EPIDEMIOLOGICAL STUDY ON AFLATOXIN M1 IN BOVINE MILK SAMPLES FROM PUNJAB AND ITS POSSIBLE ASSOCIATION WITH RUMEN LIQUOR PARAMETERS

Thesis

Submitted to Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

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in
VETERINARY PUBLIC HEALTH AND EPIDEMIOLOGY
(Minor Subject: Veterinary Microbiology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, **"EPIDEMIOLOGICAL STUDY ON AFLATOXIN M1 IN BOVINE MILK SAMPLES FROM PUNJAB AND ITS POSSIBLE ASSOCIATION WITH RUMEN LIQUOR PARAMETERS"** submitted for the degree of **M.V.Sc.**, in the subject of **Veterinary Public Health and Epidemiology** (Minor subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Hanul Thukral** (L-2018-V-81-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

The present study was carried out with the objectives to estimate the prevalence of AFM1 in bovine milk from commercial and household dairy establishments across all the districts of Punjab and to find the association between AFM1 excretion in milk and indicators of rumen fermentation among target animals. A total of 402 milk samples (cattle milk-266 and buffalo milk-136) were analyzed using commercial ELISA and representative samples were validated by HPLC-FLD technique. A total of 120 rumen liquor samples were analyzed for physico-chemical parameters, rumen metabolites and rumen microflora count as indicators of rumen fermentation. The results revealed that 56.22% of the milk samples exceeded the MPL of EC and 13.43% exceeding the MPL of FSSAI for AFM1 in milk. Of all the districts, Hoshiarpur (100%) and Moga (100%) had the highest number of samples above EC-MPL, whereas Muktsar (64.7%) and Moga (65%) had the highest number of samples above FSSAI-MPL. With respect to the mean concentration of AFM1 in milk, Ludhiana (0.89±1.47 ppb) was found to be most contaminated and Kapurthala (0.01±0.009 ppb) the least. On analysis of species variation, buffalo milk (prevalence: 57.35%; mean concentration: 0.42±0.9 ppb) was found to have higher prevalence and mean concentration of AFM1 than cattle milk (prevalence: 56.39%; mean concentration: 0.193±0.3 ppb). Furthermore, milk from commercial dairy farms (prevalence: 65.67%; mean concentration: 0.344±0.65 ppb) was more contaminated than milk from household dairy establishments (prevalence: 48.26%; mean concentration: 0.195±0.65 ppb). The multivariable analysis of risk factors identified 'above average per day milk yield' (>13 kg/day) (OR: 2.432), 'poor animal hygiene scores' (i.e., > 1) (OR: 1.94) and 'intensive dairy farming system' (OR: 3.12) to be risk factors for AFM1 concentration above EC-MPL in milk. The analysis further revealed that 'increase in age of animal' (OR: 0.83) and 'addition of aflatoxin binder to animal feed' has significant protective effect on the excretion of AFM1 in milk. Among the cattle breeds, 'non-descript' (OR: 11.55), 'HF cross' (OR: 4.03) and 'HF' (OR: 3.91) breed had higher excretion of AFM1 in milk as compared to 'Sahiwal' (OR: 1.51) and 'Jersey breed' (OR: 1). The rumen liquor analysis showed that the pH (r = 0.38), MBRT (r = 0.43), SAT (r = 0.31) and ammonia nitrogen content (r = 0.34) of rumen liquor increased with increasing concentration of AFM1 in milk, whereas, TVFA's content (r = -0.25), TBC (-0.43) and TPC (r = -0.14) of rumen liquor decreased with increasing concentration of AFM1 in milk of bovines.

Keywords: Aflatoxin M1; Bovines; ELISA; HPLC; India; Milk; Punjab; Risk factors; Rumen liquor

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Signature of Major Advisor **Signature of the student**

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ABBREVIATIONS

%	:	Percent
μg	:	Microgram
μg/kg	:	Microgram per kilogram
μm	:	Micrometer
+ve	:	Positive
<	:	Less than
>	:	Greater than
±	:	Plus-minus
× g	:	gravity
≤	:	Less than equal to
≥	:	Greater than equal to
ACN	:	Acetonitrile
AF	:	Aflatoxins
AFB1	:	Aflatoxin B1
AFB2	:	Aflatoxin B2
AFG1	:	Aflatoxin G1
AFG1	:	Aflatoxin G1
AFM1	:	Aflatoxin M1
ALARA	:	As Low As Reasonably Achievable
b.w.	:	body weight
CI	:	Confidence Interval
dl	:	decilitre
e.g.	:	For example
EC	:	European Commission
EC	:	European Commission
EDI	:	Estimated Daily Intake
ELISA	:	Enzyme Linked Immunosorbent Assay
EN ISO	:	European Norms International Organization for Standardization
et al	:	et alia ((Latin- and others)
Etc.	:	et cetera
EU	:	European Union
FAO	:	Food and Agriculture Organization

FDA	:	Food and Drug Administration
Fig.	:	Figure
FLD	:	Fluorescence Detection/Detector
FSSAI	:	Food Safety and Standards Authority of India
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
gm	:	Gram
HBV	:	Hepatitis B Virus
HCC	:	Hepatocellular Carcinoma
HF	:	Holstein Friesian cattle
HPLC	:	High Performance Liquid Chromatography
i.e.	:	That is
IACs	:	Immuno-Affinity Columns
IAEC	:	Institutional Animal ethics Committee
IARC	:	International Agency for Research on Cancer
JECFA	:	Joint Expert Committee on Food Additives
kg	:	Kilogram
l or L	:	Litre (s)
LRT	:	Likelihood Ratio Test
Max.	:	Maximum
MBRT	:	Methylene Blue Reduction Test
mg	:	Milligram (s)
min	:	Minute (s)
Min.	:	Minimum
ml	:	Millilitre(s)
mm	:	Millimeter (s)
MPL	:	Maximum Permissible Limit
NDDB	:	National Dairy Development Board
ng	:	Nanogram
nm	:	Nanometer (s)
No.	:	Number
NOAEL	:	No Observed Adverse Effect Limit
O.D.	:	Optical Density
^{o}C	:	Degree Celsius
OR	:	Odds Ratio

pH	:	Potential of hydrogen
ppb	:	Parts per billion
ppm	:	Parts per million
rpm	:	Revolutions per minute
RT or t_R	:	Retention Time
SAT	:	Sedimentation Activity Test
SD	:	Standard Deviation
sec	:	Second (s)
SRL	:	Strained Rumen Liquor
TBC	:	Total Bacterial Count
TPC	:	Total Protozoal Count
TVFA’s	:	Total Volatile Fatty Acids
UHT	:	Ultra High Temperature
v/v	:	Volume by volume
viz	:	Videlicet
WHO	:	World Health Organization

CHAPTER – I

INTRODUCTION

In today's world of ever increasing population and globalization, the food safety and security need to be addressed appropriately. Ensuring safety of food has been a major focus of concern for governments around the world. Food safety and food security are closely inter-linked. Unsafe food creates a vicious cycle of disease and malnutrition, particularly affecting the vulnerable group, in addition compromising trade and tourism, national economies and ultimately sustainable development. According to World Health Organization (WHO), food-borne illnesses around the world affect an estimated 600 million people (almost 1 in 10 people) each year resulting in 4,20,000 deaths and loss of 33 million Disability-Adjusted Life Years (DALYs) (WHO 2020), where the low and middle income countries being hit the hardest.

Both microbiological and chemical hazards are of concern for ensuring safe food. Among chemical hazards of natural origin, the contamination of food and feed by mycotoxins, fish products by phycotoxins and edible plant species by their plant toxins have been recently characterized by the WHO as significant sources of food-borne illnesses (WHO 2002), with mycotoxins grabbing the most attention. Mycotoxins are toxic secondary metabolites naturally produced by fungi (Ismail *et al* 2016a). Fungi can grow on a variety of agricultural crops either before harvest or after harvest, during storage, on/in the food itself often under warm, damp and humid conditions and it is estimated that 25-50% of the world's food crops are contaminated by mycotoxins (Cardwell and Miller 1996). The primary entry point of mycotoxins in the food chain is the fungal infection of crops. Human exposure can then happen either directly via consumption of infected food or indirectly via food of animal origin that are fed contaminated feed (milk, eggs, meat). Out of 450 different types of mycotoxins and their metabolites identified till date (Njoroge *et al* 2017), the most commonly encountered mycotoxins that poses significant health risk to both humans and livestock include aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and nivalenol/deoxynivalenol (WHO 2018).

Aflatoxins are amongst the most poisonous mycotoxins produced by members of three sections of *Aspergillus* genus; section *Flavi* (*Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. toxicarius* and *A. parvisclerotigenus*), section *Ochraceorosei* (*A. ochraceoroseus* and *A. rambellii*), and section *Nidulantes* (*Emericella astellata* and *E. venezuelensis*) (Pildain *et al* 2008) with species of section *flavi*; *A. flavus* and *A. parasiticus* being the most potent and frequently encountered aflatoxin-producing fungi in agricultural commodities because of their ubiquitous distribution and their versatility to grow and produce aflatoxins under different environmental conditions (Okoth *et al* 2018, Frisvad *et al* 2019). There are more than 20 known types of aflatoxins out of which aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are naturally occurring aflatoxins while other are derivatives of these four (Liu and Wu 2010). Amongst them, AFB1 is the most toxic, mutagenic, teratogenic and carcinogenic (group 1) (Iqbal *et al* 2014) aflatoxin with their order of toxicity being AFB1>AFG1>AFB2>AFG2 (Kumar *et al* 2017). These fungi can grow on a variety of agricultural crops with maize, peanuts, cottonseed and coconut being the most prone one's (Cornea *et al* 2011) and the production of toxin depending upon a variety of factors like moisture content, temperature, storage period, contamination rate, damage during harvest, processing and transport (Scudamore 2005).

The aflatoxin M1 (AFM1) is the C_4 monohydroxylated metabolite of AFB1 that occurs in milk and milk products of animals consuming feed contaminated with AFB1 (Sharma *et al* 2020). Milk is considered as one of the major source of AFM1 in human food chain evoking global concern over food safety. Not only is AFM1 secreted in milk, urine and feces of dairy animals (Kang'ethe *et al* 2009) but its presence in human breast milk (Fakhri *et al* 2019) and transplacental transfer has also been reported in humans (Maxwell *et al* 1989). AFM1 is ten-fold less toxic than AFB1 but the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has re-classified AFM1 from group 2B to group 1 human carcinogen due to its toxicity and widespread occurrence in milk (IARC 2012). As AFM1 has binding affinity for casein in milk, therefore higher concentrations are observed in cheese and skim milk (Chavarría *et al* 2017). Also, studies have shown that, AFM1 is quite stable during milk pasteurization, cold storage as well as during the preparation of various dairy products (Codex Alimentarius Commission 2001,

Badea *et al* 2004). The fungi responsible are abundant in the tropical countries with gradually decreasing in the warm temperate to being uncommon in cool temperate zones. Studies on the seasonal variation of AFM1 revealed higher concentrations in the rainy and winter season than in summer season (Ismail *et al* 2016b, Patyal 2019).

Aflatoxin exposure is associated with both acute and chronic conditions. Aflatoxicosis came into light after the first ever recorded outbreak in turkeys (Turkey "X" disease) in England in 1960 (Blount 1961) followed by a number of outbreaks in both humans and animals in India (Krishnamachari *et al* 1975, Tandon *et al* 1977, Krishna *et al* 1991, Chahota *et al* 2000, Mahajan *et al* 2002, Reddy and Raghavender 2007) as well in rest of the world (Ngindu *et al* 1982, Lye MS *et al* 1995, Centers for Disease Control and Prevention 2004, Azziz-Baumgartner *et al* 2005, Probst *et al* 2007, Pereyra *et al* 2008, Arnot *et al* 2012, Wouters *et al* 2013, Kaleibar *et al* 2013, Sohooa *et al* 2015, Guterres *et al* 2017 and Kamala *et al* 2018). Primarily liver is the main target organ as it is the predominant site of aflatoxin metabolism. Extensive studies on aflatoxins has proved it to be an extremely carcinaogenic (Ostry *et al* 2017), hepatotoxic (Yilmaz *et al* 2020), genotoxic (Theumer *et al* 2018), immune-modulating (Shirani *et al* 2018, Baldissera *et al* 2019), growth impairing (Chen *et al* 2018, Liverpool-Tasie *et al* 2019), teratogenic (Smith *et al* 2017) and mutagenic (McCullough*et al* 2019) compound. Animal exposure to aflatoxins is associated with liver damage, teratogenicity, tumours, decreased reproductive performance and reduced production (Akande *et al* 2006). In humans, several epidemiological studies have linked an increased risk of hepatocellular carcinoma (HCC) among hepatitis B virus carriers who were positive for aflatoxin biomarkers (Wu *et al* 2009) with aflatoxins playing a possible causative role in 4.6–28.2% of all global HCC cases each year (Liu *et al* 2010). The ingestion of aflatoxins at high levels in a single dose or repeatedly for a short period of time induces acute intoxication in humans and animals with typical symptoms including jaundice, lethargy, nausea, edema, hemorrhagic necrosis of liver tissues, bile duct hyperplasia, and eventually death (in 10-60% of cases) subsequent to severe liver damage (Benkerroum, 2020). Furthermore, infants and young population are more susceptible to AFM1 due to their low body weight, high metabolic rate, and incomplete development of excretory organs (Lombard 2014), in addition to the fact that a high percentage of them consume milk as the sole source of their diet. Chronic exposure of children to AFM1

has been associated with malnutrition, stunted growth, liver cancer and underweight during infancy or later in life.

A number of studies have revealed that adult ruminants are more resistant to the effects of aflatoxins than monogastric animals and young/immature ruminants (Mathur *et al* 1976, Upadhaya *et al* 2009, Khodabandehloo *et al* 2019); this can be due to the fact that rumen may have some detoxifying capacity on aflatoxins. This was confirmed by a study conducted by Intanoo *et al* (2018) in which they isolated several bacterial and yeast strains from rumen liquor of cattle which were shown to degrade aflatoxins to some extent *in vitro*. In animals before undergoing hepatic metabolism, aflatoxin is first encountered by the diverse ruminal fauna and flora which acts as the first line of defense against aflatoxins by their degradation and detoxification. After ingestion, AFB1 is degraded in rumen into aflatoxicol (18 times less toxic than AFB1) and the remaining is absorbed in the digestive tract and converted to AFM1 in liver, the same being excreted through milk (Nidhina *et al* 2017). The extent of AFB1 conversion into AFM1 varies greatly and ranges from 0.3% to 6.2% (Var and Kabak 2009) depending upon a number of factors such as breed, feed, health, digestion rate and lactation stage of animal (Duarte *et al* 2013). On the downside, the degraded aflatoxicol can almost entirely convert back into its more toxic parent AFB1 or AFM1 in liver (Benkerroum, 2020). AFB1 and AFB2 can also be produced by rumen microflora in ruminal environment, so rumen also acts as a source of AFM1 in milk of ruminants with ruminal pH also governing the production of alfatoxins (Nidhina *et al* 2017).

For further better understanding of the complex interaction between rumen and aflatoxins, several studies were conducted to access the effect aflatoxins have on fermentation efficiency of rumen. AFB1 was shown to inhibit the growth and alter the morphology of the rumen micro-organisms *in vitro* (Mathur *et al* 1976). This is further supported by the fact that ammonia nitrogen concentration increases and volatile fatty acids (VFA's) production decreases with increase in AFB1 concentration (Xiong *et al* 2015, Khodabandehloo *et al* 2019 and Jiang *et al* 2020). Studies regarding role of pH shows variable results with some claiming high toxin concentration during low ruminal pH (Nidhina *et al* 2017) while others during high ruminal pH (Khodabandehloo *et al* 2019). Since VFA's are necessary for energy and

milk production in ruminants, it can be the reason for decrease in weight gain and production losses in affected animals. Also aflatoxins cause cessation or decrease in rumen motility after 24-32 hours of exposure which may further cause delay in aflatoxin elimination (Cook *et al* 1986). Therefore, studies on the aflatoxin biosynthetic pathway can be helpful to assess the ability to eliminate or reduce mycotoxin contamination and its adverse effects in both animals and humans.

Efforts to reduce human and animal exposure to aflatoxins have resulted in the establishment of regulatory limits and monitoring programme worldwide. The rationale for adopting specific regulations is mostly based on risk analysis studies. The Food Safety and Standards Authority of India (FSSAI), Food and Drug Administration (FDA) of USA and *Codex Alimentarius* of FAO/WHO have established the Maximum Permissible Limit (MPL) of 0.5 µg/L or ppb of AFM1 in bulk milk (FDA 2000, FSSAI 2011 and Codex Alimentarius Commission 2017) while the European Union established the MPL at 0.05 µg/L (EC 2010). As hazard assessment approach does not apply for toxins where genotoxity and carcinogenicity are the basis of concern as with the aflatoxins, therefore "No Observed Adverse Effect Limit" (NOAEL) can't be established for such compounds, instead Joint FAO/WHO Expert Committee on food Additives (JECFA) recommends maximum limits to be set "As Low As Reasonably Achievable" (ALARA) in order to protect animal and public health (FAO 2004). In recent years, regulatory limits seem to be a practical compromise between the need to have carcinogen-free product and the economic consequences of setting regulatory limits (Pohland *et al* 1992). Currently the limits of AFM1 are highly variable depending upon the degree of development and economic standing of the countries.

India leads the world in terms of livestock population (535.78 million) and milk production with an output of 187.7 million tonnes per annum, accounting for 22% of the global production (NDDB 2019 and FAO 2020). As India being a tropical country, the climatic conditions favor the growth of aflatoxin producing fungi on agricultural crops and stored animal feed. Sharma and Parisi (2017) reported that aflatoxin contamination in India in food and feed is highest when humidity is above 13% and temperature between 24^0C and 37^0C. The dairy industry in India is governed largely by unorganized sector relying heavily on the small scale dairy farmers,

thereby decontamination must be addressed at the source itself. The available physical and chemical methods of mycotoxin detoxification are restricted by problems concerning safety issues, possible losses in the nutritional quality, limited efficacy and cost implications (Intanoo *et al* 2018), but recent studies suggests the use of gastro-intestinal microbials in detoxification process as a promising alternative (Kabak and Var 2008, Nguyen *et al* 2020). However little information is available on the interactions between rumen microbes and aflatoxins, though the impact of rumen on degradation of aflatoxins is proven (Kabak and Var 2008).

The state Punjab of India is known for its high milk production and consumption with the ranking first in the country for per capita availability of milk with 1181 grams/day compared to the national average of 394 grams/day (NDDB 2019). Considering the high availability and consumption, milk is the major constituent of diet in the state, especially of infants which are highly susceptible to the adverse effects of AFM1 (Lombard 2014). In addition, the climatic conditions in state favor the growth of aflatoxins in animal feed and thereby its excretion through milk. To the best of our knowledge, very limited studies have been conducted on the presence of aflatoxins in milk in Punjab as well as in India. Therefore, a number of systematic epidemiological studies need to be carried out in order to analyse the status of aflatoxins in milk with the aim to for in better understanding of risk factors and evidence based control measures to mitigate this public health hazard. Thus, taking into account all the points discussed above, the present research proposal was formulated with the following objectives:

1. To estimate the prevalence of Aflatoxin M1 in cattle and buffalo milk samples from commercial dairy farms and household animals of Punjab.
2. To find the association, if any, between the excretion of Aflatoxin M1 in milk of bovines and their rumen liquor parameters.

CHAPTER – II

REVIEW OF LITERATURE

Mycotoxins are the toxic secondary metabolites naturally produced by fungi (Ismail *et al* 2016). Aflatoxins, mainly produced by *Aspergillus flavus* and *A. parasiticus*, are one of the most toxic mycotoxins (Okoth *et al* 2018, Frisvad *et al* 2019). Aflatoxins are a group of more than 20 related metabolites with aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) being naturally occurring aflatoxins while other are derivatives of these four (Liu and Wu 2010, Ahmad *et al* 2019). Amongst them, AFB1 is most toxic, mutagenic, teratogenic and carcinogenic (group 1) aflatoxin (Kumar *et al* 2017, McCullough *et al* 2019). The contaminated crops are considered as the primary entry point of AFB1 in the food chain. When milk animals consume AFB1-contaminated feedstuffs, the toxin is metabolised in the liver and upon hydroxylation, a lesser toxic metabolite, Aflatoxin M1 (AFM1) is excreted in milk and urine (Sharma *et al* 2020). AFM1 is tenfold less toxic than AFB1 but the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has re-classified AFM1 from group 2B to group 1 human carcinogen due to its toxicity and widespread occurrence in milk (IARC 2012). Human exposure can happen either directly via consumption of infected food or indirectly via food of animal origin.

2.1 Occurrence of AFM1 in milk

2.1.1 Occurrence of AFM1 in milk: Indian scenario

Dhavan *et al* (1995) reported data on incidence of Aflatoxins in various Animal feeds in India from period of 1983 to 1993. Data included 4818 samples of agricultural commodities, comprising cereals, oilseed cakes, compound feeds, and other ingredients for analysis. Highest incidence of aflatoxin contamination was observed in groundnut cake (96.35%) and deoiled groundnut cake (96.20%), and the highest level of aflatoxin B1, 8260 ppb, was observed in maize.

Rastogi *et al* (2004) collected 87 samples in categories of infant milk food (18), infant formula (17), milk based cereal weaning food (40) and liquid milk samples (12) from Lucknow market and found incidence of AFM1 contamination to be 87.3%. Infant milk products (65–1012 ng/l) were found to be more contaminated

than liquid milk (28–164 ng/l). 99% of contaminated samples exceeded European Communities (EU) recommended limits and 9% of samples exceeded FSSAI (Food Safety and Standards Authority of India) limit. AFB1 contamination in cattle feedstuff was estimated by extrapolation of AFM1 data and was found to be of range from 1.4 to 63.3µg/kg with a mean of 18 µg/kg which is much higher than the EU regulation (5 µg/kg).

Siddappa *et al* (2012) analyzed 45 UHT (Ultra High Temperature) milk samples from markets of Karnataka and Tamil Nadu by reversed phase HPLC (High Performance Liquid Chromatography) using fluorescent detector. On analysis, all samples were found contaminated with AFM1 and 38% of them exceeded 0.5 µg/kg, the maximum permitted limit prescribed by the *Codex Alimentarius* Commission and by the mandatory regulations of the country, the FSSAI Regulations, 2011.

Kanungo *et al* (2014) conducted a survey of AFM1 in packaged milk and infant formula milk samples from Goan market, India using HPLC and ELISA. They collected 72 samples (infant formula milk food-18 and packaged milk samples-54). On analysis, all samples were positive for AFM1 and exceeded the European Communities recommended limits (50 ng/L) and 75% of the samples exceeded *Codex Alimentarius*, Food Safety and Standards Authority of India (FSSAI) and US Food and Drug Administration recommended limits (500 ng/L). The range of contamination of AFM1 was found lower in infant milk formula (501–713 ng/L) than liquid milk (511–809 ng/L). The methods were also compared for their performance, and ELISA was found to be most suitable for analysis of low-level AFM1 contamination in milk.

