	75	Elkak *et al.,* (2012)
Morocco	Pasteurized milk	54	48	Zinedine *et al.,* (2007)
	Raw cow milk	48	13	El Marnissi *et al.,* (2012)
Pakistan	White cheese	119	93	Iqbal *et al.,* (2015)
	Cheese cream	150	89	
	Yoghurt	96	59	

	Butter	74	33	
	Yoghurt	96	32	
	Butter	70	35	
	Ice cream	79	27	
	Raw cow milk	168	168	Hussain and Anwar, (2008)
	Raw buffalo milk	360	153	
	Raw cow milk	120	63	
	Buffalo milk	55	19	Hussain *et al.,* (2010)
	Cow milk	40	15	
	Goat milk	30	6	
	Sheep milk	24	4	
	Camel milk	20	0	
	Buffalo milk	94	46	Iqbal *et al.,* (2011)
	Cow milk	84	42	
	Milk	232	177	Sadia *et al.,* (2012)
	Raw cow milk	107	76	Iqbal *et al.,* (2015)
	Raw cow milk	104	39	Iqbal *et al.,* (2015)
	UHT milk	84	35	
	Raw milk	106	100	Ismail *et al.,* (2016)
Portugal	Raw milk	31	25	Martins and Martins, (2000)
	UHT milk	70	60	
	Raw milk	598	394	Martins *et al.,* (2005)
	Natural yoghurt	48	2	
	Yoghurt with pieces of strawberries	48	16	Martins and Martins, (2004)
	Pasteurized milk	4	0	Duarte *et al.,* (2013)
	UHT milk	36	17	
Serbia	Raw, Pasteurized	50	38	Skrbi *et al.,* (2014)

	and sterilized milk			
	Cow milk	150	148	Kos *et al.*, (2014)
	Goat milk	10	8	
	Donkey milk	5	3	
	Raw milk	678	56	
	Heat treated milk	438	32	
	Milk products	322	37	
South Africa	Bulk milk	90	90	Dutton *et al.* , (2012)
	Raw milk from rural subsistence (RSFs)	125	107	Mulunda and Mike, (2014)
	Raw milk from commercial dairy farms (CDFs)	100	26	
South Korea	Raw cow milk	100	48	Lee *et al.,* (2009)
Spain	Raw cow milk	92	5	Rodríguez *et al.,* (2003)
	UHT milk	72	68	Cano-Sancho *et al.,* (2010)
	Cheese	72	0	Cano-Sancho *et al.,* (2010)
	Yoghurt	72	2	
Sudan	Raw milk	35	35	Ali *et al.,* (2014)
Syria	Raw milk	74	70	Ghanem and Orfi, (2009)
	Raw sheep milk	23	13	
	Raw goat milk	11	7	
	Pasteurized cow Mmilk	10	10	
Turkey	Raw milk	90	79	Bakirci, (2001)
	Pasteurized milk	85	75	Celik *et al.,* (2005)

	UHT milk	129	75	Unusan, (2006)
	UHT milk	27	24	Gürbay *et al.,* (2006)
	UHT milk	100	67	Tekinsen and Eken, (2008)
	UHT milk	40	8	Kabak and Var, (2008)
	Raw milk	45	41	Kocasari *et al.,* (2012)
	Pasteurized milk	45	30	
	Raw milk	176	53	Golge, (2014)
	White cheese	100	82	Sarimehmetoglu and Kuplulu, (2004)
	Kashar cheese	100	81	
	Tulum cheese	100	85	
	Processed cheese	100	79	
	White cheese	23	9	Gurses *et al.,* (2004)
	Kashar cheese	14	6	
	Tulum cheese	11	7	
	Civil cheese	9	4	
	Lor cheese	6	2	
	White cheese	200	10	Yaroglu *et al.,* (2005)
	Kashar cheese	200	12	
	Processed cheese	200	8	
	White cheese	94	86	Aycicek *et al.,* (2005)
	Cheese	49	44	
	Kashar cheese	53	47	
	Butter	27	25	
	Kashar cheese	132	109	Tekinsen and Eken, (2008)
	Turkish white brined cheese	70	5	Er *et al.*, (2010)
	White-brined urfa cheese	127	36	Kav *et al.,* (2011)
	Turkish white brined cheese	193	159	Ardic *et al.,* (2009)

	White cheese	20	14	Ertas *et al.,* (2011)
	Kashar cheese	20	8	
	Tulum cheese	20	16	
	Yoghurt	50	28	
	Dairy dessert	50	26	
	White cheese	45	42	Kocasari *et al.,* (2012)
	Butter	45	39	
	Yoghurt	45	20	
	Ice cream	45	34	
	Milk powder	45	42	
	Kashar cheese	147	144	Gul and Dervisoglu, (2014)
	Raw Milk	104	51	
Thailand	Raw cow milk	240	240	Ruangwises and Ruangwises, (2010)
	Pasteurized milk	120	-	Suriyasathaporn and Nakprasert, (2012)
China	Milk Samples	734	590	Xiong *et al.,* (2020)
India	Milk Samples	150	46	Sharma *et al.,* (2020)
Indonesia	Milk Samples	42	38	Sumantrai *et al.,* (2019)
Kenya	Raw Milk	96	96	Kuboka *et al.,* (2019)
Lebanon	Raw Milk	11	10	Daou *et al.,* (2020)
Mexico	Fluid Milk	55	11	Quevedo-Garza *et al.,* (2020)
Morocco	Milk Samples	40	14	Alahlah *et al.,* (2020)
Pakistan	Fresh Milk	960	672	Akbar *et al.,* (2019)
Pakistan	Raw Milk	210	195	Ahmad *et al.,* (2019)
Brazil	Milk Samples	60	17	Contecotto *et al.,* (2021)
China	Milk Samples	547	431	Xiong *et al.,* (2020)
India	Market Milk	408	56	Kaur *et al.,*(2021)
Morocco	Milk Samples	67	09	Mannani *et al.,* (2021)

2.9 DETECTION METHODS OF AFLATOXIN M_1

The toxicity and potency of aflatoxins make them the primary health hazard as well as responsible for losses associated with contamination of processed foods and feeds. Determination of aflatoxins concentration in food stuff and feeds is thus very important. However, due to their low concentration in foods and feedstuff, analytical methods for detection and quantification of aflatoxins have to be specific, sensitive, and simple to carry out. Several methods including chromatography, mass spectroscopy, enzyme-linked immunesorbent assay (ELISA), and biosensors etc. have been described for detecting and quantifying aflatoxins in foods. Chromatography is one of the most popular methods to analyze mycotoxins such as aflatoxins.

The most common techniques of chromatography are gas chromatography (GC), liquid chromatography (LC), high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Among these methods, LC, TLC and HPLC are the most widely used quantitative methods in research and routine analysis of aflatoxins (Skendi *et al.,* 2016). The liquid chromatography is separative method which offers good sensitivity, high dynamic range, versatility and soft ionization conditions that permit access to the molecular mass of intact biological molecules. LC is usually coupled to fluorescence detection stage (FLD), UV absorption and amperometric detection with pre-column derivatization or post-column derivatization. Extraction and clean up procedures for aflatoxins analysis typically rely on solid phase extraction (SPE) with different absorbent materials. A particular case of SPE is immunoaffinity columns. Improvements have been made in techniques based on LC, such as: TLC and reversed-phase high performance liquid chromatography (RP-HPLC).

2.9.1 Thin-layer chromatography

Thin-layer chromatography was first used by de Iongh *et al.* (1994) and has been regarded by the Association of Official Analytical Chemist (AOAC) as the method of choice since 1990. Thin-layer chromatography is one of the most widely used separation techniques in aflatoxins analysis. It consists of a stationary phase made of either silica or alumina or cellulose immobilized on an inert material such as glass or plastic, called the matrix. The mobile phase is comprised of methanol: acetonitrile: water mixture (Betina *et al.,* 1985; Kotinagu *et al.,* 2015),

which carries the sample along as it moves through the solid stationary phase. In TLC, distribution of aflatoxins between mobile and stationary phases is based primarily on differences in solubility of analytes in two phases. Different analytes, depending on their molecular structures and interaction with stationary and mobile phases, either adhere to stationary phase more or remain in mobile phase, thereby allowing for quick and effective separation. Thin-layer chromatography has been widely used in the determination of aflatoxins in different foods and as low as 1–20 ppb of aflatoxins has been reported.

2.9.2 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is the most popular chromatographic technique for separation and determination of organic compounds. About 80 % of organic compounds in the world are determined using HPLC (Zhang *et al.*, 2015). In addition, HPLC is one of the most common methods to detect and quantify aflatoxins in food. It has been used jointly with techniques such as UV absorption, fluorescence, mass spectrometry and amperometric detectors. Sulyok *et al.,* (2014) analyzed AFB_1, B_2, G_1 and G_2 based on HPLC and amperometric detection, and reported that it is possible to detect 5 ng/L of all four aflatoxins. The HPLC technique makes use of a stationary phase confined to either a glass or a plastic tube and a mobile phase comprising aqueous/organic solvents, which flow through the solid adsorbent (Golge *et al.*, 2016; Kemboi *et al.*, 2020). When the sample to be analyzed is layered on top of the column, it flows through and distributes between both mobile and stationary phases. This is achieved because components in the samples to be separated have different affinities for two phases and thus move through the column at different rates. The liquid (mobile) phase emerging from the column yields separate fractions containing individual components in the sample. In practice, HPLC technique employs a stationary phase such as C-18 chromatography column, a pump that moves mobile phase(s) through column, a detector that displays retention times of each molecule, and mobile phases (Li *et al.*, 2015; Campagnollo *et al.*, 2020). The sample to be analyzed is usually injected into stationary phase and analytes are carried along through stationary phase by mobile phase using high pressure delivered by a pump. The analytes are distributed differently within stationary phase through chemical as well as physical interactions with stationary and mobile phases (Gul and Dervisoglu, 2014; Alahlah *et al.*, 2020).

The time at which a specific analyte elutes is recorded by a detector as its retention time. The retention time depends on the nature of the analyte and composition of both stationary and mobile phases .There are several detectors such as fluorescent detector, ultraviolet detector and diode array detector that can be used in HPLC for aflatoxin analysis in food (principally in milk, cheese, corn, peanuts and nuts). Commonly, quantification of aflatoxins is made by a fluorescence detector that takes advantage of fluorescence properties of aflatoxins under determined wavelength. As a result, researchers have been focused on improving these fluorescence properties to develop more sensitive methods than commonly used so far. Currently techniques such as pre-column derivatization and post column derivatization are commonly used to improve aflatoxins fluorescence properties. They also have a clean-up stage to obtain a more pure sample, permiting a better quantification. Some of the common methods used in the clean-up stage are: immunoaffinity column and solid phase extraction (Pleadin *et al.*, 2015; Hajmohammadi *et al.*, 2020; da Cruz *et al.*, 2021; de Souza et al., 2021).

2.9.3 Frontier infrared spectroscopy

Frontier infrared spectroscopy technique is another useful method analysis of aflatoxin. This technique depends on the modification in molecular vibrations upon irradiation generated by infrared radiations. These generated vibrations within the bonds of molecules can be measured. Since the atomic size, length of the bond, and strength of the bond differ greatly from molecule to molecule, the rate at which a particular bond absorbs infrared radiation differ from bond to bond and in the mode of vibration. In some times the various bonds of molecules should vibrate at different frequencies, in tandem with the type of bond excited. So when an infrared spectrometer is used in analysis of a compound, infrared radiations has the ability to covering a variety of diverse frequencies are passed through the sample and radiant energy absorbed by the types of bonds in the molecules is measured. A spectrum is then produced normally consisting of plot of % transmittance against the wave number. The use of fourier infrared spectroscopy has been reported for analysis of aflatoxins in peanuts and peanut cake by Mirghani *et al.,* (2001). Pearson *et al.*, (2001) also used fourier infrared spectroscopy to detect aflatoxin in single corn kernels and found 95 % of kernels analysed were correctly categorized.

2.9.4 Ion mobility spectrometry

Ion mobility spectrometry is a technique that is used in characterization of chemicals on the basis of speed acquired by gas-phase ions in an electric field. This technique has been used to determine concentration of aflatoxins, as evidenced by the work of Sheibani *et al.*, (2008) in which they detected and quantified concentration of AFB_1 and B_2 in pistachio. It has certain advantages in common with FT-NIR, as require low detection limit, fast response, simplicity, portability, low cost. Detection of aflatoxins in a sample, evaporation and mixing it with a carrier gas. Then it enters into ion mobility spectrometer (IMS) where mixture is ionized and passed through an electric field gradient, where ions of different substances will travel at different speeds. The study by Sheibani *et al.*, (2008) showed that this technique was unable to quantify as low as 0.25 ng/L.

2.9.5 Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) technique is currently used in the detection of AFM_1 in milk and milk products (Mohajeri *et al.*, 2013) and most of the commercially available ELISA kits which is used to dectect aflatoxins is based on competitive immunoassay pattern. These ELISA kit are having enzymes such as horseradish peroxidase and alkaline phosphatase for analysis of Aflatoxins. The reason behins to use ELISA method because it offers a numorus advantages *i.e.* it perform on a 96-well assay platform, which can means a large number of samples simultaneously; it is cheap and easy to use and do not require extra treatment of samples and there are no health hazards allied with enzyme labels isotopes (Golge, 2014; Salah-Abbsa *et al.*, 2015).

2.9.6 Radioimmunoassay

Radioimmunoassay (RIA) was the first immunoassay technique to be developed for detection of insulin in human blood. Radioimmunoassay has also been used for analysis of aflatoxins in food samples. Anwar and Hussain, (2008) reported use of solid phase radioimmunoassay technique in determination of AFB_1 in peanut with detection limit of 1 ppb. Similarly, radioimmunoassays have been used for qualitative and quantitative determination of AFB_1 levels. The major advantage of radioimmunoassay is its ability to perform multiple analyses simultaneously with high levels of sensitivity and specificity.

2.9.7 Biosensor

The term biosensors refers generally to a small, portable and analytical device based on the combination of recognition of biomolecules with an appropriate transducer, with the ability to detect chemical or biological materials selectively with high sensitivity. The principle of detection is based upon specific binding of analyte of interest to complementary biorecongnition element immobilized on a suitable support medium. The binding of analyte with element, which result specific interaction leading in a change of one or more physico-chemical properties such as: pH, electron transfer, mass, or heat transfer.

These changes can be detected and measured by a transducer. Depending on method of signal transduction, biosensors can be divided into different groups: electrochemical, optical, thermometric, piezo-electric or magnetic. In case of aflatoxin detection, electrochemical and optical are the most commonly used (Rodríguez Velasco *et al.,* 2003). Sanli *et al.,* (2012) presented an immune-affinity fluorometric biosensor where sample was filtered through a column containing sepharose beads to which polyclonal aflatoxin-specific antibodies were joined. The beads with attached aflatoxins were subsequently rinsed to remove any impurities and interference. Posterior, an eluant solution was passed through beads causing antibodies to release bound aflatoxins. The analyte was collected and placed in a fluorometer.

This system consists essentially of two subsystems a fluidics subsystem in charge of mechanical-handling and processing and an electro-optical system that add a miniature fluorometer. Wang *et al.,* (2013) demonstrated an implementation of long range surface plasmon (LRSP) - enhanced fluorescence spectroscopy (SPFS) in an immunoassay based biosensor for highly sensitive detection of AFM_1 in milk samples. The fluoropore-labeled molecules captured on the sensor are excited with surface plasmons (SPs) and emitted fluorescence light is measured.

The system takes the advantage of the electromagnetic intensity improvement occurring upon the resonance excitation of SPs that increase intensity of fluorescence signal. Rastogi *et al.,* (2004) demonstrated a novel approach where a highly sensitive microplate sandwich ELISA was developed and integrated with magnetic nanoparticles (MNPs) which could detect ultra trace amount of AFM_1 in milk. Sandwich type immunoassay is an effective bioassay due to its high

specificity and sensitivity. Magnetic nanoparticles were used as an affinity capture column, wherein immobilized antibodies on their surface could capture AFM_1 from milk sample (Xiong *et al.,* 2018). There are numourn detection methods are available for analysis of aflatoxins in foods and feeds. Among of them chromatographic based methods like TLC and HPLC are considered to be gold standard and most extensively used techniques in detection of aflatoxins. But, they are having lots of cumbersome while sample preparations, and use of expensive equipments.

On the above facts used techniques having limitations and it is necessary to develop more sensitive and better techniques for detection of aflatoxins. Furthermore, some new analytical methods which are based on spectroscopy and immunochemistry are added to earlier chromatographic methods.Moreover, immunoassays emerged as superior alternatives for routine and on-site detection of aflatoxins. The consistant improvement in analytical chemistry and recent advancements in immunochemistry heaving a bright side to development of more specific, sensitive, simple, and rapid immunoassays which have become the method of preference for on-site and routine analysis of aflatoxins in foods and feeds (Taheur *et al.,* 2021; Daou et al., 2020; Muaz *et al.,* 2021).

2.10 ELIMINATION OF AFM_1

Milk treatments commonly used in dairy industry can be divided into two distinct processes: those that do not involve separation of milk components, such as heat treatment, low-temperature storage, and yoghurt preparation; and processes that involve separation of milk components, such as concentration, drying, as well as cheese and butter production. The stability of AFM_1 during heat processing of milk, such as pasteurization and heating directly on fire for 3-4 hours has been studied (Siddappa *et al.,* 2012; Jalili and Scotter, 2015; Daou *et al.,* 2020; Gavahian *et al.,* 2021). Although results of these studies remained inconsistent as, most of reports indicated that heat treatments do not change the amount of AFM_1 in these products appreciably.

Studies on stability of AFM_1 in milk during cool or frozen storage also showed variable results, but storage of frozen contaminated milk and other dairy products for few months did not appear to affect the AFM_1 content (Picinin *et al.,* 2013). The manufacture of cultured dairy

products, such as kefir and yoghurt, also did not significantly decrease AFM_1 content (Rodríguez Velasco *et al.,* 2003; Moretti *et al.,* 2018; Shi *et al.,* 2018; Khaneghah *et al.,* 2021).

The few studies that addressed partitioning of AFM_1 during cream and butter processing confirmed that a small proportion of AFM_1 is carried over to cream and a yet smaller proportion to butter. No loss of AFM_1 occurred as the remainder was found in skim milk and buttermilk, respectively. The manufacture of cheese involves several steps, and AFM_1 does not appear to be degraded in first phase *i.e.* conversion of milk into pressed curd, as its amount in whey and curd remained approximately same as that of original milk (Campagnollo *et al.,* 2020; Nunes *et al.,* 2020). The AFM_1 predominantly interacted with casein, as cheese curd contained higher concentration than whey. The association of AFM_1 with casein is also manifested in a higher concentration in cheese than in the milk from which the cheese is made.

Sulyok *et al.,* (2014) expressed concentration of AFM_1 in milk divided by concentration of AFM_1 in cheese as the enrichment factor. On the basis of several studies, these researchers concluded that enrichment factor is 2.5-3.3 in many soft cheeses and 3.9-5.8 in hard cheeses. During second phase of cheese manufacture *i.e.* ripening, some discrepancies were found in stability of AFM_1, but, in general, it did not appear to be degraded during ripening.

Several possibilities for eliminating or inactivating AFM_1 in milk, involving chemical and physical treatment, have been investigated.

2.10.1. Chemical methods

The chemicals that have been studied for their ability to degrade AFM_1 are limited to those that are permitted as food additives: sulfites, bisulfites, and hydrogen peroxide. When raw milk naturally contaminated with AFM_1 was treated with 0.4 % potassium bisulfite at 25°C for 5 h, its concentration decreased by 45 %, Whereas, higher concentrations of bisulfite were found to less effective (Santini *et al.,* 2013). Aflatoxin M_1 in naturally contaminated milk did not get affected by the presence of 1 % hydrogen peroxide at 30°C for 30 min, but addition of hydrogen peroxide at a concentration of 0.05-0.1% with lactoperoxidase resulted 50 % reduction in AFM_1 concentration.

2.10.2. Physical methods

Physical processes that have been explored to remove AFM_1 from milk include adsorption, separation, heat treatment, ultrasonic treatment, and irradiation. Five per cent bentonite in milk adsorbed 89 % of AFM_1. The effects of ultra-violet radiation with and without hydrogen peroxide showed reduction in AFM_1 concentration to the rate of (3.6–100 %), depending on length of time, to which milk was exposed to radiation, volume of treated milk, presence of hydrogen peroxide, and other aspects of experimental design. Fink-Gremmel, (2008) reported that various heat–time treatments caused reductions in the AFM_1 concentrations of milks between 12 % and 40 %. Choudhary *et al.*, (1997) studied the effect of various heat treatments on AFM_1 content of cow's milk and reported that sterilization of milk at 121°C for 15 min caused 12.21 % degradation of AFM_1, whereas boiling decreased AFM_1 by 14.50 %. They concluded that destruction of AFM_1 depends on time and temperature combination of the heat treatment applied. In an investigation conducted by Bakirci, (2001), it was observed that pasteurization caused a decrease in the level of AFM_1 at the rate of 7.62 %.

The chemical and physical treatments described are not readily applicable in dairy industry, at least at present, as little is known about their biological safety, or nutritional quality of the treated products. Moreover, costs of these processes may be considerably higher which make them prohibitive for large-scale application. If AFM_1 cannot be destroyed or removed readily, it can be excluded from milk only by eliminating AFB_1 from the diet of animals.

Therefore, microorganisms, especially bacteria, have been studied for their potential to either degrade aflatoxins or reduce their bioavailability. In recent years, scientists have focused on identification and application of natural products for inactivation of aflatoxins. Among these, probiotic lactic acid bacteria have been identified as a safe means to reduce aflatoxins from food and feeds. Furthermore, probiotic bacteria exert a number of other beneficial health effects, and thus make them even more suitable additives to food and feeds (Dashti *et al*., 2009).

2.11 PROBIOTIC

Probiotic - the word is derived from the Greek and means "*for life*" and was first used by Lilley and Stillwell to describe substances secreted by one microorganism to stimulate the growth of another - as an antonym for 'antibiotic' (Lilly and Stillwell, 1965). In 1974, Parker

defined probiotic “as organisms and substances, which contribute to intestinal microbial balance” (Parker, 1974). Fuller, (1989) redefined probiotics as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. "The World Health Organization defined probiotics as "live micro-organisms which, when administered in adequate amounts, confer health benefit on the host". Probiotic are also documented as a microbial dietary adjuvant that directly constructively affects physiology of host by modulating mucosal and systemic immunity as well as improving nutritional and microbial balance in the gestro intestinal tract.

The cellular complex of lactic acid bacteria (LAB) which are projected as a probiotic-active substance which has the capability to interact with host mucosa and resulting in beneficially modulate immune system independent of LAB’s viability. Lactic acid bacteria widely used in various fermented foods since antiquity. The preservative and health benefits of such traditional fermented foods have been renowned for thousands of years. A number of *Lactobacillus* sp, *Bifidobacterium* sp, *Saccharomyces boulardii*, and some other microbes have been proposed to be used as probiotic strains, *i.e.* live microorganisms as food supplement in order to benefit health.

The health claims range from rather vague as regulation of bowel activity and increasing of well-being. To be more specific, such as exerting antagonistic effect on gastroenteric pathogens *viz., Clostridium difficile, Campylobacter jejuni, Helicobacter pylori* and rotavirus, neutralising food mutagens produced in colon, shifting immune response towards Th_2 response, and thereby alleviating allergic reactions, and lowering serum cholesterol (MacFarlane and Cummings, 2002).

2.12 AFLATOXIN M_1 BINDING PROPERTY OF PROBIOTIC LAB

The group of lactic acid bacteria occupies a central role in food fermentation processes and has a long and safe history of their application and consumption of fermented foods. Various compounds produced by LAB have been suggested to be responsible for inhibitory effects on fungal growth such as lactic, acetic, propionic acid, phenyl lactic, bacteriocine, hydrogen peroxide, hydroxylated fatty acids and various phenolic compounds (Mandal *et al*., 2007; Chung *et al.,* 1989). The most efficient way to prevent contamination of foods with mycotoxins is to

avoid growth of mycotoxigenic fungi, however, contamination of various commodities with toxigenic moulds is unavoidable under certain environmental conditions.

Several strategies have been applied to destroy aflatoxins or reduction of their bioavailability in contaminated foods. Aflatoxin may be degraded by physical and chemical treatment. There is currently no acceptable method to counteract AFM_1 occurrence in milk and dairy products. Thus, a practical and effective method is needed to be developed for the detoxification of AFM_1 contaminated milk and milk products. Some strains of lactic acid bacteria have been reported to be effective in removing AFB_1 and AFM_1 from contaminated liquid media and milk (El-Nezami *et al.,* 1998; Lee *et al.,* 2009; Peltonen *et al.,* 2001; Zinedine *et al.,* 2007; Min *et al.,* 2020). Hernandez-Mendoza *et al*., (2009) reported that LAB and bifidobacteria are of particular interest for reducing the bioavailability of aflatoxins due to their GRAS status and use as probiotics.

The stability and strength of binding of probiotics to toxins is also a key consideration for evaluation of strains ability to reduce aflatoxin bioavailability. Several bacterial strains, of food or human origin, have been tested for their ability to bind aflatoxins and other mycotoxins to their surface. El-Nezami *et al.,* (1998) found that gram positive bacteria (five strains of *Lactobacillus* and one *Propionibacterium*) were more efficient in removing aflatoxin from liquid medium than gram negative *E. coli*.

Pierides *et al.,* (2000) investigated the ability of dairy strains of lactic acid bacteria to remove AFM_1 from contaminated phosphate buffer saline, skim and full cream milk. All tested strains, whether viable or heat-killed, could reduce AFM_1 content of a liquid medium, which indicated that bacterial viability is not prerequisite to toxin removal and suggests involvement of a cell wall-related physical phenomenon instead of metabolic degradation. In this study, viable *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *L. gasseri* ATCC 33323 binds 77 ± 0.4, 75.2 ± 1.2 and 51.4 ± 1.9 % AFM_1 respectively, after 4 hours of incubation.

Nevertheless, heat inactivated bacteria showed 57.8±3.3, 51.6±3.0 and 61.5±0.7 % binding ability, after 15-16 hours of incubation period in PBS. Furthermore, *L. rhamnosus* GG and *L. rhamnosus* LC-705 reported approximately 80 % binding with AFB_1 within 0 h (El-Nezami *et al.,* 1998).In addition, Bovo *et al.,* (2012), also evaluated the capability of live and

heat killed lactic acid bacteria to remove AFM_1 in PBS and skim milk. They were found that tested strains shows AFM_1 binding in range of 12.42 to 45.67 % for heat killed and 5.60 to 33.54 % for viable bacteria exposed to AFM_1 for 15 min or 24 hours, respectively.

Sarimehmetoglu and Kuplulu, (2004) reported binding ability of yoghurt bacteria with AFM_1 in PBS and milk. They reported that *L. delbrueckii* subsp. *bulgaricus* CH-2 bound 18.70 and 27.56 % in PBS and milk, respectively, while *S. thermophilus* ST-36 29.42 and 39.16 % in PBS and milk, respectively. Aflatoin M_1 was bound at the level of merely 14.82 % in yogurt. The results indicated that binding ability of *S. thermophilus* ST-36 was higher than *L. delbrueckii* subsp. *bulgaricus* CH-2 in both PBS and reconstituted milk. The AFM_1 binding ability of strains was higher in milk than in PBS.

The levels of AFM_1 in probiotic yogurts showed a significant reduction during refrigerated storage. The AFM_1 levels were lower in MY1821 cultured (*L. acidophilus, B. lactis, L. casei, S. thermophilus* and *L. bulgaricus*) yogurt as compared to ABY3 (*L. acidophilus, B .lactis, S. thermophilus* and *L. bulgaricus*). The percent loss of AFM_1 in milk was 41 and 49 % at the end of storage for ABY3 and MY1821 yogurt, respectively (Montaseri *et al.,* 2014). El Khoury *et al*., (2011) investigated AFM_1 reduction by *L. bulgaricus* and *S. thermophilus* in PBS liquid medium and during yogurt making. The kinetic of AFM_1 removal was evaluated every 2 hours upto 14 hours in PBS assay and every 1 h for 6 hours during yogurt production. *L. bulgaricus* showed high percentage of AFM_1 binding ability (87.6 % after 14 hours) as compared to *S. thermophilus* (70 % after 14 hours) in PBS.

Furthermore, results showed that decrease in AFM_1 of PBS cultures was proportional to bacterial growth during the incubation period. The binding ability of AFM_1 by *L. bulgaricus* and *S. thermophilus* also investigated during the making of yogurt. *L. bulgaricus* reduced 46.1 % of AFM_1 after 2 hours of incubation. The binding levels of AFM_1 by *L. bulgaricus* increased with increased time to reach 58.5 % after 6 hours of incubation. As expected *S. thermophilus* showed lower binding ability *i.e.* 22.6 and 37.7 % removal after 2 hours and 6 hours, respectively, during yogurt preparation.

Investigations on removal of AFM_1 from spiked milk during yoghurt production and storage were carried out. Three sets of yoghurt were produced from spiked milk (50 mg/L). Set

A acted as control using yoghurt culture (*S. thermophilus* and *L. bulgaricus*). Set B was fermented with 50 % yoghurt culture (*S. thermophilus* and *L. bulgaricus*) and 50 % *L. plantarum*. Set C was fermented with 50 % yoghurt culture (*S. thermophilus* and *L. bulgaricus*) and 50 % *L. acidophilus*. The samples were collected during different storage times (1, 3, 5 and 7 days) at 5^{o}C to determine ability of stains to reduce AFM_1.

They reported that set B showed highest AFM_1 removal capability to *i.e.* 31.5, 51.8, 66.0, 87.8 % on 1, 3, 5 and 7 day of storage, respectively. Yoghurt fermented by 50 % yoghurt culture (*S. thermophilus* and *L. bulgaricus*) and 50 % *L. plantarum* recorded highest reduction in the levels of AFM_1 at the end of storage period (Elsanhoty *et al.,* 2014). Elgerbi and Aidoo, (2006) assessed the ability of strains of *Lactobacillus* spp., *Lactococcus* spp. and *Bifidobacterium* spp. to bind AFM_1 in solution. They found that the percentage of AFM_1 bound by these strains ranged from 0 to 14.6 % after 24 h and 4.5 to 73.1 % after 96 h.

Corassin *et al.,* (2013), evaluated the ability of *Saccharomyces cerevisiae* and pool of three lactic acid bacteria (*L. rhamnosus, L. delbrueckii spp. bulgaricus* and *B. lactis*), alone or in combination, to bind AFM_1 in UHT (ultra high temperature) skim milk spiked milk 0.5 ng AFM_1/mL. All LAB pool (10^{10} cells/mL) and *S. cerevisiae* (10^{9} cells/mL) cells were heat-killed (100^{o}C/1 hours) and then used for checking the effect of contact time (30 min or 60 min) on toxin binding in skim milk at 37^{o}C. The mean percentages of AFM_1 bound by LAB pool in milk were 11.5 ± 2.3 % and 11.7 ± 4.4 % for 30 min and 60 min, respectively. Compared to LAB pool, *S. cerevisiae* cells had higher capability to bind AFM_1 in milk (90.3 ±0.3 % and 92.7 ± 0.7 % for 30 min and 60 min, respectively).

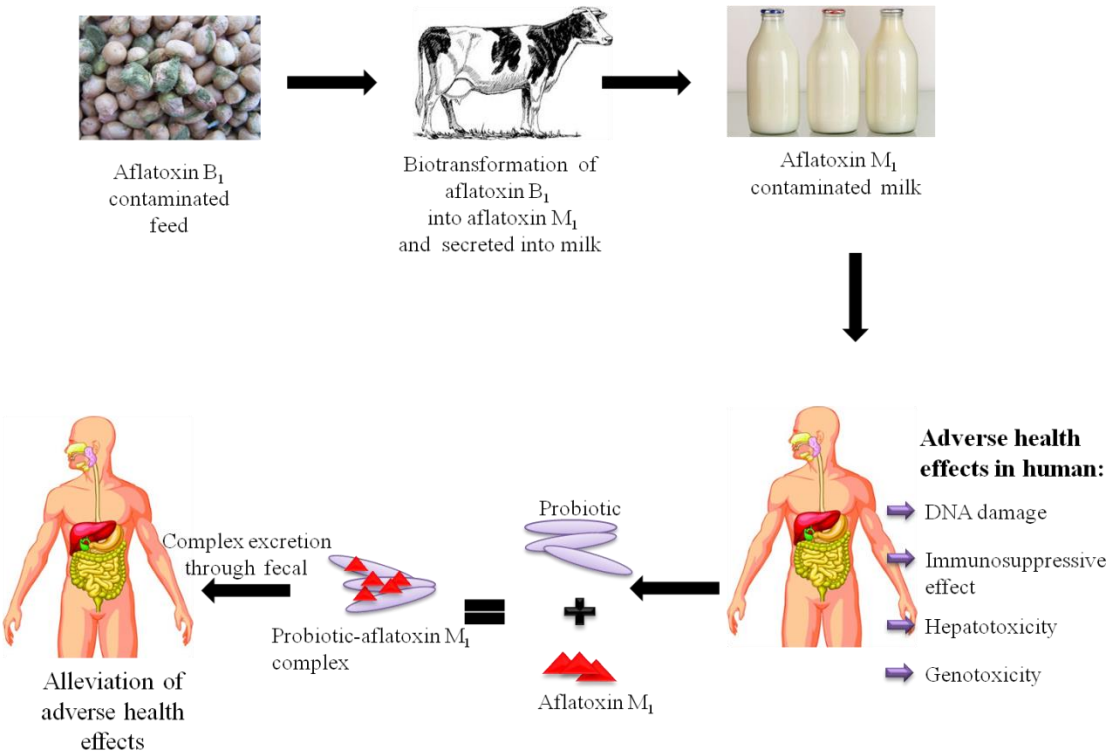
When using *S. cerevisiae* + LAB pool, a significant increase was observed in AFM_1 binding *i.e.* 100.0 % during 60 min contact time. These results indicated that heat killed *S. cerevisiae* cells, alone or in combination with LAB pool used, has potential application to reduce concentrations of AFM_1 in milk.

Additionally, Elsanhoty *et al.,* (2014) also reported that lactic acid bacteria had the potential to bind AFM_1 and can lower toxin levels up to the safe limits. They also reported that AFM_1 content decreases by decreasing the pH of spiked broth.

Serrano-Nino *et al.*, (2013) screened five probiotics strains for their AFM_1 binding abilities in PBS. They noticed binding of AFM_1 ranged from 19.95 to 25.43 %. Interestingly, binding process proceeded quickly which ranged from 19.95 to 25.28 % at 0 h. Salah-Abbes *et al.*, (2013) evaluated binding properties of *L. plantarum* MON03 and *L. rhamnosus* GAF01, isolated from Tunisian artisanal butter to AFM_1 in PBS and reconstituted milk (0.05, 0.10, and 20 ng/mL) after 0, 6, and 24 hours at 37°C. They observed AFM_1binding abilities of *L. plantarum* MON03 and *L. rhamnosus* GAF01 strains in PBS and reconstituted milk ranged from 16.1-78.6 % and 15.3-95.1 %, respectively.

Salah-Abbes *et al.*, (2015) investigated binding ability of live and heat killed *L. plantarum* MON03 in PBS spiked with 50000 ng/mL of AFM_1, after 0, 12 and 24 hours at 37°C. They also found that, AFM_1-binding by *L. plantarum* MON03 increased in a time-dependent manner. Live *L. plantarum* MON03 binds from 25.9 to 93.1 % of AFM_1 after 0-24 hours, while heat-killed *L. plantarum* MON03 binds 25.9 to 55.1 % after 0-24 hours of incubation in PBS.

Fig. 2.3 Origin and reduction of AFM_1 in milk and milk products



Recently, Irena Barukcic *et al.,* (2018) studied reduction of aflatoxin M_1 concentrations using selected probiotic (*Lactobacillus casei*LC-01) and nonprobiotic (yoghurt culture YC-380) culture. The tested cultures caused remarkable reductions of AFM_1. Probiotic cultures were more effective that achieving a reduction level of approximately 58 %. The nonprobiotic cultures *i.e.* yoghurt culture YC-380 reduced level of approximately 41 %.

In addition, Red *et al.,* (2018) investigating the ability of *B. animalis lactis* and *L. bulgaricus* at concentrations of 10^8 and 10^9 cfu/mL to detoxify skim milk that was spiked with 0.25 ng/mL, 0.5 mg/mL, and 0.75 mg/mL AFM_1 at 30 min, 60 min, 120 min, and 24 hours, and at 4 and 37 °C. The highest level of AFM_1 binding to 10^8 cfu/mL of *L. bulgaricus* was 57.5 ± 5.2 % after 24 hours, at 37 °C.

Omeiza *et al.,* (2018) investigate AFM_1 reduction by using pool of lactic acid bacteria (*L.bulgaricus,* S. *thermophilus, L. rhamnosus* and *L. plantarum*) and they found 17.4, 30.0 and 41.1 % reduction of AFM_1 in 12 hours, 48 hours and 72 hours, respectively.

Hashemi *et al.,* (2018) studied the ability of *Lactobacillus* strains such as *L. plantarum* LP3, *L. plantarum* PTCC 1058, *L. plantarum* AF1 and *L. plantarum* LU5 to remidiate AFM_1 and OTA in fermented cream during 24 hours fermentation. In addition, the antifungal activity of the same lactobacilli has also been investigated against the *A.s flavus* PTCC 5004, *A. parasiticus* PTCC 5018, *A. nidulans* PTCC 5014 and *A. ochraceus* PTCC 5060. The results of this study showed that the number of cells of all lactobacilli strains was increased by 64–70 % during fermentation. In the presence of these *Lactobacillus* strains the amount of AFM_1 and OTA decreased significantly ($P \leq 0.05$) in the range of 26-52 % and 32-58 %, respectively, after 24 hours of fermentation. Amongst *Lactobacillus* strains, *L. plantarum* LU5 showed the highest antifungal activities.

Recently, Goncalves *et al.,* (2021a) microorganisms as a biological method to reduce AFM_1 they studied the binding efficiency of *L. rhamnosus* and *Lactococcus lactis*, and a yeast strain, *Saccharomyces cerevisiae* with AFM_1 on second day and after thirty days in the Minas Frescal cheese and they found On second day, a reduction of AFM_1 was of 74 % and after thirty days that was 100 %. Furthermore, da Cruz *et al.,* (2021) conduct a study to evaluate the aflatoxin M_1 removal efficiency of probiotic fruit-derived *Lactobacillus* isolates, namely, *L.*

paracasei 108, *L. plantarum* 49, and *L. fermentum* 111from a phosphate buffer solution with viable and non-viable cells (heat-killed; 100°C, 1 h) after interval of 1 and 24 hours at 37°C. They found viable and non-viable cells of all examined isolates were capable of removing AFM_1 in PBS with removal percentage values in the range of 73.9-80.0 % and 72.9-78.7 %, respectively. Viable and non-viable cells of all examined *Lactobacillus* isolates had similar abilities to remove AFM_1.

Muaz *et al.,* (2021) conduct an experiment with the objective to enhance the AFM_1 binding efficiencies of bacterial cells belonging to *L. paracasei* and *B. coagulans* in the presence of activated carbon (CAC), bentonite (CBENT) and sorbitan monostearate (CSP60). The reduction of AFM_1 was found to be directly proportional to the concentration of microbial cells. Heat killed and acid treated *L. paracasei* successfully reduced AFM_1 in milk spiked at 0.2 µg/L to 89 % and 100 % in CBENT, 84 % and 90 % in CAC, 59 % and 47 % in CSP60, respectively. Among treatments involving *B. coagulans,* acid treated CSP60 proved to be least effective showing 44.6 % reduction, while CBENT for both acid and heat treated along with acid treated CAC proved to be most effective by removing 100 % AFM_1.

Recently Martinez *et al.,* (2021) also investigated the binding efficiency of lactic acid bacteria and they were found that tested strains significantly adsorbed AFM_1 in milk at percentages that varied from 19 % to 61 %. Among of them *L. rhamnosus* RC007 showed the highest adsorption percentage (61 %) as compared with other tested strain *i.e. P. acidilactici* RC005*, P. pentosaceus* RC006*, S. cerevisiae* RC016, *S. boulardii* RC009*, K. marxianus* VM003.

2.13 BACTERIAL-AFLATOXIN M_1 COMPLEX STABILITY

The complex stability of bacterial cells and AFM_1 having utmost importance as far as bioavailability of AFM_1 is concerned as AFM_1 and bacterial complex can be upturned during milk processing or its digestion in the human body. Hence, exploration of complex stability is of prime importance. It was suggested that weak non-covalent connections among AFM_1 and bacterial cell well components is one of the main reason in release of AFM_1 after washing (Ismail *et al.,* 2016; Serrano-Nino *et al.,* 2013).

The differences in amounts of bounded AFM_1 released after washing could be due to binding of AFM_1 by bacterial cells appears extremely strain specific, or cross linked interactions between AFM_1 molecules present in the cell walls of two different bacteria (Hernandez-Mendoza *et al.,* 2009). Seldom reports are available with reference to the stability of AFM_1-bacteria complex. Kabak and Var, (2008) found that binding between AFM_1 and bacteria was not irreversible because after washing of bacteria-AFM_1 complexes, only small amounts (5.62-8.54 %) of AFM_1 were released back into buffered solution. Haskard *et al.,* (2001) documented twelve live and heat killed bacterial strains twards their complex stability with AFB_1.

They were found that at the end of fifth extraction process there is up to 71 % of total AFB_1 retain their comples stability with heat killed bacteria which is the hifher retained amount of AFB_1. They were also declared that these binding are of reversible in nature; however, its stability is of strain specific and also affected by the nature of treatment and environmental conditions. In the same way, Shahin, (2007) also investigate the complex stability of viable and non viable (heat-treated) *L. lactis* and *S. thermophilus;* after three washed there is no released detectable amount of AFB_1 was observed. Furthermore, lowest toxin complex stability was observed by Bovo *et al.* (2012) as they found major release of bound AFM_1 from 40.57 to 87.37 % after washes with PBS, similarly, AFB_1 bound by *L. amylovorus* CSCC 5160, *L. amylovorus* CSCC 5197 and *L. rhamnosus* Lc 1/3 showed release of toxin back into solution to the tune of 94.4, 74.2, and 27.8 %, respectively, (Peltonen *et al.,* 2001).

In contrast, *B. bifidum* NRRL B-41410 strain exhibited most stable AFM_1-microbial complex as merely ~1.4 % reduction of AFM_1 after washing with PBS (Serrano-Nino *et al.,* 2013). Hernandez-Mendoza *et al.,* (2009) observed, AFB_1-bacteria complex formed by *L. reuteri* NRRL1417 and *L.casei* Shirota was significantly more stable than those formed by *B. bifidum* NCFB2715, *L. johnsonii* NCC 533, and *L. casei* defensis DN-114-001. After 4 hours of incubation *L. reuteri* NRRL1417 and *L.casei* Shirota retained bound 85.3 and 93.8 % of the AFB_1, respectively and binding increased to 90 % at 12 hours incubation.

Recently da Cruz *et al.,* (2021) examin aflatoxin M_1 retention capacity of the viable and heat-killed cells of *L. paracasei* 108, *L. plantarum* 49, and *L. fermentum* 111 after washing with PBS and they found percentage values of recovered AFM_1 from viable and heat-killed cells were in the range of 13.4 ± 1.5–60.6 ± 1.6 % and 10.9 ± 1.2 %–47.9 ± 1.5 %, respectively. The highest

values of recovered AFM_1 after 1 and 24 hours were found for *L. fermentum* 111 and *L. paracasei* 108, respectively, for both viable and heat-killed cells.

Similiarly, *L. paracasei* and *B. coagulans* was studied for their complex stability with aflatoxin M_1in the presence of activated carbon (CAC), bentonite (CBENT) and sorbitan monostearate (CSP60) and found CBENT and CAC (acid and heat killed) among both bacterial strains showed the formation of most stable complex with AFM_1 showing no release of detectable AFM_1 after couple of phosphate buffer saline washings. Among other treatments, CSP60 of heat killed cells formed most stable complex for both *L. paracasei* and *B. coagulans* with 19 % and 22 % release of initially bound AFM_1, respectively. The results showed that the combination of microbial cells with activated carbon and bentonite may be used as an efficient and effective strategy to mitigate the problem of AFM_1 in milk (Muaz *et al.,* 2021).

2.14 MECHANISM OF AFLATOXIN M_1 REDUCTION BY LACTIC ACID BACTERIA

The mechanism of aflatoxins removal by lactic acid bacteria is still not well understood, it has been suggested that instead of metabolic degradation, aflatoxins bind to bacterial cell wall components (El-Nezami *et al.,* 1998; Lahtinen *et al.,* 2004). Cell wall components such as polysaccharide and peptidoglycan have been recognized to be the most important elements responsible for binding of aflatoxins by lactic acid bacteria (Peltonen *et al.,* 2001; Hernandez-Mendoza *et al.,* 2009; Assaf *et al.,* 2018).

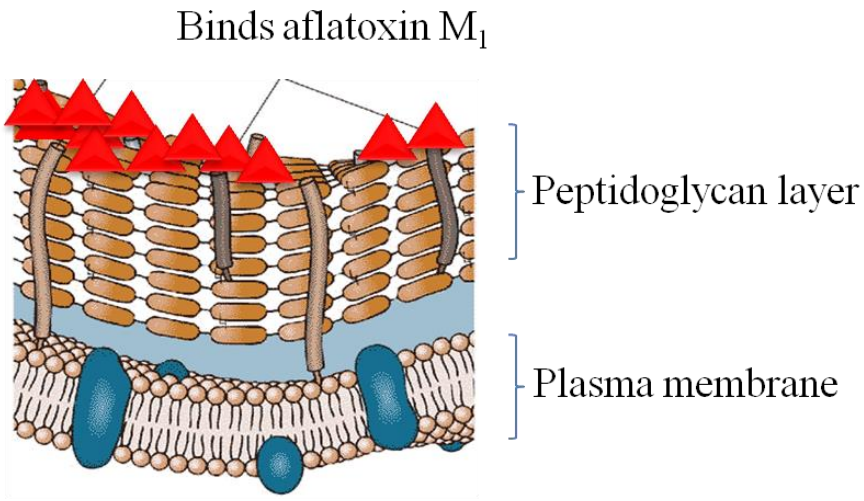
To reveal the precise binding sites for AFB_1 on the surface of *Lactobacilli*, bacterial cells were subjected to various physical, chemical and enzymatic treatments. They concluded that cell wall polysaccharides and peptidoglycanes were responsible for binding of AFB_1 to cell surface of *L. rhamnosus* GG and *L. rhamnosus* LC705 (Haskard *et al.,* 2000; Haskard *et al.,* 2001). These observations was further confirmed by Lahtinen *et al.,* (2004) who studied different cell wall components (exopolysaccharides, cell wall isolates and peptidoglycans) of *L. rhamnosus* GG and concluded that peptidoglycans are most likely binding sites for AFB_1.

Heat treatment may denature proteins, that may lead to the formation of maillard products while acid treatment may break glycosidic linkages in polysaccharides; amine linkages in peptides and proteins which increased pore size in peptidoglycan layer of bacterial surface (Haskard *et al.,* 2001). Serrano-Nino *et al.,* (2013) provided information that teichoic acids also

play asignificant role in AFB_1 binding on bacterial cell wall. Cell surface hydrophobicity conjectured as play a vital role in the mechanisms of binding.

But in contrary of Kabak and Var, (2008) demonstrated no correlations between hyrophobicity of the bacterial cell surface & AFM_1-binding of bacteria. Therefore, it is probable that numerous factors are involved in AFM_1 toxins binding. Hamad *et al.,* (2017) reported that SEM analysis showed that the microorganism's surface is able to adsorb the toxin particles that appear as a small dot on the microorganism's cell walls. Abdelmotilib *et al.,* (2018) reported in their study that non-viable strains of the yeasts *Kluyveromyces lactis* and *S. cerevisiae* showed changes in the cell wall after adsorption of AFM_1.

Fig. 2.4 Binding of AFM_1 on bacterial cell wall



Guan *et al.,* (2021) stated that adsorption means that due to the special structure on the microbial cell wall, aflatoxins interect with non-covalant bonds (the main effect is that of Van dar Waals forces), which make it easier to bind, redusing the bioavailbility of aflatoxins in the GIT tarct, anf protect the body from toxin infection.

2.15 REDUCTION OF AFLATOXIN M_1 BIOACCESSIBILITY BY LACTIC ACID BACTERIA

Illustration of any destructive possessions on the specific tissue or organ of the body, aflatoxins first released from their matrix and then absorbed in the intestinal cells. The term bioaccessibility or bioavailability of toxins is defined as the quantity of ingested toxins that

released from their food and feed matrix in the gastrointestinal tract and thus becomes accessible for intestinal absorption (Versantvoort *et al.,* 2005). Several studies have been performed to examine bioaccessibility of various aflatoxins from different food matrices (Kabak and Ozbey, 2012; Serrano-Niño *et al.,* 2013). Both *in-vitro* and *in vivo* methods can be used for assessment of bioaccessibility of aflatoxins.

In-vitro methods for bioaccessibility assessment are simple, rapid, and low-cost with high reproducibility; whereas, *in-vivo* method are more convincing, but difficult to perform and time-consuming (Avantaggiato *et al.,* 2004). Versantvoort *et al.* (2005) determined bioaccessibility of AFB_1 from peanut slurry and ochratoxin A from buckwheat showed high bioaccessibility of 94 % and 100 %, respectively.

Kabak and Ozbey, (2012) studied the bioaccessibility of different aflatoxins from seven food matrices range ranged from 85.1 to 98.1 % for AFB_1, 83.3 to 91.8 % for AFB_2, 85.3 to 95.1 % for AFG_1 and 80.7 to 91.2 % for AFG_2. The bioaccessibility of AFM_1 in spiked and naturally contaminated milk samples ranged from 80.5 to 83.8 % and from 81.7 to 86.3 %, respectively. (Kabak and Ozbey, 2012); but recently Serrano-Nino et *al.,* (2013) found 100 % bioaccessibility of AFM_1 in artificially contaminated milk. It has been observed that bioaccessibility of aflatoxins depends on several factors, such as food matrices, mode of entry, extent of contamination and type of contamination *i.e.* spiked versus naturally contaminated food materials (Kabak *et al.,* 2009).

Serrano-Nino *et al.,* (2013), assessed the potential of five probiotic strains *viz.*, *L. reuteri* NRRL B-14171, *L. acidophilus* NRRL B-4495, *L. johnsonii* NRRL B-2178, *L. rhamnosus* NRRL B-442, *B. bifidum* NRRL B-41410 to reduce the bioaccessibility of AFM_1 by follow *in-vitro* digestion model. They observed decrease in bioaccessibility of AFM_1 by 22.72 to 45.17 %. In addition, Kabak and Ozbey, (2012) evaluated six probiotic strains to reduce AFM_1 bioaccessibility under simulated gastrointestinal conditions. They observed 15.5-31.6 % reduction in bioavailability of AFM_1 in comparison with the control. Furthermore, after the addition of six probiotic strains to the digestion model resulted in reductions in aflatoxins bioaccessibility *viz.,* 18.1-35.6 % for AFB_1, 17.3-35.5 % for AFB_2, 13.5-31.9 % for AFG_1, and 10.5-33.6 % for AFG_2 (Kabak and Ozbey, 2012). The 37 and 73 % of reduction in

bioaccessibility of AFB_1 and ocratoxin A, respectively, was observed in the presence of probiotic bacteria (Kabak *et al.*, 2009).

3.0 MATERIALS AND METHODS

3.1 CHEMICALS AND REAGENTS

The analytical grade chemical and reagents used for whole study were purchased from different manufactures like, competitive ELISA kit for detection of AFM_1 (Shenzhen Lvshiyuan Biotech. Ltd. China). Aflatoxin M_1, potassium thiocyanate (KSCN), sodium bicarbonate ($NaHCO_3$), urea, glucose, sodium sulphate (Na_2SO_4), monopotassium phosphate (KH_2PO_4), magnesium chloride ($MgCl_2$), Potassium chloride (KCl), bile salts, uric acid, D-glucosamine hydrochloride, D-glucuronic acid and anhydrous Na_2HPO_4 were obtained from HiMedia Pvt. Ltd. Mumbai, India.

Sodium phosphate (NaH_2PO_4) and ammonium chloride (NH_4Cl) were procured from (SRL, Mumbai, India) and sodium chloride (NaCl) and sodium hydroxide (NaOH) were obtained from (Fisher Scientific, Mumbai, India). In addition, α-amylase, mucin, pepsin, pancreatin, lipase and bovine serum albumin were obtained from Sigma-Aldrich, USA.

High performance liquid chromatography (HPLC)-grade chloroform and calcium chloride ($CaCl_2$) were purchased from Merck, Mumbai, India.

The phosphate buffered saline (PBS) was prepared in the laboratory by dissolving 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g, Na_2HPO_4 and 8 g NaCl in 1000 mL water and its pH was adjusted to 7.3 with 0.1 N NaOH.

3.2 BACTERIAL CULTURES

Thirty lactobacilli cultures previously isolated and identified up to genus level in our laboratory were used in this research project. The lactobacilli cultures used in present study have been mentioned in **Table 3.1**.

Table: 3.1 List of lactobacilli cultures along with their sources

S. No.	Lactobacilli cultures	Sources
1.	*Lactobacillus plantarum* BM70	Buffalo milk
2.	*Lactobacillus plantarum* BM71	Buffalo milk

3.	*Lactobacillus plantarum* CAM94	Camel milk
4.	*Lactobacillus plantarum* CAM95	Camel milk
5.	*Lactobacillus plantarum* CM62	Cow milk
6.	*Lactobacillus plantarum* CM63	Cow milk
7.	*Lactobacillus plantarum* CM67	Cow milk
8.	*Lactobacillus plantarum* CM66	Cow milk
9.	*Lactobacillus plantarum* CM68	Cow milk
10.	*Lactobacillus plantarum* CM69	Cow milk
11.	*Lactobacillus plantarum* HD48	Homemade *dahi*
12.	*Lactobacillus plantarum* HD51	Homemade *dahi*
13.	*Lactobacillus plantarum* MDD53	Market made *dahi*
14.	*Lactobacillus plantarum* MDD56	Market made *dahi*
15.	*Lactobacillus plantarum* MDD54	Market made *dahi*
16.	*Lactobacillus plantarum* HIF33	Human infant feces
17.	*Lactobacillus plantarum* HIF81	Human infant feces
18.	*Lactobacillus plantarum* HIF82	Human infant feces
19.	*Lactobacillus plantarum* HIF104	Human infant feces
20.	*Lactobacillus plantarum* HAF84	Human adult feces
21.	*Lactobacillus plantarum* HAF85	Human adult feces
22.	*Lactobacillus plantarum* LD59	Local Dairy *dahi*
23.	*Lactobacillus plantarum* CRD 2	Local *dahi*
24.	*Lactobacillus plantarum* CRD 7	Local *dahi*
25.	*Lactobacillus plantarum* CRD 121	Local *dahi*
26.	*Lactobacillus rhamnosus* CRD 4	Local *dahi*
27.	*Lactobacillus rhamnosus* CRD 9	Local *dahi*
28.	*Lactobacillus rhamnosus* CRD 11	Local *dahi*
29.	*Lactobacillus bulgaricus* 11B	Local *dahi*
30.	*Lactobacillus rhamnosus* GG	Old stock (Synbiotic functional foods & bioremediation research laboratory, ICAR-National Dairy Research Institute (Deemed University), Karnal-132002, Haryana, India)

3.2.1 Maintenance, preservation, and propagation

Lactic cultures were activated twice at their optimum growth condition in MRS broth before any experiment. Loopful of overnight grown culture was streaked on MRS agar plates to obtain well isolated pure colonies. These plates were also used for maintaining these cultures up to one month. The lactic cultures were maintained by subculturing in sterilized yeast glucose litmus chalk milk (YGLM) medium on fortnightly basis and stored at 4^{o}C in refrigerator. Long term preservation of cultures was carried out in 40 % sterilized glycerol solution. The glycerol was sterilized at 121^{o}C /15 min/ 15 psi (twice at one-day interval). Glycerol stocks were prepared using overnight grown active cultures and stored at -20^{o}C.

3.2.2 Determination of culture morphology and purity

Purity and morphology of cultures were determined by Gram's and negative staining as well as catalase test.

3.2.2.1 Gram's staining

Gram's staining was performed as per standard method, briefly as follow:

- Thin smear of loopful culture was prepared on grease free glass slide, air dried and heat fixed.
- Slide was flooded with crystal violet staining reagent for about 1 minute.
- The slide was washed using a gentle, indirect stream of tap water for about 10 seconds. The slide was flooded with a mordant (Gram's iodine) for one minute.
- The slide was again washed in a gentle, indirect stream of tap water for about 10 seconds.
- Slide was then flooded with decolorizing agent (alcohol) for 15 seconds. This can also be done by adding a drop by drop to the slide until decolorizing agent stop running with primary stain from slide.
- The slide was flooded again using counterstain safranin for about 30-60 seconds.
- Slide was washed under gentle and indirect stream of tap water to a point where no color appears in the effluent and then air dried.

3.2.2.2 Negative staining

Loopful of freshly overnight grown culture was transferred on grease free glass slide and mixed with one drop of nigrosin solution. Thin smear was prepared and allowed to air-dry. Slide was viewed in 100 X objective magnification using oil immersion. Bacterial cells appear bright against dark background. Precaution was taken to keep the smear as thin as possible so that cells can be clearly differentiated based on morphology.

3.2.2.3 Catalase test

To find out if a particular bacterial isolate is able to produce catalase enzyme. A small inoculum of bacterial culture was transferred to glass slide and mixed with 3 % hydrogen peroxide solution. The rapid release of effervescence of oxygen indicated positive test. Lack of effervescence was negative indication. Slide method was used to perform catalase test. Few drops of freshly overnight grown culture or an isolated colony from MRS agar plate was placed on clean grease free slide and added few drop of 3 % hydrogen peroxide. This was mixed with the help of loop and observed for the effervescence production due to liberation of free oxygen.

OBJECTIVE I

3.3 SCREENING OF PROBIOTIC *LACTOBACILLUS* STRAINS FOR AFLATOXIN M_1 BINDING ABILITY

3.3.1 AFM_1 binding assay in PBS with live probiotic *Lactobacillus* strain

The AFM_1 binding assay was performed according to the procedure described by Abbes *et al.,* (2013) with slight modifications. Stock solutions of AFM_1 was prepared in acetonitrile and final concentration of test AFM_1 (10 ng/mL) was made through dilution with PBS. One milliliter of active culture of each probiotic strain (10^9cfu/mL) was centrifuged at 5000 rpm for 10 min and the bacterial pellet was washed twice with 1 mL of sterile ultrapure water (Milli-Q, Millipore, Merk, India).