Nile *et al* (2016) analyzed a total of 600 samples of milk from different species [buffalo (150), cow (150), goat (150) and sheep (150)] from Maharashtra state of India for the presence aflatoxin M1 (AFM1) by using high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) methods. AFM1 presence was found in buffalo (38.6%), cow (45.3%), goat (33.3%) and sheep (36.6%) milk. The mean value of AFM1 was 0.026 µg/L in buffalo, 0.018 µg/L in cow, 0.014 µg/L in goat, and 0.017 µg/L in sheep milk. AFM1 concentration was found higher in milk obtained from urban and semi-urban areas and low in rural areas

Patyal *et al* (2020a) analyzed 230 different types of milk samples constituting raw, pasteurized and ultra-high temperature treated milk from Punjab by ELISA and HPLC-FLD method. The percentage of samples above the MPL established by the European Commission (EC) and Food safety and standard authority of India (FSSAI) were found to be 45% and 38% respectively.

Sharma *et al* (2020) collected 150 milk samples from markets of Hisar, Haryana and analyzed using HPLC. Out of these 31 exceeded the maximum limit of 0.5 μg/kg given by FSSAI, India with pasteurized milk samples more contaminated than those sold by local traders and vendors. They estimated dietary intake of AFM1 for adults in Haryana was 0.005, 0.003, 0.002 μg/kg b.w./day through consumption of pasteurized milk, milk from local traders and milk from local vendors, respectively. Whereas, for Indian population, dietary intake values were recorded as 0.002 μg/kg b.w./day for pasteurized milk and 0.001 μg/kg b.w./day for both milks from traders and vendors.

2.1.2 Occurrence of AFM1 in milk: Global scenario

Bakirci (2001) analyzed 90 raw milk samples from Van, Turkey for AFM1 contamination and found toxin in 79 (87.77%) samples out of which 35 samples (44.3%) crossed the maximum permissible limit set by the Turkey Government (0.05 ppb). Statistical evaluations showed that there were significant differences ($P<0.05$) between the mean concentrations of AFM_1 of milk samples taken from March to April and March to May. Further analyses of those samples showed that there were no statistical differences between AFM_1 contents of bulk milk and pasteurized milk, skim milk, yoghurt, buttermilk, and whey. AFM_1 contents of white-pickled cheese and Kashar cheese samples were higher than those of bulk milk samples (*p-value* = <0.01) whereas those of cream and butter samples were lower (*p-value* = <0.01).

Roussi *et al* (2002) surveyed milk from Greece for presence of AFM1. A total of 114 samples of pasteurized, UHT and concentrated milk from supermarkets and 52 raw milk samples from cow, sheep and goat were collected during 1999-2000. Another batch of milk samples containing 54 samples of pasteurized milk, 23 samples of bulk-tank raw milk and 55 raw milk samples from cow, sheep and goat were collected during 2000-2001. Analysis of a total of 297 samples was carried out via HPLC. In the first sampling, AFM_1 contamination in pasteurized, UHT, concentrated

and cow, sheep and goat raw milk was 85.4, 82.3, 93.3, 73.3, 66.7 and 40%, respectively, with only one cow raw milk sample and two concentrated milk samples exceeding the EC limit of 50 ng l^{-1}. In the second sampling, AFM1 contamination in pasteurized, bulk-tank and cow, sheep and goat raw milk were 79.6, 78.3, 64.3, 73.3 and 66.7%, respectively, with only one cow and one sheep raw milk samples exceeding the limit of 50 ng l^{-1}.

Velasco *et al* (2003) did a survey to estimate the presence the AFM1 in raw milk from dairy farms in Leon, Spain. 92 milk samples were analyzed using ELISA and HPLC. AFM1 was detected in 3.3% of the samples, all below the maximum limit permitted by the European Union.

Nakajima *et al* (2004) surveyed commercial pasteurized milk across Japan for the occurrence of AFM1 during 2001-2002. 208 milk samples were collected during winter season from 11 regions of the country (9-45 samples/region). On analysis with HPLC, AFM_1 was detected in 207/208 samples (99.5%) at 0.001–0.029 $\mu g\, kg^{-1}$, with a mean of 0.009 $\mu g\, kg^{-1}$. No significant difference of the level of AFM_1 contamination was observed among the regions.

Celik *et al* (2005) showed presence of AFM1 in pasteurized milk. 85 pasteurized milk samples were analyzed by ELISA out of which 75 samples (88.23) were contaminated with the toxin and 48 samples (64%) exceeded the maximum limit of 50 ng/L given by *Codex Alimentarius*.

Sassahara *et al* (2005) analyzed 42 raw milk samples from Paraná state, Brazil for AFM1 contamination. Out of 42, 10 (24%) were positive for AFM1 contamination and 3 (7%) were above the maximum limit given by *Codex Alimentarius* of 0.5 ppb.

Bognanno *et al* (2006) conducted a survey of AFM1 in ovine milk from farms of Sicily, Italy using HPLC with a fluorescent detector. AFM_1 was present in 81% of samples, ranging from 2-108 ng/L. Three samples were above the legal limit of 50 ng/L. Milk from stabulated ewes (35.27ng/L) has higher concentration of toxin than in milk from grazing ewes (12.47ng/L).

Diaz *et al* (2006) surveyed milk from retail market in Bogotá, Colombia for the presence of AFM1. They analyzed 241 milk samples using HPLC during 2004-2005 and AFM1 was detected in 69.2% samples in 2004 and 79.4% of samples in

2005 with levels of 0.7 to 213.0 ng l^{-1} in 2004, and 10.6 to 288.9 ng l^{-1} in 2005. All samples were below the national permissible limit of 400 ng/L.

Unusan (2006) collected 129 samples of commercial UHT milk from markets of Central Anatolia, Turkey and were analyzed for AFM1 using ELISA. 75/129 samples (58.1%) came out positive for AFM1 with mean concentration of 108.17 ng/L. 68/129 samples (53%) were below the limit, the remaining 61/129 (47%) were well above the limit permitted by the EU.

Gallo *et al* (2008) performed two animal trials. In 1st trial, the plasma levels of AFB1, AFB2, AFG1, AFG2 and AFM1 were monitored in 7 lactating Holstein dairy cows following feeding a single oral bolus of a naturally contaminated corn meal (4.89 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2). Blood samples collected at 0 and 5, 10, 15, 20, 25, 30 minutes after treatment were analyzed by HPLC for presence of toxins. AFB1 in plasma peaked (33.6 ng/L) as soon as 20 minutes after treatment. The plasma AFM1 was already detectable at 5 minutes (10.4 ng/L) and peaked at 25 minutes (136.3 ng/L). In trial 2, same aflatoxin doses were provided to eight Holstein dairy cows through vaginal implant. Blood samples were collected at 0 and 15, 30, 60, 180, 360 minutes after treatment and individual milk samples of six milkings, one before and five after treatment, were also collected. In results of trial 2, only AFB1 and AFM1 were detectable in plasma, starting from the first sampling time (15 minutes), with values of 10.7 and 0.5 ng/L, respectively. The AFB1 peaked at 30 minutes (23.9 ng/L). Concentration of AFM1 in milk was highest in the first milking (203 ng/L) after treatment and decreased to starting values 36 hours after treatment. Trial 2 suggested that AFB1 absorption may also take place in mouth or esophageal mucous membranes before reaching rumen.

Hussain and Anwar (2008a) collected 169 milk samples from five different species of mammals (buffaloes; 55, cows; 40, goats; 30, sheep; 24, and camels; 20 from the area of Faisalabad district of the Punjab province of Pakistan to be analyzed for AFM1 contamination by HPLC using fluorescent detector. AFM_1 contamination percentage in buffalo milk, cow milk, goat milk, and sheep milk has been found to be 34.5%, 37.5%, 20%, and 16.7%, respectively with no contamination in the camel milk.

Hussain *et al* (2008b) surveyed different localities in the central areas of Punjab, Pakistan for the occurrence of AFM1 in milk of cows and buffaloes using HPLC. They collected 480 milk samples (360 of buffalo and 120 of cow. The percentage of AFM_1 contamination in buffalo milk and cow milk was 42.5% and 52.5%, respectively. The mean value of AFM_1 was 0.027 $\mu g\ L^{-1}$ in buffaloes' milk and was 0.044 $\mu g\ L^{-1}$ in cows' milk. In both types of milk, level of AFM_1 concentration was higher in milk samples obtained from urban and semi-urban areas and it was least in milk from rural areas.

Ghanem *et al* (2009) found the incidence of AFM1 in milk from Syrian market. 126 milk samples were collected (raw cow milk; 74, raw sheep milk; 23, raw goat milk; 11, pasteurized cow milk; 10 and powdered milk; 8) and analyzed using ELISA. 80% of samples tested positive for AFM1 (>20 to 765 ng/l). A total of 38% of samples exceeded the Syrian and 52% exceeded the European Union tolerance limits with 59% of cow, 24% of sheep and 14% goat milk samples exceeding the European Union limit. Pasteurized milk was found to be more contaminated than raw milk.

Shundo *et al* (2009) collected 125 milk samples (powdered milk, pasteurized milk and ultra-high treated milk) from São Paulo, Brazil and were analyzed for AFM1 using HPLC-FLD. AFM_1 was found in 119 (95.2%) samples (10 to 200 ng/kg) with mean concentration of 31 ng/kg. The average daily intake estimated for AFM_1 was 1 ng/kg b.w. per day for children and 0.188 ng/kg b.w. per day for adults.

Pei *et al* (2009) studied levels of AFM1 in milk and milk products from supermarkets across north-east China by indirect competitive ELISA using monoclonal antibodies for AFM1. Monoclonal antibodies were isolated and characterized after fusion of myeloma cells with spleen cells isolated from BALB/c mice that had been immunized with AFM1 carboxymethyl oxime conjugated with bovine serum albumin (BSA). 135 samples were collected (raw milk; 12, powdered milk; 15, liquid milk; 104, cheese; 4) and after analysis, AFM1 was detected in only in 97 samples; 55 (41%) samples contained AFM1 at levels that ranged from 0.32–0.50 ng/ml, 24 (18%) samples contained 0.16–0.32 ng/ml, and 18 (13%) samples contained 0–0.16 ng/ml.

Elzupir *et al* (2010) collected 44 bulk dairy cattle milk samples from Khartoum State of Sudan and analyzed for the presence of AFM1 using HPLC with fluorescent detector and found AFM1 contamination in 95% (42/44) of samples with average concentration being 2.07 μg/L.

Rahimi *et al* (2010) collected 311 raw milk samples (cow, water buffalo, camel, sheep, and goat) from Ahvaz, Iran to be analyzed for AFM1 contamination by using competitive ELISA. Results showed AFM1 contamination in 42.1% of the samples (43.3 ± 43.8 ng/kg). The incidence rates of AFM1 in raw cow, water buffalo, camel, sheep, and goat milks were, 78.7%, 38.7%, 12.5%, 37.3%, and 27.1%, respectively. All samples complied with the Iranian national standard and FDA limit (500 ng/l), but in 36% of raw cow milk, 8% water buffalo milk, 3.9% sheep milk, and 5.7% raw goat milk samples exceeded the maximum tolerance limit accepted by European union/*Codex Alimentarius* Commission (50 ng/l).

Bilandžić *et al* (2010) collected 61 milk samples for small milking farms during February, March and April (winter–spring season), and June, July and September (summer–autumn season) for analysis of AFM1 by ELISA. Winter-spring season recorded higher AFM1 level (35.8–58.6 ng/l) than summer–autumn season (11.6–14.9 ng/l) with only 1.6% of samples exceeding the European Union limit.

Iqbal *et al* (2011) analyzed 178 milk samples (buffalo-94 and cow-84) for AFM1 from Punjab and the North West Frontier Province (NWFP) of Pakistan (n=89 in each province) by HPLC-FLD. Overall, 46% of buffalo and 49% of cow milk samples were contaminated with AFM1 from Punjab as compared with 52% and 51% for milk samples from NWFP, respectively.

Asi *et al* (2012) studied the effect of seasonal variability, lactation times and differentiation in species on the level of AFM1 in Punjab, Pakistan. They collected 356 milk samples from five different species (buffalo, cow, goat, sheep and camel). On analysis, contamination was found higher in winter season than in summer for all species. 55, 56, 32, 58 and 27% of winter milk samples of buffalo, cow, goat, sheep and camel exceeded the EU maximum limit (0.05 μg/kg), compared with 38, 33, 21, 36 and 14% of summer milk samples, respectively. Milk from morning milking was found to be more contaminated than from evening milking. The percentage of

morning milk samples exceeding the EU maximum limit was 72, 67, 69, 71 and 44% for buffalo, cow, goat, sheep and camel in contrast for evening milks percent (39, 30, 18, 33 and 25%), respectively. Concentrate-fed animal's milk was more contaminated than grazing animals.

Iqbal *et al* (2013) studied the effect of seasonality on the AFM1 level in milk and milk products. 221 samples of milk and milk products during winter and 212 were collected during summer season and analyzed using HPLC. Results showed higher contamination in winter season than in summer. 45% samples were contaminated (40% of raw milk, 51% of UHT milk, 37% of yogurt, 60% of butter and 43% of ice cream) in winter and 27, 24, 25, 34 and 17% of samples were found above the recommended limit for AFM_1, respectively. However, from summer season 32% samples were found to be contaminated (36% of raw milk, 31% of UHT milk, 29% of yogurt, 40% of butter and 24% of ice cream) and 23, 23, 18, 20 and 5% of samples were found above the permissible limit for AFM_1, respectively.

Picinin *et al* (2013) studied aflatoxin M1 (AFM1) contamination from Minas Gerais State, Brazil, in different climate conditions. A total of 129 raw milk samples were collected from dairy farms in three distinct periods (dry period, transition period and rainy period), and analyzed by enzyme-linked immunoabsorbent assay (ELISA) as screening test. Samples with AFM1 at concentrations above 0.05 mg/L were confirmed by liquid chromatography with fluorescence detection (HPLC-FD). All the analyzed samples showed positive result for AFM1. 18 samples (13.95%) showed contamination with AFM1 above the permitted limit of 0.05 mg/L established by *Codex Alimentarius* and European Commission. Milk contamination with AFM1 was significantly affected by climatic conditions, and the highest values were seen in dry period.

Golge (2014) analyzed a total of 176 samples of raw milk obtained from dairy plants of Adana province of Turkey in 2012 for the presence of aflatoxin M1 (AFM1) by high performance liquid chromatography with fluorescence detection (HPLC-FD). The limits of detection (LOD) and quantification (LOQ) of the analytical method were 0.021 µg/kg and 0.025 µg/kg respectively. Aflatoxin M1 was detected in 53 out of 176 samples analysed (30.1%). The ranges for positive samples were 0.042-0.552, 0.033-1.01, 0.047-0.150 and 0.025-0.102 µg/kg in autumn, winter, spring and summer

seasons, respectively. Thirty samples of raw milk (17%) were above the legal limits of Turkey and EC regulations.

Kos *et al* (2014) analyzed the presence of AFM1 in milk of 150 cows, 10 goats, 5 donkeys, 10 breast milk samples of human and one infant formula sample using Enzyme Linked Immunosorbent Assay (ELISA) method. AFM1 was detected in 98.7% of analyzed cow milk samples in concentrations ranging from 0.01 to 1.2 mg/kg and 129 (86.0%) cow's milk samples contained AFM1 in concentration greater than maximum residue levels (MRL) of 0.05 mg/kg defined by European Union (EU) Regulation. Other types of milk revealed the presence of AFM1 in 80.0% goats, 60.0% donkeys and 60.0% of breast milk samples.

Kamkar *et al* (2014) collected 129 raw milk samples (60 of cow, 60 of buffalo) from Shush, Iran and analyzed them using ELISA for AFM1 contamination. AFM_1 was detected in 44/60 (69%) cow milk samples (3.6–419 ng/l) and in 46/60 (79%) buffalo milk samples with a mean of 116 ng/l (13–423 ng/l). 28%of cow and 52% of buffalo milk samples exceeded the 50 ng/l limit set by the European Union.

Kara *et al* (2014) surveyed milk from dairy farms of Afyonkarahisar, Turkey for the presence of AFM1. 126 buffalo and 124 cow milk samples were collected and analyzed using HPLC. AFM1 was only detected in buffalo milk (in 34 out of 126 samples).

Senerwa *et al* (2016) did a cross-sectional survey on the aflatoxin contamination of milk and dairy feeds from 5 countries of Kenya representing different agro-climatic conditions (Kwale, Isiolo, Tharaka-Nithi, Kisii and Bungoma). Dairy feed concentrates and cattle milk samples were collected two times a day from 285 dairy farmers in five countries and were analyzed for AFB1 and AFM1 using competitive ELISA. The dairy feed concentrates from farmers had AFB1 levels ranging from less than one part per billion (ppb) to 9661 ppb and the positive samples ranged from 47.8 to 90.3%. Aflatoxin M1 prevalence in milk was lowest in Kwale (13.6%) and highest in Tharaka-Nithi (65.1%). The proportion of milk samples with AFM1 above the Codex Alimentarius standard varied from 3.4% (Kwale) to 26.2% (Tharaka-Nithi); the highest was 6999 ppt.

Armorini *et al* (2016) surveyed milk for presence of AFM1 from Bologna town of Italy. A total of 58 milk samples were collected, 22 from organic and 36 from conventional production and were analyzed using HPLC. 35/58 samples were positive for AFM1, 11 organic and 24 conventional but no sample exceeded the MPL set by EU of 0.05 µg/kg. AFM1 contamination was of range of 0.009 - 0.026 ng/ml.

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 aflatoxin M_1 by using an ELISA from west part of Iran. Frequency of aflatoxin M_1 and its concentration ranges in the ELISA positive samples were determined by HPLC with fluorescence detection. Result of study showed aflatoxin M_1 contamination was 84.3%, 44.6% and 65.3% for cow, goat and sheep raw milk samples respectively.

Miocinovic *et al* (2017) did a survey for the occurrence of AFM1 in raw milk and dairy products Serbia in 2015 and also compared with the data from previous 2 years. The study included 1207 raw milk and 997 dairy products samples collected in different seasons during the year. The AFM1 level exceeded the European Union permissible limit in 29.3% of raw milk and 4.2% of milk product samples. The highest level of AFM1 in raw milk was found during the autumn season. While comparison of the data from 3 years, 2015 showed the lowest level of contamination.

Asghar *et al* (2018) conducted a survey of AFM1 in milk from markets of Karachi, Pakistan. 156 milk samples were collected and analyzed using ELISA. AFM_1 was detected in 143 (91.7%) samples, ranged from 20 to 3090 ng L^{-1} with a mean level of 346.2 ng L^{-1}. Seasonal variation showed AFM1 levels to be higher samples collected during summer than in winter.

Goncalves *et al* (2018) analyzed AFM1 in 112 milk samples (i.e. whole, skimmed, semi-skimmed - liquids and powders) collected from southern Brazil and city of Assomada by using HPLC-FLD method. Out of 62 milk samples from southern Brazil, 68% showed positive result for AFM1 (range: 40-3670 ng/l). Among the 50 samples from Assomada city, AFM1 was detected in 76% of the samples (range: 32-2896 ng/l).

Xiong *et al* (2018) did a cross-sectional survey of aflatoxins in dairy cow feed and milk from Central China. 174 feed, 111 UHT milk and 131 pasteurized milk

samples were collected and feed was analyzed for AFB1 by HPLC and milk for AFM1 using ELISA. 35.1% of feed samples tested positive for AFB1 and 6.3% samples were above the EU limit of 20 ppb. 73.6% of milk samples were positive for AFM1 (Average value being 100.0 ng/L). The AFM1 content was above the EU's legal limit of 50 ng/L in 1.8% of UHT milk samples and in 59.5% of pasteurized milk samples.

Tahira *et al* (2019) collected a total of 570 milk samples (340 raw, 105 ultra-heat treated (UHT), 65 pasteurized, 40 dried and 20 condensed milk samples) and they were analyzed for AFM1 by ELISA. They found 100% incidence of AFM1 in UHT (0.35±0.28ng/ml) and pasteurized (0.11+0.03ng/ml). 86.66% raw milk samples were contaminated with AFM1 (0.52±0.42ng/ml) and 66.66% of dried milk samples (0.03+0.02ng/ml) with zero condensed milk samples positive. Season variation showed highest prevalence (100%) in autumn season followed by winter (81.81%), summer (80%) and spring season (62.06%) respectively.

Pour *et al* (2019) conducted a meta-analysis on AFM1 contamination in milk and dairy products from Iran with inclusion of 70 articles. The prevalence of AFM_1 was found to be 64% (39.7 ng/l) in raw milk, 95% (62.3 ng/l) in pasteurized milk, 71% (60.1 ng/l) in sterilized milk, 59% (5.5 ng/l) in breast milk and 72% (82.3 ng/kg) in dairy products. Overall 9% of milks and 10% of dairy products had AFM_1 in concentrations exceeding the permitted level of Iranian standards (500 ng/l) and for *Codex Alimentarius* standards (50ng/L), 25% of milks and 18% of dairy products exceeded their standards.

Venâncio *et al* (2019) studied the effect of seasonality on AFM1 occurrence in milk. They collected 40 samples of raw milk during the summer (five samples from a subtropical climate and 14 from a temperate climate) and winter (six samples from a subtropical climate and 15 from a temperate climate) in 2017 and analyzed using ELISA. 87.5% samples were positive for AFM1 with average concentration being 16.66 ng/L and all samples were below the *Codex Alimentarius* standard of 50 ng/L and no significant differences in the levels of AFM1 between the properties located in the two climate zones, in both summer and winter.

Ahmad *et al* (2019) conducted a survey for the presence of AFM1 in raw and processed milk from Lahore and Multan province of Pakistan. On analysis they found that contamination of raw milk samples from Lahore was 90% and from Multan was

92%. The dietary exposure data of AFM_1 among six different groups was calculated, which indicated that the male children population was the most vulnerable group to AFM_1, up to 6.68 ng L^{-1} per day and the least affected one was the female group above 20 years of age with 1.13 ng L^{-1} per day.

Abyaneh *et al* (2019) collected 45 milk samples (raw, pasteurized and UTH milk) from markets of different cities of Tehran, Iran and they were analyzed for AFM1 by HPLC. On analysis, it was found that 36/45 samples (80%) were positive for AFM1 and no sample exceeded the Iran legal limit of 0.1 ppb. On the basis of the average milk intake, the mean daily exposure to AFM1 was estimated between 0.03 ng/ Kg BW per day and 0.06 ng/ Kg b.w. per day.

2.2 Aflatoxins and rumen

Polan *et al* (1974) did an experimental study on 4 lactating cows in latin square design and they were fed 10, 50, 250 and 1250 ppb of AFB1 containing concentrate for 14 days and no AFB1 for 56 days between treatment. It was found that AFM1 level increased to day 4 with little change through day 14 with no AFM1 in milk 2 days after stopping the treatment. By regression analyses it was found that a minimum of 46 ppb of AFB1 is required for AFM1 to be detectable in milk.

Mathur *et al* (1976) studied whether rumen organisms can metabolize aflatoxin B1 to other fluorescent compounds and the effect of aflatoxin B1 on the morphology and growth of rumen microorganisms by collecting rumen liquor samples and mixing them with 50ug/ml AFB1 in dimethyl sulfoxide and incubating for 24hrs at 37^0C. No fluorescent metabolites were found by chromatography which separate aflatoxin B1 from them. Rumen bacteria (mixed cultures and *Streptococcus bovis)* incubated with aflatoxin B1 grew in long chains instead of cells in control cultures with mainly doublets with some short chains.

Cook *et al* (1986) studied the clinical and pathological effects of acute aflatoxicosis and its effect on rumen motility by feeding aflatoxin produced from *Aspergillus parasiticus* NRRL 2999 to 4 cattle in feed. Aflatoxin altered the frequency and amplitude of rumen motility and the effect was dose dependent. AFM1 was detected in rumen content of all study subjects 2 hours after aflatoxin ingestion.

Frobish *et al* (1986) selected 32 lactating Holstein cows and fed them AFB1 contaminated cottonseed meal as 20% of their ration (equivalent to 0, 20, 48 and 104 µg/kg in complete feed). In observation they found out that AFM1 appeared in milk of all 32 cows within 12hrs of treatment and toxin concentrations came to a steady state condition of 0.35, 0.63 and 1.61 µg/L for treatments of 20, 48 and 104 µg AFB1/kg, respectively at 24 h and returned to the *Codex Alimentarius* limit of 0.5 µg/L or lower within 24 h after removal of the contaminated feed. The ratio of AFB1 in the feed conversion to AFM1 in the milk was found to be 66:1 and the mean percent of daily AFB1 intake that was transferred to AFM1 was 1.74.

Upadhya *et al* (2009) did a comparative study on the AFB1 degrading ability of cattle and goats. Both the groups were fed 60% timothy and 40% commercial diet with free access to water. Rumen fluid from donor animals was incubated with 80 ppb of AFB1 at 39^{o}C and degradation was measured after 3 hours. Rumen liquor from goats was found to have higher degradation capacity for AFB1 than from cattle.

Jiang *et al* (2012) studied the effect of AFB1 on ruminal fermentation at AFB1 dose rates of 0, 320, 640, 960 ng/ml on ruminal fermentation of substrates high in alfalfa hay (HA, alfalfa hay: maize meal = 4:1) and ryegrass hay (HR, ryegrass hay: maize meal = 4:1). AFB1 decreased the Ammonia N concentration, gas production rate in rumen and volatile fatty acids production. After 72 h incubation, 0.831 (HA) and 0.842 (HR) of included AFB1 (960 ng/ml) was degraded demonstrating the AFB1 degrading ability of rumen.

Nidhina *et al* (2017) isolated and identified the pathogenic microbes present in the rumen liquor. The screened fungal organism was identified as *Aspergillus flavus*. Fungal toxin was extracted using immuno-affinity column (IAC) and quantified by High Performance Liquid Chromatography (HPLC). The organism had ability to grow under aerobic and anaerobic conditions and also produce aflatoxin B1 and aflatoxin B2. The aflatoxin B1 production under aerobic condition was 0.902 ± 0.08 mg/ml culture broth and under anaerobic condition was 0.925 ± 0.2 mg/ml culture broth. Aflatoxin B2 was more compared to aflatoxin B1 and the quantity was 14.472 ± 1 under aerobic condition and 1.467 ± 0.3 under anaerobic condition and the rumen liquor from which the isolation was carried out also indicated the presence of aflatoxin B1 (3.964 ± 0.5 mg/ml) and B2 (1.170 ± 0.6 mg/ml). Hence, the study

suggests the ability of microbial flora present inside the rumen to produce aflatoxin. The study also revealed that lower ruminal pH favors aflatoxin production and vice versa.

Intanoo *et al* (2018) isolated bacterial and yeast strains from rumen liquor possessing AFB1 degrading capacity. In the experiment, three yeast and three bacteria isolates were selected that are active in both aerobic and anaerobic conditions. Up to 85% of AFB_1 was detoxified by yeast isolates and up to 60% AFB_1 reduction was evident by bacteria isolates. Two yeast isolates were identified as *Kluyveromyces marxianus* and one isolate as *Pichia kudriavzevii*. The three bacteria isolates were identified as *Enterococcus faecium*, *Corynebacterium phoceense* and *C. vitaeruminis*.

Khodabandehloo *et al* (2019) studied the effects of AFB1 on ruminal fermentation parameters *in-vitro*. In the experiment, two concentration ranges (0, 0.5, 1, and 1.5 μg/ml of rumen inoculum as low and 0, 5, and 10 μg/ml as high concentration ranges) were used to evaluate AFB1 effect. In the low concentration range, the half-time of asymptotic gas production (T1/2) increased and the fractional rate of gas production (μ) decreased with AFB1 dosage. However, in the high concentration range, the asymptotic gas production (A) and T1/2 decreased. pH and ammonia nitrogen concentrations increased and volatile fatty acids concentration decreased with increase in AFB1 concentration with propionate concentration increased at the cost of acetate.

2.3 Health implications of aflatoxins

Shank *et al* (1971) analyzed autopsy specimens for aflatoxins from 23 children in Thailand who died from acute encephalopathy and fatty degeneration of the viscera (EFDV) and from 15 children who died of unrelated causes as controls. Aflatoxin B_1 was detected in 22/23 EFDV cases. The highest levels detected were 93 μg B_1/kg in a liver specimen, 123 μg/kg in stool, 127 μg/kg in stomach and intestinal contents and 8 μg/ml in bile with trace amounts in brain, kidney and urine. None of the urine specimens from controls tested positive for any of the aflatoxins, but very little amounts of aflatoxin B_1 (1–4 μg/kg tissue) could be demonstrated in some autopsy specimens from 11/15 control subjects.

Krishnamachari *et al* (1975) did an outbreak investigation of hepatitis affecting humans and dogs in western India. Case presentation included jaundice,

rapidly developing ascites, portal hypertension, and a high mortality-rate. Liver necropsy from cases showed bile duct proliferation and giant cells. The cases were found to be associated with ingestion of aflatoxin contaminated maize.

Dvořáčková *et al* (1977) studied 27 cases of Reye syndrome (encephalitic syndrome with fatty degeneration of the viscera), all children between 3 days-8 years of age. They divided cases into 3 groups. In the first group, 20 children who died within 2-10 days after the first symptoms of the disease appeared. In their liver diffuse fatty degeneration was found. In the second group, 3 children who died within 1-2 months after the acute onset of the disease with their liver showing fibrosis with bile duct proliferation and steatosis were found. In the third group, 4 children died within 2-4 months after the first symptoms with liver showing cirrhosis. Laboratory analysis of all cases confirmed no bacterial or viral infection, but in the most of them, direct contacts with viral infections were proved. In the liver specimens of the children in all 3 groups the presence of aflatoxin B1 chromatographically and spectrophotometrically was found suggesting the possible etiological role of AFB1 in Reye syndrome.

Tandon *et al* (1977) did a retrospective epidemiological survey to study the epidemic of jaundice due to toxic hepatitis in three adjoining districts of Northwest India during the period November and December, 1974. It affected dogs and humans. The disease had a sub-acute onset starting with high fever, followed by rapidly progressive jaundice. Ascites appeared simultaneously and soon became quite massive. Hepatomegaly was recorded when ascites decreased. Liver function tests suggested cholestatic jaundice. The mortality rate was 10% in humans and 100% in dogs. Liver histology was characterized by edema and collagenization of the central veins, cholangiolar proliferation, moderate to severe ballooning of the hepatocytes, perisinusoidal fibrosis, cholestatis, and cirrhosis with reverse lobulation. Analysis of the data suggests that some food toxin may have been a factor in the outbreak of this unusual epidemic of toxic hepatitis.

Ryan *et al* (1979) studied 8 clinical cases of Reye's syndrome whose blood and autopsy samples were analyzed for presence of AFB1 by HPLC. AFB1 was isolated from 7 cases. AFB1 was detected in blood samples of 2 patients suffering from acute phase of the disease. In autopsy specimens of liver, AFB1 was detected in 6 cases.

Willis *et al* (1980) reported an attempted suicide by a 25 year old woman by ingesting 5.5 mg of AFB1 over 2 days and 6 months later 35 mg more over 2 weeks. After the 1st episode she had a transient, non-pruritic, macular rash, nausea and headache. After the 2nd occasion she reported nausea only. Fourteen years later she remained well.

Ngindu *et al* (1982) described an outbreak of acute hepatitis occurred in 1981 in Kenya affecting 20 people, out of which 12 died. Two families, from which 8 of 12 sick members, died were eating maize which contained 12000 ppb of aflatoxin B_1. Liver tissue at necropsy contained up to 89 ppb of this mycotoxin.

Stubblefield *et al* (1983) studied the fate of AFB1 in 2 Holstein cows after ingestion of 0.35 mg of purified AFB1/kg body weight/day for 3 consecutive days. Cow 1 slaughtered 1 day after end of 3-day trial and cow 2 post 3-day trial was fed aflatoxin-free rations for 7 additional days before slaughter. On analysis, AFB1 and AFM1 was found in tissue samples of brain, gallbladder and bile, heart, intestine, kidney, liver, lung, mammary gland, skeletal muscle, spleen, supra-mammary lymph nodes and tongue, and non-tissue samples of blood, feces, milk, rumen content, and urine with kidney, liver, and mammary gland had the highest concentrations of total aflatoxins (57.9, 13.2, and 25.1 ng/g, respectively), and aflatoxin M1 concentration 40 times more than the aflatoxin B1 level in kidney. In contrast, in cow 2, only kidney, liver and intestine from tissue samples and milk and urine from non-tissue samples showed the presence of aflatoxins.

Coulter *et al* (1984) did a survey for the occurrence of aflatoxins in human breast milk from Sudan. Breast milk of 99 mothers was analyzed. Only AFM1 and AFM2 were found in human breast milk. AFM1 occurred alone in 13 milks, (mean concentration = 19 pg/ml), AFM2 in 11 milks (mean concentration 12·2 pg/ml), and in 13 samples both AFM1 and AFM2 were detected. No aflatoxin was detected in subcutaneous abdominal wall fat removed during Caesarian section from 15 women, but was present in three out of 14 bloods taken during anesthesia**.**

Maxwell *et al* (1989) conducted a cross-sectional study in humans to find out whether AFM1 crosses human placental barrier and its presence in human breast milk. AFM1 and AFM2 were detected in 37% of 99 Sudanese, 28% of 191 Kenyan

and 34% of 510 Ghanaian breast milk samples. Analysis of cord blood samples from 282 babies in Ghana, 101 babies in Kenya and 78 babies in Nigeria showed aflatoxins in 31%, 37% and 12% respectively. Mother's blood obtained at delivery in 83 Kenyan cases and 77 Nigerian cases showed aflatoxins in both maternal and cord blood specimens in 14 Kenyan and 7 Nigerian instances. 3 undiagnosed still births reported during the study were also positive for aflatoxins in both core and maternal blood samples.

Denning *et al* (1990) studied the trans placental transfer of aflatoxins by measuring AFB_1, AFG_1 and AFQ_1 by ELISA in human cord sera obtained at birth and in serum obtained immediately after birth from the mother. For this study, 35 women were enrolled from Thailand. Of the 35 samples of cord sera, 17 (48%) contained aflatoxin in concentrations from 0.064 to 13.6. By comparison only two (6%) of 35 maternal sera contained aflatoxin (mean concentration = 0.62 nmol/ml), demonstrating possible trans-placental transmission of aflatoxins.

Aguilar *et al* (1993) studied the mutagenesis of codons 247-250 of p53 by rat liver microsome activated AFB1 in human HCC cells HepG2 by restriction fragment length polymorphism/polymerase chain reaction genotypic analysis. They found that AFB1 preferentially induces the transversion of G→T in the third position of codon 249 which is found in approximately half of hepatocellular carcinoma (HCC) from regions in the world with high contamination of food with AFB1.

Lye *et al* (1995) recorded an outbreak of aflatoxins in which 13 children in Perak, Malaysia died of acute hepatic encephalopathy in October, 1988. Epidemiologic investigations determined that the children had eaten a Chinese noodle, loh see fun, hours before they died. The attack rates among those who had eaten the noodles were significantly higher than those who had not ($P < 0.0001$). All the cases were geographically scattered in six towns in two districts along the route of distribution of the noodle supplied by one factory in Kampar town. Aflatoxins were confirmed in postmortem samples from patients.

Cusumano *et al* (1996) did *in-vitro* experiments to study the effect of aflatoxins on the human immune system. In the study, monocytes were exposed to different concentrations (0.1-1pg/mL) of AFB1 and parameters like phagocytosis, microbicidal activity, superoxide production and intrinsic antiviral activity were

recorded. Phagocytosis and microbicidal activity were significantly impaired by AFB_1 at doses as low as 0.1 pg/ml. But intrinsic antiviral activity or superoxide production were not affected by AFB1.

Yu *et al* (1997) studied the effect of aflatoxin exposure on the risk of developing hepatocellular carcinoma in chronic hepatitis B virus carriers. For this, urinary AF metabolites were measured for 43 HCC cases and 86 controls in a cohort of 7342 men in Taiwan. Thirty hepatocellular carcinoma cases and 63 controls were also tested for AFB_1-albumin adducts. Results showed the odds ratio (OR) between chronic hepatitis B virus carriers and AFB_1-N^7-guanine adducts in urine to be 6.0, compared with chronic hepatitis B virus carriers who were negative for AFB_1-albumin adducts and urinary AFB_1-N^7-guanine, no elevated risk was observed for those who were positive for either marker. But an extremely high risk of hepatocellular carcinoma among those having both markers was found (OR=10.0).

Abulu *et al* (1998) studied possible association between aflatoxin exposure and jaundice in neonates. For this study, cord blood samples were collected from 164 neonates (14 neonates without jaundice and 150 with jaundice). The cord blood samples on analysis for aflatoxins, found neonates with jaundice having high mean concentration of AFB1 (32.3 ng/ml and 35.6 ng/ml) with rate of detection higher in wet (81.8%) than dry season (50.0%).

Rossano *et al* (1999) studied the effect of AFB1 on the immune response by incubating monocytes with concentrations of AFB1 ranging from 0.01 to 1.0 pg/mL. After incubation for different time periods, monocytes were activated with bacterial lipopolysaccharide. Immune response was measured by cytokine levels via immunoassay and mRNA via cDNA amplification. Monocytes treated with AFB1 resulted in a decrease in IL-1, IL-6 and TNFα release already at a concentration of 0.05 pg/mL. The gene expression of the cytokines considered was immensely affected by treatment with AFB1 (AFB1 completely blocked the transcription of IL-1α, IL-6 and TNFα mRNAs, while it did not affect β-actin mRNA at the concentrations used. It therefore appears that AFB1 exerts its effect on cytokine release through selective inhibition of specific mRNA, without affecting general protein synthesis.

Chahota *et al* (2000) described an outbreak of aflatoxicosis and chlamydiosis in a poultry farm in Himachal Pradesh, India. Mortality rate was 65%, 25% and 32%

among broiler chicks (1-2 week of age), layer chicks (2-3 weeks of age) and broilers (8-15 weeks of age), respectively. Tissue samples of 20 dead birds were taken randomly and their feed samples were also analyzed for aflatoxins. *C. psittaci* was isolated from 30% (6/20) birds. The concerned feed was found to be contaminated with aflatoxins ranging from 100 ppb to 500 ppb. Case presentation included anorexia, depression, prostration, soiled vents, nervous signs such as torticollis, spasm of neck muscles and death with legs extended to the posterior. Post mortem examination revealed generalized paleness of the musculature, distended gall bladder, sub-cutaneous hemorrhages in thigh, breast and congestion of liver, lungs, intestine, kidneys and heart.

Chen *et al* (2001) performed a cross-sectional epidemiological study to find the association between hepatitis B virus (HBV) infection and AFB_1 exposure. 200 adolescents from 20 townships of Taiwan were tested for hepatitis B surface antigen (HBsAg) and AFB_1-albumin adducts. Results showed the AFB_1-albumin adduct level higher in HBsAg-positive compared with HBsAg-negative subjects.

Turner *et al* (2003) studied the effect of aflatoxins on the immune system in children between 6-9 years of age in Gambia, Africa. A total of 472 children were selected for the study and their serum aflatoxin-albumin (AF-alb) adducts, secretory IgA (sIgA) in saliva, cell-mediated immunity (CMI), determined using the CMI multitest where test antigens are applied to the skin and antibody responses to both rabies and pneumococcal polysaccharide vaccines was measured. AF-alb adducts were detected in 93% of the children. sIgA was markedly lower in children with detectable AF-alb compared with those with no detectable levels. Antibody response to one of four pneumococcal serotypes, but not rabies vaccine, was weakly associated with higher levels of AF-alb. There was no association between CMI responses to test antigens and AF-alb.

Gong *et al* (2004) conducted a longitudinal study (8 months) in Benin, West Africa to access post weaning exposure to aflatoxin in Impairment of child growth. In the study, 200 children, 16-37 months age) were included from four villages, two with high and two with low aflatoxin exposure (50 children per village). Serum aflatoxin–albumin (AF-alb) adducts, anthropometric parameters, plasma levels of vitamin A and zinc, information on food consumption, and various demographic data were

measured. AF-alb adducts increased markedly in three of the four villages, with the largest increases in the villages with higher exposures. Children who were fully weaned at recruitment had higher AF-alb than did those still partially breast-fed (major weaning food being maize-based porridge). There was a strong negative correlation between AF-alb and height increase over the 8-month follow-up after adjustment for age, sex, height at recruitment, socioeconomic status, village, and weaning status, thus emphasizing on the association between aflatoxin and stunting.

Lewis L *et al* (2005) conducted a cross-sectional survey to access the maize contamination with aflatoxins in markets of Kenya after an aflatoxicosis outbreak in Kenya in 2004 affecting 317 and killing 125. 350 maize products from 243 maize vendors in 65 markets were collected and analyzed. 55% of samples exceeded the Kenyan regulatory limit of 20 ppb (35% above 100 ppb and 7% above 1000 ppb), therefore indicating the potential high exposure of toxin in the affected population.

Mwanda *et al* (2005) described a case report of acute aflatoxicosis: A 17-year-old boy presented with vomition, features of infection and gastrointestinal tract symptoms. Physical examination revealed a very ill looking pale patient with abdominal distension, tenderness and rectal bleeding and easy bruisability. Laboratory investigations showed abnormal liver function tests, pancytopenia and elevated serum levels of aflatoxins. Management consisted of supportive care with antibiotics and antifungal therapy, transfusion of red blood cells and fresh frozen plasma. His recovery was uneventful. The literature on human aflatoxicosis shows that the presentation may be acute, sub-acute and chronic.

Strosnider *et al* (2006) did a case-control study on 40 case-patients of acute aflatoxicosis from an aflatoxicosis outbreak in Kenya in 2004. 80 healthy participants were randomly selected as controls. Questionnaires regarding maize storage and consumption were distributed among participants and maize (for total aflatoxins) and blood samples (for aflatoxin B1-lysine albumin adducts and hepatitis B surface antigen) were obtained from them. Maize from case-households had higher aflatoxin concentration than control households. Case patients had positive hepatitis B titers more often than controls. Case patients stored wet maize inside their homes rather than in granaries more often than did controls. Concluded that aflatoxin

concentrations in maize, serum aflatoxin B1-lysine adduct concentrations, and positive hepatitis B surface antigen titers were all associated with case status.

Liu and Wu (2010) did a risk assessment study to determine the global burden of Hepatocellular carcinoma attributable to aflatoxin exposure. In this study, they collected global data on food-borne aflatoxin levels, consumption of aflatoxin-contaminated foods, and hepatitis B virus (HBV) prevalence. They also calculated the cancer potency of aflatoxin for HBV-postive and HBV-negative individuals, as well as the uncertainty in all variables. Results showed that of the 550,000–600,000 new HCC cases worldwide each year, about 25,200–155,000 (4.6–28.2%) may be attributable to aflatoxin exposure (with most affected parts being sub-Saharan Africa, Southeast Asia, and China).

Arnot *et al* (2012) described an outbreak of aflatoxicosis in dogs in S. Africa. During 2011, approximately 100 dogs were presented at Onderstepoort Veterinary Academic Hospital with typical signs of icterus, haematemesis, melaena, haematochezia, gastro-enterorrhagia and hepatosis. Histopathological examination revealed fatty hepatosis and bile duct proliferation with presence of a blue-grey granular material within the bile ducts. 124 dog food samples from cases were analyzed for aflatoxins. Aflatoxin contamination in suspected food ranged from < 5 mg/kg to 4946 mg/kg.

Kaleibar *et al* (2013) investigated an outbreak of aflatoxicosis in calves. A farm in Iran fed alfalfa hay and sunflower cakes to two hundred 4-10 month-old male Holstein calves, out of which 35 calves died 4 months after the typical case presentation which included general unthriftiness and diarrhea with firm, pale livers and hydrothorax in Necropsy findings. Laboratory findings revealed lymphopenia, monocytosis, high levels of serum sorbitol dehydrogenase (SDH), alkaline phosphatase (ALP) and SGOT in the serum and congestion, necrosis, fatty change and megalocytosis in the necropsied livers. The concerned feed was found to be contaminated with AFB1 (1,130 μg of AFB_1/kg of feed). A co-infection with Bovine viral diarrhea was also diagnosed.

Umar *et al* (2015) investigated an outbreak of aflatoxicosis in a bovine herd in Pakistan. A total of 45 animals were affected out of which 15 died. The case presentation included anorexia, depression, photosensitization and diarrhea.

Postmortem examination revealed hemorrhages on viscera, blood exudation from natural orifices and prolapse. The concerned feed was corn rich and on culturing showed the presence of *Aspergillus flavus* and *Aspergillus parasitticus.* AFB1 quantification revealed toxin level in feed to be as high as 33,500 ppb.

Kamala *et al* (2018) did an outbreak investigation of an unknown disease in central part of Tanzania. A cluster of 68 cases was identified between May to November, 2016. Out of total 68 cases, 20 died with case fatality rate being 30%. Case presentation was jaundice (n=60), abdominal pain (n=59), vomiting (n=56), diarrhea (n=34) and ascites (n=32). From the epidemiological survey, home grown maize was found to be common in all cluster families which was found to be contaminated with aflatoxins (10-51,100 μg/kg). Analysis of serum aflatoxin-adduct levels was also found to be higher in cases than in control group.