The washed bacterial pellet was resuspended in 1 mL of PBS (pH 7.3) spiked with 10 ng/mL of AFM_1 and after vortexing for 15 seconds incubated at 37°C for 6, 12, and 24 h. The AFM_1 binding efficiency of *Lactobacillus* strains were examined by removal of cell biomass by

centrifugation at 5000 rpm for 10 min at 10°C. The unbound AFM_1 in the supernatant was determined by ELISA. Cell-free PBS spiked with 10 ng/mL of AFM_1 was used as positive control and cells suspended in non spiked PBS as negative control.

3.3.2 Live probiotic *Lactobacillus* strain-AFM_1 complex stability assay

Probiotic lactobacilli-AFM_1 complex stability was evaluated by washing it three times with PBS (AFM_1 free). The amount of AFM_1 released after each wash was determined by ELISA. The experiment was performed in triplicates as given by Hernandez-Mendoza *et al.,* (2009) with slight modifications.

The bacteria-AFM_1 complex was suspended in 1 mL of AFM_1 free PBS, vortexed for 15 second and incubated at room temperature for 5 min. Thereafter, bacterial cell biomass was centrifuged at 5000 rpm for 10 min at 10°C and supernatant obtained was used for analysis of released AFM_1.The same procedure was repeated thrice.

3.3.3 Chloroform treatment assay

A chloroform treatment assay was performed to study the mode of interaction between *Lactobacillus* strains and AFM_1 as per the method given by Haskard *et al.,* (2001) with some modifications. The chloroform treatment was performed as follows: Bacterial pellets with bound AFM_1 were resuspended in 1 mL of AFM_1 free chloroform and incubated for 30 min at 37°C. After incubation it was vortexed for 5 min and again incubated at 37°C for 30 min. After repeated (two times) vortexing and incubation process, cell biomass was pelleted out at 7000 rpm/15 min at 10°C and the supernatant was quantified for released AFM_1 by ELISA.

3.3.4 *In- vitro* digestion model for estimation in reduction of AFM_1 bioaccessibility by live lactobacilli strains

In-vitro digestion model was used to appraise the efficacy of selected probiotic *Lactobacillus* strains to reduce AFM_1 bioaccessibility under simulated GIT conditions. The *in-vitro* bioaccessibility assay was carried out with slight modifications in the procedure of Kabak and Ozbey, (2012). The modifications include such as before commencement of bioaccessibility assay bacterial cells were allowed to form complex with AFM_1 at 37°C for 24 hours. Subsequently, bacterial-AFM_1 complex was subjected to simulated gastrointestinal conditions.

Experimental groups were established as follows: skim milk free from AFM_1 and probiotic bacteria; skim milk spiked with 10 ng/mL AFM_1; skim milk containing AFM_1 and probiotic bacteria (10^9 CFU/mL).

The experiment was performed in triplicate. The digestion process was started from saliva phase for 10 min. and the gastric conditions for 2 hours, followed by simulated small intestine conditions for 2 hours at 37°C. The digestion process was started by taking 4.5 mL of each experimental group were placed in digestion tubes. The simulated mouth conditions (pH 6.5) were created by transferring 6 mL of artificial saliva into digestion tubes. The tubes were incubated horizontally in a shaking water bath at 37°C for 10 min at 80 rpm.

Following addition of 12 mL of gastric juice (pH 2.0). These tubes were placed horizontally in a shaking water bath at 37°C for 2 hours at 80 rpm. The last step, 12 mL of simulated duodenal juice (pH 7.5) was added simultaneously, and the mixture was incubated under similar conditions for 2 hours. Following intestinal digestion, the chyme (supernatant) and the digested matrix (pellet) were obtained by centrifugation at 5000 rpm for 10 min at 4°C. The quantity of AFM_1 in the supernatant was measured by ELISA.

Table: 3.2 Composition of digestive juices

Parameter	Saliva	Gastric juice	Duodenal juice	Bile juice
Composition (per litre)	0.9 g KCl	2.75 g NaCl	7.01 g NaCl	5.26 g NaCl
	0.2 g KSCN	0.27 g NaH_2PO_4	3.39 g $NaHCO_3$	5.79 $NaHCO_3$
	0.9 g NaH_2PO_4	0.82 g KCl	0.08 g KH_2PO_4	0.38 g KCl
	0.57 g $NaSO_4$	0.4 g $CaCl_2.2H_2O$	0.56 g KCl	0.15 ml HCl (37 %)
	0.3 g NaCl	0.31 g NH_4Cl	0.05 g $MgCl_2$	0.25 g urea
	1.7 g $NaHCO_3$	6.5 ml HCl (37 %)	0.18 ml HCl (37 %)	0.22 g $CaCl_2.2H_2O$
	0.2 g urea	0.65 g glucose	0.1 g urea	1.8 g BSA
	290 mg α-amylase	0.02 g glucuronic acid	0.2 g $CaCl_2.2H_2O$	30 g Oxbile
	15 mg uric acid	0.085 g urea	1 g Bovine serum	-

			albumin (BSA)	
	25 mg mucin	0.33 g glucosamine hydrochloride	9 g pancreatin	-
	-	1 g BSA	1.5 g lipase	-
	-	2.5 g pepsin	-	-
	-	3 g mucin	-	-
pH	6.5±0.1	2±0.1	7.5 ±0.1	7.5±0.1
Transit Time	10 min	2 h	2 hours	2 hours

3.3.5 AFM_1 binding assay in milk with heat killed probiotic *Lactobacillus* strain

The AFM_1 binding assay was performed according to the procedure of Peltonen *et al.,* (2001) with some modifications. Stock solutions of AFM_1 were prepared in acetonitrile, and the final concentration of test AFM_1 (10 ng/mL) was made through the dilution with AFM_1 free skim milk. One milliliter of each probiotic *Lactobacillus* strain active culture (10^9cfu/mL) was centrifuged at 5000 rpm for 10 min; bacterial pellets were washed twice with 1 mL of sterile ultrapure water (Milli-Q- Millipore, Merk, India).

Washed bacterial pellets were subjected to autoclave (121°C for 15 min) and re-suspended in 1 mL of skim milk spiked with 10 ng/mL of AFM_1, vortexed for 15 second and bacterial-AFM_1 suspensions were incubated at 37°C for 6, 12, and 24 hours. Subsequently, cells were removed by centrifugation (5000 rpm, 10 min) and supernatant containing unbound AFM_1 collected and analyzed by ELISA. Cell-free skim milk spiked with 10 ng/mL of AFM_1 was used as positive control and cells suspended in non spiked skim milk acted as negative control.

3.3.6 Complex stability assay with heat killed probiotic *Lactobacillus* strain

The complex stability of AFM_1 and heat killed probiotic *Lactobacillus* strains were evaluated. The experiment was performed in triplicates as per method of Kabak and Var, (2008) with slight modifications.

The pellets obtained by centrifugation were suspended in 1 mL of AFM_1 free PBS, vortexed for 15 second and incubated at 37°C for 10 min. Thereafter, bacterial cell biomass was

centrifuged at 5000 rpm for 10 min at 10°C and supernatant obtained was used for the analysis of released AFM_1. The same procedure was repeated thrice.

3.3.7 *In-vitro* digestion model for estimation in reduction of AFM_1 bioaccessibility by heat killed probiotic *Lactobacillus* strains

In-vitro digestion model was used to appraise the efficacy of selected heat killed probiotic *Lactobacillus* strains to reduce AFM_1 bioaccessibility under simulated gastrointestinal conditions. The *in-vitro* bioaccessibility assay was carried out with slight modifications in the procedure of Kabak and Ozbey, (2012).

The modifications include before commencement of bioaccessibility assay heat killed bacterial cells were allowed to form complex with AFM_1 at 37°C for 24 hours. Subsequently, bacterial-AFM_1 complex were subjected to simulated gastrointestinal conditions. Experimental groups were established as follows: skimmed milk free from AFM_1 and probiotic bacteria; skimmed milk spiked with 10 ng/mL AFM_1; skimmed milk containing AFM_1 and heat killed probiotic bacteria (10^9 cfu/mL). The experiment was performed in triplicate.

The digestion process was started from saliva phase for 10 min. and the gastric conditions for 2 hours, followed by simulated small intestine compartment for 2 hours at 37°C. To start the digestion process, 4.5 mL of each experimental group were placed in digestion tubes. For the simulated mouth compartment (pH 6.5), 6 ml of artificial saliva was added into digestion tubes; incubated horizontally in a shaking water bath at 37°C, 80 rpm for 10 min.

Later, 12 mL of gastric juice (pH 2.0) was added. The tubes were placed horizontally in a shaking water bath at 37°C and 80 rpm for 2 hours. Finally, 12 mL of simulated duodenal juice (pH 7.5) was added simultaneously, and the mixture incubated under similar conditions for 2 hours. Following intestinal digestion, the chyme (supernatant) and the digested matrix (pellet) were obtained by centrifugation at 5000 rpm for 10 min at 4°C. The quantity of AFM_1 in the supernatant was measured by ELISA. All the experiments were performed in triplicate.

3.3.8 Quantitative analysis of AFM_1 by ELISA

The quantitative analysis of AFM_1 was determined by competitive ELISA using AFM_1 test kit. The AFM_1 standard (50 µL) and test samples (50 µL) were added into the separate well

in duplicate of micro-titer plate precoated with AFM_1 antibodies. Subsequently, enzyme conjugate (50 µL) and antibody working solution (50 µL) were added into each well.

Plate was gently shaked and incubated at 25°C for 30 min. After the washing step, 50 µL of each substrate A and substrate B were added to the wells and incubated at 25°C for 15 min. The reaction was stopped by addition of 50µL of stop solution and the absorbance was measured at 450 nm in ELISA plate reader (Perkin Elmer VictorX3,2030 Multilabel Reader, USA). The absorption intensity was established to be inversely proportional to AFM_1 concentration in the sample.

OBJECTIVE II

3.4 EVALUATION OF SELECTED PROBIOTIC *LACTOBACILLUS* STRAIN FOR REDUCTION OF AFLATOXIN M_1 BIOACCESSIBILITY (*IN VITRO* AND *IN VIVO*) AND IT'S PREVENTIVE ROLE AGAINST AFLATOXINM_1 IMMUNOTOXICITY AND GENOTOXICITY

3.4.1 Animals

Female Balb/C mice used in this study were obtained from small animal house of ICAR-National Dairy Research Institute (Deemed University), Karnal-132001, Haryana, India. The animals were about 8-9 weeks old and were approximately of average body weight (~25 g).

All mice were acclimatized for 1 week prior to any experimental treatments. They were housed in polypropylene cages (6 or 8 animals/cage). The animal cages were kept in well ventilated and air-conditioned room maintained at small animal house facility with relative humidity of 40±5 % at 22±2°C. Under 12 hours lighting and 12 hours dark cycle. The animals were maintained in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) as well as from Committee for the Purpose of Control & Supervision of Experiments on Animal (CPCSEA).

3.4.2 Grouping of animals and feeding schedules

The experiments were completed in two phases (phase 1 and 2).

3.4.3 PHASE 1 (Standardization of AFM_1 dose in mice)

The phase 1 study was assigned for AFM_1 dose standardization to determine the toxic effect at different concertration of AFM_1 on mice. The animals were divided into 3 groups of 4

mice each and fed permitted *ad libitum* diet and water (**Table 3.3**). The AFM_1 was orally administered by gavage for standardization of the dose for 15 days.

Table: 3.3 Standardization of AFM_1 dose

Group	No. of animals	Standardization of AFM_1 dose for 15 days	
I	4	Normal control group (NCG)	Sacrifice of all mice after 15 days
II	4	AFM_1 dose @ 0.5 ppb	
III	4	AFM_1 dose @ 1.0 ppb	

3.4.4 PHASE 2

Table: 3.4 Experimental design and sacrifice schedule

Group	No. of animals	Experimental design		
		30 days		**31st Day**
NCG	6	Basal diet (Control)	Administration of AFM_1 and probiotic culture through gavage to each mice	Sacrifice of all mice after 30 days analysis as per table 3.5
PCG	6	Basal diet + AFM_1		
LGG	6	Basal diet +LGG		
AFM_1-LGG	6	Basal diet + LGG + AFM_1		
CRD7	6	Basal diet + CRD7		
AFM_1-CRD7	6	Basal diet + CRD7+ AFM_1		

NCG= Normal Control Group, PCG= Positive Control group, LGG= *L. rhamnosus* GG Group, AFM_1+LGG= Aflatoxin M_1 + *L. rhamnosus* GG Group, CRD7= *L. plantarum* CRD7 Group, AFM_1+CRD7= Aflatoxin M_1 + *L. plantar.*

The phase 2 study was assigned for efficacy evaluation of selected probiotic *L. plantarum* CRD7 for reduction of AFM_1 bioaccessibility and its preventive role against toxicity. The mice were randomly divided into six major groups *i.e.* normal control group (NCG), AFM_1 group (PCG), LGG group, AFM_1- LGG group, CRD7 group and AFM_1- CRD7 group fed with

respective experimental diets. Six mice were taken in each group, used to investigate the effects of oral administration of *L. plantarum* CRD7 for reduction of AFM_1 bioaccessibility and its preventive role against toxicity. The grouping of animals and sacrifice schedule has been given in **Table 3.4**. The body parts of animals were analyzed for the parameters indicated in **Table 3.5**.

Table: 3.5 Parameters studied for animal experiments

S. No.	Parameter studied
1.	Body weight
2.	Analysis the effect of probiotic on AFM_1 bioaccessibility
3.	Determination of immunotoxicity
4.	Determination of genotoxicity
5.	Determination of hepatotoxicity
6.	Determination of oxidative stress
7.	Histo pathological examinations

3.4.5 Animal sacrifice

Animals were sacrificed as per schedule mentioned in **Table 3.3 & Table 3.4**. All preparations like proper color coding and marking of tubes for sample collection including blood, liver, thymus and spleen were done two days prior to sacrifice day. Histopathological analysis of liver was carried out. Two sets of tubes; one having 5 mL phosphate buffer saline (PBS) and other having 10 % formalin solution were prepared. On the day of sacrifice, body weight was recorded as well as feces were collected prior to sacrifice. Sacrifice was done using cervical dislocation after giving anesthesia using diethyl ether. Immediately after dissection, blood and tissues were collected in respective tubes carefully and all samples were kept in ice at 4°C till next use. Tissue samples for the purpose of histopathology were sent on the same day in formalin solution to histopathologist. Blood was used immediately after its collection for liver

enzyme estimation and other biochemical parameters as per instructions given in the respective kits.

3.4.6 Blood collection

Various biochemical parameters indicative of liver function were studied analyzing blood obtained from cardiac puncture method immediately after opening the diaphragm. The blood was stored in pre-sterilized 2 mL microcentrifuge tube. Tubes were marked properly *w.r.t.* group name, animals and sample number. Blood was allowed to coagulate by keeping it in tilted position at room temperature *i.e.* 37°C for about 30 minutes. It was later centrifuged at 5000 rpm for 5 minutes to obtain blood serum. Prolong centrifugation was avoided to prevent RBC lysis. The supernatant obtained after centrifugation was collected in separate 2 ml microcentrifuge tube and stored at 2-8°C. The liver enzyme as well as other components remains stable in serum up to 3-5 days at 2-8°C.

3.4.7 Organ collection

Liver

Liver was excised out and transferred into 5 ml PBS for further biochemical analysis. For histopathological analysis livers were transferred into 10 ml of 10 % formalin solution.

Thymus

Thymus excised out and transferred into 5 ml PBS to check the viability of thymus cells.

Spleen

Spleen dissected out and transferred into 5 ml PBS to check the viability of spleen cells.

3.4.8 *In vivo a*ssessment of AFM_1 bioaccessibility

*In vivo a*ssessment of AFM_1 bioaccessibility was carried out as per procedure of Mykkanen *et al.,* (2005). To access the aflatoxin M_1 bioaccessability in mice model, feces of mice were collected after 12 hours of dosing and were subjected for ELISA technique to analyze amount of AFM_1 released with probiotic *Lactobacillus* strain.

3.4.8.1 Sample preparation

Fecal samples were accessed for release of AFM_1. The fecal material were homogenized with chloroform and then centrifuged at 5000 rpm for 15 min.
The supernatant material was collected and after filtration subjected for quantitative analysis.

3.4.8.2 Quantitative analysis of AFM_1 by ELISA

The quantitative analysis of AFM_1 was determined by competitive ELISA using AFM_1 test kit. The AFM_1 standards (50 µL) and test samples (50 µL) were added into separate in duplicate wells of micro-titer plate precoated with AFM_1 antibodies. Subsequently, enzyme conjugate (50 µL) and antibody working solution (50 µL) were added into each well.

Plate was gently shaked and incubated at 25°C for 30 min. After the washing step, 50 µL of substrate A and substrate B were added to the wells and incubated at 25°C for 15 min. The reaction was stopped by addition of 50 µL of stop solution and the absorbance was measured at 450 nm in ELISA plate reader (Perkin Elmer VictorX3,2030 Multilabel Reader, USA). The absorption intensity was established to be inversely proportional to AFM_1 concentration in the sample.

3.4.9 Biochemical analysis

3.4.9.1 Determination of immunotoxicity

To determine immunotoxicity blood, thymus and spleen were collected and analyzed for total leucocytes count, different leucocytes count, viable splenocyte and viable thymocytes.

3.4.9.1.1 Total Leucocytes Count

Leucocytes or white blood cells are the effectors cells of the immune system and these circulate throughout the bloodstream and lymphatic system. An infection or a physical injury results in an inflammatory response, which induces increased production of leucocytes or white blood cells for resolving the injury or infection. Due to this association between leucocytes or white blood cells and inflammatory response, leucocytes or white blood cells count is a valuable metric for diagnosis and prognosis of several diseases.

Blood sample were diluted to 1:20 with diluting fluid. The cork of the tube was tightly closed and suspension was mixed by rotating in a cell-suspension mixer for at least 1 minute. The Neubauer counting chamber was filled by using a Pasteur pipette (the depth of the fluid should be 0.1 mm). The 'W' marked areas were focused (each having 16 small squares) by turning objective to low power (10 X). Cells were counted in all four W marked corners.

Calculations: Number of white blood cells/mm^3 of blood = (number of white cells counted * Dilution) / (Area counted * Depth of fluid). [Where, Dilution = 20; Depth of fluid = 0.1 mm (constant; area counted 4 * 1 sq. mm = 4 sq. mm).

3.4.9.1.2 Different Leucocytes Count

Fluctuation in numbers of different leucocytes counts is also a sign of physical illness and infection in the body. To determine different leucocytes count polychromic staining solution (Leishman stain) are used that contains methylene blue and eosin. These basic and acidic dyes induce multiple colours when applied to cells. Methanol acts a fixative and also as a solvent. The fixative makes them adhere to the glass slide. The basic components of white blood cells (cytoplasm) are stained by acidic dye and they are describing as eosinophilic or acidophilic. The acidic components (nucleus & nucleic acid) of the cells take blue to purple shades by basic dye and they are called basophilic. The neutral components of the cell are stained by the dyes.

A thin blood smear was prepared by spreading a drop of blood evenly on a clean and dry glass slide and dries the smear at room temperature. Smear was covered with the Leishman staining solution by adding 10 – 15 drops on the smear by using a Pasteur pipette. Equal amount of buffer solution was added on the slide. Smear was washed using running tap water.

Stained smear was examined under the oil immersion objective (100 X) in the microscope. Field was examined by moving the slide systematically. The types of leukocytes seen in each field were recorded and result was given as percentage (%) of the cells.

3.4.9.2 Splenocytes and Thymocytes assessment

Spleen and thymus cellularity were determined after dispersion of the tissues into single-cell suspension in RPMI 1640 culture medium (Gibco) as described by Nohara *et al.*, (2002). Briefly, thymocytes and spleen cells were prepared in complete RPMI 1640 containing 12 mM

HEPES (pH 7.1), 50 mM 2- mercaptoethanol, 100 U penicillin/ml, 100 mg streptomycin/ml, and 10 % FCS, by passing each isolated tissue through a stainless steel mesh. The spleen and thymus cells were further treated with 0.84 % [w/v] ammonium chloride/EDTA solution to eliminate red blood cells. After a final centrifugation/washing step, cell viability was determined by MTT assay.

The MTT 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format (Mosmann,1983).

50 µL of sample and 50 µL of MTT solution were added into each well. The 96 well plates were incubated at 37°C for 3 hours. After incubation, 150 µL of MTT solvent was added into each well. Sample plate was wrapped with foil and shake on an orbital shaker for 15 minutes. The absorbance was measured at 590 nm within 1 h.

3.4.9.3 Determination of hepatotoxicity

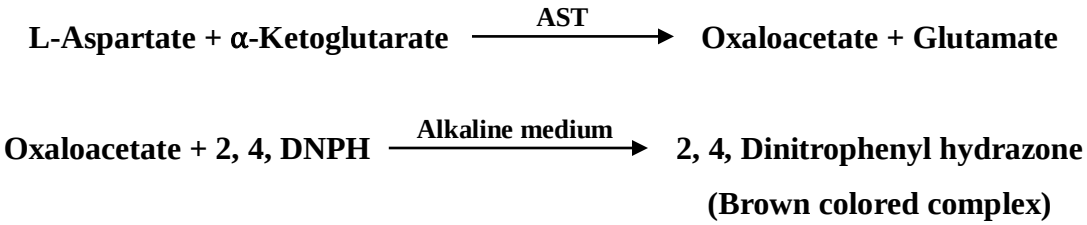
Liver function tests (LFTs) or liver enzyme tests are group of blood tests that detects any kind of inflammation and damage to the liver. They also indicate about healthy functioning of liver. Hepatotoxicity was determined by assessing the level of enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) in blood plasma.

3.4.9.3.1 Aspartate transaminase (AST) estimation

Serum glutamic oxaloacetate transferase (SGOT) or aspartate transaminase (AST) or aspartate aminotransferase (ASAT) is a pyridoxal phosphate (PLP)-dependent transaminase enzyme (EC 2.6.1.1). AST catalyzes the reversible transfer of α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism. The AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Serum AST and ALT (alanine transaminase) levels, and their ratio (AST/ALT ratio) are commonly measured as clinical biomarkers for liver health.

Principle

The estimation of this enzyme is based on the principle that enzyme catalyzes the transfer of amino group between L-aspartate and α-ketoglutarate. Aspartate transaminase converts L-aspartate and α-ketoglutarate to oxaloacetate and glutamate. The oxaloacetate formed in previous step reacts with 2, 4, dinitrophenylhydrazine (2, 4, DNPH) to produce a hydrazone derivative, which in an alkaline medium that produces a brown colored complex whose intensity is measured at 450 nm. Normal range falls between 8 - 40 Units/L.



Diagnostically, it is almost always measured in units/liter (U/L). Experiments were performed using fresh serum following the instructions given in the manual of the kit.

Procedure: The concentration of enzymes in serum was determined by using commercial enzymatic kits (Reckon Diagnostic P.Ltd) as per the standard protocol recommended.

3.4.9.3.2 Alanine transaminase (ALT) estimation

The enzyme alanine aminotransferase (ALAT) or alanine transferase (ALT) is widely reported in a variety of animal tissues. The major source of ALT is of hepatic origin which has led to the application of ALT determinations in the study of hepatic diseases. Elevated ALT serum levels have been reported in hepatitis cirrhosis and obstructive jaundice. Levels of ALT are only slightly elevated in patients following a myocardial infarction. UV methods for ALT determination were first developed by (Wroblewski and Ladue, 1956). The method was based on the oxidation of NADH by lactate dehydrogenase (LDH). In 1980, International Federation of Clinical Chemistry (IFCC) recommended a reference procedure for the measurements of ALT based on the Wroblewski and Ladue procedure.

Principle

The estimation of ALT is based on the principle that it catalyze transfer of amino group between L-alanine and α-ketoglutarate that results in the formation of pyruvate and L-glutamate. The increase in pyruvate is measured in a subsequent indicator reaction which is catalyzed by lactate dehydrogenase:

$$\textbf{L-Alanine + α-Ketoglutarate} \xrightarrow{\text{ALT}} \textbf{Pyruvate + L-Glutamate}$$

$$\textbf{Pyruvate + NADH + } H^{+} \xrightarrow{\text{LDH}} \textbf{Lactate + NAD+ + } H_2O$$

The pyruvate formed in the first reaction is reduced to lactate in presence of lactate dehydrogenase (LDH) and NADH. The activity of ALT is determined by measuring the rate of oxidation of NADH at 340 nm. Endogenous sample pyruvate is converted to lactate by LDH during the lag phase prior to measurement. Experiment was performed using fresh serum following the instructions given in the manual of the kit.

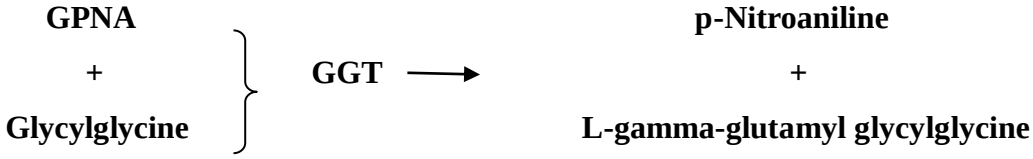
Procedure: The concentration of enzymes in serum was determined by using commercial enzymatic kits (Reckon Diagnostic P.Ltd) as per the standard protocol recommended.

3.4.9.3.3 Gamma-glutamyl transferase (GGT) estimation

Gamma glutamyl transferase (GGT) is one of the most sensitive enzyme of the hepatobiliary system. Elevated serum gamma-GT levels are indicative of the disease of the liver, biliary tract and pancreas. In cases of metastatic carcinoma, viral hepatitis, chronic hepatitis, cholelithiasis, cholangitis and cholecystitis, gamma-GT levels are found to be elevated. Since GGT activity is not elevated in any bone disorders, the assay is considered as a valuable diagnostic aid for differentiation between bone and liver disease in conjuction with alkaline phosphatase.

Gamma glutamyl transferase (GGT) catalyzes the transfer of the gamma glutamyl group from a gamma glutamyl peptide to an amino acid of another peptide. In this method, L-gamma-glutamyl-p-nitroanilide (GPNA) as a substrate since end product is colored and it permits a direct reaction rate measurement without deproteinization. Gamma - glutamyl-p-nitroanilide (GPNA) and glycylglycine are converted by the action of GGT to p - nitroaniline and L - gamma glutamyl

glycylglycine. The rate of increase in absorbance at 405 nm due to the release of p-nitroaniline is directly proportional to the GGT activity.



Procedure: The concentration of enzymes in serum was determined by using commercial enzymatic kits (Reckon Diagnostic P.Ltd) as per the standard protocol recommended.

3.4.9.4 Determination of oxidative stress

The oxidative stress was estimated in liver collected from experimental animals as describes in section **3.4.7.** The tissue homogenate was prepared and oxidative stress variables *i.e.* activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured as follows.

3.4.9.4.1 Preparation of tissue homogenate

The tissues were lysed by the exposure to chilled water and vigorous shaking. Twenty percent homogenate was prepared and used freshly for the measurement of oxidative stress variables.

For preparation of liver homogenate, methodology of Cohen *et al.*, (1970) was adopted. The liver was weighed (1 g) and homogenized (10 strokes) in the cold isotonic buffer **(**A stock solution was prepared by dissolving 180 g NaCl, 27.3 g Na_2HPO_4 and 4.86 g $NaH_2PO_4.H_2O$ in distilled water, pH adjusted to 7.4 and a final volume was made to two liters. Nine milliliter of stock solution was diluted with distilled water and volume made to 100 mL to obtain isotonic solution).

The homogenate was centrifuged for 10 min at 700 g to remove nuclei and cell debris. The supernatant was used for assaying oxidative measures.

3.4.9.4.2 Estimation of total protein in liver homogenate

Protein was estimated according to the method of Lowry *et al.* (1951).

Reagents:

Lowry's reagent

Solution A: 2 % Na_2CO_3 in 0.1N NaOH;

Solution B: 0.5 % $CuSO_4$.$5H_2O$ in distilled water, Solution C: 1 % sodium potassium tartarate in distilled water.
Forty eight mL of solution A was mixed with 1 mL each of solution B and C.

Folin-Ciocalteu (FC reagent)

This reagent was obtained as stock solution (2N). It was diluted with distilled water to 1 N before use.

Procedure

To 0.1 mL of the liver homolysate sample solution, 0.9 mL of distilled water and 5 mL of Lowry's reagent was added. It was mixed well and allowed to stand for 15 min at 37°C and then 0.5 mL of Folin-Ciocalteu reagent was added. The contents were shaken vigorously and allowed to stand for 30 min in dark for maximum development of color. Absorbance was measured at 660 nm and total protein content was calculated from the standard curve prepared using bovine serum albumin as standard.

3.4.9.4.3 Catalase activity estimation

The catalase activity was assayed by the method of Aebi, (1984).

Reagents:

Phosphate buffer (50 mM, pH 7.0):

a) **Solution A**: 8.90 g of Na_2 $HPO_4.2H_2O$ dissolved in distilled water and volume made to one litre.

b) **Solution B**: 6.81 g of KH_2PO_4 dissolved in distilled water and volume made to one liter. To solution A, solution B was added until the pH reached to 7.0.

c) **H_2O_2 (30 mM):** Diluted 0.34 mL of 30 percent H_2O_2 with phosphate buffer to 100 mL.

d) **Triton X-100 (10 %)**: 10 mL of Triton X-100 was added to 90 mL of 1 % isotonic buffer.