2.4 Risk factors associated with AFM1 in milk

Malissiova *et al* (2013) studied the presence of AFM1 in ewe's and goat's milk from Thessaly, Greece and the potential associated risk factors under both organic and conventional milk production schemes. 243 milk samples were screened using ELISA and confirmed by HPLC. For risk factors analysis, a detailed questionnaire was filled out at the time of sampling from dairy farmers. Only 1.7% of milk samples were above the EU maximum tolerable limit of 0.05 ppb with organic farms having higher AFM1 contamination than conventional farms. Risk factors analysis revealed use of warehouse for feed storage (OR 2.69, 95%CI 1.25-5.79), winter season (OR 2.58, 95%CI 1.07-6.24) and feeding field pea (OR 4.17, 95%CI 1.41-12.32) as statistically significant associated risk factors.

Michlig *et al* (2016) studied the incidence of AFM1 in milk and risk factors associated with the presence of AFM1 in milk in Argentina. 160 bulk milk tank samples from dairy farms were analyzed for the presence of AFM1 using UHPLC-MS/MS method and survey for the potential risk factors for AFM1 was done through a questionnaire applied to dairy farmers. Incidence of AFM1 was 38.8% with all the samples below the maximum permissible limit prescribed by the *Codex Alimentarius* (0.5 ppb). Risk factor analysis showed commercial feed consumption (OR= 4.630, p-value = 0.001), soybean consumption (OR= 3.542, p-value = 0.019) and cotton seed

consumption (OR= 3.542, p-value = 0.019) to be associated with high levels of aflatoxin in milk.

Patyal *et al* (2020b) tested 189 raw pooled milk samples from Punjab for presence of AFM1 by competitive ELISA and HPLC-FLD and investigated for associated risk factors for AFM1 in milk. The percentage of samples above the MPL established by the European Commission (EC) and Food safety and standard authority of India (FSSAI) were found to be 50.8% and 36.5% with mean concentration of 0.917µg/L. The risk factor analysis revealed feeding of readymade concentrate feed and leftover household cereals, longer feed storage duration and feed storage quality to be significantly associated with presence of AFM1 in farm milk.

CHAPTER – III

MATERIALS AND METHODS

The present study was conducted with the mandate to determine the occurrence of AFM1 in milk of cattle and buffaloes from commercial and household dairy establishments across 22 districts of Punjab and to determine the associated risk factors related with AFM1 excretion in milk of bovines. The study also included the analysis of rumen liquor of targeted animals to find any association between AFM1 excretion in milk and rumen parameters. The details of the materials used and methodologies adopted to carry out the present study are described below and have been systematically represented in Fig. 1:

3.1 Study area

The present study was carried out in all the 22 districts of Punjab. The details of district-wise sample collections have been discussed below:

3.2 Collection of samples

3.2.1 Milk samples

3.2.1.1 Sample size estimation

As per 19th Indian Livestock census-2012, the total 'in-milk' bovine population of Punjab was 28,78,881 ('in-milk' cattle: 9,18,868 and 'in-milk' buffaloes: 19,60,013). The sample size was estimated by using Epi Info 7 software of the CDC by considering total study population as 28,78,881 bovines and the prevalence of aflatoxin M1 as 50%. On assuming 95% confidence interval and 5% confidence limit, the sample size output was found to be 384 'in-milk' bovines.

3.2.1.2 Sample collection

A total of 402 bovine milk samples were collected from cattle (n=266) and buffaloes (n=136) from 27 commercial dairy farms (farms having ≥10 animals) and 46 dairy household (having <10 animals) across all the districts of Punjab, India by using proportionate sampling method during the period of July 2019 to February 2020.

From each animal, 50 ml of the milk sample was collected aseptically in sterile centrifuge tubes and were transported from to the laboratory in ice-box on the day of collection. The samples were stored at -20^{o}C until further analysis. The district-wise numbers of samples are provided in Table 1.

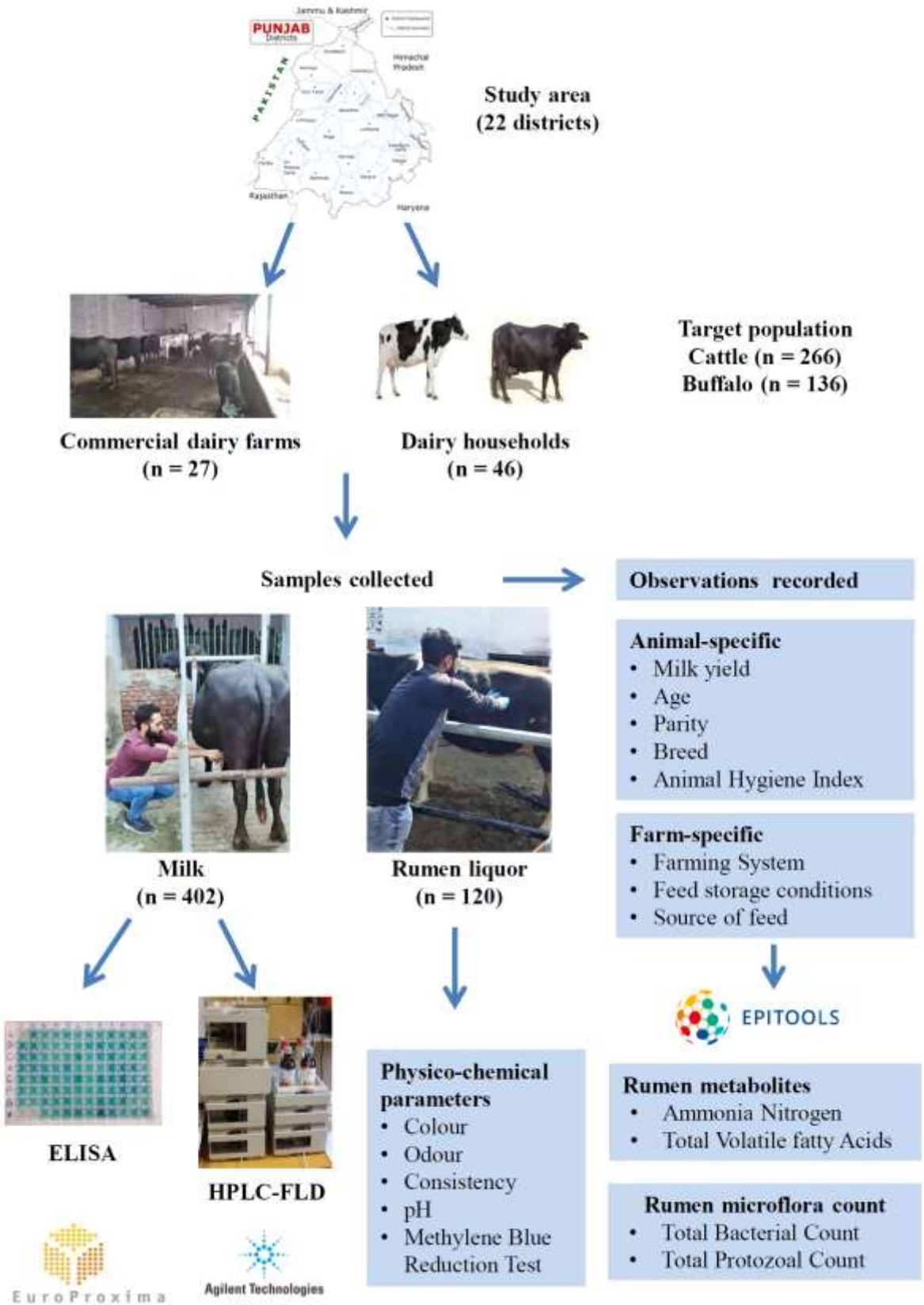


Fig. 1: Schematic representation of the methodology used

Table 1: District-wise distribution of collected samples of milk and rumen liquor

S. No.	Districts	Total Bovine Population	Total 'in-milk' Bovine Population	No. of milk samples collected	No. of rumen liquor samples collected
1.	Amritsar	530970	201768	33	8
2.	Barnala	233134	88590	13	4
3.	Bathinda	381472	144959	21	5
4.	Faridkot	193373	73481	11	4
5.	Fathegarh Sahib	209600	79648	12	4
6.	Ferozepur + Fazilka	751494	285567	40	10
7.	Gurdaspur + Pathankot	543621	206576	27	8
8.	Hoshiarpur	408794	155341	20	6
9.	Jalandhar	387556	147271	20	6
10.	Kapurthala	195477	74281	10	4
11.	Ludhiana	671419	255139	34	10
12.	Mansa	328631	124879	20	5
13.	Moga	333577	126759	20	6
14.	Mohali	186840	70999	11	4
15.	Muktsar	274658	104370	17	5
16.	Navanshahr	169840	64539	9	3
17.	Patiala	450801	171304	30	7
18.	Rupnagar	209184	79489	11	4
19.	Sangrur	657876	249992	22	9
20.	Tarn Taran	469131	178269	21	8
	Total	**7587448**	**2883230**	**402**	**120**

3.2.2 Rumen liquor samples

3.2.2.1 Sample size estimation

The rumen liquor samples were collected from 25% of the total study subjects (at least 100 bovines) during the time of milk sample collection of respective animals. The ethical permission for the collection of rumen liquor samples has been taken from the Institutional Animal ethics Committee (IAEC) (IAEC/2019/188-221).

3.2.2.2 Collection of rumen liquor

A total of 120 rumen liquor samples (25% of number of collected milk samples) were collected across all the districts of Punjab. The district-wise details of the numbers of rumen liquor samples have been provided in Table 1. From individual animal, 15 ml of the rumen liquor was collected in sterile centrifuge tube by needle puncture of rumen at the left paralumbar fossa using 20 ml syringe with 16-gauge needle under aseptic conditions in the supervision of the regional veterinary doctor. The collected samples were strained through double layer of muslin cloth as described by Lengemann and Allen (1955) and labeled properly as 'strained rumen liquor' (SLR).

The preservation of rumen liquor was done in saturated solution of mercuric chloride and in 8% formaldehyde solution separately. For analysis of rumen metabolites, 2-3 drops of mercuric chloride were added per 4-5 ml of rumen liquor sample and for rumen microflora count, 1 ml of formaldehyde was added per 1 ml of rumen liquor sample. The samples were immediately stored in ice box after preservation and in -20^{o}C upon reaching laboratory for further analysis.

3.3 Animal and farm level observations

The relevant animal and farm related risk factors for AFM1 excretion in milk samples were reviewed and adopted from the previous studies (Malissiova *et al* 2013, Michlig *et al* 2016 and Patyal *et al* 2020b). The following animal and farm specific parameters were recorded during the dairy farm visits for analysis of the risk factors (Table 2).

Table 2: Proforma for animal and farm related factors

Date of visit:						
District:						
Village:						
	Farm Id: **Sample ID:**					
Animal-specific factors	**Milk Yield** (kgs/day)					
	Age (in years)					
	Parity					
	Breed					
	Animal Hygiene Index* (details provided below)					
Farm-specific factors	**Type of farm** (Commercial/Household)					
	Source of feed (Marketed / self-cultivated feed with or without binder)					
	Farming System (Intensive/Semi-intensive/Extensive)					
	Feed Storage Conditions (Open/Covered)					

***Animal Hygiene Index:** The animal hygiene indexing was carried out as described by Cook (2002). The method charts the soiling and manure contamination in three main areas: lower leg (rear only), udder and the upper leg (rear only), and flank. All study animals were scored on a scale of 1 to 4 during sample collection, lower scores being indicative of cleaner body regions and vice-versa. The details of the hygiene scoring indexes have been provided in Table 3.

Table 3: Details of hygiene score index

Animal Hygiene Index	Body regions		
	Lower leg	**Udder**	**Upper leg**
1	Little or no manure above the coronary band	No manure present	No manure present
2	Minor splashing of manure above the coronary band	Minor splashing of manure near the teats	Minor splashing of manure
3	Distinct plaques of manure above the coronary band	Distinct plaques of manure on the lower half of udder	Distinct plaques of manure with hair showing through
4	Solid plaque of manure extending high up the leg	Confluent plaques of manure encrusted on and around the teats	Confluent plaques of manure

3.4 Experimental set-up and materials used

The description of experimental set-up and different materials used for conducting the present study are as follows:

3.4.1 Experimental set-up

The experimental and analysis work was carried out at:

1. School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab.
2. Department of Veterinary Physiology and Biochemistry, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab.

3.4.2 Equipment used:

The equipment used for the present study were vacuum concentrator (Vacufuge® plus, Eppendorf™ AG, Germany), vortex mixer (Spinix®, Tarsons India Ltd.), vaccum manifold (Water Corporation, USA), electronic weighing balance (ME104E, Mettler Toledo India Pvt Ltd.), -20°C refrigerator (Samsung India Ltd.), refrigerated centrifuge (Eppendorf™, USA), deep freezer (Vestfrost™ solutions, Denmark), Merck millipore water purification system (Direct-Q3™, Merck KGaA, Darmstadt, Germany), water bath, sonicator, micropipettes (Finnipipette F1, Thermo Scientifics, Finland) and filtration assembly. The other equipment pertaining to specific usage has been described in the concerned sections.

3.4.3 Glassware and plasticwares

The glassware from Borosil Glass Works Ltd. (India) and plasticwares comprising polypropylene tubes (Corning, USA), micropipette tips (Imperial Bio-Medic, India), microfuge tubes, PTFE syringe filters (Randisc™, Avantor perfromance materials India Ltd.) and disposable syringes (Dispovan) were used in the present study. Chromatographic auto sampler glass vials (amber coloured) were purchased from Agilent Technologies Ltd. (USA). The glasswares were thoroughly cleaned with hot water and Extran® (Merck, Darmstadt, Germany), rinsed with deionized milli-Q water followed by drying in hot air oven at 80°C before use. To remove any possible aflatoxin adsorption sites, glasswares were soaked in 2 M sulphuric acid for 1 day and then rinsed well with distilled water to remove all traces of acid.

3.4.4 Reagents and solvents

All reagents used were of analytical research grade procured from Sigma-Aldrich (VETEC™), Merck Life Sciences (EMPARTA®), Avantor performance materials (RANKEM™) and Fischer Scientifics (QUALIGENS®), India. All the solvents used were of chromatography grade and purchased from Merck and RFCL Ltd. (India). Solvents were redistilled before use and their purity and stability was ensured by running reagent blanks along with actual analysis.

3.5 Detection and quantification of AFM1 in milk samples

3.5.1 AFM1 analysis of milk samples by ELISA

A microtiter plate based competitive enzyme-linked immunosorbent assay was used for AFM1 quantification in milk samples. Aflatoxin M1 ELISA Test Kits (Catalog No. 5121AFM) procured from EuroProxima B.V., The Netherlands were used having 5pg/ml limit of detection (LOD) for milk samples and cross-reactivity with AFM1 and AFM2 being 100% and <20%, respectively. The kit contained 96-wells microtiter plate precoated with anti-AFM1 antibodies, 20x concentrated rinsing buffer (30 ml), 100x concentrated conjugate solution (150 µl) with dilution buffer (15ml), 12ml substrate and 15 ml stop solution and eight standard solutions with AFM1 concentrations of 0, 6.25, 12.5, 25, 50, 100, 200 and 1000 pg/ml. The optical density was measured by using Thermo Scientific™ Multiskan™ GO microplate spectrophotometer. Thereby, the testing of the milk samples was carried out as per the manufacturer's instructions as described below:

3.5.1.1 Preparation of milk samples

The milk samples were centrifuged at 2000×g for 10 min at 4°C after being thawed to room temperature in water bath and fat layer was removed from the top by using spatula.

3.5.1.2 ELISA method of AFM1 detection in milk

The dummy plate to be used was first thoroughly rinsed with rinsing buffer and dried on paper towel before use. The 200 µl of defatted milk samples were added in the dummy plate in duplicate in different wells and from there 100 µl of them were transferred to the antibody coated micro-titer plate using multi-channel pipette. The 100 µl of standard solutions (0, 6.25, 12.5, 25, 50, 100, 200, 600, 800 and 1000 pg/ml) were then added in duplicate in respective wells of the micro-titer plate. The 600 and 800 pg/ml standard solution was prepared by dilution of 1000 pg/ml solution. After sealing and manually shaking the microtiter plate for few seconds, it was incubated for one hour in dark at room temperature (25°C). During this, the free AFM1 in the milk samples would bind to the anti-AFM1 antibodies coated on the wells. After incubation, the solution in wells of the microtiter plate was discarded and the wells were washed 3 times with rinsing buffer followed by drying on paper towel and then 100 µl of conjugate solution (Horseradish peroxidase labeled AFM1) was pipetted into each well except wells H1 and H2 (Blank). After sealing, manual shaking and incubating for another 30 minutes in dark at room temperature, the wells were again emptied and washed 3 times with rinsing buffer. This would remove the unbound conjugate from the wells. The final incubation of 30 minutes in dark at room temperature was done after adding 100 µl TMB substrate solution into all wells. During the incubation, TMB substrate solution would bind to the HRP-labeled AFM1 that is bound to the anti-AFM1 antibodies on the microtiter plate to produce visible color change. A 100 µl of stop solution was then added to each well of microtiter plate to stop the enzymatic reaction. After that the absorbance/optical density (O.D.) values were read immediately at 450 nm in the microplate spectrophotometer.

3.5.1.3 AFM1 quantification

The concentration of AFM1 in milk samples was calculated by using regression equation obtained from calibration curve built by plotting values of %

maximal absorbance of standards on Y-axis versus the analyte equivalent concentration (pg/ml) on logarithmic X-axis (Fig. 2).

$$\% \text{ maximal absorbance} = \frac{\text{O. D. of standard or sample}}{\text{O. D. of zero standard}} \times 100$$

The O.D. of the standards and samples were averaged and corrected by subtracting the mean O.D. of blank wells before computation. The O.D. is inversely proportional to the AFM1 concentration in the sample.

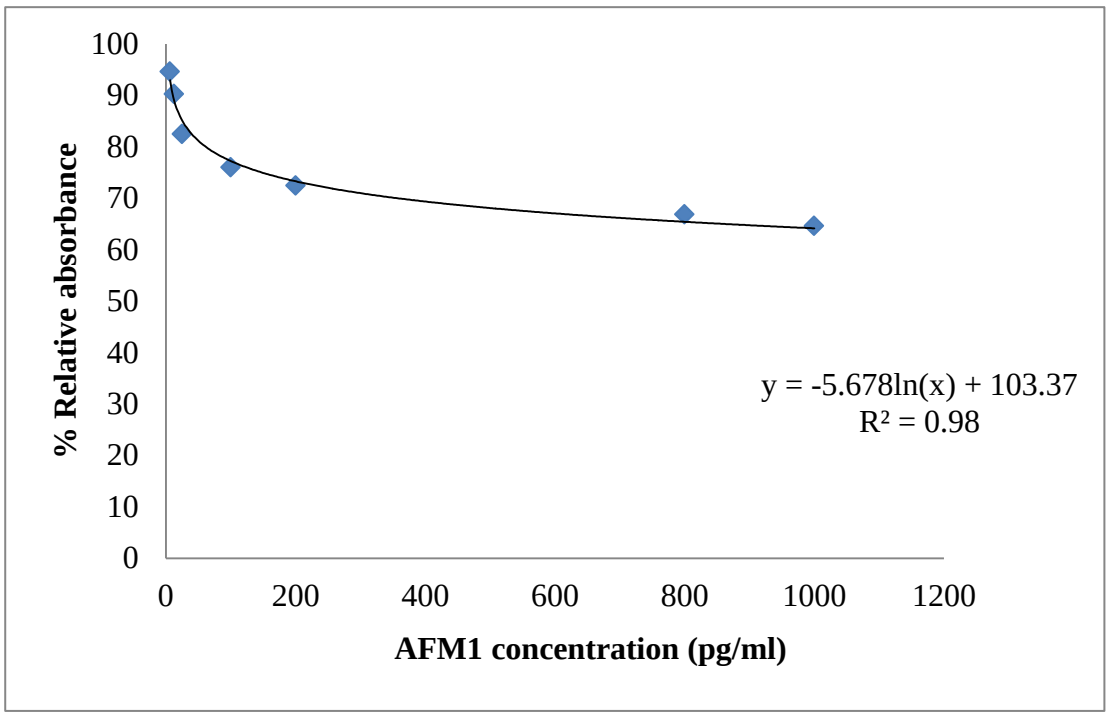


Fig. 2: Standard calibration curve for AFM1 on logarithmic scale

3.5.2 AFM1 analysis of milk samples by HPLC

The 10% of representative positive milk, which were in the non-compliant category with EC-MPL (i.e., >0.05 ppb) by ELISA were further confirmed for AFM1 via High Pressure Liquid Chromatography with fluorescent detector (HPLC-FLD) method. In addition, 5% of samples which were found to be ELISA negative (<0.005 ppb) for AFM1 were selected randomly and confirmed via HPLC-FLD.

AFM1 standard (CRM46319) having 98% purity, previously procured from Sigma Aldrich (Supelco), Co, USA was used. The AFM1 standard solution made in 10% acetonitrile solution (ACN) and mobile phase composed of water and ACN (67:33, v/v) in isocratic conditions were used in HPLC analysis. Before HPLC

analysis, both water and ACN were filtered through 0.45 µm membrane filters under vacuum and degassed by ultrasonication for 20 min.

3.5.2.1 Preparation of sample

Milk samples (50 ml) for HPLC analysis were first heated in water bath at 35°C-37°C for 6-7 minutes and were then centrifuged at 2000×g for 15 minutes. The fat layer separated was removed by using spatula.

3.5.2.2 Extraction and Clean-up of samples by Immunoaffinity Column (IAC)

After preparation of the milk samples, extraction and clean-up was done using IACs (AflaStar™ M1 R – Immunoaffinity columns) procured from Romer Labs Inc., 1301 Stylemaster Drive Union, USA. Immunoaffinity columns were placed on vacuum and 50 ml milk sample was passed through the column at the flow rate of 1-3 ml/min until air comes out through the column. Then the column was rinsed with 20 ml of distilled water to remove impurities and dried by applying vacuum. After this, 100% HPLC grade methanol was used to elute AFM1 from the IAC. Approximately 1.5-3 ml methanol was allowed to pass through the column at the flow rate of 1 drop per second in a clean acid-treated glass beaker. The eluate was evaporated to near dryness in vacuum concentrator and was reconstituted with 1 ml of mobile phase. Finally, the reconstituted eluate was filtered through 0.22 µm syringe filter and was stored in amber coloured HPLC glass vial for further analysis.

3.5.2.3 Quantification of AFM1 in milk samples by HPLC analysis

After the extraction and clean-up of milk samples, HPLC analysis was done using Agilent 1260 infinity HPLC system operated via OpenLAB EZChrom software. The sample was passed through Waters® Spherisorb® C-18 column (250 × 4.6 mm i.d., 5 µm particle size) at 40°C in mobile phase constituting water and ACN (67:33, v/v) under isocratic conditions at flow rate of 1.2 ml/min. The run time was fixed at 10 minutes and FLD detector was used to detect the presence of AFM1 at the excitation and emission wavelength of 365 nm and 435 nm respectively. The identification and quantification of AFM1 was done by comparing retention time and peak area of the sample chromatogram with those of calibration standards run under the same operating conditions. The method used for analysis is summarized in Table 4.

Table 4: HPLC-FLD method used for detection of AFM1 in milk samples

Parameters	Specifications
Sampler	
Injection Volume	20 µl
Quaternary pump	
Flow rate	1.2 ml/min
Solvents	Water: ACN (67:33)
Stop time	10 min
Column compound	
Temperature	40^{o}C
FLD Detector	
Excitation wavelength	365 nm
Emission wavelength	435 nm

3.6 Analysis of rumen liquor samples

The present study included the analysis of 120 rumen liquor samples for (1) Physico-chemical parameters [colour, odour and consistency, pH, Methylene Blue Reduction Time (MBRT), Sedimentation Activity Time (SAT)]; (2) Rumen metabolites [Total Volatile Fatty Acids (TVFA's) and ammonia nitrogen]; and (3) Rumen microflora count [Total Bacterial Count (TBC) and Total Protozoal Count (TPC)]. The materials used and methodologies adopted for the analysis are described below:

3.6.1 Physico-chemical parameters

3.6.1.1 Colour, odour and consistency

Colour, odour and consistency of rumen liquor samples were immediately recorded after its collection as described by Garry (2002).

3.6.1.2 pH of rumen liquor

pH of rumen liquor was determined immediately after collection by digital pH meter (Systronics digital pH meter 802).

3.6.1.3 Methylene blue reduction test (MBRT)

Methylene blue reduction test was done according to the method described by Dirksen (1979). This test is a measure of microbial metabolism/activity in rumen

liquor. 0.25 ml of 0.03% methylene blue solution was added in 5 ml of freshly collected rumen liquor in a test tube and incubated in water bath at 39^{o}C. Time taken for the reduction of methylene blue by the microbial constituents of rumen liquor i.e. for discoloration of sample, was noted using a plain rumen fluid as a basis for comparison.

3.6.1.4 Sedimentation activity test (SAT)

Sedimentation activity test was performed according to the method adopted by Dirksen (1979). Freshly collected rumen liquor was observed in glass test tubes kept in water bath at 39^{o}C. Majority of the fine constituents begin to settle, and the larger and fibrous food particles are carried upward by gas bubbles from fermentation forming a broad, foamy layer on top. The time required for sedimentation and floatation was referred to as sedimentation activity time.

3.6.2 Rumen metabolites

3.6.2.1 Total volatile fatty acids (TVFA)

Total volatile fatty acids concentration in rumen fluid was estimated by the method of Barnett and Reid (1957). A total of 1 ml of SRL was transferred into the Markham's micro-Kjeldahl distillation apparatus and 1 ml of scaribrick buffer (10% potassium oxalate and 5% oxalic acid in equal volumes) was added to it. The cup was made air tight with stop cork and by adding some water in it. Steam distillation was carried and approximately 75 ml distillate was collected. To the distillate, few drops of phenolphthalein indicator were added and titrated against standard 0.01 N NaOH solution.

$$TVFA's\ (mEq/L) = Amount\ of\ standard\ base\ used\ (ml) \times 10$$

3.6.2.2 Ammonia nitrogen (NH_3-N)

Conway micro diffusion technique of Conway (1957) was used to estimate NH3-N in SRL. In the inner chamber of Conway cell, 1 ml of 2% boric acid solution containing mixed indicator was taken and 1 ml of clear SRL was pipetted into the outer compartment. Further, 1 ml of 50% potassium carbonate solution was then slowly added into the outer compartment opposite to SRL. After covering the micro-diffusion cell, it was gently rotated clockwise and anticlockwise at a horizontal plain

to mix contents of outer chamber followed by incubation for 1 hour at 37°C. Thereafter, contents of the inner chamber were titrated against standard 0.01N H_2SO_4 solution. Simultaneously, a blank of 1 ml distilled water was also titrated against standard acid.

$$Ammonia\ nitrogen\ \left(\frac{mg}{dl}\right) = \frac{Amount\ of\ standard\ acid\ used \times 0.14}{Volume\ of\ sample\ taken\ (ml)} \times 100$$

3.6.3 Rumen microflora count

3.6.3.1 Total bacterial count

Total bacterial count was determined as per the method of Gall *et al* (1949) using nigrosin slide technique. The preserved sample of SRL was thawed and shaken vigorously in order to separate microbes from feed particles and to break microbial clumps. The thawed rumen liquor sample was centrifuged at 3000 rpm for 5 min and the supernatant was serially diluted in 1:10000 ratio with distilled water. The diluted bacterial suspension was mixed well and 0.01 ml of diluted suspension was taken onto a clean grease free glass slide. A loopful of saturated nigrosin stain was added to 0.01 ml of diluted suspension. The sample was mixed and uniformly spread over 2×2 cm area of glass slide with the platinum loop. The procedure was performed in the biosafety level-2 cabinet. The smear was dried immediately over a preheated (about 60°C) hot plate. Bacterial counting was done in total 30 microscopic fields from 2x2 cm area of stained smear under 100x objective of microscope. The total bacterial count per ml of rumen liquor was calculated by the formula given below:

$$TBC\ per\ ml\ of\ sample = Number\ of\ bacteria\ per\ field \times Number\ of\ microscopic\ field\ (1000) \times Dilution\ factor\ (10^6)$$

3.6.3.2 Total Protozoal count (TPC)

Rumen protozoal count was done as per the method described by Naga and El-Shazly (1969). A total of 5 ml of sample was taken through wide bore (3.5 mm) pipette into a test tube. Then 15 ml of normal saline solution (0.85%) was transferred and thereafter 5 ml Lugol's iodine was added. The solution was mixed gently and 0.1 ml of sample was transferred swiftly to a dry clean slide and spread under a glass cover of known area (24×60 mm). A total of 30 fields were counted per slide both for

ease and accuracy and total protozoal count per ml of rumen liquor was calculated by the formula given below:

$$TPC\ per\ ml\ of\ sample = Number\ of\ protozoa\ per\ field \times Number\ of\ microscopic\ field\ (1000) \times Dilution\ factor\ (100)$$

3.7 Statistical analysis

All the data entry and the computation of mean, standard deviation, maximum and minimum concentrations were done using Microsoft® Excel 2010. The computation of measure of association as Odds Ratio for univariable analysis of risk factors for occurrence of AFM1 in milk was performed using Epitools software (Sergeant, ESG, 2009). Various risk factors of AFM1 excretion in milk were used as predictors which were determined using crude univariate measures of effect as Odds Ratio. The covariates associated with risk of AFM1 excretion in milk at 20% were imputed in a logistic regression model to determine independent predictors of AFM1 excretion in milk. The model was constructed using backward stepwise approach using Likelihood Ratio Test (LRT) for covariate selection. The analyses were conducted using SPSS version 24.0 (SPSS Inc., IBM, NY, USA). Further, the correlation between various indicators of rumen fermentation and excretion of AFM1 in milk were computed as Pearson Correlation Coefficient (r) by using Microsoft® Excel 2010.

CHAPTER – IV

RESULTS AND DISCUSSION

In the present study, milk samples of cattle and buffalo from all the districts of Punjab were analyzed for AFM1 by using competitive Enzyme Linked Immuno-sorbent Assay (ELISA) and representative samples were confirmed by High Pressure Liquid Chromatography with fluorescent detector (HPLC-FLD). The animal- and farm-specific risk factors associated with the excretion of AFM1 in milk were also identified. In addition, analysis of rumen liquor samples of targeted animals was performed to find any possible association between AFM1 excretion in milk and indicators of rumen fermentation.

4.1 Detection and quantification of AFM1 in bovine milk samples

A total of 402 milk samples were analyzed for the presence of AFM1 by competitive Enzyme Linked Immuno-sorbent Assay (ELISA). The milk samples with AFM1 concentration above the detection limit of ELISA kit (i.e., 0.005 ppb) were considered positive. Of all the samples whose concentrations were above 0.05 ppb and 0.5 ppb were considered under non-compliant category with respect to European Commission (EC) maximum permissible limit (MPL) and Food safety and Standards Authority of India (FSSAI) MPL for AFM1 respectively. The representative ELISA positive and negative samples were further confirmed via High Pressure Liquid Chromatography with fluorescent detector (HPLC-FLD) method.

4.1.1 Prevalence of AFM1 in bovine milk from Punjab

Covering all the districts of Punjab (n=22), 402 milk samples were collected and analyzed for the presence of AFM1. Out of the total, 79.1% (318/402) were found positive (i.e., above the detection limit of ELISA kit of 0.005 ppb) with 56.22% (226/402) of samples exceeding the maximum permissible limit (MPL) set by the European Commission (EC) (i.e., 0.05 ppb), whereas, 13.43% (54/402) of samples were found to be above the MPL set by Food Safety and Standards Authority of India (FSSAI) (i.e., 0.05 ppb). The AFM1 concentration in milk ranged from 0.005 to 6.832 ppb with the mean concentration ± standard deviation (SD) of 0.269±0.07 ppb.

In recent past, various studies from different parts of India reported high prevalence of AFM1 in milk samples, for example, the occurrence of AFM1 in

accordance with FSSAI limits in Haryana (20.6%) (Sharma *et al* 2020), Karnataka (38%) and Tamil Nadu (17.3%) (Siddappa *et al* 2012), Goa (75%) (Kanungo & Bhand 2014), Punjab (36.5%) (Patyal *et al* 2020b) and Uttar Pradesh (9%) (Rastogi *et al* 2004) were found to be ranged between 9% to 75%. The countries near the tropics are at higher risk as they provide relatively favorable environmental conditions for growth of aflatoxigenic fungi. Several reports from Pakistan (Asghar *et al* 2018, Ahmad *et al* 2019), Brazil (Picinin *et al* 2013), Kenya (Senerwa *et al* 2016) and China (Xiong *et al* 2020) also reported higher AFM1 prevalence. However, apart from tropical countries, the aflatoxins in milk remain a public health issue in other countries like Italy (Serraino *et al* 2019), Greece (Roussi *et al* 2002) and Croatia (Bilandžić *et al* 2017).

The high prevalence and levels of AFM1 in milk samples of bovines could mainly attributed to high prevalence and concentration of AFB1 in feed and feed ingredients of dairy animals in Punjab (Patyal 2019). Other possible reason for the high prevalence of AFM1 levels in milk could be due to large scale feeding of stored concentrated feed based on corn, wheat and cotton seed to the dairy animals which has been reported as risk factor for AFM1 in milk (Michlig *et al* 2016). The inadequate storage conditions of these feeds with poor ventilation can lead to the growth of aflatoxigenic fungi as reported earlier (Asi *et al* 2012 and Nile *et al* 2016). In addition, the sample collection time period of the present study included the rainy and winter season, both of which are reported to support the growth of aflatoxigic fungi on feed leading to high AFM1 levels in milk (Ismail *et al* 2016 and Patyal 2019).

4.1.2 District wise prevalence of AFM1 in bovine milk from Punjab

Of all the 22 districts of Punjab, all the tested milk samples were found positive for AFM1 from Barnala, Faridkot, Hoshiarpur, Moga, Mohali and Rupnagar with the lowest positivity rate from Bathinda district (38%). With respect to the AFM1 concentration in milk, the samples were found to be most contaminated from the Ludhiana district of Punjab with the mean concentration of 0.89±1.47 ppb, while least contaminated from Kapurthala (0.01±0.009 ppb). Moreover, with respect to EC-MPL, Hoshiarpur (100%) and Moga (100%) districts of Punjab had the highest

prevalence of AFM1, whereas, Muktsar (64.7%) and Moga (65%) had the highest prevalence of AFM1 with respect to FSSAI-MPL. Prevalence and mean concentration of AFM1 in each district has been presented in Table 5, Fig. 3 and 4. A comparative district-wise study comprising of 3 districts of Punjab by Patyal (2019) reported 56%, 57.5% and 56% AFM1 prevalence in farm milk samples from Ludhiana (0.747±0.59 ppb), Bathinda (0.854±0.73 ppb) and Amritsar (0.871±0.69 ppb) respectively.

4.1.3 Species-wise prevalence of AFM1 in milk

In present study, a total of 402 milk sample collection included 266 cattle and 136 buffalo milk samples. The comparison of the prevalence was done by considering the EC-MPL guidelines (AFM1 MPL of 0.05 ppb) as it is stringent and widely accepted for trade related issues. The prevalence of AFM1 with respect to EC-MPL was found slightly higher in buffalo milk [57.35% (78/136)] as compared to cattle milk [56.39% (150/266)]. The mean AFM1 concentration in buffalo milk (0.42±0.9 ppb) was also found to be higher than that of cattle milk (0.193±0.3 ppb). The buffalo milk had 16.91% (23/136) of milk samples in the non-compliant category with respect to FSSAI-MPL in comparison to 11.65% (31/266) samples of cattle milk. The mean concentration of AFM1 was found to be significantly higher in buffalo than in cattle milk (t-test: p-value = 0.0001). However, the difference between prevalence of AFM1 between the species was found to be non-significant (Chi-square test: p-value = 0.854). The prevalence and mean concentration of AFM1 in cattle and buffalo milk has been represented in Fig. 5.

The results of the present study were in the line with earlier reports of Iqbal *et al* (2011) and Asi *et al* (2012) who reported high AFM1 prevalence and mean concentration in buffaloes than in cattle. However, Hussain *et al* (2008b) reported high AFM1 prevalence and levels in cattle as compared to buffalo. In addition, Nile *et al* (2016) found high AFM1 prevalence in cattle but high mean concentration in buffalo. Since, the targeted samples of buffalo and cattle milk were from the same farms, thereby feed as a source of aflatoxins is assumed to be common for both species. Therefore, the high AFM1 contamination in buffalo milk than in cattle milk can be related to high feed intake in buffaloes in comparison to cattle leading to increase in aflatoxin ingestion per gram of feed in buffaloes and thus excretion in milk.

Table 5: District-wise prevalence and mean concentration of AFM1 in bovine milk from Punjab

Sl. No.	District	% positive samples (>0.005 ppb)	% samples above EC-MPL (>0.05 ppb)	% samples above FSSAI-MPL (>0.5 ppb)	Mean Concentration (ppb) ± standard deviation
1.	Amritsar	57.6% (19/33)	24.2% (98/33)	0% (0/33)	0.04±0.07
2.	Barnala	100% (13/13)	84.6% (11/13)	15.4% (2/13)	0.303±0.2
3.	Bathinda	38% (8/21)	19% (4/21)	0% (0/21)	0.024±0.04
4.	Faridkot	100% (11/11)	81.8% (9/11)	18.2% (2/11)	0.376±0.5
5.	Fatehgarh Sahib	91.7% (11/12)	91.7% (11/12)	8.3% (1/12)	0.199±0.2
6.	Ferozepur + Fazilka	70% (28/40)	22.5% (9/40)	0% (0/40)	0.035±0.04
7.	Gurdaspur + Pathankot	55.6% (15/27)	14.8% (4/27)	0% (0/27)	0.034±0.06
8.	Hoshiarpur	100% (20/20)	100% (20/20)	5% (1/20)	0.336±0.1
9.	Jalandhar	85% (17/20)	65% (13/20)	2/20 (10%)	0.123±0.17
10.	Kapurthala	50% (5/10)	0% (0/10)	0% (0/10)	0.01±0.01
11.	Ludhiana	97% (33/34)	70.6% (24/34)	35.3% (12/34)	0.89±1.47
12.	Mansa	70% (14/20)	65% (13/20)	15% (3/20)	0.237±0.21
13.	Moga	100% (20/20)	100% (20/20)	65% (13/20)	0.643±0.36
14.	Mohali	100% (11/11)	54.5% (6/11)	0% (0/11)	0.173±0.18
15.	Muktsar	82.4% (14/17)	82.4% (14/17)	64.7% (11/17)	0.662±0.5
16.	Nawanshahr	88.9% (8/9)	77.8% (7/9)	0% (0/9)	0.18±0.15
17.	Patiala	96.7% (29/30)	83.3% (25/30)	0% (0/30)	0.19±0.14
18.	Rupnagar	100% (11/11)	81.8% (9/11)	0% (0/11)	0.171±0.1
19.	Sangrur	77.3% (17/22)	54.5% (12/22)	27.3% (6/22)	0.5±0.9
20.	Tarn Taran	66.7% (14/21)	33.3% (7/21)	4.7% (1/21)	0.12±0.21

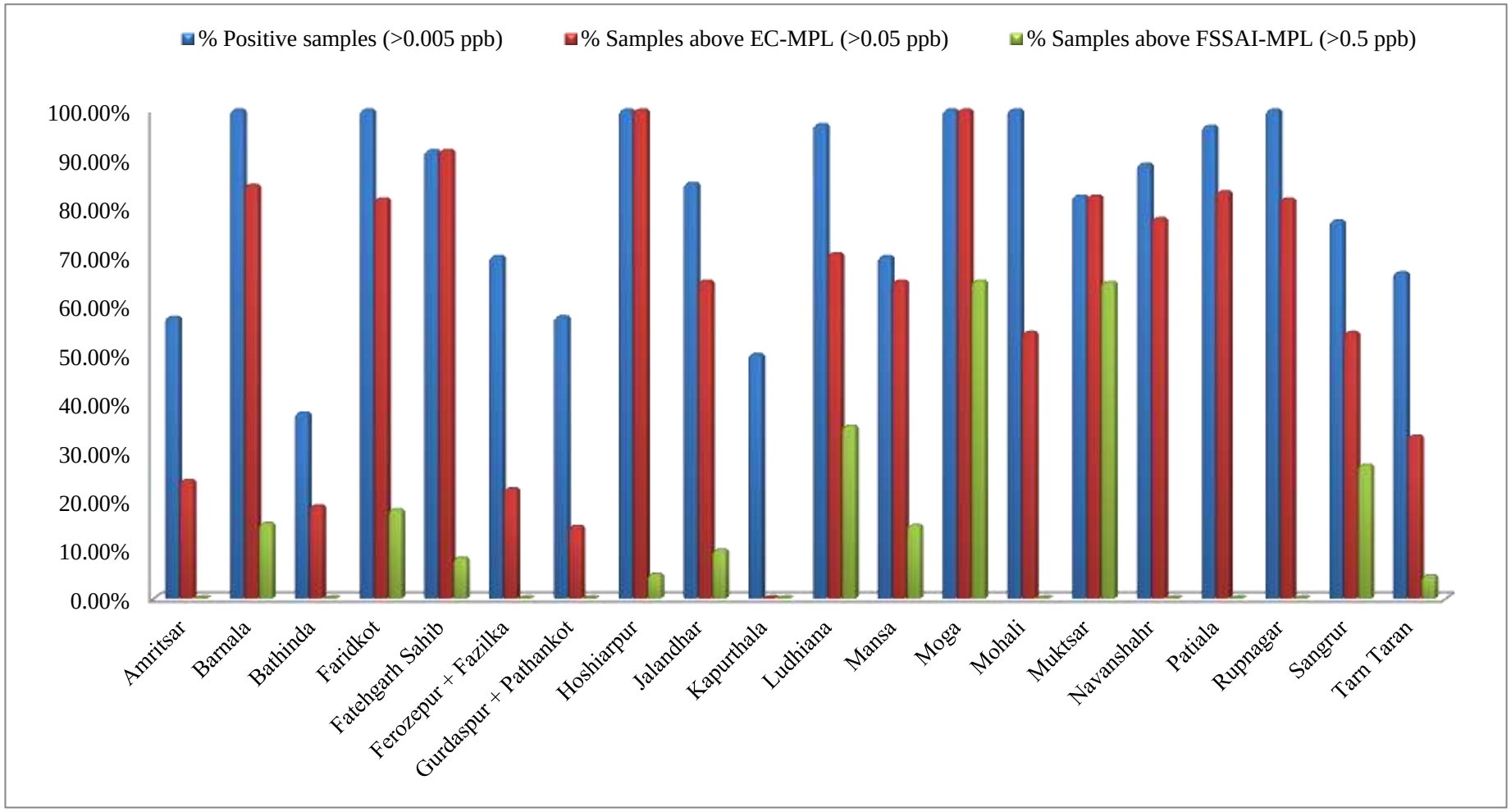


Fig. 3: District-wise prevalence of AFM1 in bovine milk from Punjab

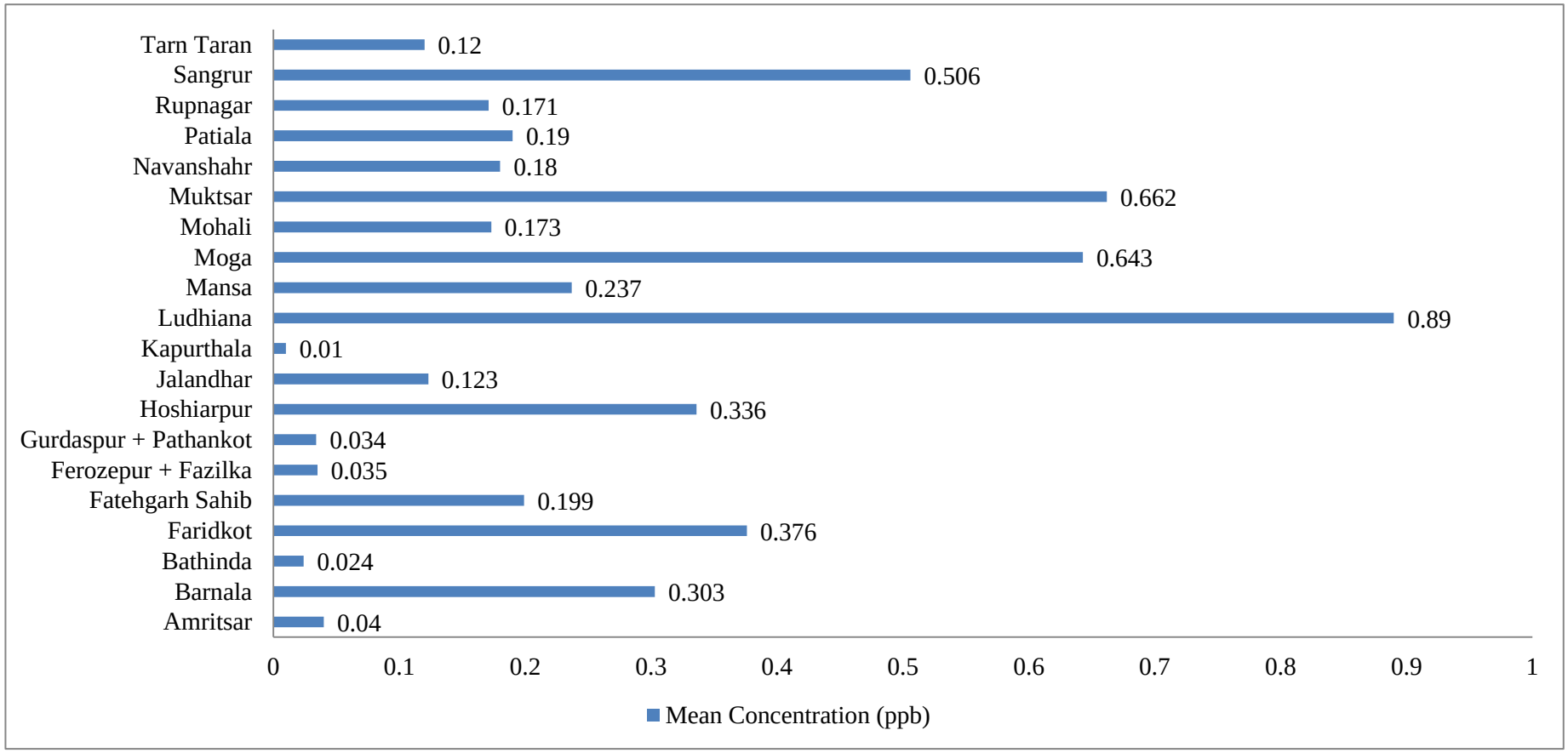


Fig. 4: Mean concentration (ppb) of AFM1 in bovine milk from different districts of Punjab