Procedure:

The reaction mixture in total volume of 3 mL contained 2 mL of liver homogenate in appropriate dilution with phosphate buffer (50 mM, pH 7.0) and 1 ml of H_2O_2 (30 mM) at room temperature. The blank contained 1 mL phosphate buffer instead of substrate (H_2O_2) and 2 mL appropriately diluted liver lysate. The reaction was started by addition of H_2O_2. The decomposition of H_2O_2 was measured by decrease in absorbance at 240 nm using specord 200 double beam UV/visible spectrophotometer (Analytikjena, Germany). The initial absorbance was approximately A=0.500 and decrease in absorbance was followed for 1 min. The difference in absorbance (ΔA 240) per unit time was the measure of catalase activity. The enzyme activity was calculated using an extinction coefficient of 0.00394 litres/mM/mm and expressed as µmoles of H_2O_2 consumed/min/mg protein. Catalase activity was observed in liver homogenate expressed as µmol of H_2O_2 consumed/min/mg protein.

3.4.9.4.4 Superoxide dismutase (SOD) estimation

The SOD activity was assayed by the method of Marklund and Marklund, (1974).

Reagents

A) **Tris-HCl buffer (50 mM with 1mM Diethylene Triamine Penta (DETAP) Acetic Acid, pH 8.2)**
   Tris (605 mg) and DETAP acetic acid, 39.33 mg) were dissolved in distilled water and pH was adjusted to 8.2 using HCl (1N) and total volume made to 100 mL.
B) **Pyrogallol (2 mM)**
   Pyrogallol (25.2 mg) was dissolved in 100 mL of HCl (10 mM).

Procedure

The reaction mixture contained 0.2 mL of appropriately diluted RBC lysate or liver homogenate. Total volume was made to 3 mL by adding Tris-HCl buffer and 0.2 mL of pyrogallol. A blank was prepared without addition of sample. The rate of auto-oxidation of pyrogallol was taken as increase in absorbance at 420 nm against a reference cuvette containing 3 mL Tris-HCl buffer. The increase in absorbance was approximately 0.02 per min in the absence of SOD. The inhibition of pyrogallol auto-oxidation was brought about

by SOD, which was employed for the determination of enzyme activity. A unit of enzyme was defined as the amount of enzyme that inhibits the reaction by 50 %.

3.4.9.4.5 Glutathione Peroxidase (GPx) estimation

The enzyme activity of GPx was estimated spectrophotometrically using the method of Burk and Lawrence (1976). GPx was assayed by the couple enzyme method utilizing excess glutathione reductase that couples oxidation of NADPH to the rate of reaction of the peroxidase with H_2O_2 and glutathione (GSH).

The reaction mixture consisted of 50 mM potassium phosphate buffer pH 7.0, 1 mM EDTA, 1 mM Sodium azide (NaN_3), 0.2 mM NADPH, 1 EU/mL glutathione reductase, 1 mM GSH and 1.5 mM cumene hydroperoxide in total volume of 1 mL.

0.1 mL of sample was added to 0.8 mL of above mixture and allowed to incubate for 5 min at room temperature before initiation of reaction. Reaction was initiated by addition of 0.1 mL of cumene hydroperoxide and absorbance was recorded at 340 nm for 5 min. For blank, distilled water was used. The oxidation of NADPH was monitored by change in absorbance at 340 nm. The enzyme activity was calculated using extinction coefficient of 6.22×10^3 liters/ mol/ cm.

3.4.10 Determination of genotoxicity

3.4.10.1 Comet assay

Comet assay has been performed as per the method of Abd-Allah *et al*., (1999). A single cell gel assay (also term comet assay) is a very useful micro-electrophoresis technique for evaluation of DNA damage and repair in individual cell. The technique has been developed to visualize DNA damage induced by various methods such as radiation, oxidative damage, chemicals and toxins.

Reagents

1X PBS (Ca^{++} and Mg^{++} free), NaOH pellets, dimethyl sulfoxide, Dimethyl sulfoxide (DMSO), 10X TBE buffer, 0.5 mM EDTA, dH_2O, methanol, glacial acetic acid, lysis solution, comet low melting agarose, alkaline unwinding solution (pH >13), electrophoresis solution, EtBr solution.

3.4.10.1.1 Tissue preparation

A small piece of tissue was placed for 1-2 min in 1X PBS+20 mM EDTA. The tissue was cut in size 12mm^3 using dissecting scissor. Cell suspension was recovered by avoiding material contamination. The mixture was centrifuged at 5000 rpm for 15 min and final concentration of the suspension would be adjusted to 1x 10^5 cells/mL. Mix the cell suspension in ice cold 1X PBS (Ca^{++} and Mg^{++} free).

Procedure

1. Cell suspension was mixed with molten low melting agarose (at 37^{o}C) in a ratio of 1:10.
2. The 75µL of cell suspension was transferred on comet slide and spread properly.
3. Slide was placed in dark for 10 min.
4. The slide was immerse with lysis solution and left at 2-8^{o}C for 30-60 min.
5. Slide was washed with tap water and then immerse in freshly prepared alkaline unwinding solution for 20-60 min.
6. The excess buffer was removed by washing the slide with tap water and then immerse in 1X TBE buffer for 5min.
7. Slide was transferred into a horizontal electrophoresis apparatus.
8. Slide was placed in gel tray and cover with 1X TBE buffer.
9. Slide was washed with tap water and then dip in 70 % ethanol for 5 min.
10. After drying slid was proceed for EtBr solution.

3.4.10.1.2 Data analysis

Under fluorescence microscope EtBr treated DNA was easily detectable. In healthy cell, the DNA is confined into the nucleoid. Undamaged DNA is super coiled and thus do not migrate very far under the influence of electric current. In the cell those have accrued damage to the DNA, alkaline treatment unwind the DNA and releasing those fragment which migrate from the nucleoid when subjected to an electric field. The negative charge DNA migrates towards the anode and the extrusion length reflects increasing relaxation of super coiling which is indicative of damage. When using TBE and electrophoresis buffer the length of the comet tail may be correlated with DNA damage. The characteristic of the comet tail including length, wide and DNA content may also be useful in assessing quantitative difference in the type of DNA damage.

3.4.11 Histopathology of liver tissue of mice

Livers was fixed for 1h in 10 % formalin saline and immediately embedded in paraffin followed by sectioned at 5 µm thicknesses using a rotary microtome. Sections were stained with hematoxylin-eosin (H&E) for light microscopy examination.

3.4.12 Statistical Analysis

The results were expressed as mean ± SEM. Statistical significance (<0.05) was tested by employing analysis of variance (ANOVA) and comparison between means were made by Tukey's multiple comparison test. For computation of data, software application programs like Microsoft Excel, Graph Pad 7.0, and SPSS 10.0 were used.

4.0 RESULT AND DISCUSSION

Aflatoxins are one of the highly toxic secondary metabolites derived from polyketides produced by certain kinds of fungi (moulds) that are found naturally all over the world. Two closely related species of fungi are mainly responsible for producing aflatoxins of public health significance: *Aspergillus flavus* and *A. parasiticus.* These moulds, normally found on dead and decaying vegetation, can invade food crops under favorable conditions typically found in tropical and subtropical regions, including high temperatures and high humidity (Payne *et al.,* 1998).

Human and animals can be exposed to aflatoxins by eating contaminated cereal crops including wheat, walnut, corn, cotton, peanuts and tree nuts (Abrar *et al.,* 2013). These lead to serious threats to human and animal health by causing various complications such as hepatotoxicity, teratogenicity, and immunotoxicity. In addition, long-term or chronic exposure to aflatoxins has several health consequences: aflatoxins are potent carcinogens and may adversaly affect all organs, especially liver and kidney. These cause liver cancer, and other types of cancer. Aflatoxin B_1 and AFM_1 are also known to be carcinogenic to humans.

The potency of aflatoxin to cause liver cancer is significantly enhanced in presence of infection with hepatitis B virus (HBV). Aflatoxins are mutagenic (affect DNA), genotoxic, and have the potential to cause birth defects in children such as stunted growth. Although these data have yet to be confirmed because other factors also contribute to growth faltering e.g. low socioeconomic status, chronic diarrhoea, infectious diseases, malnutrition. These may decrease resistance to infectious agents such as HIV, tuberculosis etc. (Alborzi *et al.,* 2006).

Milk and dairy products are considered as integral part of human diet as these are consumed in every home by all age groups because of high nutritional value and health promoting attributes. However, these may act as a vehicle of toxic contaminants such as aflatoxins, which cause various physiological risk effects in consumers especially in most sensitive group (Scaglioni *et al.,* 2014; Kang *et al.,* 2009). AFM_1 is stable in raw milk as well as heat treated milk.

AFM_1 is more specifically a problem of food safety. Therefore, there is need to develop strategies or innovative solutions for reduction and minimizing health risks of AFM_1. The best

strategy to minimize AFM_1 menace is use of certain probiotic strains, able to bind and form complex with AFM_1, thereby increase elimination of this complex from the gut through feces. Thus, this biological strategy prevents absorption of aflatoxins in human and animal bodies through the gastrointestinal tract, improve AFM_1 decontamination from the body and minimize potential risks of AFM_1 (Barukcic *et al.*, 2018). Keeping in view these reasons, present study was aimed to find a competent strain of probiotic *Lactobacillus* which would be able to reduce and control AFM_1 toxicity from contaminated milk and milk products.

4.1 DETERMINATION OF PROBIOTIC CULTURES PURITY

Purity of all the thirty lactobacilli cultures was ascertained by examining their colony morphology and microscopic examinations *i.e.* Gram's and negative staining.

4.1.1 Morphological examination

Colony morphology of lactobacilli cultures were examined by visual observations by cultivating them on MRS agar plates at 37°C for 24-48 hours. Generally lactic cultures give either pearl like white convex, opaque, round or rice like elongated or embedded colonies. All cultures developed characteristic *Lactobacillus* colonies on MRS agar suggestive of their purity and thus were designated as *Lactobacillus* species **(Plate 4.1 A)**.

4.1.1.1Gram staining

Staining is an important technique used in microbiology to determine purity of microbial culture and differentiate them on the basis of morphology (size, shape and arrangement). The bacteria present in an unstained smear are invisible when viewed using a light microscope. Once stained, the morphology and arrangement of bacteria can be clearly depicted. Gram staining is differential staining techniques that characterize bacteria into two groups: Gram-positive and Gram-negative. Gram-negative bacteria are decolorized by alcohol, losing purple color of primary stain, while Gram-positive bacteria retain purple color due to presence of large peptidoglycan layer. After decolorization step, a counter stain is used to impact a pink color to decolorized Gram-negative organisms. The cultures were examined under the microscope on the basis of their response to Gram's staining described under section **3.2.2.1**. All cultures were

observed Gram's positive as purple, rods of varying size under microscope, thereby confirmed their purity **(Plate 4.1 C)**.

4.1.1.2 Negative staining

Negative staining was performed by the method described under section **3.2.2.2**, often used in microbiology laboratories for ascertaining purity of cultures. The background is stained and actual specimen remains unstained, thus creating a contrast due to which specimen appears bright against dark background. Nigrosin or India ink is most frequently used dye for this purpose. The negative staining is particularly useful for determining cell size and arrangement. It can also be used to stain cells that are too delicate to be heat-fixed. For bright field microscope, negative staining is typically performed using a black ink fluid such as nigrosin. All the cultures were found pure in negative staining based on road like structures **(Plate 4.1 B)**.

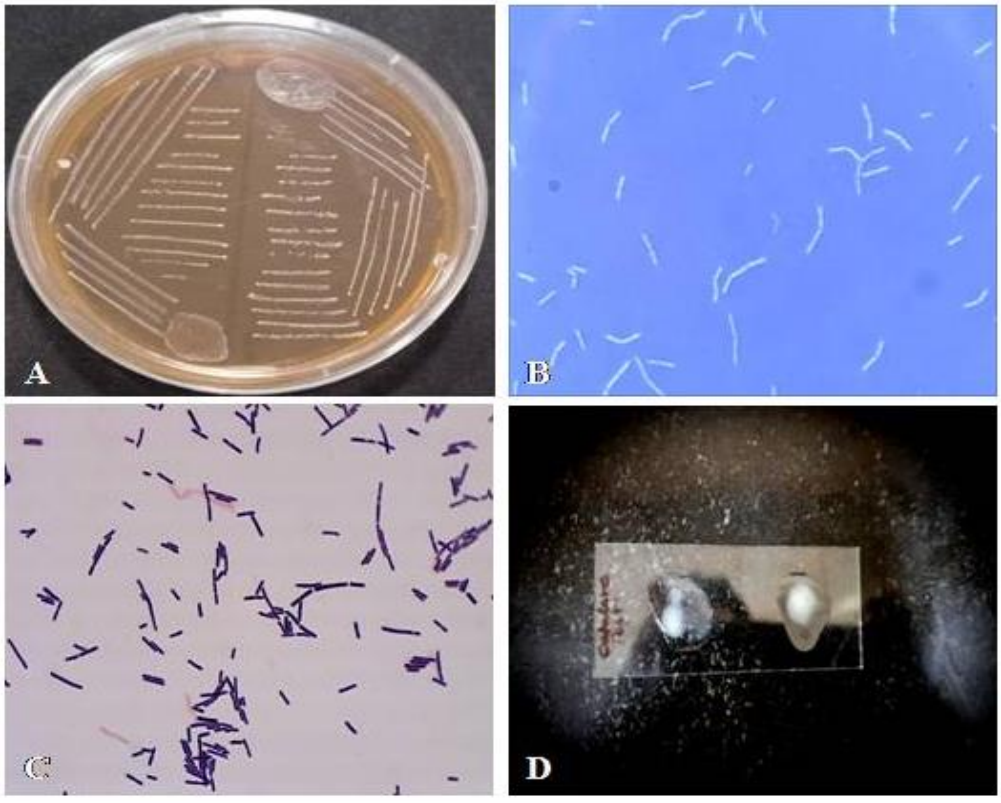


Plate (A) Colony morphology of *L. plantarum* CRD7 on MRS agar
(B) Microscopic view of *L. plantarum* CRD7 (Negative staining)
(C) Microscopic view of *L. plantarum* CRD7 (Gram's staining)
(D) Catalase test negative for *L. plantarum* CRD7

4.1.1.3 Catalase test

It was performed by the method describe under section **3.2.2.3**. Catalase, an extracellular enzyme secret by many microorganisms, is a characteristic enzyme of pathogenic bacteria hence, its presence or absence can make it as an important diagnostic tool. The enzyme catalyse the breakdown of hydrogen peroxide to produce molecular oxygen that generate vigorously with effervescence, when a microbial culture was mixed with an equal volume of 3 % hydrogen peroxide. Absence of any effervescence is indicated as negative for the catalase enzyme production. All the lactic cultures used in present investigation were found negative for catalase enzyme suggestive of their purity and food grade quality microbes **(Plate 4.1 D)**.

OBJECTIVE I

SCREENING OF PROBIOTIC *LACTOBACILLUS* STRAINS FOR AFLATOXIN M_1 BINDING ABILITY

4.2 AFM_1 BINDING BY LIVE AND HEAT KILLED PROBIOTIC *LACTOBACILLUS* STRAINS IN PHOSPHATE BUFFER SALINE (PBS) AND SKIM MILK

There are several biological, chemical, and physical methods documented in the literature to eradicate, inactivate, and avert fungal growth and production of toxin. Although, investigated methods have not been completely adopted, especially in under developed countries, because to comply, they need high expenses, affect nutritional value of the foods and feeds, tainted organoleptic characteristics of the products, difficulties to use, and other unknown effects to human health. On the other hand, traditionally used indigenous food microorganisms such as bifidobacteria and lactobacilli may have the potential to prevent adverse health impacts of aflatoxins (Bhat *et al.,* 2010; Di Natale *et al.,* 2009).

4.2.1 Aflatoxin M_1 binding efficiency of live probiotic *Lactobacillus* strains in PBS

There are many recent reports on probiotic effects of these bacteria, which are increasingly used in preparation of fermented dairy foods (Oatley *et al.,* 2000). However, very little information is available regarding binding ability of AFM_1 by lactic acid bacteria (LAB) strains as compared to AFB_1.

Thirty probiotic *Lactobacillus* strains were evaluated in the present study for their AFM_1 binding ability in PBS solution and results have been presented in **Fig 4.1 (A, B & C)** and **Table 4.1.** The AFM_1 binding ability of probiotic *Lactobacillus* strains was found to be highly strain dependent which ranged between 2.46±2.31 - 64.16±5.60 %. The AFM_1 binding on bacterial cells increased with increase in incubation period 6, 12 and 24 hours. Regardless of the strain variance, AFM_1-binding by the employed strains increased in a time-dependent manner.

Highest binding of AFM_1 was shown by *L. plantarum* CRD7 (64.16±5.60 %) followed by *L. rhamnosus* CRD9 (61.03±3.90 %), *L. plantarum* CM63 (54.16±3.78 %), *L. plantarum* BM71 (53.30±0.1 %) and *L. plantarum* HIF81 (56.84±3.86 %), respectively after 24 hours of incubation at 37°C. In contrast, *L. plantarum* HIF33 showed least binding *i.e.* 9.8±2.17 % after 24 hours of incubation at 37°C. Five strains *viz., L. plantarum* CRD7, *L. rhamnosus* CRD9, *L. plantarum* CM63, *L. plantarum* BM71 and *L. plantarum* HIF81 displaying highest AFM_1 binding ability were selected for subsequent detailed studies.

4.2.2 Aflatoxin M_1 binding efficiency of heat killed probiotic *Lactobacillus* strains in skim milk

These selected probiotic *Lactobacillus* strains were subjected to autoclave prior to evaluate their binding efficiency in skim milk. The results for ability of heat killed probiotic lactobacilli strains to bind AFM_1 in skim milk are presented in **Fig 4.2.** and **Table 4.2.** The amount of AFM_1 bound was strain specific as observed in case of live cells with the percent binding ranged from 15.79±3.75 - 60.06±3.08 %, depending on incubation period *i.e.* 6, 12 and 24 hours.

The observations on AFM_1-binding by heat killed probiotic *Lactobacillus* strains showed incremant in a time-dependent manner. The binding of AFM_1 by heat killed probiotic *Lactobacillus* stains *viz., L. plantarum* CRD7, *L. rhamnosus* CRD9, *L. plantarum* CM63, *L. plantarum* BM71, *L. plantarum* HIF81 at 10^9 cfu/mL in skim milk was 60.06±3.08, 57.23±6.61, 49.01±3.18, 55.96±4.99 and 53.4±3.36 %, respectively. *L. plantarum* CRD7 was found to be the best binder and *L. plantarum* CM63 was least AFM_1 binder.

These findings demonstrated potential of probiotic lactobacilli to detoxify AFM_1 contaminated milk, fermented and non fermented dairy products through the application of live

and heat killed probiotic strains of LAB both in fermented and non fermented dairy foods. The extent of AFM_1 binding by probiotic *Lactobacillus* strains obtained in this study are in agreement with the previous reports on AFM_1 binding properties of LAB.

Abbes *et al.,* (2013) evaluated AFM_1 binding ability of *L. plantarum* MON03 and *L. rhamnosus* GAF01 isolated from Tunisian artisanal butter in PBS and reconstituted milk spiked with various concentrations *viz.* 0.05, 0.10, and 0.20 µg AFM_1/mL after 0, 6, and 24 hours at 37°C. AFM_1 binding abilities of *L. plantarum* MON03 and *L. rhamnosus* GAF01 strains in PBS and reconstituted milk ranged from 16.1±23.5-78.6±5.12 % and 15.3±1.20-95.1±6.23 %, respectively.

Salah-Abbes *et al.,* (2015) also investigated the binding potential of *L. plantarum* MON03 in PBS contaning 50µg/mL of AFM_1, after interval of 0, 12 and 24 hours at 37°C. They observed that, AFM_1-binding by *L. plantarum* MON03 increased with the increase in incubation time. These observations showed that binding may be altered with time dependent manner. *L. plantarum* MON03 showed binding in the range from 25.9 and 93.1 % with AFM_1 after 0 and 24 hours of incubation period. Present study also demonstrated that all tested probiotic strains has the ability to remove AFM_1 in the range of 2.46±2.63-64.16±4.10 % at different time of incubation.

In present investigation AFM_1 binding significantly ($p<0.05$) differed among both live as well as heat killed strains *viz.*, *L. plantarum* CRD2, *L. rhamnosus* CRD4, *L. plantarum* CRD7, *L. rhamnosus* CRD9, *L. rhamnosus* CRD11, *L. bulgaricus* 11B, *L. plantarum* CRD121, *L. plantarum* HIF33, *L. plantarum* HD48, *L. rhamnosus* GG, *L. plantarum* HD51, *L. plantarum* MDD53, *L. plantarum* MDD54, *L. plantarum* MDD56, *L. plantarum* LD59, *L. plantarum* CM62, *L. plantarum* CM63, *L. plantarum* CM66, *L. plantarum* CM67, *L. plantarum* CM68, *L. plantarum* CM69, *L. plantarum* BM70, *L. plantarum* BM71, *L. plantarum* HIF81, *L. plantarum* HIF82, *L. plantarum* HAF84, *L. plantarum* HAF85, *L. plantarum* CAM94, *L. plantarum* CAM95 and *L. plantarum* HIF104 at different incubation time. Our findings clearly demonstrated that contact time is one of the key factors for binding of AFM_1 by different probiotic *Lactobacillus* strains.

Pierides *et al.,* (2000) documented a safe and convenient method to remove AFM_1 by using biological approach (specific lactic acid bacteria) from liquid media and they found that viable *L. rhamnosus* GG binds AFM_1 by 77±0.4 %, *L. rhamnosus* LC-705 (75.2±1.2 %) and *L. gasseri* ATCC33323 (51.4±1.9 %) after 4 hours incubation in liquid medium. However, after heat inactivation these same bacteria exhibited low AFM_1 binding ability *i.e.* 57.8±3.3, 51.6±3.0 and 61.5±0.7 %, after 15-16 hours incubation periods in PBS.

Furthermore, *L. rhamnosus* GG and *L. rhamnosus* LC-705 bound approximately 80 % AFB_1 within 0 h (El-Nezami *et al.,* 1998). Similarly, Bovo *et al.,* (2012), also evaluated ability of live and heat killed lactic acid bacteria (*L. plantarum, E. avium, P. pentosaceus, L. bulgaricus, L. rhamnosus, B. lactis* and *L. gasseri*) to remove AFM_1 in PBS and skim milk. They reported that tested strains bound AFM_1 in the range of 12.42±2.14 - 45.67±1.23 % for heat-inactivated bacteria and 5.60±2.10 - 33.54±5.12 % for viable bacteria when exposed to AFM_1 for 24 hours.

Sarimehmetoglu and Ruangwises, (2004) analyzed commonly used yogurt bacteria for their binding ability to AFM_1 in PBS and skim milk. They observed *L. delbrueckii* subsp. *bulgaricus* CH-2 bound 18.70±5.41 and 27.56±3.15 % in PBS and skim milk, while *S. thermophilus* ST-36 bound 29.42±3.14 and 39.16±352 % in PBS and skim milk, respectively. Correspondingly, the defensive effect of various bacterial strains against AFM_1 reduction has also been described by El Khoury *et al.,* (2011) who investigated ability of *L. bulgaricus* and *S. thermophilus* used in the manufacturing of Lebanese traditional foods to decrease the levels of AFM_1. They reported high AFM_1 binding ability *i.e.* 87.6±4.02 % after 14 hours, observed in case of *L. bulgaricus* and on the other hand *S. thermophilus* reduced 70±3.02 % of AFM_1 in PBS. Interestingly, *L. bulgaricus* bound 38.7±1.26 % of AFM_1 after 2 hours of incubation and *S. thermophilus* showed 19.8±4.25 % removal of AFM_1. Moreover, in combintion they reduced 26.1±5.41 % after 2 hours and 68±5.34 % after 14 hours.

The maximum microbial AFM_1 binding potential has been reported by Corassin *et al.,* (2013). They studied a pool of three heat killed lactic acid bacteria, *Saccharomyces cerevisiae* and a combination of these two, possessed binding percentages of AFM_1 in skim milk as 11.7±2.30, 92.7±5.21 and 100±5.26 %, respectively. The pool of *S. cerevisiae* and LAB was found to be most effective for aflatoxin remediation. Elsanhoty *et al.,* (2014) also reported that

strains of lactic acid bacteria show the potential to adhere with AFM_1 and reduce the toxin level up to a recommended safe limit.

Serrano-Nino *et al.,* (2013) investigated the potential of five probiotic strains for their ability to bind with AFM_1 in liquid mediun (PBS). They found that amount of bound AFM_1 ranged from 19.95±3.21 to 25.43±5.42 %.

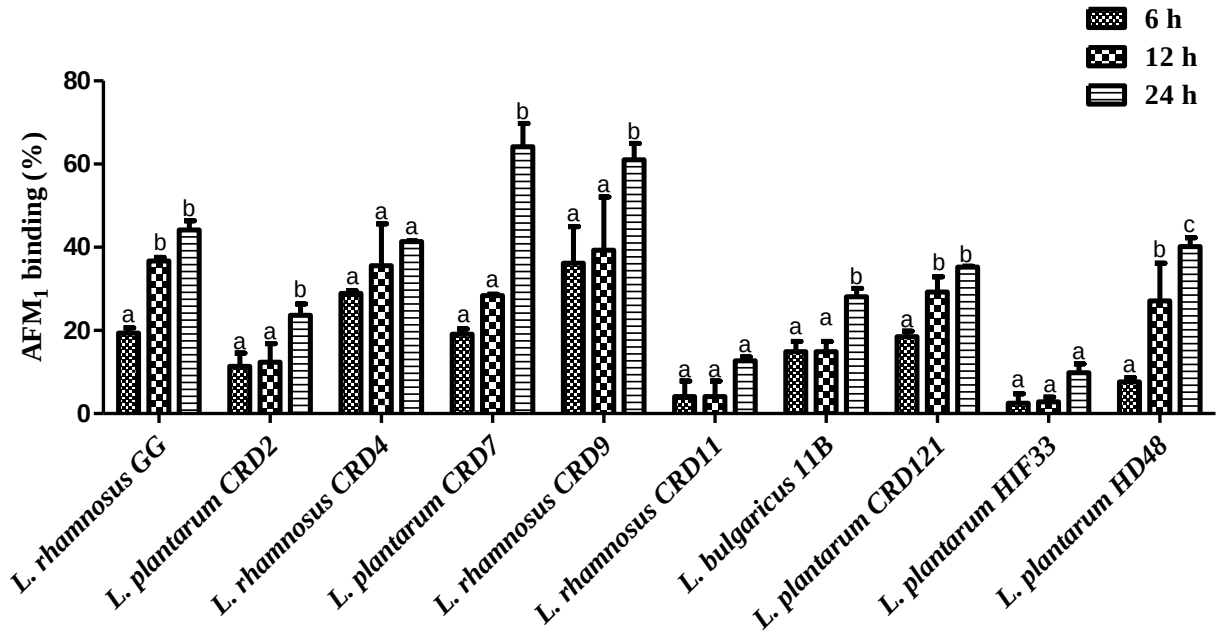
Goncalves *et al.,* (2021) microorganisms as a biological method to reduce AFM_1 they studied the binding efficiency of *L. rhamnosus* and *Lc. lactis*, and a yeast strain, *S. cerevisiae* with AFM_1 on second day and after thirty days in the Minas Frescal cheese and they found on second day, a reduction of AFM_1 was of 74 % and after thirty days that was 100 %. Furthermore, da Cruz *et al.,* (2021) conducted a study to evaluate the AFM_1 removal efficiency of probiotic fruit-derived *Lactobacillus* isolates, namely, *L. paracasei* 108, *L. plantarum* 49, and *L. fermentum* 111from a phosphate buffer solution with viable and non-viable cells (heat-killed; 100°C, 1 h) after interval of 1 and 24 hours at 37°C. They found viable and non-viable cells of all examined isolates were capable of removing AFM_1 in PBS with removal percentage values in the range of 73.9-80.0 % and 72.9-78.7 %, respectively. Viable and non-viable cells of all examined *Lactobacillus* isolates had similar abilities to remove AFM_1.

Muaz *et al.,* (2021) conduct an experiment with the objective to enhance the AFM_1 binding efficiencies of bacterial cells belonging to *L. paracasei* and *B. coagulans* in the presence of activated carbon (CAC), bentonite (CBENT) and sorbitan monostearate (CSP60). The reduction of AFM_1 was found to be directly proportional to the concentration of microbial cells. Heat killed and acid treated *L. paracasei* successfully reduced AFM_1 in milk spiked at 0.2 µg/L to 89 % and 100 % in CBENT, 84 % and 90 % in CAC, 59 % and 47 % in CSP60, respectively. Among treatments involving *B. coagulans,* acid treated CSP60 proved to be least effective showing 44.6 % reduction, while CBENT for both acid and heat treated along with acid treated CAC proved to be most effective by removing 100 % AFM_1.