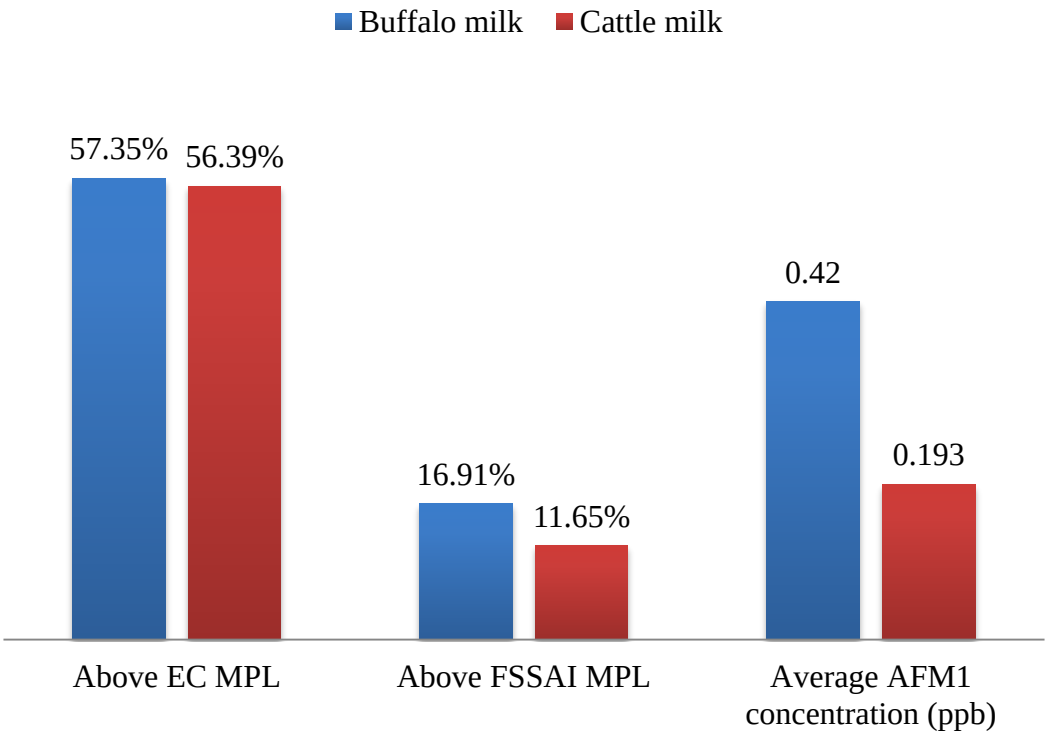


Fig. 5: Species-wise prevalence and mean concentration of AFM1 in milk

4.1.4 Comparison of prevalence of AFM1 in bovine milk on the basis of dairy farm type

During sample collection, 201 milk samples were collected from 27 commercial dairy farms and the other 201 milk samples were collected from 46 dairy households across Punjab. The prevalence of AFM1 in milk from commercial dairy farms (having ≥10 animals) was found out to be 65.67% with average AFM1 concentration of 0.344±0.65 ppb as compared to 48.26% prevalence with average AFM1 concentration of 0.195±0.65 ppb in milk from dairy households with respect to EC-MPL. The commercial dairy farms had 18.4% of milk samples in the non-compliant category with respect to FSSAI while dairy households had only 8.46%. Both the prevalence and mean concentration of AFM1 in milk from commercial dairy farms was found to be significantly higher than in milk from dairy households (Prevalence: Chi-square test, p-value = 0.0004; Mean concentration: t-test, p-value = 0.009). The prevalence and mean concentration of AFM1 in milk from commercial and dairy establishments has been represented in Fig. 6.

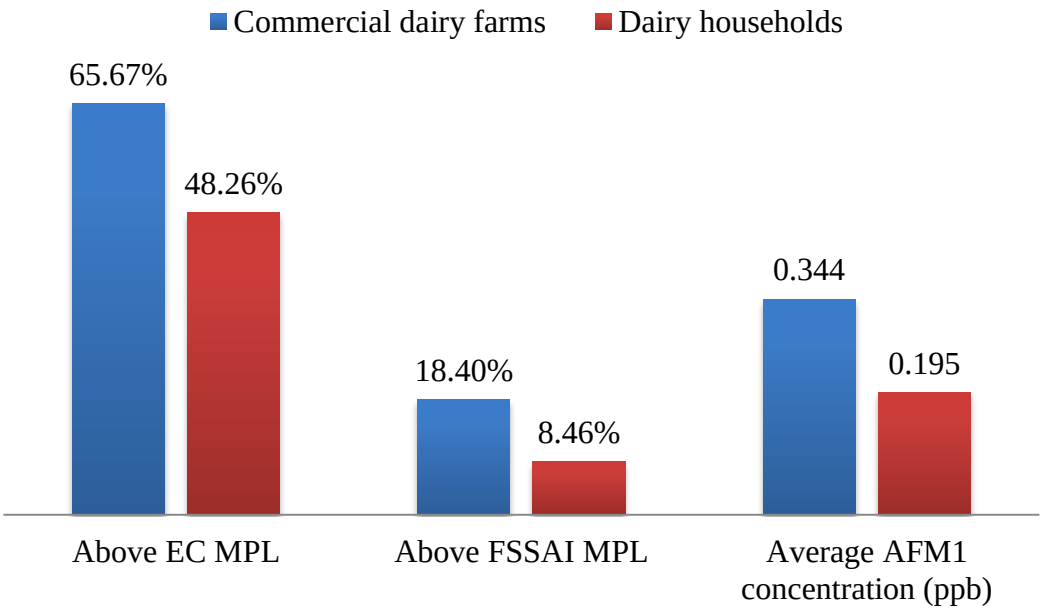


Fig. 6: Prevalence and mean concentration of AFM1 in milk based on type of dairy farm

The results of the present study were found to be in agreement with the study of Michlig *et al* (2016), which also reported intensive commercial dairy farming as a risk factor for the presence of AFM1 in milk. The main difference observed between commercial and household dairy establishments were their feeding practices. The commercial dairy farms are mainly focused on high milk yield and to achieve that they include commercial high energy concentrated feed based on cotton seed and corn as a major part of their feeding ration and these type of feeds are more prone to contamination by aflatoxigenic fungi as reported earlier (Asi *et al* 2012 and Nile *et al* 2016), thereby increasing AFM1 excretion in milk.

4.2 Confirmation of milk samples for AFM1 by HPLC-FLD

For the detection of AFM1 by HPLC, 10% of representative positive milk samples for AFM1, which were in the non-compliant category with respect to EC-MPL (i.e., >0.05 ppb) by ELISA and 5% of ELISA negative milk samples were analyzed. The results of the HPLC-FLD analysis were found to be in compliance with the results of the ELISA screening as represented in Fig. 7(a) - 7(f). Earlier reports also suggested the agreement between the ELISA and HPLC-FLD detection of AFM1 (Picinin *et al* 2013, Patyal *et al* 2020b).

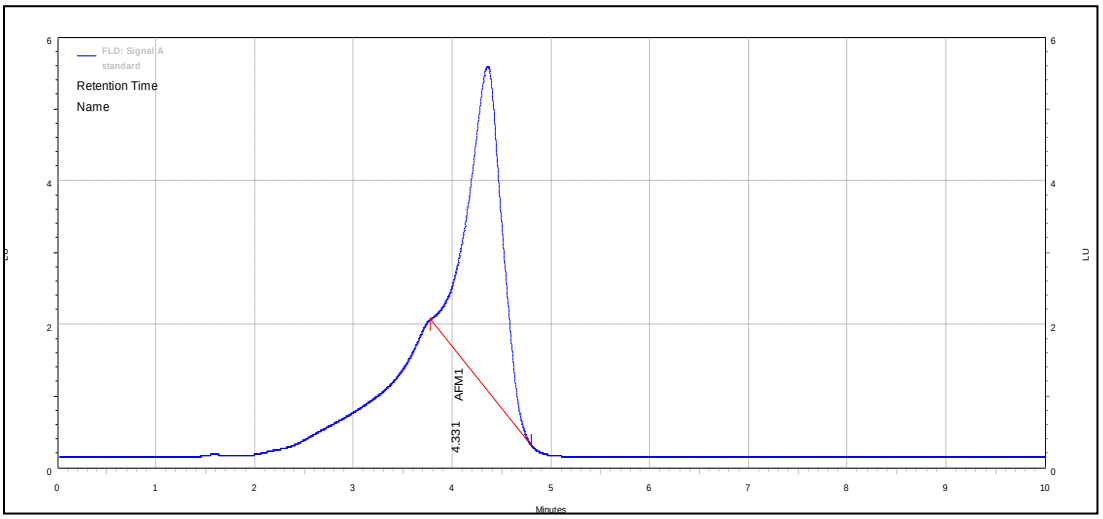


Fig 7(a): HPLC-FLD chromatogram of AFM1 standard

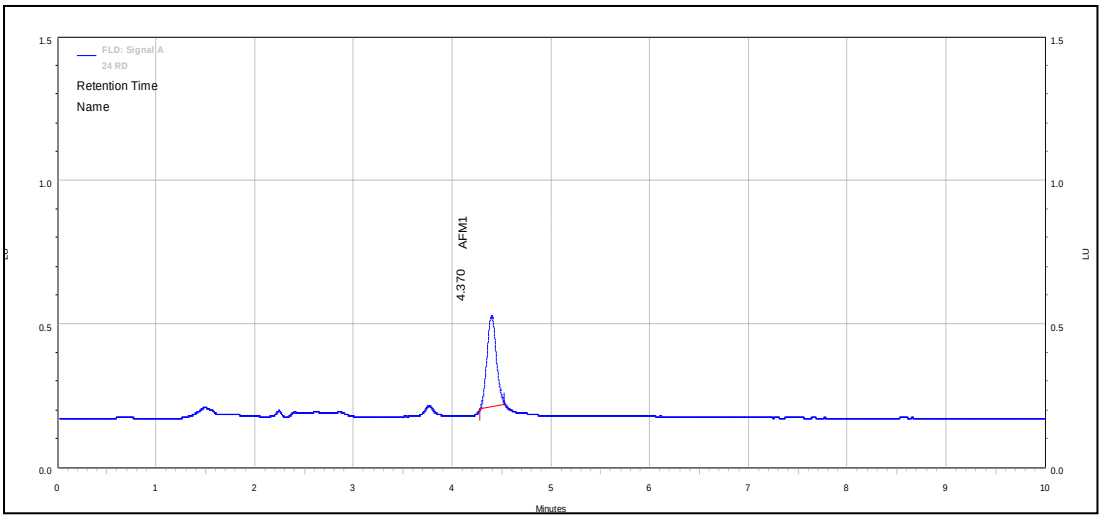


Fig 7(b): HPLC-FLD chromatogram of milk sample positive for AFM1

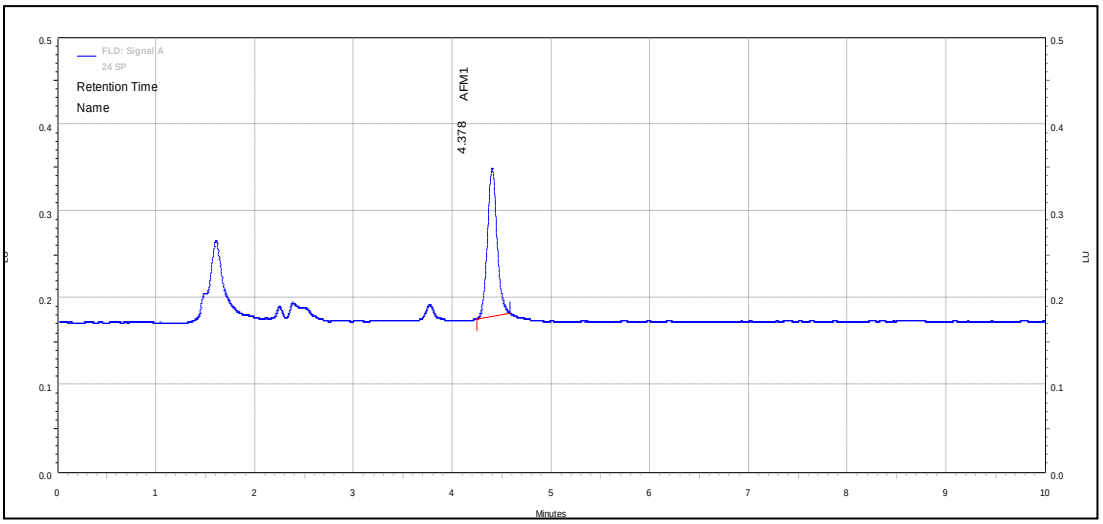


Fig 7(c): HPLC-FLD chromatogram of milk sample positive for AFM1

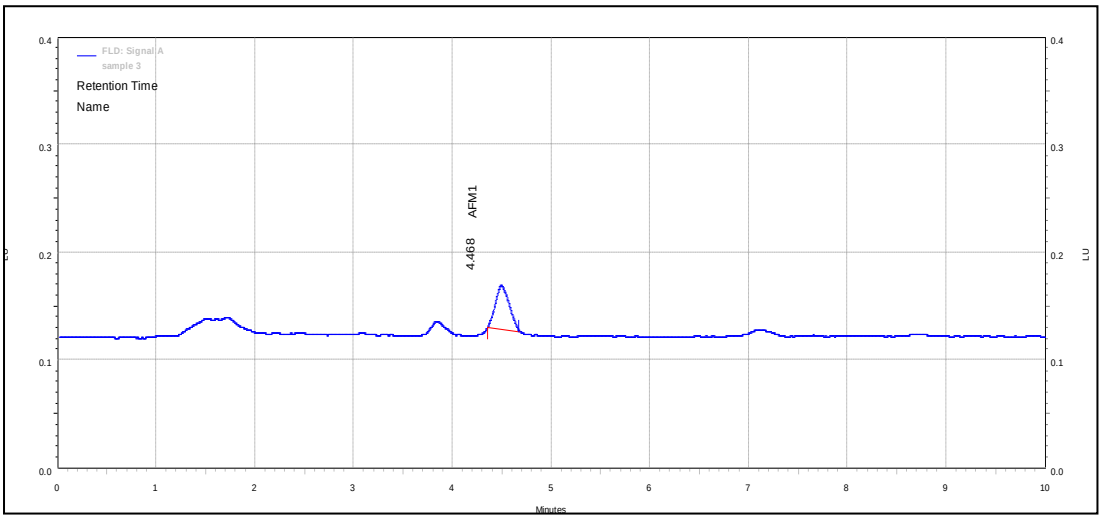


Fig. 7(d): HPLC-FLD chromatogram of milk sample positive for AFM1

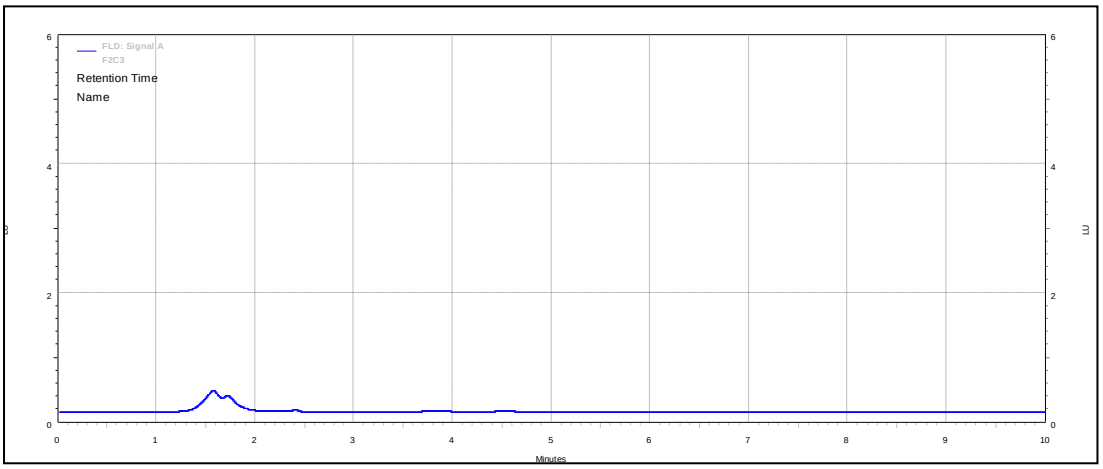


Fig 7(e): HPLC-FLD chromatogram of milk sample negative for AFM1

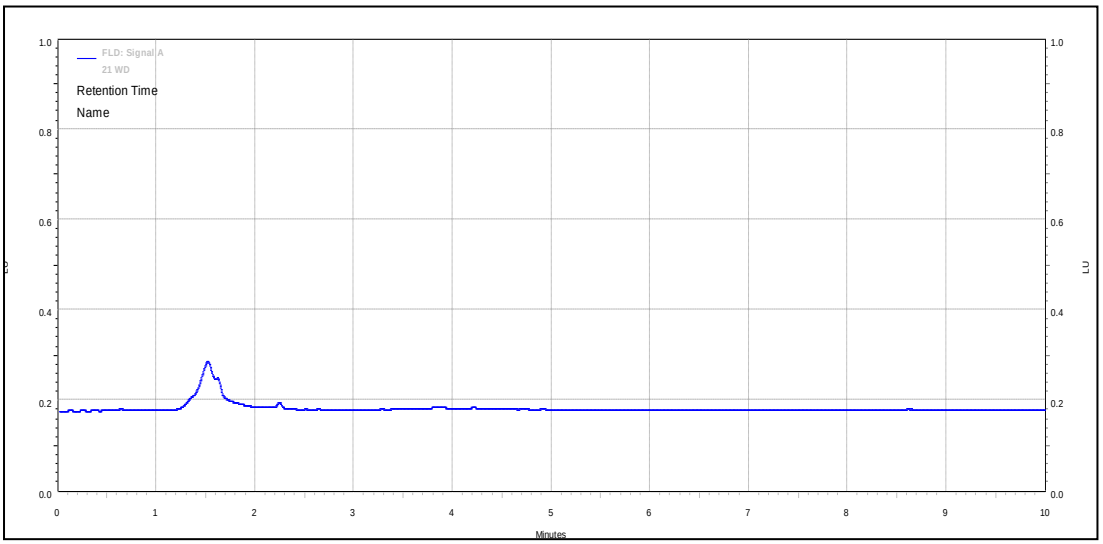


Fig 7(f): HPLC-FLD chromatogram of milk sample negative for AFM1

4.3 Univariable analysis of risk factors for AFM1 in milk

The univariable analysis of risk factors associated with AFM1 in milk has been analyzed by using Epitools software (Sergeant, ESG, 2009). The animal specific (milk yield, age, parity, breed and animal hygiene index) and farm-specific (source of animal feed, farming system and feed storage conditions) factors were taken into consideration. The odds ratio as a measure of association has been calculated for these factors in relation to AFM1 concentration in milk samples. The EC-MPL (0.05 ppb) was taken as cut-off point for the analysis due to its global acceptance in terms of trade related issues. The milk samples with AFM1 concentration above 0.05 ppb were grouped in above EC-MPL category and below 0.05 ppb were grouped in below EC-MPL category.

4.3.1 Animal-specific factors

4.3.1.1 Milk yield

The milk production/day (in kg) was recorded for 402 bovines during the sample collection. The samples having above and below to the average milk yield (i.e., 13 kg) were analyzed against the AFM1 concentration in 2×2 contingency table (Table 6).

Table 6: Association between milk yield and AFM1 concentration

Parameters	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	Total	OR (95% CI)	p-value
Milk yield above average (>13 kg)	122	55	177	2.45 (1.62-3.69)	<0.0001 (Statistically significant)
Milk yield below average (≤13 Kg)	107	118	225		
Total	229	173	402		

The animals having milk yield above the average were found to have 2.45 (95% CI: 1.62-3.69) times higher odds for above EC-MPL AFM1 concentration in milk as compared to animals having below average milk yield.

The results of the present study were found in agreement with Veldman *et al* (1992), Masoero *et al* (2007) and Britzi *et al* (2013) who also reported that high yielding bovines are associated with high carry-over rate of aflatoxins in milk.

However, Fels-Klerx *et al* (2016) reported milk yield of having minimal effect on the AFM1 excretion rate. High milk yield is a phenotype of breeding for increased production but is also dependent upon intensive husbandry and feeding practices. Therefore, further studies need to be conducted on the genotypic and phenotypic aspects of high milk yield related to high AFM1 levels in milk. However, the farm intensification and feeding of high energy concentrated diet are established risk factors for presence of AFM1 in milk in previous studies (Asi *et al* 2012, Michlig *et al* 2016) as well as in the present study. Moreover, the high yielding bovines have higher feed intake in comparison to low yielding bovines, thus ingesting higher amount of AFB1 per gram of feed might lead to increased AFM1 excretion.

4.3.1.2 Age

The mean age of 402 study animals was found to be 6 years, thereby, the animals were grouped in below average age and above average age category and were analyzed against the AFM1 concentration in 2×2 contingency table (Table 7):

Table 7: Association between age (years) of bovines and AFM1 concentration in milk

Parameters	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	Total	OR (95% CI)	p-value
Below average age (≤6 years)	127	61	188	2.29 (1.52-3.43)	<0.0001 (Statistically significant)
Above average age (>6 years)	102	112	214		
Total	229	173	402		

The animals with below average age (i.e., ≤6 years) were found to have 2.29 (95% CI: 1.52-3.43) times higher odds for AFM1 concentration above EC-MPL in milk as compared to animals with above average age (i.e., >6 years).

To the best of our knowledge, there is no related report for the association between age and AFM1 excretion in milk of bovines. The association from the present study could be due the fact that young animals might have higher feed intake than older animals, thus increasing the AFB1 intake in them via feed and also in

higher age groups, the milk yield decreases (data from present study: the average milk yield of ≤6 years age group was 13.76 kg and of >6 years age group was 12.11 kg), and milk yield is found to be directly related with AFM1 excretion in milk as reported in previous studies (Veldman *et al* 1992, Masoero *et al* 2007 and Britzi *et al* 2013) and in the present study.