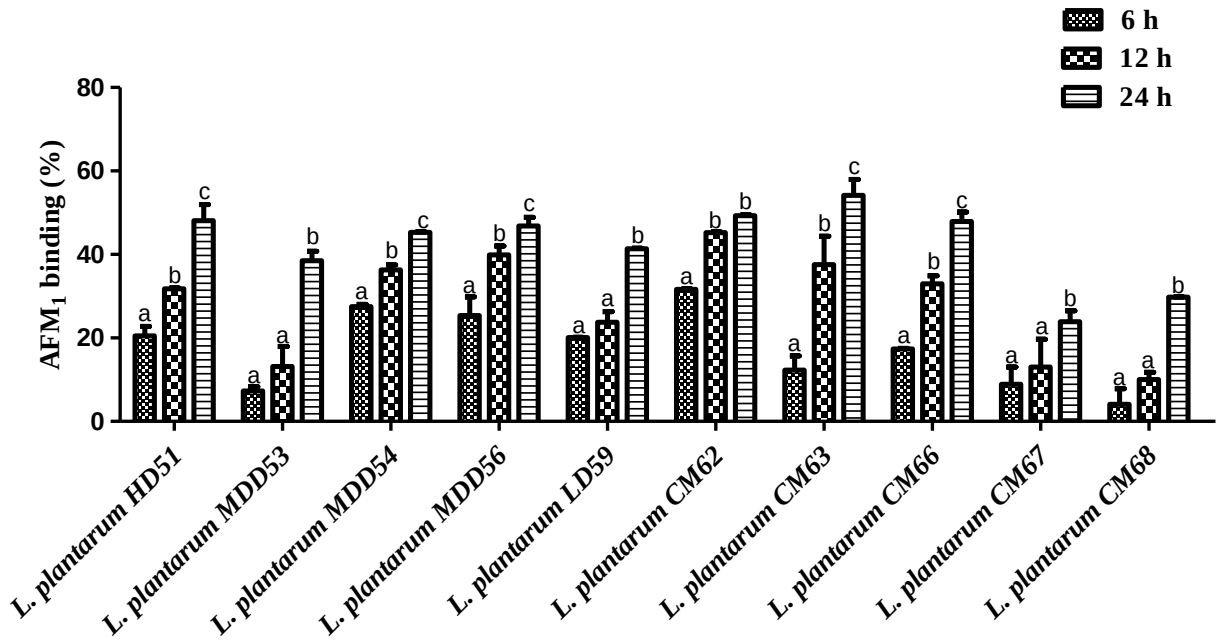
Martinez *et al.,* (2021) also investigated the binding efficiency of lactic acid bacteria and they found that tested strains significantly adsorbed AFM_1 in milk at percentages that varied from 19 to 61 %. Among these *L. rhamnosus* RC007 showed the highest adsorption percentage (61 %) as compared with other tested strains *i.e. P. acidilactici* RC005, *P. pentosaceus* RC006, *S. cerevisiae* RC016, *S. boulardii* RC009, *K. marxianus.*

Fig 4.1 AFM_1 binding ability of live probiotic *Lactobacillus* strains in PBS

(A)



(B)



(C)

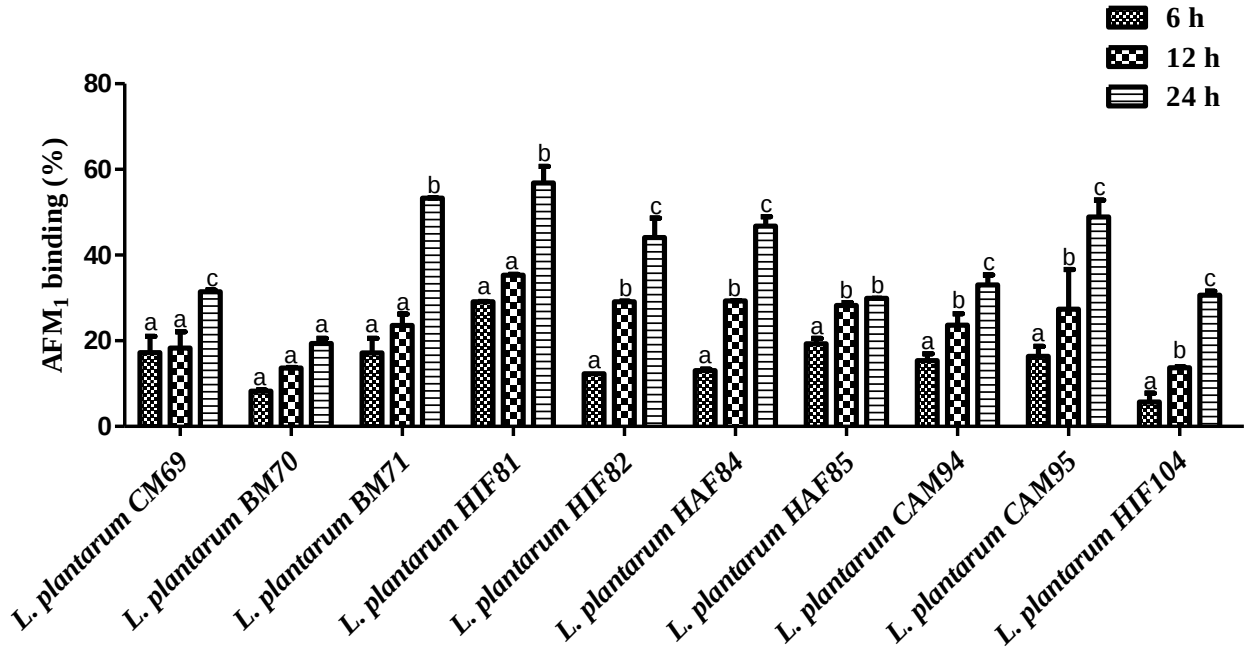


Fig 4.2 AFM_1 binding ability of heat killed probiotic *Lactobacillus* strains in skim milk

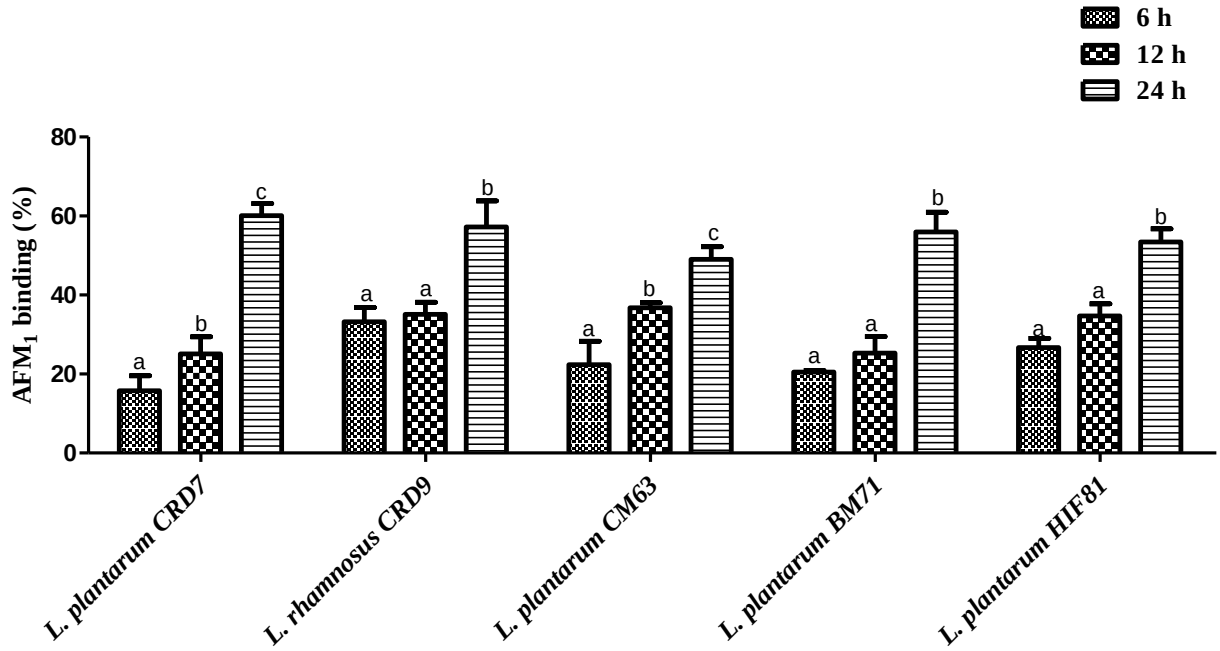


Table: 4.1 AFM_1 binding ability of probiotic *Lactobacillus* strains in PBS

***Lactobacillus* cultures**	**AFM_1 (%) binding at 6 hours**	**AFM_1 (%) binding at 12 hours**	**AFM_1 (%) binding at 24 hours**
L. rhamnosus GG	19.36 ± 1.27[a]	19.36 ± 1.27[b]	44.17 ± 2.17[b]
L. plantarum CRD2	11.4 ± 3.14[a]	12.36 ± 4.46[a]	23.60 ± 2.77[b]
L. rhamnosus CRD4	28.90 ± 0.69[a]	35.6 ± 10.05[a]	41.36 ± 0.15[a]
L. plantarum CRD7	19.06 ± 1.28[a]	28.36 ± 0.35[a]	64.16 ± 5.60[b]
L. rhamnosus CRD9	36.13 ± 8.83[a]	39.33 ± 12.77[a]	61.03 ± 3.90[b]
L. rhamnosus CRD11	4.13 ± 3.69[a]	4.13 ± 3.69[a]	12.66 ± 0.98[a]
L. plantarum CRD121	18.53 ± 1.27[a]	29.22 ± 3.64[b]	35.2 ± 0.2[b]
L. bulgaricus 11B	14.86 ± 2.47[a]	14.86 ± 2.47[a]	28.13 ± 1.93[b]
L. plantarum HD48	7.63 ± 1.00[a]	27.13 ± 9.01[b]	40.16 ± 2.14[c]
L. plantarum HD51	20.46 ± 2.31[a]	20.46 ± 2.31[b]	48.01 ± 3.89[c]
L. plantarum MDD53	7.30 ± 0.98[a]	13.16 ± 4.82[a]	38.5 ± 2.26[b]
L. plantarum MDD54	27.50 ± 0.5[a]	36.30 ± 1.21[b]	45.33 ± 0.11[c]
L. plantarum MDD56	25.4 ± 4.45[a]	39.92 ± 2.10[b]	46.83 ± 2.06[c]
L. plantarum LD59	20.13 ± 0.05[a]	23.73 ± 2.54[a]	41.36 ± 0.11[b]
L. plantarum CM62	31.66 ± 0.11[a]	45.23 ± 0.20[b]	49.3 ± 0.2[b]
L. plantarum CM63	12.30 ± 3.38[a]	37.56 ± 6.79[b]	54.16 ± 3.78[c]
L. plantarum CM66	17.33 ± 0.15[a]	32.96 ± 1.93[b]	47.87 ± 2.32[c]
L. plantarum CM67	8.86 ± 4.16[a]	13.40 ± 6.63[a]	23.90 ± 2.59[b]
L.plantarum CM68	4.13 ± 3.69[a]	10.06 ± 1.68[a]	29.73 ± 0.11[b]
L. plantarum CM69	17.24 ± 3.79[a]	18.3 ± 3.81[a]	31.43 ± 0.37[c]
L. plantarum BM70	8.26 ± 0.21[a]	13.63 ± 0.15[a]	19.34 ± 1.24[a]
L. plantarum BM71	17.16 ± 3.39[a]	23.56 ± 2.65[a]	53.3 ± 0.1[b]
L. plantarum HIF33	2.46 ± 2.31[a]	2.46 ± 2.31[a]	9.8 ± 2.17[a]
L. plantarum HIF81	29.12 ± 0.06[a]	35.3 ± 0.17[a]	56.84 ± 3.86[b]
L. plantarum HIF82	12.3 ± 2.18[a]	29.1 ± 0.1[b]	44.1 ± 4.50[c]
L. plantarum HIF104	5.7 ± 2.04[a]	13.70 ± 0.17[b]	30.66 ± 0.92[c]
L. plantarum HAF84	13.06 ± 0.30[a]	29.3 ± 0.1[b]	46.8 ± 2.16[c]

L. plantarum HAF85	19.3 ± 1.3^{a}	28.2 ± 0.69^{b}	29.86 ± 0.05^{b}
L. plantarum CAM94	15.4 ± 1.56^{a}	23.6 ± 2.68^{b}	33.03 ± 2.30^{c}
L. plantarum CAM95	16.33 ± 2.36^{a}	27.36 ± 9.29^{b}	48.87 ± 3.97^{c}

Values are expressed as Mean ± SEM

a,b,c-Values with same superscripts are nonsignificant. Values with different superscripts are significantly different

Table: 4.2 AFM_1 binding ability of selected heat killed probiotic *Lactobacillus* strains in skim milk

***Lactobacillus* cultures (Heat killed)**	**AFM_1 binding (%) at 6 hours**	**AFM_1 binding (%) at 12 hours**	**AFM_1 binding (%) at 24 hours**
L. plantarum CRD7	15.79 ± 3.75^{a}	25.12 ± 4.32^{b}	60.06 ± 3.08^{c}
L. rhamnosus CRD9	33.20 ± 3.62^{a}	35.1 ± 3.08^{a}	57.23 ± 6.61^{b}
L. plantarum CM63	22.34 ± 5.95^{a}	36.8 ± 1.21^{b}	49.01 ± 3.18^{c}
L. plantarum BM71	20.51 ± 0.40^{a}	25.26 ± 4.25^{a}	55.96 ± 4.99^{b}
L. plantarum HIF81	26.7 ± 2.33^{a}	34.7 ± 3.05^{a}	53.4 ± 3.36^{a}

Values are expressed as Mean ± SEM

a,b,c -Values with same superscripts are non significant. Values with different superscripts are significantly different

4.3 LIVE AND HEAT KILLED PROBIOTIC *LACTOBACILLUS* STRAIN-AFM_1 COMPLEX STABILITY

The complex stability of bacterial cells and AFM_1 is of utmost importance as far as the bioavailability of AFM_1 in the body is concerned. Due to reversibel nature of AFM_1 and bacterial complex AFM_1 may be released during milk processing or its digestion process in the human body. Hence, investigation on complex stability of bacteria and AFM_1 is of prime importance. It was documented that a weak non-covalent interaction exist between AFM_1 and bacterial cell components, which may be the reason for deattachment of AFM_1 after washing (Serrano-Nino *et al.*, 2013; Ismail *et al.*, 2016).

The alterations in the amount of bound AFM_1 released after washing could be due to binding of AFM_1 by bacterial cells is extremely strain specific; or the cross linked interactions between AFM_1 and molecules present in the cell walls of two distinct bacteria (Hernandez-Mendoza *et al.*, 2009). Several investigations have been conducted on AFB_1-bacterium complex

stability but limited reports are available with reference to the stability of the AFM_1-bacteria complex.

The data of the present study on live bacteria-AFM_1 complex stability *w.r.t.* effect of PBS washing on the release of AFM_1 is presented in **Table 4.3 (A).** In case of live bacterial-AFM_1 complex stability, highest percentage of AFM_1 release was observed in first wash, which was significantly ($p<0.05$) higher for all selected probiotic *Lactobacillus* strains tested and ranged from 7.40±0.23-20.45±2.10 % of the initially bound AFM_1. The percentage of AFM_1 released during the second wash ranged from 0.98±0.14 - 18.20±1.24 % which was significantly ($p<0.05$) lower than the first wash. In case of *L. plantarum* BM71 and *L. plantarum* HIF81, no detectable AFM_1 was released during second wash similarly during third wash no additional AFM_1 was released in all the tested probiotic *Lactobacillus* strains except of *L. rhamnosus* GG which showed release of 12.72±1.30 % AFM_1 indicating of weak AFM_1-LGG complex stability.

In case of heat killed bacterial-AFM_1 complex stability, the data of effect of washing on the release of AFM_1 from the heat killed bacteria-AFM_1 complex is presented in **Table 4.3 (B).** The highest release of AFM_1 was observed after first and second wash with PBS, which was significantly ($p<0.05$) higher for all heat killed probiotic *Lactobacillus* strains and released AFM_1 concentarition ranged from 27.5±2.4 - 14.3±3.03 % of initially bound AFM_1. The percentage of AFM_1 released during third wash was noticed only in case of *L. plantarum* CRD7, *L. rhamnosus* CRD9 which ranged from 7.5±3.0 - 12.2±4.36 %, respectively. Our studies also illustrated that binding of AFM_1 and bacterial cells whether it is live or heat killed was irreversible to great extent as only small amounts of AFM_1 was released back into buffered solution, especially during first and second wash of tested probiotic lactobacilli strains.

Kabak and Var, (2008) in their studies found that binding of AFM_1 with bacteria was not completely irreversible because after washing of bacteria-AFM_1 complexes, small amounts (5.62±3.12 - 8.54±5.32 %) of AFM_1 were released back into buffered solution. Haskard *et al.*, (2001) documented twelve live and heat killed bacterial strains towards their complex stability with AFB_1. They found that at the end of fifth extraction process where up to 71 % of total AFB_1 retained their complex stability with heat killed bacteria which was the higher retained amount of AFB_1. They also declared that these binding are of reversible in nature; however, its stability was strain specific which was also affected by the nature of treatment and environmental conditions.

Similarly, Shahin, (2007) also investigated the stability of complex formed among lactic cultures and AFB_1. They did not observe release of quantifiable amount of AFB_1 from *S. thermophilus* and AFB_1 complex. On the other hand, lowest complex stability was reported by Bovo *et al.* (2012) who observed major release of bound AFM_1 in the range of 40.57±1.20 - 87.37±2.01 % for live and 53.79±4.81 - 69.29±3.06 % for heat killed cells after washing with PBS.

In contrast, *B. bifidum* NRRL B-41410 strain has been documented to form the most stable AFM_1-microbial complex since as merely 1.4±3.14 % reduction of AFM_1 was measured after washing with PBS (Serrano-Nino *et al.*, 2013). Hernandez-Mendoza *et al.*, (2009) observed a significant stable complex among AFB_1 and lactic acid bacteria such as *L. reuteri* NRRL1417 and *L. casei* strain Shirota. After 4 hours of incubation *L. reuteri* NRRL1417 and *L. casei* Shirota resulted retention of bound 85.3±5.10 % and 93.8±3.02 % of AFB_1, respectively.

Recently, da Cruz *et al.*, (2021) examined AFM_1 retention capacity of the viable and heat-killed cells of *L. paracasei* 108, *L. plantarum* 49, and *L. fermentum* 111 after washing with PBS and they found percentage values of recovered AFM_1 from viable and heat-killed cells in the range of 13.4±1.5 - 60.6±1.6 % and 10.9±1.2 - 47.9±1.5 %, respectively. The highest values of recovered AFM_1 after 1 and 24 hours were found for *L. fermentum* 111 and *L. paracasei* 108, respectively, for both viable and heat-killed cells.

Similiarly, *L. paracasei* and *B. coagulans* was studied for their complex stability with AFM_1 in the presence of activated carbon (CAC), bentonite (CBENT) and sorbitan monostearate (CSP60) and found CBENT and CAC (acid and heat killed) among both bacterial strains showed the formation of most stable complex with AFM_1 showing no release of detectable AFM_1 after couple of phosphate buffer saline washings. Among other treatments, CSP60 of heat killed cells formed most stable complex for both *L. paracasei* and *B. coagulans* with 19 % and 22 % release of initially bound AFM_1, respectively. The results showed that the combination of microbial cells with activated carbon and bentonite may be used as an efficient and effective strategy to mitigate the problem of AFM1 in milk (Muaz *et al.*, 2021).Guan *et al.*, (2021) stated that adsorption occurs due to the special structure on the microbial cell wall, aflatoxins interact with non-covalent bonds (the main effect is that of Van dar Waals forces), which make it easier to bind, reducing the bioaccessibility of aflatoxins in the GIT tract, and protect the body from toxin infection.

Table: 4.3 (A) Bacterial-AFM_1 complex stability of live probiotic *Lactobacillus* strains

Probiotic *Lactobacilius* strains	AFM_1 (%) released during different washings		
	First wash	Second wash	Third wash
L. rhamnosus GG	20.45 ± 2.10^a	18.20 ± 1.24^a	12.72 ± 1.30^b
L. plantarum CRD7	16.40 ± 2.31^a	1.48 ± 1.06^b	ND*
L. rhamnosus CRD9	17.30 ± 4.58^a	0.98 ± 0.14^b	ND*
L. plantarum CM63	7.40 ± 0.23^a	1.03 ± 1.75^b	ND*
L. plantarum BM71	13.07 ± 2.24^a	ND*	ND*
L. plantarum HIF81	11.05 ± 0.19^a	ND*	ND*

ND* - Not detected

Values are expressed as Mean ± SEM

a,b,c Values with same superscripts are non significant. Values with different superscripts are significantly different

Table: 4.3 (B) Bacterial-AFM_1 complex stability of selected heat killed probiotic *Lactobacillus* strains

Heat killed probiotic *Lactobacilius* strains	AFM_1 (%) released during different washings		
	First wash	Second wash	Third wash
L. plantarum CRD7	17.5 ± 3.48^a	14.3 ± 2.82^a	7.5 ± 3.00^b
L. rhamnosus CRD9	21.05 ± 2.09^a	21.75 ± 3.73^a	12.2 ± 4.36^b
L. plantarum CM63	27.5 ± 2.4^a	14.69 ± 3.03^b	ND*
L. plantarum BM71	16.36 ± 2.85^a	15.6 ± 2.72^a	ND*
L. plantarum HIF81	16.9 ± 3.97^a	18.86 ± 2.69^a	ND*

ND* - Not detected

Values are expressed as Mean ± SEM

a,b,c –Values with same superscripts are non significant. Values with different superscripts are significantly different

4.4. CHLOROFORM EXTRACTION OF BOUND AFM_1

Although the exact mechanism of aflatoxins removal by lactic acid bacteria is still not clear, however, it has been suggested that instead of metabolic degradation, aflatoxins bind to bacterial cell wall components (El-Nezami *et al.,* 1998; Lahtinen *et al.,* 2004). Cell wall components such as polysaccharide and peptidoglycan have been suggested to be the most

important elements responsible for binding of aflatoxins by lactic acid bacteria (Peltonen *et al.*, 2001; Hernandez-Mendoza *et al.,* 2009).

In the present study AFM_1 bacterial cells complex was treated with chloroform to illustrate the mode of interaction between *Lactobacillus* strains and AFM_1. The data of current investigations on AFM_1 release from AFM_1 bound bacterial cells following chloroform treatment have been demonstrated in **Table 4.4.** Following chloroform treatment, 88.57±0.16 - 92.30±2.23 % of bound AFM_1 was released from the bacterial cells. However, no significant difference ($p<0.05$) was observed in the released amount of AFM_1 among selected probiotic *Lactobacillus* strains *viz., L. rhamnosus* GG (90.80±5.54 %), *L. plantarum* CRD7 (91.25±2.94 %), *L. rhamnosus* CRD9 (89.18±1.40 %), *L. plantarum* CM63 (88.90±4.61 %), *L. plantarum* BM71 (92.30±2.23 %) and *L. plantarum* HIF81 (88.570.16 %).

The numerous studies have been tried to investigate the particular binding sites of aflatoxins on the bacterial cell surface by subjecting the cells to various physical, chemical and enzymatic treatments. These studies have concluded that polysaccharides and peptidoglycan located on the cell wall share the major sites for binding with aflatoxins (Haskard *et al.,* 2000; Haskard *et al.,* 2001). This was further confirmed by Lahtinen *et al.,* (2004) who investicated the different cell wall components like exopolysaccharides, cell wall isolates and peptidoglycans of *L. rhamnosus* GG and found that peptidoglycan are the most likely binding sites for AFB_1. Furthermore, Serrano-Nino *et al.,* (2015) further documented that teichoic acids could be accountable for binding of AFB_1 on bacterial cell wall. The cell surface hydrophobicity is often speculated to play a persuasive role in binding mechanisms; but on the other hand findings of Kabak and Var, (2008) revealed no correlations between cell surface hydrophobicity and AFM_1-binding ability. It is expected that numerous factors are responsible in AFM_1 and possible other toxins binding with bacterial cells.

The data obtained in the current study are in concurrence with the previous report of Haskard *et al.,* (2001) who suggested that AFB_1 binds to bacterial cells by weak non-covalent interactions, such as by associating with hydrophobic pockets exist on the bacterial cell surface.

Table: 4.4 Effect of chloroform extraction on release of AFM_1 bound to probiotic *Lactobacillus* strains

Probiotic *Lactobacillus* strains	AFM_1 (%) recovered in chloroform extraction
L. rhamnosus GG	90.80±5.54[a]
L. plantarum CRD7	91.25 ± 2.94[a]
L. rhamnosus CRD9	89.18 ± 1.40[a]
L. plantarum CM63	88.90 ± 4.61[a]
L. plantarum BM71	92.30 ± 2.23[a]
L. plantarum HIF81	88.57 ± 0.16[a]

Values are expressed as Mean ± SEM
a-Values with same superscripts are non significant.

Results from ELISA, light microscopy and recovery of almost all bound AFB_1 by washing with chloroform is further confirmation of weak interactions observed in between the bacterial cells and AFB_1 instead of adhesion or degradation. The present study also documented the significant higher ($p<0.05$) recovery of AFM_1 by chloroform treatment that ranged from 88.57±0.16 - 92.30±2.23 %, of selected probiotic *Lactobacillus* strains indicate of its removal by adsorption instead of degeradation.

OBJECTIVE 2

EVALUATION OF SELECTED PROBIOTIC *LACTOBACILLUS* STRAINS FOR REDUCTION OF AFLATOXIN M_1 BIOACCESSIBILITY (*IN VITRO* AND *IN VIVO*) AND ITS PREVENTIVE ROLE AGAINST AFLATOXIN M_1 IMMUNOTOXICITY AND GENOTOXICITY

4.5 EFFICACY OF SELECTED LIVE AND HEAT KILLED PROBIOTIC LACTOBACILLUS STRAIN TO REDUCE AFM_1 BIOACCESSIBILITY IN *IN-VITRO* DIGESTION MODEL

The illustration of any destructive possessions on the specific tissue or organ of the body, aflatoxins must have to be first released from their matrix and then get absorbed in the intestinal cells. The term bioaccessibility or bioavailability of toxins is defined as the quantity of ingested toxins that get released from their food and feed matrix in the gastrointestinal tract and thus

becomes accessible for intestinal absorption (Versantvoort *et al.,* 2005). In present study, *in-vitro* bioaccessibility model, we first made AFM_1-bacterial cells complex through AFM_1 binding assay; subsequently this complex was tested for AFM_1 bioaccessibility as well as complex stability under simulated *in-vitro* gastrointestinal tract conditions. In case of heat killed probiotic *Lactobacillus* strains, prior to experiment, all selected probiotic cultures were subjected to autoclave condition to kill them completely. The results of the reduction in AFM_1 bioaccessibility by selected strains of live probiotic lactobacilli have been depicted in **Table 4.5 (A).**

The observed bioaccessibility of AFM_1 was significantly ($p<0.05$) reduced by using the selected probiotic strains which ranged from 21.56±1.66 to 52.84±3.34 %. The highest reduction *i.e.* 52.84±3.34 % was noted for *L. plantarum* CRD7 and lowest 21.56±1.66 % for *L. rhamnosus* GG. On the other hand, 94.32±2.15 % of AFM_1 bioaccessibility was recorded in AFM_1spiked skim milk at the end of digestion assay in the positive control.

The results on the reduction of AFM_1 bioaccessibility in the presence of heat killed probiotic *Lactobacillus* strains are shown in **Table 4.5 (B).** This is the first demonstration of effect on bioaccessibility of AFM_1 in the presence of heat killed probiotic lactobacilli strains. In current investigation bioaccessibility of AFM_1 was significantly ($p<0.05$) reduced by heat killed probiotic *Lactobacillus* strains in *in-vitro* digestion model. The reduction in relative AFM_1 bioaccessibility ranged from 32.20±2.73-50.70±1.90 %. The *L. plantarum* BM71 showed highest (50.70±1.90 %) while lowest reduction (32.20±2.73 %) in AFM_1 bioaccessibility was observed in case of *L. plantarum* HIF81 in spiked skim milk. However, 96.15±2.61 % of AFM_1 bioaccessibility was recorded in skim milk at the end of digestion assay in the positive control.

Hence, we may conclude that tested both of live and heat killed probiotic strains reduced significant higher ($p<0.05$) AFM_1 bioaccessibility as well as the AFM_1-bacterial complex is most stable under simulated GIT conditions. Comparable results were also documented by Serrano-Nino *et al.,* (2013), who assessed the ability of probiotic strains such as *L. acidophilus* NRRL B-4495, *L. rhamnosus* NRRL B-442, *L. reuteri* NRRL B-14171, *L. johnsonii* NRRL B-2178 and *B. bifidum* NRRL B-41410 to diminish the bioaccessibility of AFM_1 using *in-vitro* digestion model and observed reduction of AFM_1 bioaccessibility to a level of 22.72±3.21-45.17±2.41 %.

Several other studies have reported the bioaccessibility of various aflatoxins in different food matrices (Kabak and Ozbey, 2012; Serrano-Niño *et al.,* 2013). Both *in-vitro* and *in vivo* methods can be used for assessment of bioaccessibility of aflatoxins. *In-vitro* methods for bioaccessibility assessment are simpler, rapid and economic with high reproducibility, on the other hand *in vivo* bioaccessibility assessment are more convincing but difficult to perform and time-consuming process (Avantaggiato *et al.,* 2003).

Versantvoort *et al.* (2005) reported the bioaccessibility of AFB_1 from peanut slurry and ochratoxin A from buckwheat as 94±0.21 % and 100±2.75 %, respectively. Kabak and Ozbey, (2012) in his study reported the bioaccessibility of AFM_1 in spiked and naturally contaminated milk samples as 80.5±0.40 - 83.8±0.51 % and 81.7±2.03 - 86.3±4.01 %, respectively, although another recent study by Serrano-Nino *et al.,* (2013) found 100±0.4 % bioaccessibility of AFM_1 in artificially contaminated milk. Bioaccessibility of aflatoxins depends on several factors, such as food matrices, mode of entry, extent of contamination and type of contamination *i.e.* spiked versus naturally contaminated food materials (Kabak *et al.,* 2009).