4.3.1.3 Parity

The total study subjects (i.e. 402) were grouped into parity group A (1st to 3rd parity) and parity group B (4th to 7th parity) based on the below- and above- average parity (i.e. 3rd parity). These groups were analyzed in relation to the AFM1 concentration in 2×2 contingency table (Table 8):

Table 8: Association between parity of bovines and AFM1 concentration in milk

Parameters	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	Total	OR (95% CI)	p-value
Parity group A (1st to 3rd parity)	183	118	293	1.85 (1.18-2.92)	0.0104 (Statistically significant)
Parity group B (4th to 7th parity)	46	55	109		
Total	228	174	402		

The bovines under parity group A were found to have 1.85 (95% CI: 1.18-2.92) times higher odds for AFM1 concentration above EC-MPL in milk as compared to bovines under parity group B.

To the best of our knowledge, there are no related reports that studied the association between parity and AFM1 excretion in milk of bovines. The association from our present study could be explained on the basis of the fact that in higher parity groups, the milk yield of an animal decreases and vice-versa (data from present study: the average milk yield of 1st to 3rd parity group was 13.36 kg and of 4th to 7th parity group was 12.44 kg) and milk yield is directly related with AFM1 excretion in milk as

reported in previous studies (Veldman *et al* 1992, Masoero *et al* 2007 and Britzi *et al* 2013) and in present study.

4.3.1.4 Breed

The breed data of cattle population (n = 266) in the study were also considered for analysis of risk factor for AFM1 in milk. The five major cattle breeds which were recorded in the present study were Holstein Friesian (HF), Cross of HF, Jersey, Sahiwal and non-descript cattle. The lowest AFM1 contamination was found to be in Jersey, therefore, the odds of Jersey breed for AFM1 was taken as baseline value (i.e., 1) and the remaining cattle breeds were analyzed in relation to the AFM1 concentration (Table 9).

Table 9: Association between breeds of cattle and AFM1 concentration in milk

Breed	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	OR (95% CI)	p-value
Jersey	4	11	1 (baseline value)	
Sahiwal	11	20	1.51 (0.39-5.9)	0.79 (Statistically non-significant)
HF	27	19	3.91 (1.08-14.14)	0.06 (Statistically non-significant)
HF cross	88	60	4.03 (1.23-13.26)	0.03 (Statistically significant)
Non-descript	21	5	11.55 (2.57-51.95)	<0.00001 (Statistically significant)

With respect to the AFM1 levels of Jersey breed, the non-descript cattle had the highest odds for AFM1 concentration above EC-MPL in milk (OR= 11.55; 95% CI: 2.57-51.95) followed by HF cross (4.03; 95% CI: 1.23-13.26), HF (3.91; 95%CI: 1.08-14.14) and Sahiwal (1.51; 95% CI: 0.39-5.9) breed. However, only the odds of HF cross (p-value = 0.03) and non-descript (p-value = 0) breeds were found to be statistically significant. To the best of our knowledge, no comparative data was found

in relation to the association between AFM1 excretion in milk and type of cattle breed, so further large scale multi-centric epidemiological studies need to be conducted to understand the effect of genotypic and phenotypic aspects of different breeds on the metabolism and excretion of AFM1 in milk before arriving at any concluding remark.

4.3.1.5 Animal Hygiene Index

During the sample collection, each animal was given a hygiene score *via.*, visual observation criteria as described in Table 3 (the hygiene score 1 being the best and 4 being the worst). The lowest AFM1 contamination was found to be in hygiene score 1 group, so the OR of hygiene score 1 group was taken as baseline value (i.e., 1) and the higher hygiene scores were analyzed in relation to the AFM1 concentration in Table 10.

Table 10: Association between hygiene score of bovines and AFM1 concentration in milk

Parameters	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	OR (95% CI)	p-value
Hygiene Score 1	37	46	1 (baseline value)	
Hygiene Score 2	61	53	1.43 (0.81-2.53)	0.27 (Statistically non-significant)
Hygiene Score 3	73	39	2.33 (1.3-4.6)	0.01 (Statistically significant)
Hygiene Score 4	58	35	2.06 (1.13-3.76)	0.03 (Statistically significant)

The animals having hygiene score 3 and 4 were found to have 2.33 (95% CI: 1.3-4.6; p-value: 0.01) and 2.06 (95% CI: 1.13-3.76; p-value: 0.03) times higher odds for AFM1 concentration above EC-MPL in milk as compared to animals with hygiene score 1. The milk from the animals with hygiene score 2 (1.43; 95% CI: 0.81-2.53) was also found to be more contaminated with AFM1 than hygiene score 1 animals,

however, the variation was found to be statistically non-significant (p-value: 0.27). To the best of our knowledge, no comparative data was found in relation to association between AFM1 excretion in milk and animal hygiene index. The higher hygiene index of animals directly reflects the poor farm management conditions which directly or indirectly can cause fungal growth on farm premises including feed and thus leading to high aflatoxin ingestion and excretion by animals.

4.3.2 Farm-specific factors

4.3.2.1 Farming system

For risk factor analysis, the dairy farming system across Punjab was observed under two major categories; i.e., Intensive farming (based on complete stall feeding, high labor requirement with limited land use) and semi-intensive (animals are both grazed and stall fed, land use is more with limited labor requirement) and they were analyzed against the AFM1 concentration in 2×2 contingency table (Table 11):

Table 11: Association between type of dairy farming system and AFM1 concentration in milk

Parameters	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	Total	OR (95% CI)	p-value
Intensive farming system	202	111	313	4.18 (2.51-6.94)	<0.0001 (Statistically significant)
Semi-intensive farming system	27	62	89		
Total	229	173	402		

The intensive dairy establishments were found to have 4.18 (95% CI: 2.51-6.94) times higher odds for AFM1 concentration above EC-MPL as compared to semi-intensive dairy establishments. The results of our study were in line with the previous study of Michlig *et al* (2016) who also reported that farm intensification is associated with high AFM1 levels in milk of bovines. The high excretion of AFM1 in milk from intensive dairy farms could be due to excessive stall feeding practices (Asi *et al* 2012) where concentrated feed is fed to animals mainly consisting of commercial feed, cotton seed and corn which are also reported as risk factors for presence of AFM1 in milk (Michlig *et al* 2016). In addition, intensive dairy farms in the region

were observed to house mainly high yielding cattle breeds and high milk yield has been reported as a risk factor for the presence of AFM1 in milk of bovines in the present study and also in previous studies (Britzi *et al* 2013).

4.3.2.2 Feed storage conditions

During sample collection, the site and conditions under which feed was stored were visited and observations were broadly categorized as covered feed storage (a dedicated establishment with roof for feed storage with a single entry point) and open feed storage (feed being stored on- or off- farm site with no dedicated establishment). The observations recorded were analyzed against the AFM1 concentration in 2×2 contingency table (Table 12):

Table 12: Association between feed storage conditions of dairy farm and AFM1 concentration in milk

Parameters	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	Total	OR (95% CI)	p-value
Covered storage	188	143	331	1.04 (0.62-1.75)	0.99 (Statistically non-significant)
Open storage	41	30	71		
Total	229	173	402		

On analysis, the covered feed storage conditions were found to have 1.04 (95% CI: 0.62-1.75) times higher odds for AFM1 excretion above EC-MPL in milk, however, the association was found to be non-significant (p-value = 0.99). Thereby, we can conclude that both types of the feed storage conditions were found to present nearly equal risk for excretion of AFM1 above EC-MPL in milk and further large scale studies need to be conducted to deduce any association. The results of present study were in contrast to Malissiova *et al* (2013) who reported feed storage in warehouses to be a risk factor for presence of AFM1 in milk.

4.3.2.3 Source of feed

During sample collection, the sources of feed for bovines were recorded from the dairy farmers. The farmer's responses were grouped into 3 categories: (1) Marketed feed (pre-prepared packed feed bought from market but not having aflatoxin binders), (2) Self-formulated feed (the ingredients of feed are either grown

by farmers or purchased from local vendors and mixed) and (3) Feed + aflatoxin binder (Commercial aflatoxin binders were mixed along with feed of animals). The marketed feed (without binders), self-formulated feed and feed + aflatoxin binder categories were analyzed against AFM1 concentration in Table 13.1 and 13.2.

Table 13.1: Association between source of feed (marketed or self-formulated) in dairy farm and AFM1 concentration in milk

Parameters	AFM1 conc. above EC-MPL	AFM1 conc. below EC-MPL	Total	OR (95% CI)	p-value
Self-formulated feed	88	52	140	1.1 (0.7-1.71)	0.77 (Statistically non-significant)
Marketed feed (without binder)	125	81	206		
Total	213	133	346		

Table 13.2: Association between addition of aflatoxin binders in animal feed and AFM1 concentration in milk

Parameters	AFM1 conc. above EC-MPL	AFM1 conc.below EC-MPL	OR (95% CI)	p-value
Feed + aflatoxin binder	16	40	1 (baseline value)	
Marketed feed (without binder)	125	81	3.86 (2.03-7.34)	<0.0001 (Statistically significant)
Self-formulated feed	88	52	4.23 (2.16-8.3)	<0.0001 (Statistically significant)

The marketed (without binder) and self-formulated feed category were found to have nearly equal odds (OR= 1.1 (95% CI: 0.7-1.71) for excretion of AFM1 concentration above EC-MPL in milk. The feed + aflatoxin binder category was found to be least contaminated amongst the 3 categories, so odds ratio of feed + aflatoxin binder category was taken as baseline value (i.e., 1) and the marketed feed

(without binder) and self-formulated feed were found to have 3.86 (95% CI: 2.03-7.34) and 4.23 (95% CI: 2.16-8.3) times higher odds for AFM1 concentration above EC-MPL in milk than feed + aflatoxin category, respectively. The results of present study were in contrast to Michlig *et al* (2016) who reported commercial feed to be a risk factor for presence of AFM1 in milk. Though previous studies by Aslam *et al* (2016) and Ullah *et al* (2016) have reported that the addition of aflatoxin binders to feed could significantly lower the AFM1 levels in milk.

4.4 Multivariable analysis of risk factors for presence of AFM1 in milk

The variables with p-value of <0.2 in univariable analysis were used for building logistic regression models using independent predictors of milk yield (in kg/day), age (in years), parity (in numbers) and hygiene index of individual animal (range:1-4), farming system (intensive vs. semi-intensive) and source of feed for dairy farm in respect to 'excretion of AFM1 in milk'. The analysis was performed using SPSS version 24.0 (SPSS Inc., IBM, NY, USA) and the details of the results of multivariable analysis and model summary have been presented in Table 14. The risk factors identified for AFM1 concentration above EC-MPL included: above average per day milk yield (>13 kg/day) (adjusted OR: 2.432), higher animal hygiene scores (>1) (adjusted OR: 1.94), intensive dairy farming system (adjusted OR: 3.12) and animal feed (both marketed and self-formulated) without aflatoxin binder (adjusted OR: 4.67). Whereas, the increase in the age of the animal was observed as protective factor with adjusted OR of 0.83 and the increase in parity was found to be non-significantly associated with AFM1 excretion (adjusted OR: 1.03; p-value 0.79).

The results of multivariable analysis of risk factors of the present study are in agreement with Veldman *et al* (1992), Masoero *et al* (2007) and Britzi *et al* (2013) for higher milk yield and with Michlig *et al* (2016) for farm intensification and feeding of commercial feed as risk factors for presence of AFM1 in milk.

Table 14: Multivariable analysis for risk factors analysis

Variables in the Equation								
	B	**S.E.**	**Wald**	**Df**	**Sig.**	**Exp(B)**	**95% C.I. for EXP(B)**	
							Lower	**Upper**
Milk Yield (Kg/day)	0.889	0.238	13.9	1	0.0001	**2.43**	1.524	3.880
Age (years)	-0.186	0.071	6.93	1	0.008	**0.83**	0.723	0.954
Parity	0.028	0.106	0.069	1	0.793	**1.03**	0.836	1.264
Animal Hygiene Index	0.665	0.276	5.78	1	0.016	**1.94**	1.131	3.343
Farming System	1.14	0.280	16.5	1	0.0001	**3.12**	1.800	5.397
Source of feed	1.54	0.359	18.4	1	0.0001	**4.67**	2.311	9.445
Constant	-1.78	0.560	10.1	1	0.001	**0.17**		

Model Summary			
Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	464.583[a]	0.191	0.257
Estimation terminated at iteration number 4 because parameter estimates changed by <0.001.			

4.5 Correlation between AFM1 excretion in milk of bovines and their rumen liquor parameters

The association between excretion of AFM1 in milk of bovines and their rumen liquor parameters *viz.* (1) Physico-chemical parameters [(pH, Methylene Blue Reduction Time (MBRT), Sedimentation Activity Time (SAT), colour, odour and consistency)], (2) Rumen metabolites [(Total Volatile Fatty Acids (TVFA's) and Ammonia Nitrogen)] and (3) Rumen microflora count [(Total Bacterial Count (TBC) and Total Protozoal Count (TPC)] was analyzed using Microsoft® Excel 2010. The Pearson Correlation Coefficient (r) was calculated between the AFM1 concentrations in milk of bovines and their respective rumen liquor parameters. The correlation coefficient was categorized into weak (0-0.25), fair (0.25-0.5), good (0.5-0.75) and excellent (0.75-1) correlation based on r value as described earlier (Cohen 2013). The details of data on rumen liquor parameters and AFM1 in milk are presented in Table 15.

Table 15: Details of various rumen liquor parameters and AFM1 excretion in milk

Sample ID	AFM1 in milk (ppb)	Colour	Odour	Consistency	pH	MBRT (sec.)	SAT (min.)	TVFA's (mEq/ml)	NH3-N (mg/dl)	TBC ($\times 10^9$)	TPC ($\times 10^5$)
SRL 1	0.612	Normal	Normal	Normal	7.8	270	8.6	110	6	7.4	6.4
SRL 2	0.580	Normal	Normal	Normal	8.6	260	6.7	102	4	6.5	7.1
SRL 3	1.102	Normal	Normal	Normal	7.7	310	8.3	81	10	5.8	5.2
SRL 4	1.531	Abnormal	Abnormal	Normal	8.6	340	9.1	74	12	4.1	4.3
SRL 5	0.221	Normal	Normal	Normal	6.6	220	9.7	100	10	10.7	7.2
SRL 6	0.660	Normal	Normal	Normal	7.6	210	7.4	105	6	6.8	6.5
SRL 7	0.023	Normal	Normal	Normal	8.7	170	4.6	125	2	17.9	8.6
SRL 8	0.003	Normal	Normal	Normal	8	170	3.2	110	6	17.8	16
SRL 9	0.003	Normal	Normal	Normal	7.7	110	3.2	98	6	34	9.4
SRL 10	0.040	Normal	Normal	Normal	7.8	270	7.4	94	4	32	19.3
SRL 11	0.015	Normal	Normal	Normal	8.1	165	7	120	6	18	5.15
SRL 12	0.105	Normal	Normal	Normal	7.5	108	5.8	69	12	22	9.2
SRL 13	0.005	Abnormal	Normal	Normal	6.8	180	6.8	128	4	21.9	17.45
SRL 14	0.015	Normal	Normal	Normal	7.2	230	6.3	105	3	26.3	13.5
SRL 15	0.079	Normal	Normal	Normal	7.4	110	3.3	75	4	21.5	2.6
SRL 16	0.023	Abnormal	Normal	Normal	7.5	50	5.4	100	4	34	8.3
SRL 17	0.066	Normal	Normal	Normal	7.9	75	2.4	68	2	14.3	16.9
SRL 18	0.021	Normal	Normal	Normal	6.5	80	4	91	6	19.1	17.3
SRL 19	0.371	Normal	Abnormal	Normal	7.2	130	6	103	5	9.7	12.5

Sample ID	AFM1 in milk (ppb)	Colour	Odour	Consistency	pH	MBRT (sec.)	SAT (min.)	TVFA's (mEq/ml)	NH3-N (mg/dl)	TBC ($\times 10^9$)	TPC ($\times 10^5$)
SRL 20	0.079	Normal	Normal	Normal	7.1	70	5	123	3	13.6	1.35
SRL 21	0.002	Normal	Normal	Normal	7.4	60	5.3	131	8	22	1.55
SRL 22	0.134	Abnormal	Normal	Normal	6.6	170	7.5	91	3	11.6	18.3
SRL 23	0.061	Normal	Normal	Normal	6.6	114	2.5	127	10	14.3	0.9
SRL 24	0.006	Normal	Normal	Normal	7.8	86	3.5	112	2	18.9	4.1
SRL 25	0.094	Normal	Normal	Normal	6.8	57	4.3	98	6	16.4	7.9
SRL 26	0.047	Normal	Normal	Normal	6.7	210	8	118	18	21.5	7.9
SRL 27	0.094	Normal	Normal	Normal	6.7	48	3	116	5	19.8	11.3
SRL 28	0.038	Normal	Abnormal	Normal	7.3	40	3.2	90	11	19.8	2.15
SRL 29	0.018	Normal	Normal	Normal	7.3	360	8.5	123	6	25	8.75
SRL 30	0.139	Normal	Normal	Normal	7.3	128	7.4	120	2	18.4	8.6
SRL 31	0.082	Normal	Normal	Normal	6.6	130	5.4	112	14	34.4	20
SRL 32	0.012	Abnormal	Normal	Normal	7.2	120	4.2	85	5	32	7.9
SRL 33	0.009	Normal	Normal	Normal	6.3	174	3.8	132	18	24	6.55
SRL 34	0.006	Normal	Normal	Normal	6.8	40	3.1	126	3	18	9.5
SRL 35	0.014	Normal	Normal	Normal	7.2	80	4.8	98	6	19.5	3.85
SRL 36	0.028	Abnormal	Abnormal	Abnormal	6.2	100	4.3	95	6	12.2	2.4
SRL 37	0.015	Normal	Normal	Normal	6	60	5.3	90	4	13.7	2.85
SRL 38	0.012	Normal	Normal	Normal	6	40	3.9	103	4	10.1	7.35
SRL 39	0.012	Normal	Normal	Normal	6.5	80	8.6	96	6	19.7	4.6

Sample ID	AFM1 in milk (ppb)	Colour	Odour	Consistency	pH	MBRT (sec.)	SAT (min.)	TVFA's (mEq/ml)	NH3-N (mg/dl)	TBC ($\times 10^9$)	TPC ($\times 10^5$)
SRL 40	0.013	Normal	Normal	Normal	6.7	40	6.5	80	2	21	1.75
SRL 41	0.037	Normal	Normal	Normal	6.5	105	4.8	70	10	35	1
SRL 42	0.027	Normal	Normal	Normal	6.5	80	2.7	76	6	16.9	1.25
SRL 43	0.008	Normal	Normal	Normal	6.5	50	2.6	132	4	4.5	14
SRL 44	0.116	Normal	Normal	Normal	8.6	106	8.7	84	4	12.1	1.7
SRL 45	0.188	Normal	Normal	Normal	7.8	50	7.6	99	6	5.8	3.55
SRL 46	0.012	Normal	Normal	Normal	6.4	66	2.3	80	2	18	0.6
SRL 47	0.700	Normal	Normal	Normal	8.5	240	8.7	110	6	4.3	0.85
SRL 48	0.035	Normal	Normal	Normal	6.7	50	6.8	123	10	17.7	8.25
SRL 49	0.243	Normal	Normal	Normal	6.7	38	2.3	120	4	13.4	5.6
SRL 50	0.047	Normal	Normal	Normal	7	42	5.4	89	4	35.3	5.85
SRL 51	0.678	Normal	Normal	Normal	7.4	77	6.5	95	6	6.6	7.7
SRL 52	0.384	Normal	Normal	Normal	7.3	69	6.1	91	4	8.9	10.55
SRL 53	3.192	Normal	Normal	Normal	8.7	420	9.6	69	16	3.2	3.2
SRL 54	0.061	Normal	Normal	Normal	7.4	141	6.4	104	2	5.5	2.1
SRL 55	0.857	Normal	Normal	Normal	7.2	330	7.5	102	6	5.7	6.3
SRL 56	0.091	Normal	Normal	Normal	7.5	19	3.1	126	2	9.8	3.9
SRL 57	0.048	Abnormal	Abnormal	Abnormal	7.7	45	3.5	71	4	14.9	5.8
SRL 58	0.001	Normal	Normal	Normal	7.1	60	2.2	131	2	19.6	6.5
SRL 59	0.378	Normal	Normal	Normal	7.2	140	8.7	67	2	5.7	7.15
SRL 60	0.418	Normal	Normal	Abnormal	7.4	120	7.5	75	6	2.7	5.3

Sample ID	AFM1 in milk (ppb)	Colour	Odour	Consistency	pH	MBRT (sec.)	SAT (min.)	TVFA's (mEq/ml)	NH3-N (mg/dl)	TBC ($\times 10^9$)	TPC ($\times 10^5$)
SRL 61	0.058	Normal	Normal	Normal	6.8	34	8.6	135	6	19.3	8.35
SRL 62	0.127	Normal	Normal	Normal	7.3	72	6.8	81	4	33	4.1
SRL 63	0.572	Normal	Normal	Normal	7.4	320	7.6	74	4	8.6	5.05
SRL 64	0.433	Normal	Normal	Normal	7.5	100	1.5	69	8	12.1	2.35
SRL 65	0.292	Normal	Normal	Normal	7.4	90	8.6	73	4	10.4	6.3
SRL 66	0.570	Normal	Normal	Normal	8.5	120	8.1	76	4	6.3	12
SRL 67	0.365	Normal	Normal	Normal	8.3	360	2.5	71	4	17.5	6.7
SRL 68	0.077	Normal	Normal	Normal	7.6	69	5.2	120	6	16.7	4.4
SRL 69	0.021	Abnormal	Abnormal	Normal	6.5	380	8.9	87	4	22	1.35
SRL 70	0.489	Normal	Normal	Normal	7.5	93	2.4	94	14	8.7	2.6
SRL 71	0.187	Normal	Normal	Normal	7.1	45	3.2	68	4	18.6	3.55
SRL 72	0.471	Normal	Normal	Normal	7.9	46	8.4	70	6	19.7	2.8
SRL 73	0.358	Normal	Normal	Normal	7.6	99	3.7	68	4	16.8	2.75
SRL 74	0.261	Normal	Normal	Normal	7.8	240	7.6	90	2	26	4.65
SRL 75	0.102	Normal	Normal	Normal	7.8	185	8.6	103	4	9.1	4.9
SRL 76	0.226	Normal	Normal	Normal	7.6	65	6.7	105	4	13.7	3.45
SRL 77	0.140	Normal	Normal	Normal	7.3	245	9.6	92	4	10.1	2.15
SRL 78	0.234	Normal	Normal	Normal	7.5	240	3.4	84	2	11.9	3.85
SRL 79	0.010	Normal	Normal	Normal	7.8	125	6.8	85	6	21.9	6.25
SRL 80	0.056	Normal	Normal	Normal	7.8	65	2.6	113	7	5.9	8.8
SRL 81	0.422	Normal	Normal	Normal	8.4	60	2.1	81	4	7.7	13.3