Kabak and Ozbey, (2012) found 15.5±2.01 - 31.6±4.20 % lessening in AFM_1 bioaccessibility under investigation when six probiotic lactic strains were evaluated for their efficiency to decrease AFM_1 bioaccessibility under simulated GIT conditions. On similar line, it was observed that the reduction in bioaccessibility of aflatoxins also influnced by the type of aflatoxin and bacterial strain used and which varied with different aflatoxins in the digestion model that resulted in reduction in aflatoxins bioaccessibility of 18.1±1.05 - 35.6±3.42 % for (AFB_1), 17.3±6.10 - 35.5±0.12 % for (AFB_2), 13.5±0.52 - 31.9±1.52 % for (AFG_1) and 10.5±3.42 - 33.6±6.01 % for (AFG_2). Kabak *et al.,* 2009 reported 37±4.0 and 73±2.04 % reduction in bioaccessibility of AFB_1 and Ocratoxin A, respectively, in the presence of probiotic bacteria.

The results obtained in the current study suggest that probiotic strains of tested lactobacilli are capable of AFM_1 binding and consequently with their potent complex stability thus able to reduce its bioaccessibility in the gut which could in turn reduce the risks of adverse health effect of AFM_1 to humans. The information generated in this study could be used to design more effective biological AFM_1 removal strategy in milk and milk products contaminated

with these aflatoxins, as well as utilization of health promoting bacteria in a process of dietary detoxification in order to reduce bioaccessibility of aflatoxins in human diet.

Table: 4.5 (A) *In-vitro* reduction in AFM_1 bioaccessibility by selected live probiotic *Lactobacillus* strains in artificially spiked skim milk

Live probiotic *Lactobacillus* strains	Reduction in AFM_1 bioaccessibility (%)
L. rhamnosus GG	21.56 ± 1.66^{c}
L. plantarum CRD7	52.84 ± 3.34^{a}
L. rhamnosus CRD9	44.09 ± 5.86^{a}
L. plantarum CM63	32.61 ± 3.13^{b}
L. plantarum BM71	37.5 ± 3.51^{b}
L. plantarum HIF81	48.26 ± 4.53^{a}

Values are expressed as Mean ± SEM

a,b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

Table: 4.5 (B) *In-vitro* reduction in AFM_1 bioaccessibility by selected heat killed probiotic *Lactobacillus* strains in artificially spiked skim milk

Heat killed probiotic lactobacilli strains	Reduction in AFM_1 bioaccessibility (%)
L. plantarum CRD7	43.06 ± 5.90^{a}
L. rhamnosus CRD9	37.93 ± 2.97^{b}
L. plantarum CM63	46.71 ± 2.19^{a}
L. plantarum BM71	50.7 ± 1.90^{a}
L. plantarum HIF81	32.23 ± 2.73^{b}

Values are expressed as Mean ± SEM

a,b -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

4.6. *IN VIVO* ASSESSMENT OF AFM_1 BIOACCESSIBILITY AND PREVENTIVE ROLE OF SELECTED PROBIOTIC *L. PLANTARUM* CRD7 AGAINST AFM_1 IMMUNOTOXICITY AND GENOTOXICITY

In-vivo investigation was conducted in two phases *i.e.* phase 1 and phase 2. The phase 1 was assigned for AFM_1 dose standardization to determine toxic effects of AFM_1 at different concentration in mice. The phase 2 was assigned for efficacy evaluation of selected probiotic *L.*

plantarum CRD7 for AFM_1 bioaccessibility reduction and its preventive role against AFM_1 immunotoxicity and genotoxicity in mice.

The experimental animal model (Balb/C) was selected carefully after going through the available literature. The parameters assigned included effect on body weight, analysis of the effect of AFM_1 administration and selected probiotic *L. plantarum* CRD7 on AFM_1 bioaccessibility, immunotoxicity, hepatotoxicity, oxidative stress, genotoxicity and liver histo-pathological condition as well as efficacy of probiotic feeding to avert its adverse health effects.

4.6.1 PHASE 1 (Standardization of AFM_1 dose in mice)

The phase 1 was allocated for AFM_1 dose standardization to determine toxic effects of AFM_1 at different concentration in mice. The animals were divided into 3 groups of 4 mice each and permitted *ad libitum* diet and water. The AFM_1 dosage was orally administered by gavage for standardization of the dose as mentioned in **Table 3.3**.

4.6.1.1 Effect of oral AFM_1 administration on the body weight of mice

The effects of the dose of AFM_1 on the body weight of mice are presented in **(Fig 4.3).** The body weight gains were significantly decreased in AFM_1 groups *vs* normal control group; in contrast, the body weights of mice exposed to AFM_1 (1.0 ppb) significantly decreased ($p<0.05$) after fifteen days of study as compared to the average body weights of mice exposed to AFM_1 (0.5 ppb).

4.6.1.2 Effect of oral AFM_1 administration on immunity cells in mice

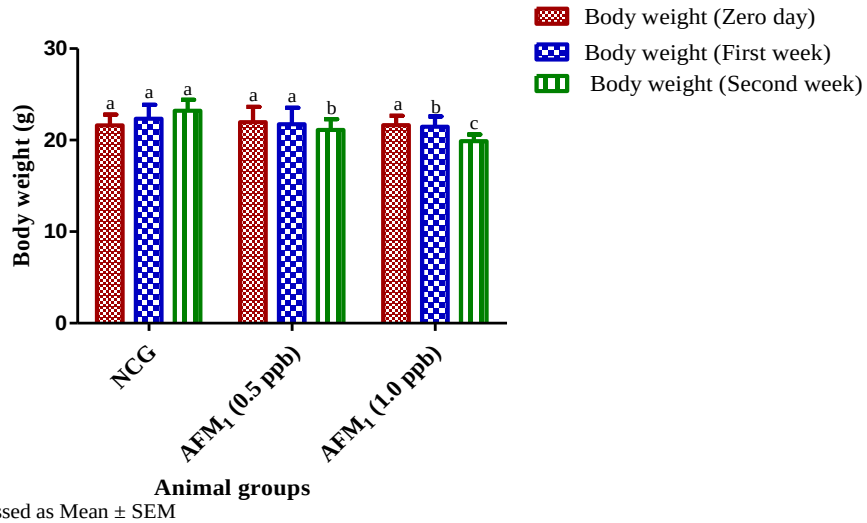
The total leucocytes counts (TLC) and differential leucocytes counts (DLC) was found significantly ($p<0.05$) decreased in the NCG *vs* AFM_1 groups. But increase in TLC and DLC were observed in the mice exposed to AFM_1 (1.0 ppb) as compared to the mice exposed to AFM_1 (0.5 ppb) concentrations and the data has been presented in **Fig 4.4 (A) & Table 4.6**.

No basophils have been observed in any group, on the other hand eosinophils were found only in the group which was exposed to AFM_1 (1.0 ppb). The significantly ($p<0.05$) higher viable splenocytes and thymocytes were observed in the AFM_1 (0.5 ppb) and NCG as compared to the mice exposed to AFM_1 (1.0 ppb) **(Fig 4.4 B)**.

4.6.1.3 Effect of oral AFM_1 administration on liver function enzymes activity

The observations of different liver function enzyme activities have been presented in **Fig 4.5**.

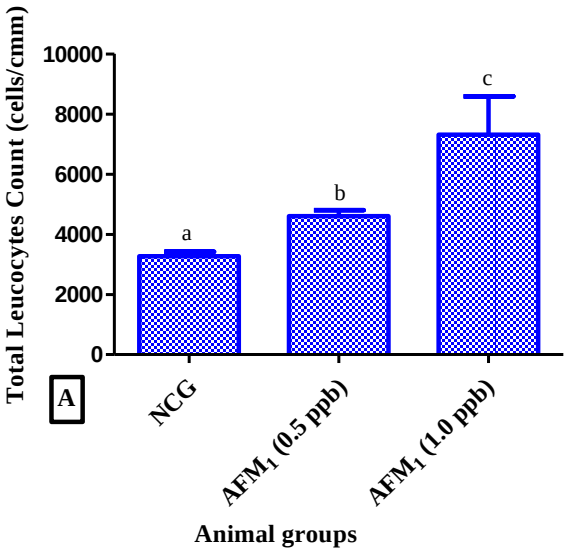
Fig. 4.3 Effect of oral AFM_1 administration on body weight of mice



Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

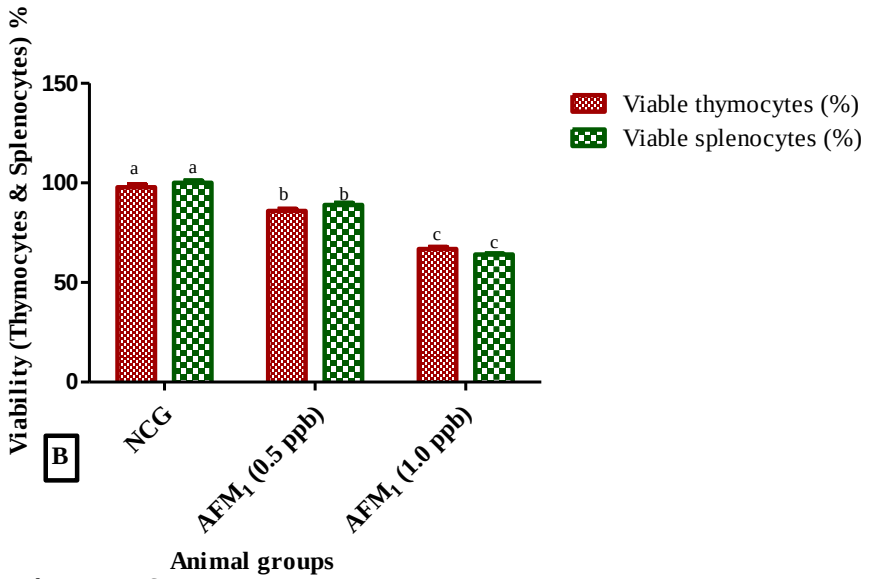
Fig. 4.4 (A) Effect of oral AFM_1 administration on total luucocytes count in mice



Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

Fig. 4.4 (B) Effect of oral AFM_1 administration on viability of thymocytes & splenocytes in mice



Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

Table: 4.6 Effect of oral AFM_1 administration on differential leucocytes count in mice

Differential Leucocytes Count (%)	NCG	AFM_1 (0.5ppb)	AFM_1 (1.0ppb)
Neutrophils	32.37 ± 4.73^a	57.76 ± 2.54^b	86.45 ± 1.76^c
Basophils	ND	ND	ND
Eosinophils	ND	ND	1.35±4.57
Lymphocytes	25.46 ± 2.07^a	34.56 ± 2.67^b	47.36 ± 4.08^c
Monocytes	1.56 ± 0.57^a	1.63 ± 0.45^a	4.25 ± 1.26^b

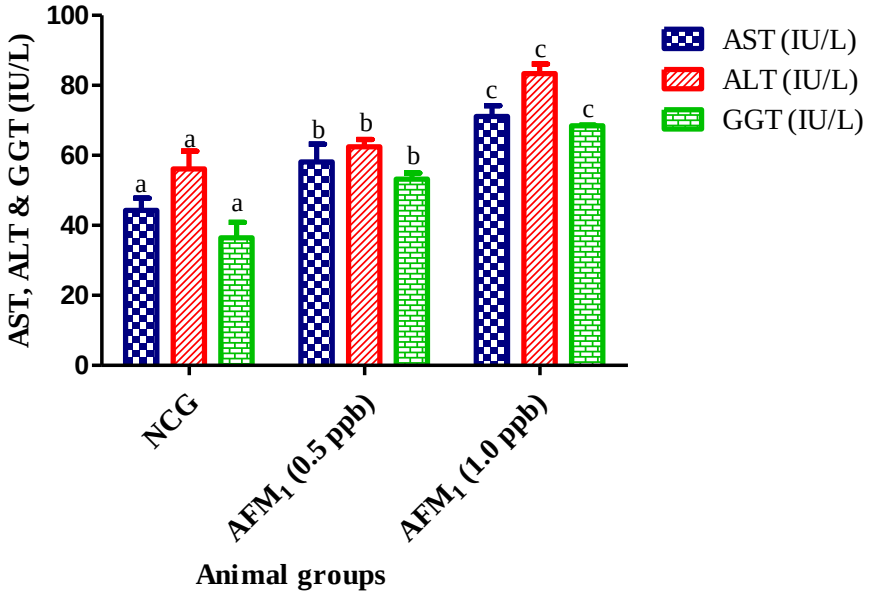
ND* - Not detected

Results are presented as average ± SD for triplicate.

a, b -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different respectively.

The activity of AST, ALT and GGT in the blood serum of AFM_1 (1.0 ppb) treated group was significantly elevated ($p<0.05$) as compared to the mice exposed to AFM_1 (0.5 ppb) and NCG.

Fig. 4.5 Effect of oral AFM_1 administration on liver function enzymes activity



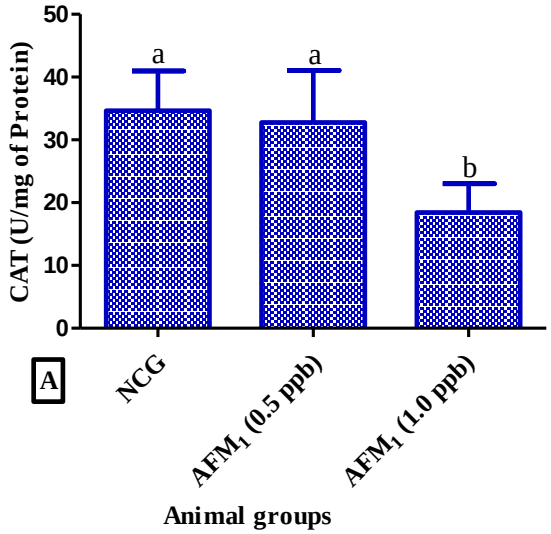
Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

4.6.1.4 Effect of oral AFM_1 administration on catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels in liver tissue homogenate

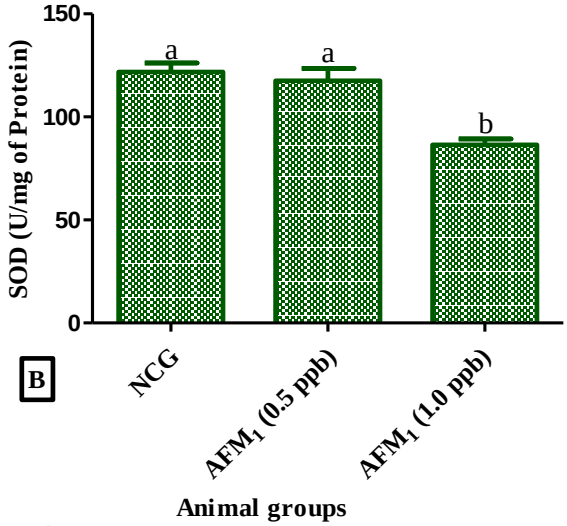
The levels of catalase, superoxide dismutase, and glutathione peroxidase in liver tissue homogenates have been depicted in **Fig 4.6 (A, B & C),** respectively. The significant decline in antioxidative strees marker enzymes (CAT, SOD, and GPx) was observed in group exposed to 1.0 ppb AFM_1. However, no significant ($p<0.05$) difference has been observed in the levels of oxidative stress marker enzymes *i.e.* CAT, SOD, and GPx in between 0.5 ppb AFM_1 treated and normal control group. Our results have demonstrated significant decrease in oxidative stress marker enzymes *i.e.* CAT, SOD, and GPx in liver of animal gropus which were exposed to 1.0 ppb AFM_1 only as 0.5 ppb dose of AFM_1 was not sufficient to induce oxidative stress in the liver cells. Present investigation on dose response reveled that 1.0 ppb AFM_1 is essential to induce toxicity in mice *w.r.t.* stress markers in liver cells.

Fig. 4.6 Effect of oral AFM_1 administration on catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in liver tissue



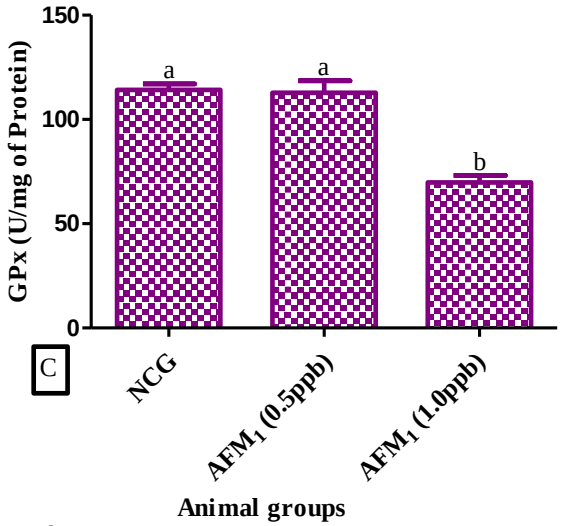
Values are expressed as Mean ± SEM

a, b -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.



Values are expressed as Mean ± SEM

a, b -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.



Values are expressed as Mean ± SEM

a, b -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

4.6.1.5 Effect AFM_1 on histopathology of liver tissue

The effects of different concentration of AFM1 on the liver histopathology of orally administered mice were also investigated. Representative photomicrographs of hepatic tissue samples of different groups *i.e.* NCG, 0.5 ppb AFM_1 and 1.0 ppb AFM_1 group have been presented in **Fig 4.7 (A, B & C).**

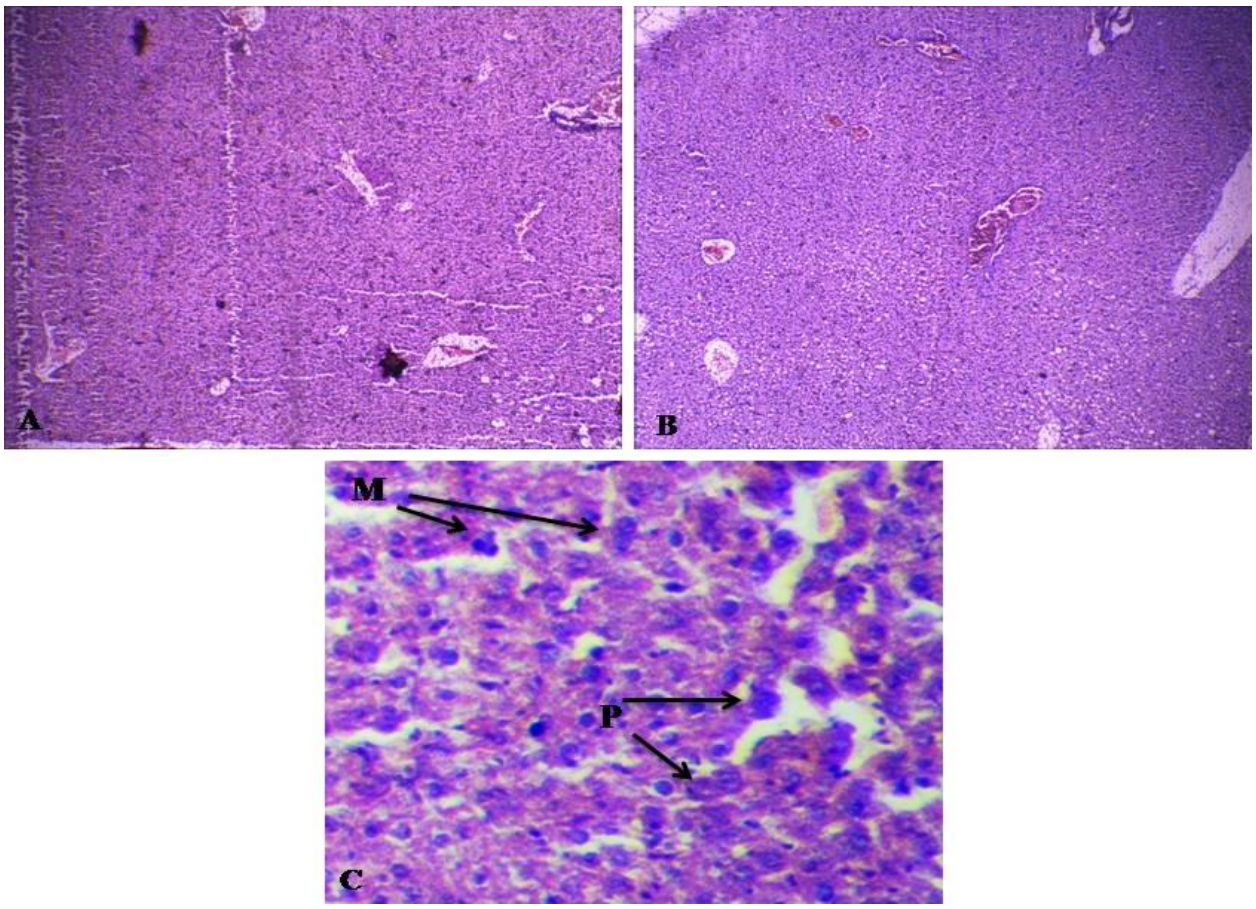
The detectable histopathological changes in the liver, including cellular carcinoma manifested by malignant lesions such as formation of plemorphic nuclei and multi-nucleated cells have been observed in the liver tissue of mice exposed with 1.0 ppb concentration of AFM_1. In contrast, no visible toxicity evidence has been observed in the liver tissue of mice exposed with the 0.5 ppb concerntation of AFM_1. In addition, normal appearance was observed of hepatic tissue in the mice of NCG.

4.6.2 PHASE 2

The phase 2 study was assigned for efficacy evaluation of selected native strain of probiotic *L. plantarum* CRD7 for reduction of AFM_1 bioaccessibility and its preventive role against its toxicity. The mice were randomly divided into six groups *i.e.* normal control group (NCG), AFM_1 group (PCG), LGG group, AFM_1-LGG group, CRD7 group and AFM_1-CRD7 group fed with respective experimental diets. Six mice were assigned to each group, used to investigate the effects of oral administration of *L. plantarum* CRD7 for reduction of AFM_1

bioaccessibility and its preventive role against toxicity. The grouping of animals and sacrifice schedule has been given in **Table 3.4** of materials and methods chapter. The body parts of animals were analyzed for the parameters as listed in **Table 3.5.**

Fig. 4.7 Histopathological analysis of liver tissue



Photomicrograph of hepatic tissue of mice (H & E staining, magnification 400X). **A**. Normal appearance of hepatic tissue of mice in the NCG; **B**. Normal appearance of hepatic tissue of mice administered with 0.5 ppb AFM_1 concentration; **C**. Hepatic tissue of 1.0 ppb AFM_1 concentration group with multi-nucleated cells and pleomorphic nuclei.

4.6.2.1 Effect of oral AFM_1 and probiotic administration on the body weight of mice

The effect of AFM_1 and probiotic administration on body weight of the mice in experimental groups has been dipcted in **Fig 4.8.** The results indicated not much variation in weight of all groups in first and second week of experimental trials. The average body weights of

mice exposed to AFM_1 alone (PCG) was found decreased significantly ($p<0.05$) after completion of final dose as compared to normal control (NCG), LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 groups. However, increase in body weight was non-significant within all treated groups *i.e.* normal control (NCG), LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 during experimental trials. On the other hand, no significant changes were recorded in the increment in body weight of mice fed with probiotic bacterial strains (*L. plantarum* CRD7 and *L. rhamnosus* GG) as compared to the normal control group. These observations revealed protective role of native probiotic *L. plantarum* CRD7 *w.r.t.* toxicity and weight management in mice model.

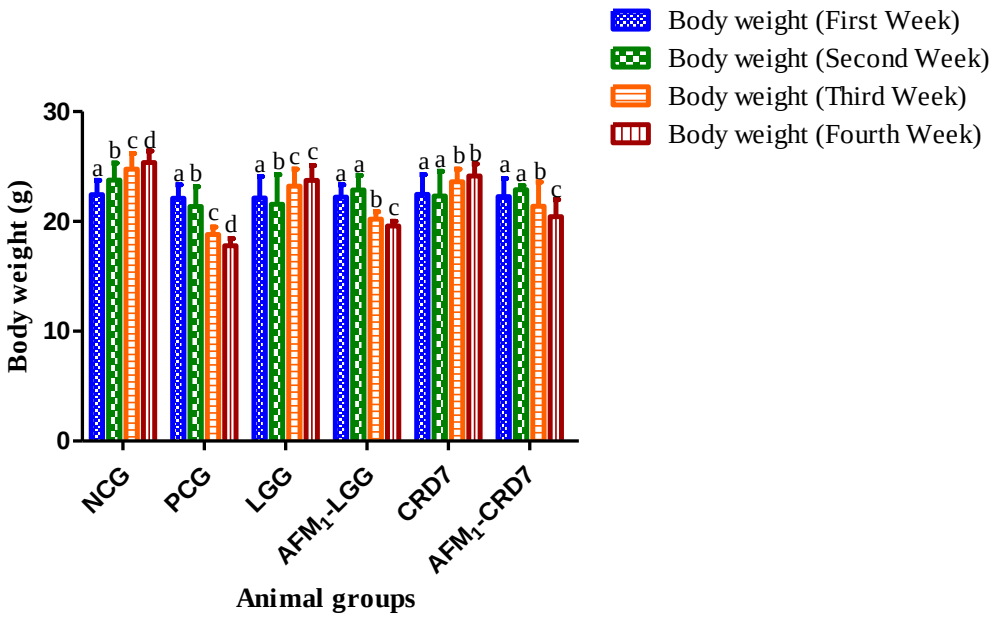
4.6.2.2 Efficacy of selected probiotic *L. plantarum* CRD7 on AFM_1 bioaccessibility in mice

Aflatoxin M_1 is an exceedingly toxic compound and it has ability to sustain various milk processing, cheese ripening and storage conditions. Hence, it may be found as contaminant in milk and dairy products with hazardous effects on human health. Many efforts have been made to detoxify toxin-contaminated foods, or to decrease its absorption at intestinal level *i.e.* bioaccessibility reduction. In this regard, several *in vitro* and *in vivo* studies have demonstrated potential of probiotic bacteria to remove aflatoxins from model systems (Wochner *et al.,* 2018).

Information about AFM_1 bioaccessibility is very important to mitigate chronic health hazards, due to frequent consumption of AFM_1 contaminated milk and milk products by all age groups. Milk and milk products are consumed worldwide in different forms and thus should be free from toxic contaminants such as enterotoxins and aflatoxins to safeguard the health of the consumers. There are seldom reports related to evaluation of probiotic bacteria for their ability to reduce AFM_1 bioaccessibility using in *in-vitro* model, as these are widely used in preparation fermented dairy foods (Roussi *et al.,* 2002; Serrano-Nino *et al.,* 2015; Kabak and Ozbey, 2012). However, very diminutive information is available on *in-vivo* evaluation of AFM_1 bioaccessibility reduction using probiotic lactic acid bacteria.

Therefore, this study was aimed to assess the ability of selected native strain of probiotic *L. plantarum* CRD7 to reduce AFM_1 bioaccessibility from artificially contaminated PBS *in-vivo*. Furthermore, our result showed that, till the second week there is no significant reduction in AFM_1 bioaccicibility has been observed in any group. After third week a significant 18.14±0.30 and 16.54±0.72 % reduction of AFM_1 bioaccicibility was observed in AFM_1-LGG and AFM_1-CRD7 groups, respectively. The significantly higher reduction in bioaccessibility of AFM_1 *i.e.*

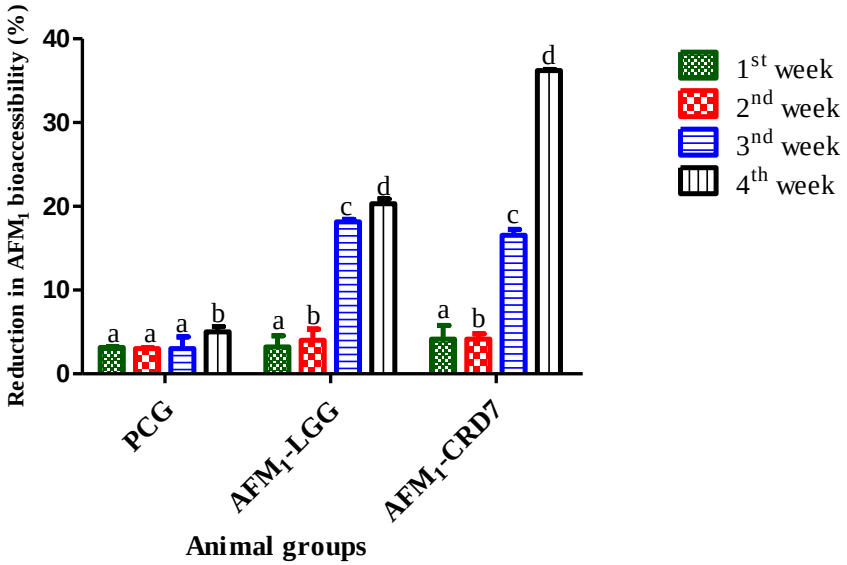
Fig. 4.8 Effect of oral AFM_1 and probiotic administration on the body weight of mice



Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

Fig. 4.9 Efficacy of selected probiotic *L. plantarum* CRD7 on AFM_1 bioaccessibility reduction in mice



Values are expressed as Mean ± SEM

a, b, c, d -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different

36.21±0.14 % was observed in case of AFM_1-CRD7 fed group ($p<0.05$) as compared to AFM_1 (5.00±0.64 %) and reference AFM_1-LGG fed group *i.e.* 20.31±0.57 % **(Fig 4.9)**. Thus, our native strain *L. plantarum* CRD7 exhibited almost 2 X higher AFM_1 bioaccibility reduction potential as that of *L. rhamnosus* GG. The results of our experiments are in agreement with Gratz *et al.*, (2006) wherein they had also studied the effect on AFB_1 and AFM_1 bioaccessibility reduction using probiotic *L. rhamnosus* GG in *in-vivo* model.

Probiotic *L. rhamnosus* GG treatment significantly increased fecal excretion of AFB_1 by 35.3±2.03-21.3±1.020 nmol/24 hours and of AFM_1 by 68.1±1.50-26.1±4.10 nmol/24 hours. This data suggest that *L. rhamnosus* GG was able to reduce additional AFB_1 and AFM_1 inside the intestinal lumens of experimental rat, most probably by binding both aflatoxins to the bacterial surface (Versantvoort *et al.*, 2005).

Kabak and Ozbey, (2012), also investigated the effect on AFM_1 bioaccessibility reduction in the presence of probiotic strains. In their study, use of probiotic bacteria resulted in reductions in AFM_1 bioaccessibility in the range of 15.5±5.01 - 31.6±2.74 %. The *L. acidophilus* NCFM 150B was the most efficient to reduce AFM_1 bioaccessibility as compared to *L. casei* strain Shirota.

Similarly, Serrano-Nino *et al.*, (2013), assessed the ability of five probiotic strains *viz.*, *L. acidophilus* NRRL B-4495, *L. reuteri* NRRL B-14171, *L. rhamnosus* NRRL B-442, *L. johnsonii* NRRL B-2178 and *B. bifidum* NRRL B-41410 to reduce the bioaccessibility of AFM_1 using *in-vitro* digestion model. They observed reduction in bioaccessibility of AFM_1 by 22.72±3.45 to 45.17±1.06 %. In addition, Kabak and Ozbey, (2012) observed that addition of six probiotic strains (*B. longum, L. rhamnosus, Bifidobacterium* species 420, *L. acidophilus* NCFM 150B and *L. casei* Shirota) to the digestion model resulted reduction in aflatoxins bioaccessibility *viz.*, 18.1±3.01-35.6±2.01 % for (AFB_1), 17.33±5.50 % for (AFB_2), 13.5±31.90 % for (AFG_1) and 10.5-33.6 % for (AFG_2). The 37 and 73 % of reduction in bioaccessibility of AFB_1 and ocratoxin A, respectively, was observed in the presence of probiotic bacteria (Kabak *et al.*, 2009).

4.6.2.3 Efficacy of selected probiotic *L. plantarum* CRD7 on AFM_1 induced immunotoxicity in mice

AFM_1 is one of the most predominant contaminant of mycotoxins in milk and milk products. It is well established that AFM_1 is heat stable and no reductions in toxin levels occur as a result of pasteurization and others high heat treatment processes. AFM_1 even has been found in UHT milk, and in some milk derivatives like yogurt and cheese (Turkoglu and Keyvan, 2019; Udovicki *et al.,* 2019). As AFM_1 is absorbed quite easily from the intestinal tract, it can give rise to a myriad of health effects, including immune system disruption and other hematologic effects (Caloni *et al.,* 2012; Abbes *et al.,* 2013).

In the current study, we have investigated in detail the ability of seclcted native probiotic *L. plantarum* CRD7 to reduce AFM_1 induced immunotoxicity. The observations of immunotoxicity have been presented in **Fig 4.10 (A, B & C)** and **Table 4.7.** The results of the blood immune cells determination studies revealed that treatment with a daily oral dose of AFM_1 induced immune system commotion as evidenced by significant ($p<0.05$) increase in total leucocytes counts (TLC) in PCG group *i.e.* 9420±6.09 as compared to NCG, LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 groups *i.e.* 3397±4.52, 3922.3±8.02, 5935.6±5.65, 3901.6±5.54 and 4479.3±0.95 cells/cmm, respectively. The data on total leucocytes counts has been presented in **Fig 4.10 (A)**.

The differential leucocytes counts (DLC) were also found significantly ($p<0.05$) increased in PCG as compared to NCG, LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 groups **(Table 4.7)**. But in case of AFM_1-LGG group the count of monocyte was slightly higher *i.e.* 5.66±2.51 as compared to group PCG *i.e.* 3.33±2.41 cells/cmm. Moreover, our findings indicated that the AFM_1 treatment also impacted the spleen and thymus cells viability. The significant ($p<0.05$) higher viable splenocytes and thymocytes were observed in NCG *i.e.* 99.84±0.11 % and 99.62±0.43 %, respectively. The viability of splenocytes and thymocytes were found decreased significantly in case of PCG group *i.e.* 42.26±3.91 and 34.32±3.49 %, respectively. The more protective effect on the viability of splenocytes and thymocytes has been observed in the AFM_1-CRD7 group *i.e.* 68.77±6.85 % and 61.83±7.92 %, respectively, as compared to the AFM_1-LGG group *i.e.* 66.16±4.56 % and 36.16±4.43 %, respectively. The detailed result of this experiment has been explained in **Fig 4.10 (B & C)**.

Similar study has been conducted by Salah-Abbes *et al.,* (2013). Wherein, Balb/C mice were randomly distributed into four treatment groups (n = 10 mice/group) and treated orally (by

gavage) daily for 15 days as follows: (1) untreated controls; (2) *L. plantarum* MON03 (LP) (10^9CFU/L, 1 mg/kg BW) alone; (3) AFM_1 only (100 mg/kg BW); (4) LP (10^9CFU/kg BW) + AFM_1 (100 mg/kg BW). They reported significant increase in total WBC levels in the blood in AFM_1-treated hosts *i.e.* $12.13x10^3$/µL as compared to LP-treated, LP-AFM_1 treated hosts *i.e.* $8.11x10^3$/µL and $8.86x10^3$/µl, respectively. Differential leucocytes counts revealed a significant decrease in leucocytes level in AFM_1-treated hosts *i.e.* $0.84x10^3$/µL as compared to LP-treated, LP-AFM_1 treated hosts *i.e.* $1.43x10^3$/µL and $1.36x10^3$/µL, respectively. Among the various cell types assessed, only eosinophil levels in LP-alone hosts were found decreased $0.08x10^3$/µL in comparison to the AFM_1-treated, LP-AFM_1 treated hosts *i.e.* $0.65x10^3$/µL and $0.15x10^3$/µL, respectively.

A suppressive effect in thymus and spleen cellularity was also observed in mice treated with AFM_1. Levels of thymocytes and splenocytes decreased significantly in AFM_1-treated *i.e.* $11.8x10^7$ and $9.5x10^7$ cell, respectively, as compared to treated with LP alone which resulted in no significant change in thymocyte and splenocyte numbers *i.e.* $26.4x10^7$ and $14.1\ x10^7$ cell, respectively. The AFM_1+LP co-treatment resulted in significant improvement in splenocyte and thymocyte counts to, $25.5x10^7$ and $14.5x10^7$ cells, respectively, which were near to normal values. These changes represented improvements *i.e.* 34.5 and 53.9% for the splenocyte and thymocyte counts, respectively which were relative to those seen in the mice in AFM_1 treated group.

These results were also in agreement with the study conducted by Abbes *et al.*, (2013). They examined the effect of *L. rhamnosus* GAF01 against the immunotoxicity induced by AFM_1. In this study, a significant decreases in the relative levels of CD4+ , CD8+ , CD54+ , and CD56+ cells was observed in the AFM_1 treated group. On the other hand, treatment with *L. rhamnosus* GAF01 did not alter blood T-cell levels. Treatment with AFM_1 + *L. rhamnosus* GAF01 resulted in a significant improvement in the total counts of immune system cells toward control levels, although these values were still different from the values of control CD4+ and CD8+ cell counts. Total WBC and RBC levels were also found decreased in only AFM_1 treated group. In contrast, total WBC and RBC counts in group treated with *L. rhamnosus* GAF01 alone were comparable to the controls. The addition of *L. rhamnosus* GAF01 to the AFM_1 treatment regimen resulted in a significant improvement in both total WBC and RBC counts.

Abbes *et al.*, (2014) also studied the possible protective role of *L. paracasei* BEJ01 against immunotoxicity and oxidative stress induced by AFB_1. They observed that exposure of spleen to AFB_1 led to increased caspase-3 activity, lipid peroxidation, and IL-10 and IL-4 mRNA levels, but decreased glutathione content and down-regulated expression of anti-oxidation protective enzymes (GPx and SOD), as well as of IFN-γ and TNF-α mRNA. Co-treatment using *L. paracasei* BEJ01 with AFB_1 suppressed levels of DNA fragmentation, normalized splenic lipid peroxidation and increased glutathione content levels, upregulated expression of GPx and SOD, and normalized mRNA levels of the analyzed cytokines.

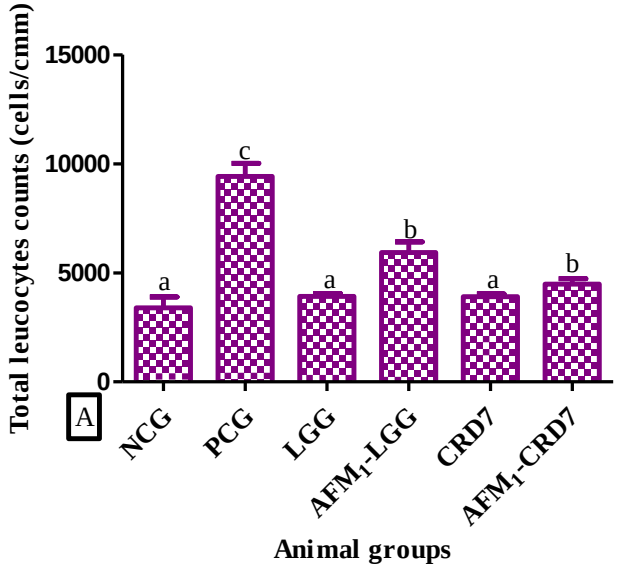
A suppressive effect on thymic and splenic cellularity was noted in mice treated with AFM_1. The levels of thymocytes and splenocytes decreased significantly as compared to control mice. Treatment with *L. plantarum* MON03 alone resulted no significant change in thymocyte and splenocyte numbers from control mice values. Compared to the organ values in the AFM_1-treated mice, the AFM_1+*L. plantarum* MON03 co-treatments resulted in significant improvement in splenocyte and thymocyte counts to near normal values.

Recently Shirani *et al.*, (2021) investigated the effects of AFM_1 on immune system and its modulation by MicroRNA (miR)-155. They found that the spleen weight was reduced in mice exposed to AFM_1 as compared to negative control. Proliferation of splenocytes in response to phytohemagglutinin-A was reduced in mice exposed to AFM_1. IFN-γ was decreased in mice exposed to AFM_1, whereas IL-10 was increased. Concentration of IL-4 did not change in mice exposed to AFM_1 as compared to negative control. Exposure to AFM_1 reduced the expression of miR-155. Significant upregulation of phosphatidyl inositol-3, 4, 5-trisphosphate 5-phosphatase 1 and suppressor of cytokine signaling 1 was observed in isolated T cells from spleens of mice treated with AFM_1, but the transcription factor c-MAF was not affected. These results suggest that miR-155 and targeted proteins might be involved in the immunotoxicity observed in mice exposed to AFM_1.

These changes represented improvements of 37.3±1.76 and 49.7±4.34 % for splenocyte and thymocyte counts respectively with relative to those seen in mice in the corresponding AFM_1 treated group. Hematologic parameter results indicated that treatment with *L. plantarum* MON03 alone did not show significant changes in blood parameters except for platelets levels that were increased. Treatment with AFM_1 alone resulted a significant increase in WBC and hemoglobin

values, as well as significant decrease in platelets and RBC levels in the blood is indicative of hematologic damage. Overall, mice co-treated with *L. plantarum* MON03 exhibited ameliorative effect on AFM_1-induced alterations (Jebali *et al.*, 2015).

Fig. 4.10 Efficacy of selected probiotic *L. plantarum* CRD7 on AFM_1 induced immunotoxicity in mice



Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

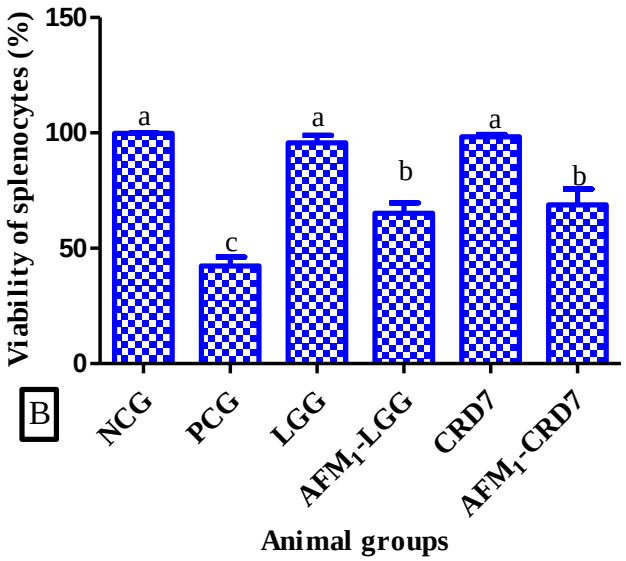
Table: 4.7. Effect of selected probiotic *L. plantarum* CRD7 on differential leucocytes count

Differential leucocytes count (%)	Experimental groups					
	NCG	PCG	LGG	AFM_1-LGG	CRD7	AFM_1-CRD7
Neutrophils	9.66±2.08 [a]	74.66±7.50 [c]	11.70±4.16 [a]	53.30±11.06 [b]	14.70±3.31 [a]	33.70±11.23 [b]
Basophils	ND	ND	ND	ND	ND	ND
Eosinophils	ND	2.33±0.57	ND	ND	ND	ND
Lymphocytes	31.33±5.50 [a]	54.30±11.23 [b]	24.33±6.02 [a]	43.70±8.73 [b]	24.30±11.5 [a]	25.33±8.08 [a]
Monocytes	2.66±2.08 [a]	3.33±2.41 [a]	2.00±2.64 [a]	5.66±2.51 [b]	ND	3.70±3.05 [a]

ND - Not detected

Values are expressed as Mean ± SEM

a, b, c –Values with same superscripts are nonsignificant. Values with different superscripts are significantly different



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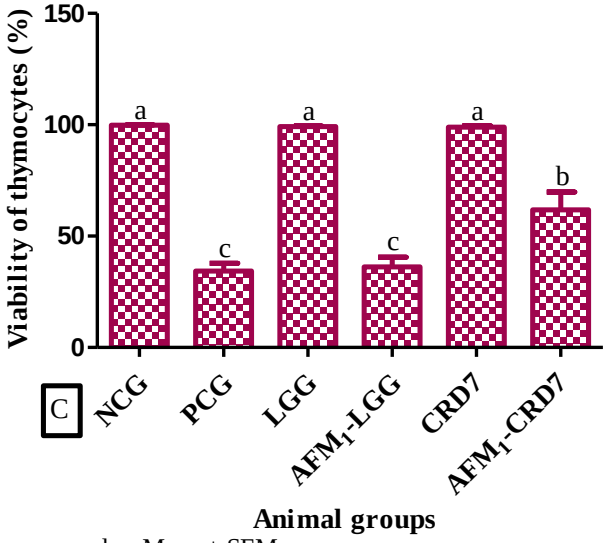


Values are expressed as Mean ± SEM

a ,b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.

4.6.2.4. Efficacy of selected probiotic *L. plantarum* CRD7 on liver function enzymes activity in serum of AFM_1 exposed mice

The liver function enzymes *viz.,* alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Gamma-glutamyl transferase (GGT) are quite sensitive for any kind of internal hepatic damage (Imperial *et al.,* 2000). In clinical practice, blood levels of GGT, ALT and AST are used to index liver injury. Gamma-glutamyl transferase present in liver and biliary epithelial cells is a sensitive marker of hepatobiliary disease. The AST, TLT and GGT levels in blood increase when the liver cell membrane is damaged and thus mark hepatocellular injury (Matloff *et al.,* 2000).

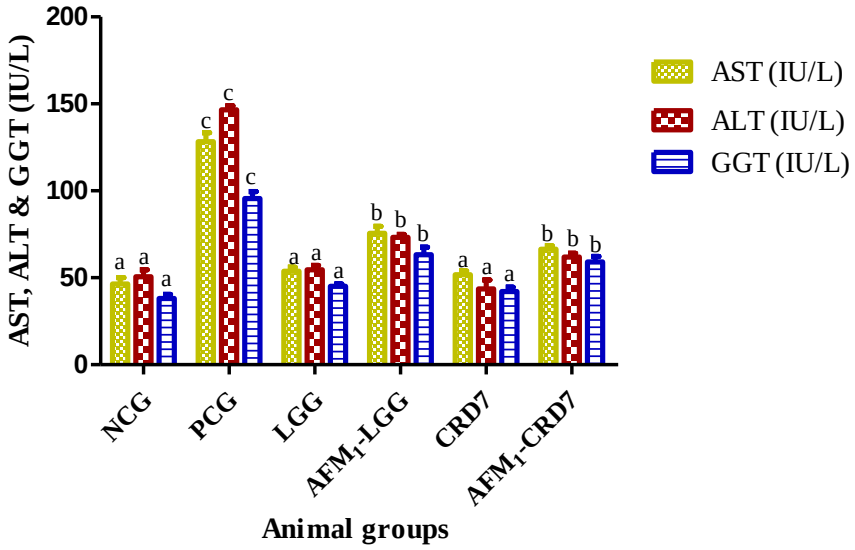
To explain their role in other diseases, GGT, AST and ALT have been proposed as surrogate markers of liver disease (Giannini *et al.,* 2001), while GGT is also seen as a marker of oxidative stress (Lee *et al*., 2009). Under diseased conditions, elevated levels of liver enzymes have been detected as a consequence of alteration in cell membrane permeability which allows leakage of these enzymes into the blood stream.

In present investigation activity of AST, ALT and GGT in the serum of PCG group was noticed significantly elevated ($p<0.05$) as compared to NCG, indicative of AFM_1 toxicity responsible for liver damage and data for the same has been presented in **Fig 4.11.** The activity of AST, ALT and GGT in blood serum of mice treated with probiotic AFM_1-CRD7 was significantly lower ($p<0.05$) as compared to PCG, which clearly indicates protective effect of probiotic *L. plantarum* CRD7 therapy that alleviated liver damage caused by AFM_1 exposure. However, no significant difference was observed in the level of AST, ALT and GGT in probiotic *L. plantarum* CRD7 and *L. rhamnosus* GG fed groups. These results reveal that our native strain of probiotic *L. plantarum* CRD7 is equally potant as that of well established probiotic *L. rhamnosus* GG. In contrast, *L. rhamnosus* GG showed poor activity as compared to our native strain of probiotic *L. plantarum* CRD7 *w.r.t.* binding and reduction in bioaccessiblity of AFM_1 *in-vivo*.

Further, a corresponding effect was observed in protection of liver from AFM_1 toxicity as it result in elevation in activity of AST, ALT and GGT in the blood serum of mice treated with AFM_1-LGG as compared to AFM_1-CRD7 treated group.

The restoration of these biological parameters in the mice further confirms the protective effects of selected probiotic lactobacilli *i.e. L. plantarum* CRD7 against chronic AFM_1 toxicity. The alleviation in AST, ALT and GGT enzymes levels represent the liver injury which has also been demonstrated by changes in histopathological analysis of liver **(Fig 4.14 and Fig 4.15)**.

Fig. 4.11. Efficacy of selected probiotic *L. plantarum* CRD7 on liver function enzymes activity in blood serum mice against AFM_1 toxicity



Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different

4.6.2.5 Effect of *L. plantarum* CRD7 on catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels in liver tissue homogenate.

The drastic increase in ROS level may also exceed the antioxidative capacity of CAT, SOD and GPx, causing exhaustion of these enzymes and reduction in their activities (Abbes *et al.*, 2014). The levels of CAT, SOD, and GPx in liver tissue homogenates have been described in **Fig 4.12 A, B & C**, respectively. The AFM_1 exposure of mice led to marked decline in activity of all the antioxidative stress marker enzymes *i.e.* CAT, SOD, and GPx. The significant decline in antioxidative stress marker enzymes (CAT, SOD, and GPx) was observed in PCG group as compared to NCG. The AFM_1-LGG treated groups showed decreased levels of antioxidative stress marker and elevated levels of CAT, SOD, and GPx in case of AFM_1-CRD7 group.

Our results have demonstrated significant decrease in antioxidative stress marker enzymes *i.e.* CAT, SOD, and GPx in liver after AFM_1 exposure. These observations clearly indicate preventive role of *L. plantarum* CRD7 against AFM_1 toxicity or alleviation of antioxidative enzymes levels. Thus, selected strain of *L. plantarum* CRD7 is a potent candidate for its application against AFM_1 toxicity due to consumption of AFM_1 contaminated milk and milk products.

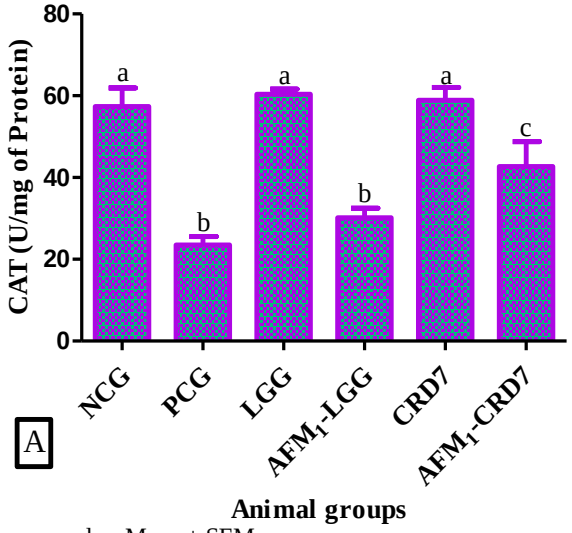
Abbes *et al.*, (2014) studied the protective role of lactic acid bacteria (*L. paracasei* BEJ01) against AFB_1 and fumonisin B_1 (FB_1) induced immunotoxicity and oxidative stress. They assessed *in-vivo* protective role of *L. paracasei* BEJ01 against AFB_1 and FB_1 induced negative effects on host splenocyte caspase-3 activity (reflecting DNA damage/cell death) and mRNA levels of select inflammation-regulating cytokines. In this study, Balb/c mice were divided into different groups each group consisted of 10 mice. These groups were treated daily for 2 weeks by oral gavage with AFB_1 (80 mg/kg BW), FB_1 (100 mg/kg BW), AFB_1+FB_1, or *L. paracasei* BEJ01 alone or in combination with the AFB_1 and/or FB_1.

After the exposures, spleens were collected for measurement of caspase-3 activity, lipid peroxidation (LP), and glutathione (GSH) content, expression of anti-oxidative protective enzymes (GPx and SOD), and mRNA levels of inflammation-regulating cytokines (IL-10, IL-4, IFN, TNF). Thymii were also excised for analysis of apoptosis. The results indicated that, in the spleen, exposure to the mycotoxins led to increased caspase-3 activity, LP, and IL-10 and IL-4 mRNA levels, but decreased GSH content and down-regulated expression of GPx and SOD as well as IFN and TNF mRNA.

Co-treatment using lactic acid bacteria with AFB_1 or FB_1 suppressed levels of DNA fragmentation, normalized splenic LP and increased GSH levels, upregulated expression of GPx and SOD, and normalized mRNA levels of the analyzed cytokines.

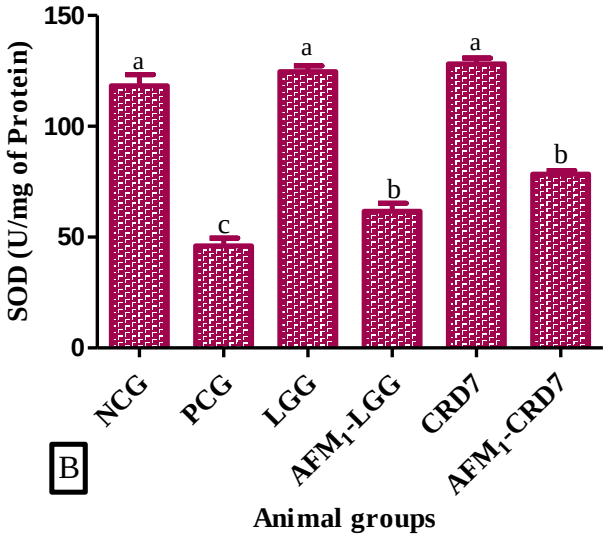
Hathout *et al.,* (2011), also evaluated the shielding role of *L. casei* and *L. reuteri* against AFB_1 induced oxidative stress in rats which were divided into six groups. The groups were treated with *L. casei* and *L. reuteri* (1×10^{11}/mL) alone and at a dose of 10 mL/kg BW or plus AFB_1 (3 mg/kg diet) for four weeks. Blood and tissue samples were tested for biochemical investigations at the end of treatments.

Fig. 4.12 Effect of selected probiotic *L. plantarum* CRD7 for antioxidative enzymes (CAT, SOD, and GPx) activity against AFM_1 liver toxicity



Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different.



Values are expressed as Mean ± SEM

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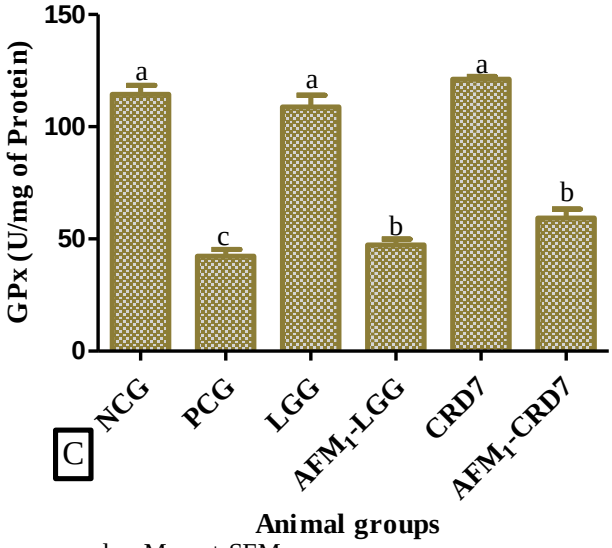


Values are expressed as Mean ± SEM

a, b, c -Values with same superscripts are nonsignificant. Values with different superscripts are significantly different

The results indicated that AFB_1 administration alone induced a significant decrease in food intake and body weight and a significant increase in transaminase, alkaline phosphatase cholesterol, triglycerides, total lipids, creatinine, uric acid and nitric oxide in serum and lipid peroxidation in liver and kidney accompanied with a significant decrease in total antioxidant capacity. Treatments with L1 or L2 succeeded to induce a significant improvement in all the biochemical parameters of the liver good health.

4.6.2.6 Efficacy of selected probiotic *L. plantarum* CRD7 on AFM_1 induced genotoxicity in mice

Genotoxicity defined as a destructive effect on a cell's genetic material (DNA, RNA) affecting its integrity. A substance that has the property of genotoxicity is known as a genotoxin. Many *in-vitro* and *in-vivo* tests for genotoxicity have been developed that detect DNA damage or its biological consequences. Many chemical carcinogens/mutagens like AFB_1, AFB_2, AFM_1, AFM_2, AFG_1, AFG_2 undergo metabolic activation to reactive species that bind covalently to DNA, to form DNA adducts, which can be detected in cells and in animal tissues by a variety of sensitive techniques. The detection and characterization of DNA adducts in human tissues provides clues to the aetiology of human cancer. AFB_1, a widespread food and feed contaminant,

is bioactivated by drug metabolizing enzymes (DME) to cytotoxic and carcinogenic metabolites like AFB_1-epoxide and AFM_1, a dairy milk contaminant.

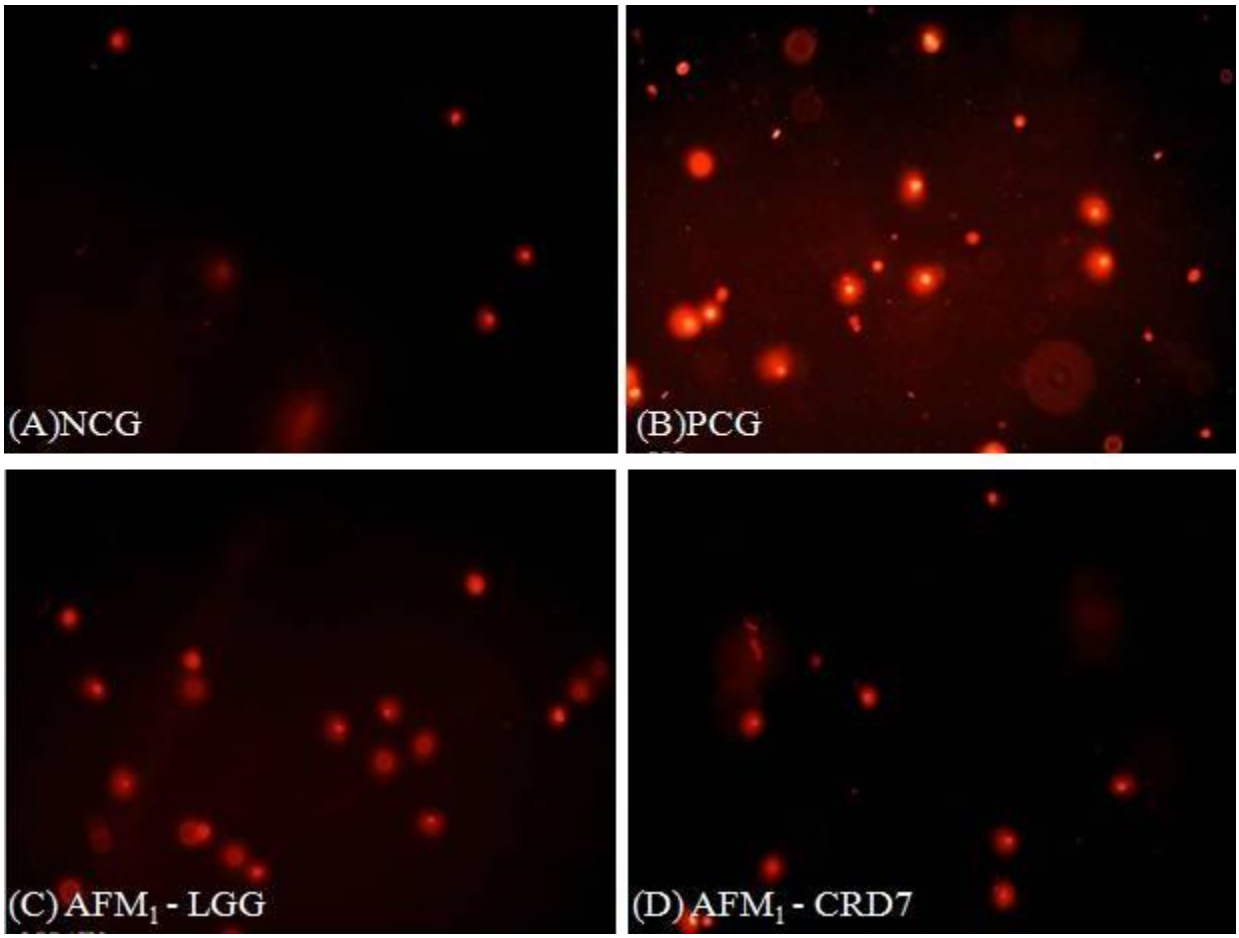
There is very scanty information in the literature regarding the genotoxic effect of AFM_1 in mice liver cells and preventive role of probiotic *Lactobacillus* strains against the genotoxicity induced by AFM_1. Therefore, the present study investigated the protective role of selective probiotic *L. plantarum* CRD7 against DNA damage, induced by AFM_1. Furthermore, our result showed significantly ($p<0.05$) higher DNA damage in the liver cells of PCG group that have been illustrated by the DNA tail length **Fig 4.13.** In contrast, no DNA damage was observed in the liver cells of NCG. The apparent protective effect was observed on the liver cells DNA in case of AFM_1-CRD7 group, and less DNA damage was observed in our native strain as compared to AFM_1-LGG group. The obtained results have been depicted in **Fig 4.13.** (NCG, PCG, LGG-AFM_1 and CRD7- AFM_1)

Zhang *et al.*, (2015), studied AFB_1 and AFM_1 induced cytotoxicity and DNA damage in differentiated and undifferentiated Caco-2 cells. They noticed that AFB_1 and AFM_1 significantly inhibited undifferentiated and differentiated cell growth, increased lactate dehydrogenase release and caused genetic damage in a time and dose-dependent manner ($p < 0.05$). In comparison, AFB_1 was found to be more toxic than AFM_1 on both undifferentiated and differentiated in cell line investigations.

All these cytotoxic outcomes might be associated with intracellular reactive oxygen species generation, leading to membrane damage and DNA strand break. Additionally, differentiated cells were found to be more sensitive to aflatoxins, which might be due to the alteration of enzymes activity during cell differentiation. In addition, the potential preventive role of *L. plantarum* MON03 against AFM_1 immunotoxicity and genotoxicity in mice were also investigated by Salah-Abbes *et al.*, (2013).

Their results showed that AFM_1 treatment cause significantly decrease in body weight gains, and induce cytotoxic and genotoxic effects which is indicated by elevation in polychromatic erythrocytes as well as micronucleation and chromosomal aberrations in the bone marrow cells. The simultaneous administration of *L. plantarum* MON03 with AFM_1 strongly reduced the adverse effects of AFM_1. Mice receiving AFM_1+*L. plantarum* MON03 co-treatment displayed no significant differences in the assayed parameters as compared to the control mice.

Fig. 4.13 Efficacy of selected probiotic *L. plantarum* CRD7 on AFM_1 induced genotoxicity (DNA damage) in mice as illustrated by comet assay



The results of present study on *in-vivo* efficacy of selected probiotic *L. plamntarum* CRD7 clearly illustrated its potential to decrease AFM_1 induced genotoxicity. Thus, the selective probiotic *L. plantarum* CRD7 can be considered as a potential candidate for use as an *in-vivo* detoxification bio-agent during contaminated food consumption *i.e.*, administration of alone probiotic and/or as a starter culture for preparation of fermented milk and milk products.

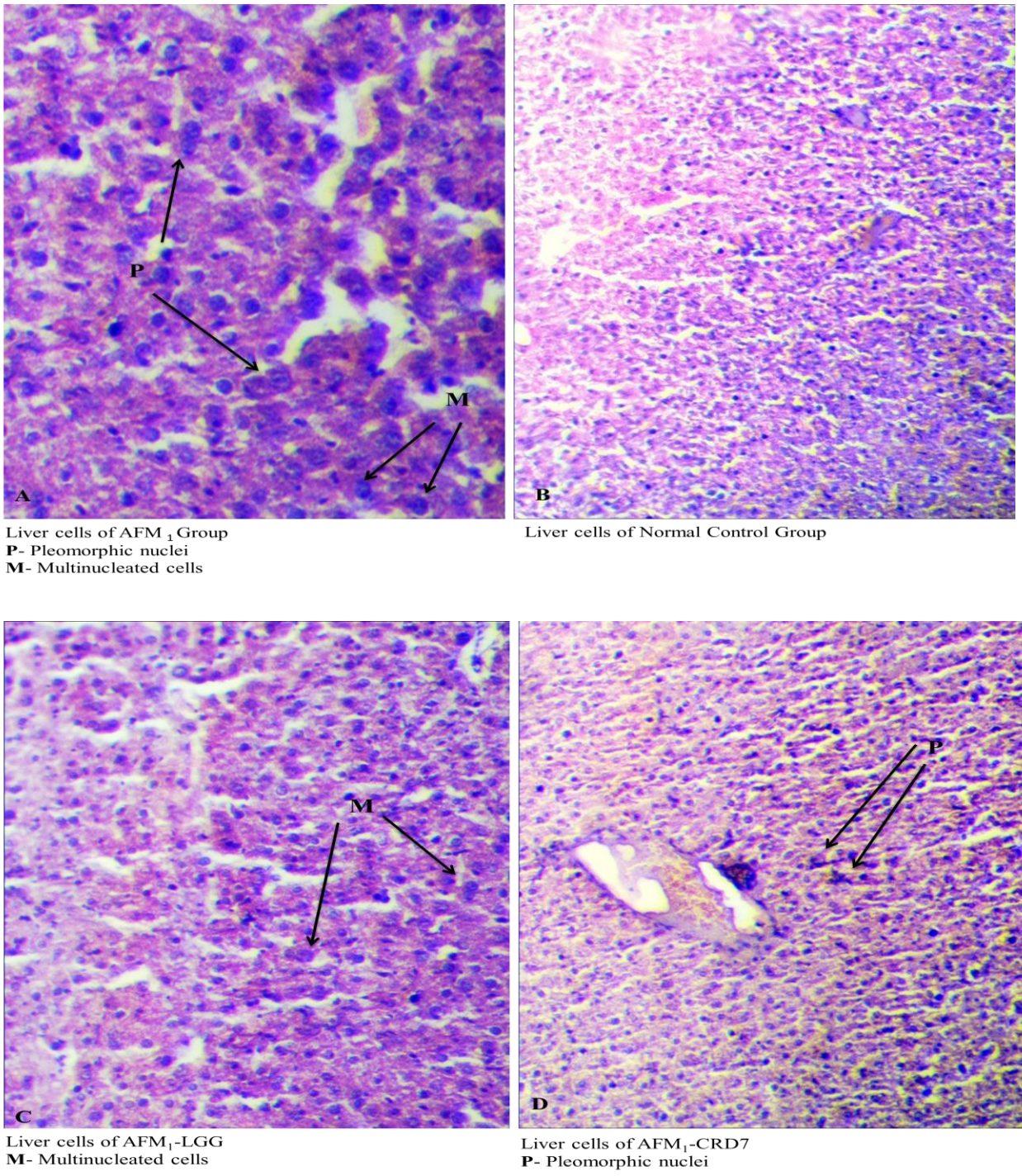
4.6.3 Effect of *L. plantarum* CRD7 on histopathology of liver tissue of AFM_1 exposed mice

Orally AFM_1 exposure of mice caused significant damage to liver tissues. Representative photomicrographs of hepatic tissue samples of groups *i.e.* NCG, PCG, AFM_1- LGG and AFM_1-CRD7 group have been presented in **Fig 4.14 (A, B, C & D) and Fig 4.15 (A, B, C & D).**

AFM_1 exposure caused histopathological changes in the liver, including cellular carcinoma manifested by malignant lesions such as formation of plemorphic nuclei and multi-nucleated cells. Co-treatment with AFM_1 + *L. plantarum* CRD7 significantly reduced such hepatic injury as only pleomorphic cells were observed and there is no multi-nucleated cells were observed. In contrast, co-treatment with AFM_1 + *L. rhamnosus* GG only multi-nucleated cells were observed in the liver tissue.

The dilated central veins in the hepatic tissue of AFM_1 exposed and AFM_1-LGG group were observed but in case of AFM_1-CRD7 and NCG group showed no evidence of dilation of central veins indicate of its effect against AFM_1 induced toxicity. In the present study normal appearance were observed of hepatic tissue in the mice of NCG.

Fig. 4.14 Histopathological analysis of liver tissue



Liver cells of AFM $_1$ Group
P- Pleomorphic nuclei
M- Multinucleated cells

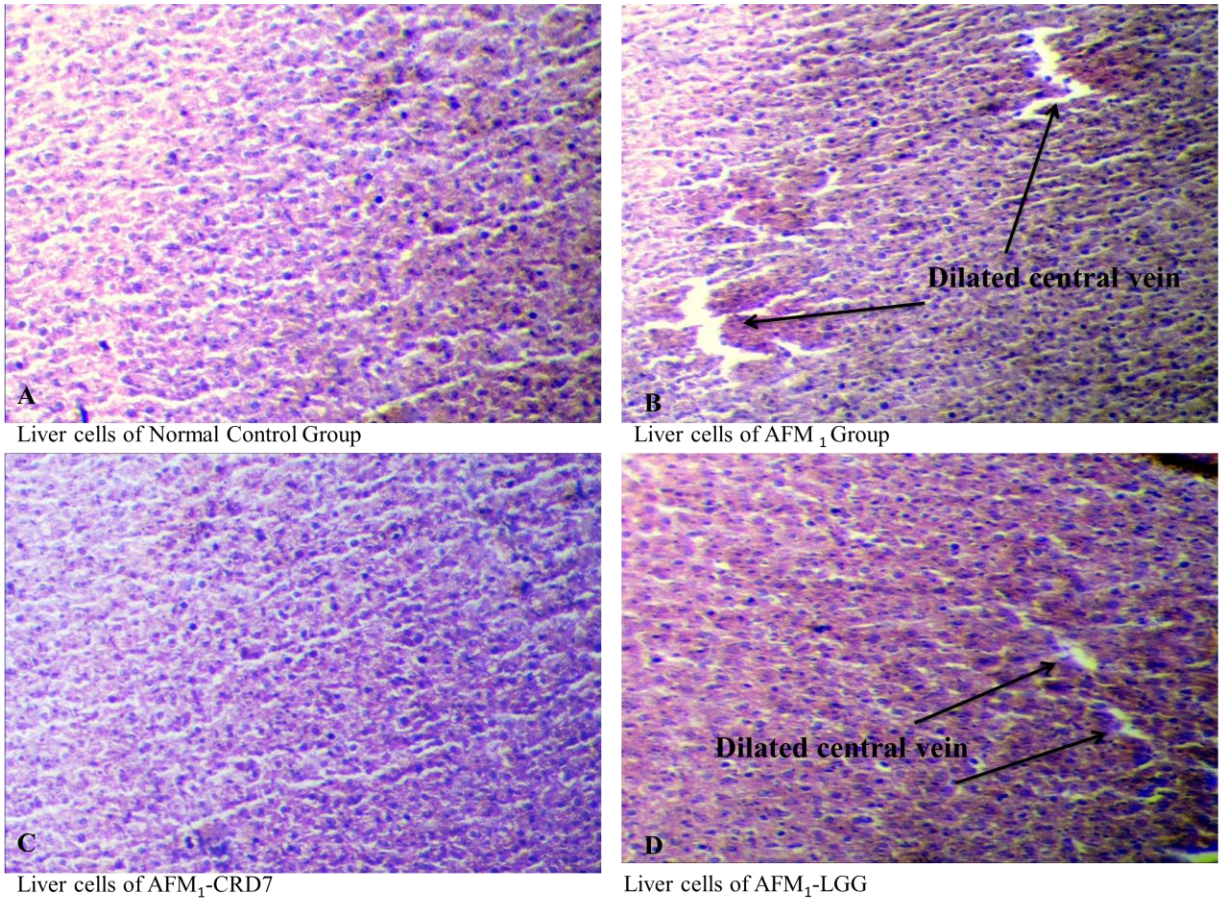
Liver cells of Normal Control Group

Liver cells of AFM_1-LGG
M- Multinucleated cells

Liver cells of AFM_1-CRD7
P- Pleomorphic nuclei

Photomicrograph of hepatic tissue of mice (H & E staining, magnification 400X). **A**. Hepatice tissue of AFM_1 group with multi-nucleated cells and pleomorphic nuclei; **B**. Normal appearance of hepatic tissue of mice in the NCG; **C**. Hepatic tissue of AFM_1- LGG group with multi-nucleated cells; **D**. Hepatic tissue of AFM_1- CRD7 group with pleomorphic nuclei.

Fig. 4.15 Histopathological analysis of liver tissue



Photomicrograph of hepatic tissue of mice (H & E staining, magnification 400X). **A**. Normal appearance of hepatic tissue of mice in the NCG; **B**. Hepatice tissue of AFM_1 group with dilated central vein; **C**. Normal appearance of hepatic tissue of mice in the AFM_1- CRD7; **D**. Hepatice tissue of AFM_1- LGG group with dilated central vein.

5.0 SUMMARY AND CONCLUSIONS

5.1 SUMMARY

Presently, use of techno-functional starter cultures in food fermentation industry is being widely explored. Techno-functional starters are those that possess at least one inherent functional property to contribute to food safety and/or offer one or more organoleptic, technological, nutritional, health advantage. Lactic acid bacteria (LAB) is the best example of techno-functional starters as they produce antimicrobial substances, exopolysacchrides, aromatic compounds, useful enzymes, or nutraceuticals as well as play a vital role in bioremediation of toxic substances (aflatoxins and heavy metals). Thus, consumption of probiotics would be helpful in maintaining health, combating intestinal disorders as well as in bioremediation of toxic compounds *vis-a-vis* AFM_1 from milk and milk products to safeguard human health.

The present study was aimed to investigate potential of probiotic *Lactobacillus* strains, previously isolated and identified from human feces as well as milk and milk products for their capability to reduce AFM_1 from suspended medium and AFM_1 bioaccessibility reduction in *in-vitro* digestion model. The selected probiotic *L. plantarum* CRD7strain was also evaluated for its efficacy in *in-vivo* to ascertain its protective effect against AFM_1 induced immunotoxicity, hepatotoxicity and genotoxicity. *L. rhamnosus* GG (LGG) was used as a reference strain to compare results of selected test strain *i.e. L. plantarum* CRD7. The salient findings of the present investigation are summarized in this section.

- Total thirty probiotic lactobacilli cultures were screened for AFM_1 binding/bioadsorption potential *in-vitro* in PBS medium. They showed a wide range of AFM_1 bioadsorption *i.e.* 2.46±2.31 - 64.16±5.60 % after incubation period of 6, 12 and 24 hours in PBS medium.
- Among thirty probiotic lactobacilli strains, five probiotic strains (*L. plantarum* CRD7, *L. rhamnosus* CRD9, *L. plantarum* CM63, *L. plantarum* BM71 and *L. plantarum* HIF81) were selected for further detailed experiments *w.r.t.* bioaccessibilty reduction under simulated gastrointestinal tract conditions.
- The selected probiotic *Lactobacillus* strains were also evaluated for their probiotic lactobacilli-AFM_1 complex stability in PBS medium. The results on stability of probiotic lactobacilli-AFM_1 complex demonstrated significant ($p<0.05$) highest percentage of

AFM_1 release in first wash, for all selected probiotic *Lactobacillus* strains which ranged from 7.40±0.23 - 20.45±2.10 % of the initially bound AFM_1. The percentage of AFM_1 released during the second wash ranged from 0.98±0.14 - 18.20±1.24 % was significantly ($p<0.05$) lower than the first wash. In case of *L. plantarum* BM71 and *L. plantarum* HIF81, no detectable $AFM_1$was released in the second wash. However, no AFM_1 was released during the third wash of all the strain used except *L. rhamnosus* GG which released 12.72±1.30 % AFM_1 even in the third wash its indicates of strong probiotic-AFM_1 complex stability of our native strains as compared to *L. rhamnosus* GG.

- Bacterial cells were treated with chloroform to illustrate mode of interaction between *Lactobacillus* strains and AFM_1. The data on mode of AFM_1 interaction with probiotic *Lactobacillus* strains showed that chloroform treatment released, 88.57±0.16 - 92.30±2.23 % of bound AFM_1 from the bacterial cells indicative of its bioadsorption instead of degradation by metabolism. However, no significant difference ($p<0.05$) was observed in the released amount of AFM_1 among selected probiotic *Lactobacillus* strains *viz.*, *L. rhamnosus* GG (90.80±5.54), *L. plantarum* CRD7 (91.25±2.94 %), *L. rhamnosus* CRD9 (89.18±1.40 %), *L. plantarum* CM63 (88.90±4.61 %), *L. plantarum* BM71 (92.30±2.23 %) and *L. plantarum* HIF81 (88.57±0.16 %).
- These selected probiotic *Lactobacillus* strains were further evaluated for their ability to reduce AFM_1 bioaccessibility in *in-vitro* digestion model. The observations showed that bioaccessibility of AFM_1 was significantly ($p<0.05$) reduced by selected probiotic strains which ranged from 21.56±1.66 - 52.84±3.34 %. The highest reduction (52.84±3.34 %) was noted for *L. plantarum* CRD7 and lowest (21.56±1.66 %) for *L. rhamnosus* GG.
- The selected probiotic *Lactobacillus* strains were also evaluated for their efficiency to bind AFM_1 in skim milk. Selected five probiotic lactobacilli strains were subjected to autoclave (121°C/15 min) prior to their use in skim milk. The selected heat killed probiotic *Lactobacillus* strains *viz.*, *L. plantarum* CRD7, *L. rhamnosus* CRD9, *L. plantarum* CM63, *L. plantarum* BM71 and *L. plantarum* HIF81) exhibited 60.06±3.08, 57.23±6.61, 49.01±3.18, 55.96±4.99 and 53.4±3.36 %, AFM_1 binding ability in skim milk, respectively.
- Selected heat killed probiotic *Lactobacillus* strains were also assessed for their complex stability in PBS medium. The highest release of AFM_1 was observed after first and

second wash with PBS, which was significantly ($p<0.05$) higher for all heat killed probiotic *Lactobacillus* strains as compared to live probiotics, which released from 27.5±2.4 - 14.30±2.82 % of initially bound AFM_1. The percentage of AFM_1 released during third wash was observed only in case of heat killed *L. plantarum* CRD7, *L. rhamnosus* CRD9 from 7.5±3.00 and 12.2±4.36 %, respectively.

- This is the first demonstration of effect on AFM_1 bioaccessibility reduction by heat killed probiotic lactobacilli strains. In current investigation bioaccessibility of AFM_1 was significantly ($p<0.05$) reduced by heat killed probiotic *Lactobacillus* strains in *in-vitro* digestion model. The reduction in relative AFM_1 bioaccessibility ranged from 32.23±2.73 - 50.7±1.90 %. *L. plantarum* BM71 showed highest (50.7±1.90 %) while *L. plantarum* HIF81 lowest reduction (32.2±2.73 %) in AFM_1 bioaccessibility in spiked skim milk.
- Swiss albino mice model was used to evaluate the selected probiotic *Lactobacillus* strains for AFM_1 bioaccessibility reduction in *in-vivo* and its preventive role against AFM_1 immunotoxicity and genotoxicity.
- The present study was conducted in two phases *i.e.* Phase 1 and Phase 2. The phase 1 was assigned for AFM_1 dose standardization to determine the effect of AFM_1 at different concentration in mice. The phase 2 was assigned for efficacy evaluation of selected probiotic *L. plantarum* CRD7 for AFM_1 bioaccessibility reduction and its preventive role against AFM_1 immunotoxicity and genotoxicity.
- In phase 1 study, animals were divided into 3 groups of 4 mice each and permitted *ad libitum* diet and water. The AFM_1 dosage (0.5 and 1.0 ppb/kg body weight) was orally administered by gavage for standardization of the dose to induce toxicity.
- The analysis parameters assigned included effect on body weight, immunotoxicity, hepatotoxicity and antioxidative stress markers.
- The average body weight of mice in experimental groups just prior to start of standardization study was kept almost equal. The result indicated that average body weights of mice exposed to AFM_1 (1.0 ppb) was significantly decreased ($p<0.05$) at the end of the study as compared to the average body weights of mice exposed to AFM_1 (0.5 ppb) and control.
- The significant ($p<0.05$) increase in total leucocytes count (TLC) and differential leucocytes counts (DLC) was observed in mice exposed to AFM_1 (1.0 ppb/kg body

weight) as compared to the mice exposed to AFM_1 (0.5 ppb/kg body weight) concentration. The higher viable splenocytes and thymocytes were observed in the AFM_1 (0.5 ppb) treated and NCG as compared to the mice exposed to AFM_1 (1.0 ppb/kg body weight).

- The activity of AST, ALT and GGT in blood serum of AFM_1 (1.0 ppb) treated group was found significantly elevated ($p<0.05$) as compared to the mice exposed to AFM_1 (0.5 ppb) and NCG.
- The significant decline in antioxidative stress marker enzymes (CAT, SOD, and GPx) was observed in group which was exposed to 1.0 ppb AFM_1. However, no significant ($p<0.05$) difference have been observed in the levels of oxidative stress marker enzymes *i.e.* CAT, SOD, and GPx between 0.5 ppb AFM_1 treated and NCG. Our results have demonstrated significant decrease in oxidative stress marker enzymes *i.e.* CAT, SOD, and GPx in liver after 1.0 ppb AFM_1 exposure only and 0.5 ppb dose of AFM_1 is not sufficient to induce oxidative stress in the liver cells.
- The phase 2 study was assigned for efficacy evaluation of selected probiotic *L. plantarum* CRD7 for AFM_1 bioaccessibility reduction and its preventive role against AFM_1 toxicity. The bacterial biomass of *L. plantarum* CRD7 was administered orally and mice were randomly divided into six groups *i.e.* normal control group (NCG), AFM_1 group *i.e.* positive control group (PCG), LGG group, AFM_1-LGG group, CRD7 group and AFM_1-CRD7 group fed with respective experimental diets having six mice in each group.
- The parameters recorded for in *in-vivo* efficacy of *L. plantarum* CRD7 was effect on body weight, AFM_1 bioaccessibility reduction, immunotoxicity, hepatotoxicity, oxidative stress, genotoxicity and liver histo-pathological examinations.
- The result of *in-vivo* efficacy of *L. plantarum* CRD7 indicated that average body weights of mice exposed to AFM_1 alone (PCG) was significantly lower ($p<0.05$) at the end of the study as compared to NCG, LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 groups. However, increase in body weight was non-significant within all treated groups *i.e.* NCG, LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 during experimental trials. On the other hand, no significant changes were recorded in increment in body weight of mice fed with selected probiotic bacterial strains (*L. plantarum* CRD7 and *L. rhamnosus* GG) as compared to the normal control group indicative of their role in weight management.

- Observations on *in-vivo* bioaccessibility reduction showsed that, till the second week no significant reduction in AFM_1 bioaccessibility was observed in any group. After third week a significant 18.14±0.30 and 16.54±0.72 % reduction of AFM_1 bioaccessibility observed in AFM_1-LGG and AFM_1-CRD7 groups, respectively. The significantly higher reduction in bioaccessibility of AFM_1 *i.e.* 36.21±0.14 % was observed in case of *L. plantarum* CRD 7 + AFM_1 (AFM_1-CRD7) fed group ($p<0.05$) as compared to AFM_1 (5.00±0.64 %) and reference *L. rhamnosus* GG + AFM_1 (AFM_1-LGG) fed group *i.e.* 20.31±0.57. These observations clearly indicates superiority of our selected native strain over established probiotic *i.e. L. rhamnosus* GG.
- The results of blood immune cells analysis revealed that treatment with a daily oral dose of AFM_1 induced immune system commotion as evidenced by significant ($p<0.05$) increase in total leucocytes counts (TLC) in PCG group *i.e.* 9420±6.09 cells/cmm as compared to NCG, LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 groups *i.e.* 3397±4.52 cells/cmm, 3922.3±8.02 cells/cmm, 5935.6±5.65 cells/cmm, 3901.6±5.54 cells/cmm and 4479.3±0.95 cells/cmm, respectively.
- The differential leucocytes counts (DLC) were also found significantly ($p<0.05$) increased in PCG as compared to NCG, LGG, AFM_1-LGG, CRD7 and AFM_1-CRD7 groups. But in case of group AFM_1-LGG, monocyte counts were slight higher *i.e.* 5.66±2.51 % as compared to group PCG *i.e.* 3.33±2.41 %.
- Moreover, our findings indicated that the AFM_1 treatment also impacted the spleen and thymus cells viability. The significant ($p<0.05$) higher viable splenocytes and thymocytes were observed in NCG *i.e.* 99.84±0.11 % and 99.62±0.43 %, respectively. The viability of splenocytes and thymocytes were found decreased significantly in case of PCG group *i.e.* 42.26±3.91 % and 34.32±3.49 %, respectively. The more protective effect on the viability of splenocytes and thymocytes has been observed in the AFM_1-CRD7 group *i.e.* 68.77±6.85 % and 61.83±7.92 %, respectively, as compared to the AFM_1-LGG group *i.e.* 66.16±4.56 % and 36.16±4.43 %, respectively.
- The activity of AST, ALT and GGT in blood serum of PCG group was significantly noticed to be elevated ($p<0.05$) as compared to NCG, which indicate that AFM_1 toxicity was responsible for liver damage. The activity of AST, ALT and GGT in blood serum of mice treated with probiotic AFM_1-CRD7 was significantly lower ($p<0.05$) as compared

to PCG group. These observations clearly indicate protective effect of probiotic *L. plantarum* CRD7 therapy that alleviated liver damage caused by AFM_1 exposure. However, between probiotic *L. plantarum* CRD7 (CRD7) and *L. rhamnosus* GG (LGG) fed groups no significant differences were observed in level of AST, ALT and GGT.

- The AFM_1 exposure of mice led to marked decline in activity of all the antioxidative stress marker enzymes *i.e.* CAT, SOD, and GPx. The significant decline in antioxidative stree marker enzymes (CAT, SOD, and GPx) was observed on PCG group as compared to NCG. The AFM_1-LGG treated groups showed decreased levels of antioxidative stress marker and elevation level of CAT, SOD, and GPx has been observed in case of AFM_1-CRD7 group. Our results have demonstrated significant decrease in antioxidative stress marker enzymes *i.e.* CAT, SOD, and GPx in liver after AFM_1 exposure. Our observations clearly indicate preventive role of *L. plantarum* CRD7 against AFM_1 toxicity or alleviation of antioxidative enzymes levels.
- Present investigation has demonstrated protective role of selected probiotic *L. plantarum* CRD7 against DNA damage, induced by AFM_1 as significantly ($p<0.05$) higher DNA damage in liver cells of PCG group was illustrated by DNA tail length. In contrast, no DNA damage was observed in the liver cells of NCG. The apparent more protective effect was observed on liver cells DNA in case of AFM_1-CRD7 groups as compared to AFM_1-LGG group.

5.2. CONCLUSIONS

Many biological, chemical, and physical methods exist to eliminate and/or inactivate as well as prevent fungal growth and toxin production. However, these applications have not been fully adopted, particularly in under developed countries, due to high cost, loss of nutritional quality, altered organoleptic characteristics of the products, practical difficulties, and unknown effects to human health. On the other hand, traditionally used indigenous food microorganisms such as bifidobacteria and lactobacilli embrace a large fraction of the normal gut flora and increasingly used in fermented dairy foods. Thus, *Lactobacillus* and *Bifidobacterium* species are widely used in food industry as probiotics would be an ideal option to prevent AFM_1 toxic effect in human body. The research findings of current study demonstrated that tested probiotic *L. plantarum* CRD7 have immense potential to remove AFM_1 and reduce its bioaccessibility *in-*

vitro and *in-vivo* in artificially contaminated skim milk as a model for a food matrix. Chloroform treatment of probiotic - AFM_1 complex also demonstrated its cell surface binding instead of absorption or degradation by probiotics.

The continuous consumption of probiotic *L. plantarum* CRD7 due to its highest AFM_1 bioadsorption ability ameliorated the immune system and liver health. Probiotic strain of *L. plantarum* CRD7 was also advantageous for reduction of antioxidative enzymes in liver. Thus, probiotic *L. plantarum* CRD7 can be used as a promising bio-therapeutic agent for AFM_1 bioremidiation from milk and milk products to avert its toxicity to safeguard human health. Hence, future research should be directed towards development of techno-functional probiotics for preparation of fermented dairy foods to provide better protection against AFM_1 toxicity in human and animals.

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