Sample ID	AFM1 in milk (ppb)	Colour	Odour	Consistency	pH	MBRT (sec.)	SAT (min.)	TVFA's (mEq/ml)	NH3-N (mg/dl)	TBC ($\times 10^9$)	TPC ($\times 10^5$)
SRL 82	0.520	Normal	Normal	Normal	7.6	65	7.6	99	8	5.9	8.85
SRL 83	0.019	Normal	Normal	Normal	7.6	65	7.3	91	4	18.7	11
SRL 84	0.008	Abnormal	Normal	Abnormal	8	135	8.6	134	4	35	10.3
SRL 85	0.010	Normal	Normal	Normal	8.4	98	8.7	77	4	19.4	5.5
SRL 86	0.019	Normal	Normal	Normal	7.3	75	7.6	71	6	22	19.4
SRL 87	0.010	Normal	Normal	Normal	6.4	50	7.2	98	6	17.5	13.9
SRL 88	0.008	Normal	Normal	Normal	6.4	114	2.4	126	4	32	22
SRL 89	0.490	Normal	Normal	Normal	6.9	80	3.2	67	4	20.4	8.3
SRL 90	0.212	Normal	Normal	Normal	6.6	43	4.8	83	4	10.4	3.75
SRL 91	0.372	Normal	Normal	Normal	6.8	60	6.9	88	4	6	5.2
SRL 92	0.394	Normal	Normal	Normal	6.4	100	7.8	108	18	12.5	2.35
SRL 93	0.012	Normal	Normal	Normal	6.1	95	2.1	110	2	28	10
SRL 94	0.236	Normal	Normal	Normal	7.5	106	7.5	72	4	20.5	0.4
SRL 95	0.143	Normal	Normal	Normal	7.7	145	2.6	71	8	10.2	0.4
SRL 96	0.257	Normal	Normal	Abnormal	7.4	175	7.5	117	6	3.5	15.8
SRL 97	0.078	Normal	Normal	Normal	7.8	190	2.5	96	2	6.7	12.3
SRL 98	0.118	Normal	Normal	Normal	7.7	250	2.6	81	2	13.7	14.3
SRL 99	0.311	Abnormal	Abnormal	Normal	8.1	250	6.5	80	4	13.4	12.65
SRL 100	0.089	Normal	Normal	Normal	7.8	225	7.4	78	4	19.5	12.3
SRL 101	0.396	Normal	Normal	Normal	7.8	310	7.6	121	4	0.67	9.35

Sample ID	AFM1 in milk (ppb)	Colour	Odour	Consistency	pH	MBRT (sec.)	SAT (min.)	TVFA's (mEq/ml)	NH3-N (mg/dl)	TBC ($\times 10^9$)	TPC ($\times 10^5$)
SRL 102	0.179	Normal	Normal	Normal	7.2	468	8.3	77	6	8.5	5.85
SRL 103	0.001	Normal	Normal	Normal	6.7	400	1.5	134	4	31.6	1.5
SRL 104	0.268	Normal	Normal	Normal	8.7	240	9.2	83	4	7	4.4
SRL 105	0.235	Normal	Normal	Normal	7.3	240	6	78	4	20.6	10.2
SRL 106	0.229	Normal	Normal	Normal	7.5	240	8.5	71	4	6.6	9.3
SRL 107	0.034	Normal	Normal	Normal	6.7	50	9.6	80	4	22	3
SRL 108	0.093	Normal	Normal	Normal	6.5	40	2.4	87	6	8	8
SRL 109	0.130	Normal	Normal	Normal	7	91	2.7	69	4	7.8	2.8
SRL 110	0.199	Normal	Normal	Normal	6.8	199	1.5	95	4	12	9.9
SRL 111	0.124	Abnormal	Abnormal	Abnormal	7.7	82	5.7	66	4	6.4	10.4
SRL 112	0.016	Normal	Normal	Normal	6.4	105	5.8	116	4	13	3.5
SRL 113	0.257	Normal	Normal	Normal	8.6	162	7.6	73	6	8.8	14
SRL 114	0.249	Normal	Normal	Normal	8.2	124	7.3	111	7	13	2.8
SRL 115	0.316	Normal	Normal	Normal	6.6	80	8	89	6	0.34	10
SRL 116	0.092	Normal	Normal	Normal	8.7	60	6.8	116	4	6	5.8
SRL 117	0.677	Normal	Normal	Normal	8.9	170	7.8	120	5	4.3	7.6
SRL 118	0.059	Normal	Normal	Normal	7.9	70	8	80	6	3.3	17.3
SRL 119	0.026	Normal	Normal	Normal	6.8	60	3	125	6	37	3.5
SRL 120	0.125	Abnormal	Normal	Normal	7.6	80	2.6	79	6	2.5	9.1

4.5.1 Rumen physico-chemical parameters:

4.5.1.1 Colour, odour and consistency

The colour, odour and consistency of rumen liquor were recorded immediately after the sample collection. The observations were grouped into 2 categories: normal and abnormal (Table 15). The normal group included, greenish-yellow to yellowish-brown colour, aromatic odour and slightly viscous consistency. The observations recorded other than normal were grouped under abnormal. On analysis, the data was found non suitable to deduct any significant association for AFM1 in milk samples with respect to colour, odour and consistency of the rumen fluid samples.

4.5.1.2 pH

The pH of rumen liquor samples was recorded immediately after collection of rumen liquor via digital pH meter (Table 15). The strength and direction of linear relationship between pH of rumen liquor samples and the AFM1 concentration in milk of respective bovines was analyzed using Pearson Correlation Coefficient (r). The correlation coefficient (r) between the two variables was found to be 0.384 with p-value of 0.000015 (statistically significant) which suggests that AFM1 excretion in milk and pH of rumen liquor are positively correlated (category: fair correlation) (Table 16).

The results of our study were in agreement with the Khodabandehloo *et al* (2019) who also reported an increase in pH of rumen liquor of rams by increasing the concentration of AFB1 in feed in an *in-vitro* study. In contrary to the results by Nidhina *et al* (2017) who reported that low ruminal pH favors aflatoxin production *in-vitro* and vice-versa. The increase in pH of rumen liquor of bovines with increasing AFM1 concentration in milk can be a result of decreasing TVFA's production (Khodabandehloo *et al* 2019 and Jiang *et al* 2020) and increasing ammonia nitrogen concentration (Khodabandehloo *et al* 2019, Jiang *et al* 2020) as a result of negative impact on the growth of rumen microflora and disruption of rumen fermentation (Fink-Gremmels 2008 and Jiang *et al* 2012) caused by increased aflatoxin concentration in rumen.

Table 16: Correlation of indicators of rumen fermentation with AFM1 in milk

	Parameters	Correlation coefficient (r)	p-value
Physico-chemical parameters	**pH**	**0.384** (positive fair correlation)	0.000015 (statistically significant)
	MBRT	**0.429** (positive fair correlation)	0.00001 (statistically significant)
	SAT	**0.312** (positive fair correlation)	0.0005 (statistically significant)
Rumen metabolites	**TVFA's**	**-0.249** (negative fair correlation)	0.006 (statistically significant)
	Ammonia nitrogen	**0.337** (positive fair correlation)	0.00017 (statistically significant)
Rumen microflora	**TBC**	**-0.429** (negative fair correlation)	0.00001 (statistically significant)
	TPC	**-0.137** (negative weak correlation)	0.136 (statistically non-significant)

4.5.1.3 Methylene Blue Reduction Time (MBRT)

The methylene blue reduction test was performed immediately with freshly collected rumen liquor samples and the data has been presented in Table 15. The strength and direction of linear relationship between MBRT of rumen liquor samples and the AFM1 concentration in milk of respective bovines was analyzed using Pearson Correlation Coefficient (r). The correlation coefficient (r) between the two variables was found to be 0.429 with p-value of <0.00001 (statistically significant) which suggests that AFM1 excretion in milk and MBRT of rumen liquor are positively correlated (category: fair correlation) (Table 16).

MBRT is the time required by the microbial population in rumen to reduce the colour imparted by the dye, therefore higher the microbial population or activity, shorter will be the reducing time and vice-versa. The previous studies (Fink-Gremmels 2008 and Jiang *et al* 2012) and the result of present study reported the inhibition of growth of rumen microbes with increasing AFB1 intake, therefore the

longer MBRT with high concentration of AFM1 in milk might be a result of the negative impact aflatoxins have on the rumen microflora.

4.5.1.4 Sedimentation Activity Time (SAT)

The SAT was recorded for rumen liquor samples immediately after collection and the data has been presented in Table 15. The strength and direction of linear relationship between the SAT of rumen liquor samples and the AFM1 concentration in milk of respective bovines was analyzed using Pearson Correlation Coefficient (r). The correlation coefficient (r) between the two variables was found to be 0.312 with p-value of 0.0005 (statistically significant) which suggests that AFM1 excretion in milk and SAT of rumen liquor are positively correlated (category: fair correlation) (Table 16).

The sedimentation activity time is a rapid evaluation of rumen microfloral activity essentially protozoal activity and an increase in SAT with increase in AFM1 excretion in milk of bovines can be due to the negative impact aflatoxin have on rumen bacterial and protozoal population (Fink-Gremmels 2008 and Jiang *et al* 2012).

4.5.2 Rumen metabolites:

4.5.2.1 Total Volatile Fatty Acids (TVFA's)

The TVFA's content (mEq) per ml of rumen liquor was determined from all the collected samples (Table 15). The correlation coefficient (r) between rumen liquor samples and the AFM1 concentration in milk was found to be -0.249 with p-value of 0.006 (statistically significant) which suggests that AFM1 excretion in milk of bovines and TVFA's content of rumen liquor are negatively correlated (category: fair correlation) (Table 16).

The findings of our study are in concordance with Mathur *et al* (1976), Jiang *et al* (2012), Khodabandehloo *et al* (2019) and Jiang *et al* (2020), which reported decrease in TVFA's concentration in rumen liquor on increasing AFB1 intake in diet. While Edrington *et al* (1994) found no differences in ruminal VFA concentrations in growing lambs fed 2.5 mg AFB1 per kg diet and Helferich *et al* (1986) also reported that AFB1 at 60–600 mg/kg did not influence the production of VFA in cattle. The decrease of volatile fatty acids production in rumen can be attributed to impaired fermentation of rumen due to decrease in population and activity of rumen microflora

as a result of high aflatoxin concentration in rumen (Fink-Gremmels 2008 and Jiang *et al* 2012).

4.5.2.2 Ammonia Nitrogen (NH_3-N)

The ammonia nitrogen content of rumen liquor samples was determined using Conway micro diffusion technique (Fig. 8) and the data has been presented in Table 15. The correlation coefficient (r) between the ammonia nitrogen content of rumen liquor samples and the AFM1 concentration in milk was found to be 0.337 with p-value of 0.00017 (statistically significant) which suggests that AFM1 excretion in milk and ammonia nitrogen content of rumen liquor are positively correlated (category: fair correlation) (Table 16).

The results of present study are in concordance with Khodabandehloo *et al* (2019), Jiang *et al* (2020). Both reported an increase in ammonia nitrogen concentration of rumen liquor on increasing AFB1 in diet of ruminants. On the contrary, Jiang *et al* (2012) reported ammonia nitrogen concentration declined with the increase in AFB1 dosage.

The increase in ammonia nitrogen concentration in rumen with increase in AFM1 excretion through milk of bovines could be due to the negative impact aflatoxin have on growth and activity of rumen microflora (Fink-Gremmels 2008 and Jiang *et al* 2012) which reduces the utilization of ammonia nitrogen by rumen microbes for bacterial protein synthesis in turn leading to rise in the ammonia nitrogen concentration of rumen. The increased ammonia nitrogen content raises the pH of rumen which further leads to increased denaturation of proteins and peptides into ammonia nitrogen.

4.5.3 Rumen microflora count:

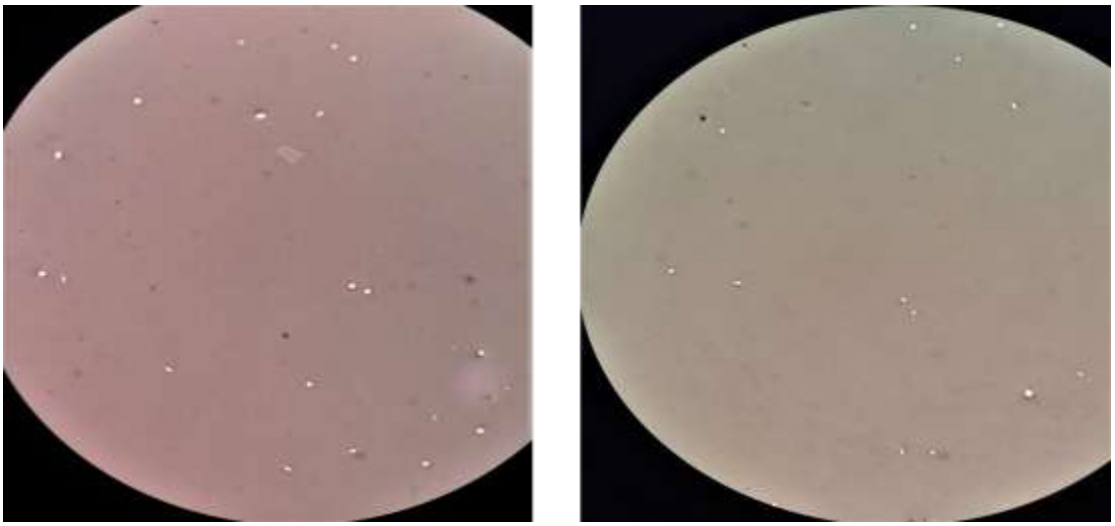
4.5.3.1 Total Bacterial Count (TBC)

The TBC of rumen liquor samples was determined by negative (background) nigrosin staining technique (Fig. 9). The data of TBC of all the rumen liquor samples has been presented in Table 15. The strength and direction of linear relationship between TBC of rumen liquor samples and the AFM1 concentration in milk of respective bovines was analyzed using Pearson Correlation Coefficient (r). The correlation coefficient (r) between the two variables was found to be -0.429 with p-value of < 0.00001 (statistically significant) which suggests that AFM1 excretion in

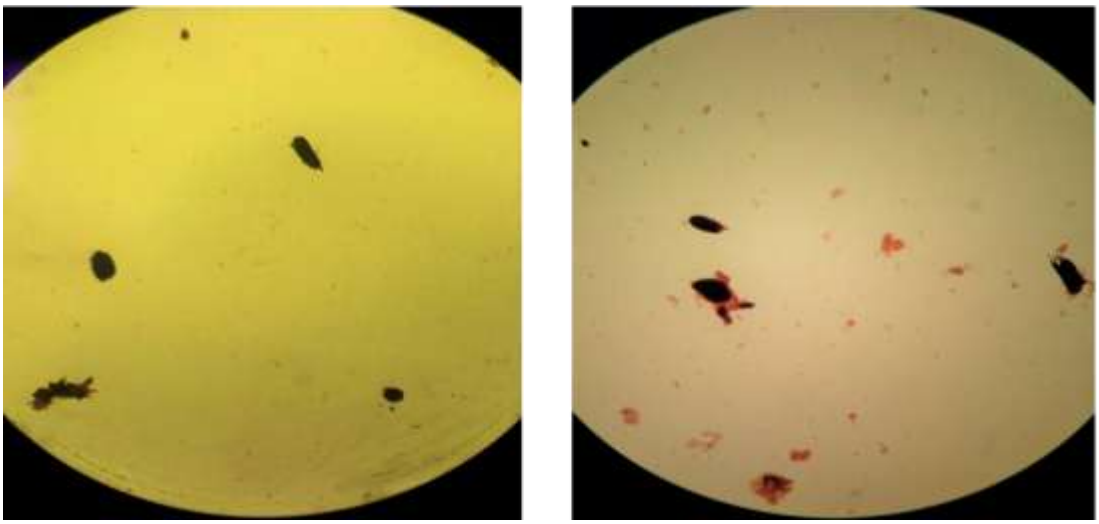
Inner compartment: 1 ml 2% boric acid solution + mixed indicator
Outer compartment: 1 ml SLR and 1 ml 50% K_2CO_3

After incubation and titration

Fig. 8: Determination of ammonia nitrogen concentration in rumen liquor by Conway diffusion method



Bacteria in rumen liquor negatively stained with nigrosine under 100x



Protozoa in rumen liquor stained brown with lugol's iodine under 10x

Fig. 9: Bacteria and Protozoa in rumen liquor visualized under microscope

milk and TBC of rumen liquor are negatively correlated (category: fair correlation) (Table 16).

The results of the present study are in agreement with Mathur *et al* (1976) who reported that AFB1 affected some rumen micro-organisms morphologically and physiologically and also inhibited their growth. Fink-Gremmels (2008) and Jiang *et al* (2012) also reported AFB1 has negative impact on rumen microflora. Further *in-vitro* and *in-vivo* studies required to get detailed knowledge on which type of bacterial population is inhibited and the associated inhibitory factors responsible for it.

4.5.3.2 Total Protozoal Count (TPC)

The TPC of rumen liquor samples were determined by staining protozoa with lugol's iodine (Fig. 9) and the data has been presented in Table 15. The strength and direction of linear relationship between TPC of rumen liquor samples and the AFM1 concentration in milk of respective bovines was analyzed using Pearson Correlation Coefficient (r). The correlation coefficient (r) between the two variables was found to be -0.137 with p-value of 0.136 (statistically non-significant) which suggests that AFM1 excretion in milk and TPC of rumen liquor are negatively correlated (weak correlation) (Table 16). However, Fink-Gremmels (2008) and Jiang *et al* (2012) also reported aflatoxin has negative impact on rumen microflora. Further experimental *in-vitro* and *in-vivo* studies are required to inspect the inhibitory factors of aflatoxin responsible for the effect.

CHAPTER V

SUMMARY AND CONCLUSIONS

Aflatoxins are mainly produced by *Aspergillus flavus* and *A. parasiticus*. They are the group of more than 20 related metabolites with AFB1, AFB2, AFG1 and AFG2 being naturally occurring. Milch animals upon consuming AFB1-contaminated feedstuffs excrete a lesser toxic form, i.e., AFM1 in milk upon metabolism in liver. Human exposure can happen either directly via consumption of infected food or indirectly via food of animal origin. Aflatoxins are established carcinogenic, hepatotoxic, genotoxic, immune-modulating, teratogenic and mutagenic compounds. The interaction of aflatoxins with ruminal microflora is known to degrade the toxins to some extent and convert them into a lesser or non-toxic form. Therefore, considering the above mentioned facts, the present study was designed to estimate the prevalence of AFM1 in milk of cattle and buffalos from all the 22 districts of Punjab and to identify any animal-specific or farm-specific risk factors associated with the presence of AFM1 in milk. For better understanding of interaction between rumen and aflatoxins, association between rumen liquor parameters and AFM1 excretion in milk has also been analyzed.

In the present study, a total of 402 milk samples were collected from cattle (n=266) and buffalo (n=136). The milk samples were analyzed for the presence of AFM1 using competitive ELISA method and representative samples were later confirmed by HPLC-FLD. For risk factor identification, a detailed questionnaire was used for recording of observations and subsequent analysis. The statistical analysis was carried out by using Epitools and SPSS software. Further, a total of 120 rumen liquor samples were collected from the study animals and their physico-chemical parameters, microflora count and rumen metabolites were analyzed in relation to AFM1 excretion in milk.

Evaluation of milk samples revealed the prevalence of AFM1 in Punjab to be 56.22% with respect to EC-MPL and 13.43% with respect to FSSAI-MPL. Of all the milk samples tested, 79.1% were found positive for AFM1, having concentration above 0.005 ppb. The AFM1 concentration in milk ranged from 0.005 to 6.832 ppb with the mean concentration of 0.269±0.07 ppb. All milk samples were found positive for AFM1 from Barnala, Faridkot, Hoshiarpur, Moga, Mohali and Rupnagar with the

lowest from Bathinda district (38%). Hoshiarpur (100%) and Moga (100%) had the highest number of samples above EC MPL whereas Muktsar (64.7%) and Moga (65%) had the highest number of samples above FSSAI MPL. With respect to mean concentration of AFM1 in milk, Ludhiana district of Punjab (0.89±1.47 ppb) was found most contaminated and Kapurthala (0.01±0.009 ppb) the least.

Species-wise evaluation of milk samples revealed the prevalence of AFM1 with respect to EC-MPL to be slightly higher in buffalo milk (57.35%) than in cattle milk (56.39%). With respect to mean concentration of AFM1 in milk, buffalo milk was found to be more contaminated than cattle milk. The buffalo milk had 16.91% of milk samples in the non-compliant category with respect to FSSAI MPL in comparison to 11.65% samples of cattle milk. The difference between the mean concentrations of AFM1 between species was found to be significant (p-value = 0.0001) in contrast to the difference between the prevalence which was found to be non-significant (p-value = 0.854).

The milk samples were also evaluated based on type of farm from which they were collected. Milk from commercial dairy farms was found to have 65.67% prevalence of AFM1 with mean concentration of 0.344±0.65 ppb as compared to 48.26% prevalence with mean concentration of 0.195±0.65 ppb in milk from dairy households with respect to EC MPL. The commercial dairy farms had 18.4% of milk samples in the non-compliant category with respect to FSSAI while dairy households had only 8.46%. Both the prevalence and mean concentration of AFM1 in milk from commercial dairy farms was found to be significantly higher than in milk from dairy households (Prevalence: chi-square test: p-value = 0.0004; Mean concentration: t-test: p-value = 0.009).

The univariable analysis of risk factors for presence of AFM1 in milk revealed above average per day milk yield (i.e., >13 kg) (OR: 2.45), below average age (i.e., ≤6 years) (OR: 2.29), animals in ≤3rd parity (OR: 1.45) and animal hygiene scores [score 2 (OR: 1.43), score 3 (OR: 2.33) and score 4 (OR: 2.06)] to be animal level risk factors for AFM1 concentration above EC-MPL in milk, whereas, intensive dairy farming system (OR: 4.18), marketed animal feed without aflatoxin binder (OR: 3.86) and self-cultivated feed (OR: 4.23), were found to farm level risk factors. The analysis of cattle breeds in relation to AFM1 excretion in milk showed non-descript (OR:

11.55), HF cross (OR: 4.3) and HF (OR: 3.91) breed to have higher excretion of AFM1 in milk in comparison to Sahiwal (OR: 1.51) and Jersey breed (OR: 1). Further multivariable analysis of the identified risk factors revealed the significant higher odds for the above average milk yield/day (>13 kg/day) (adjusted Odds Ratio: 2.432), higher animal hygiene scores (>1) (adjusted Odds Ratio: 1.94), intensive dairy farming system (adjusted Odds Ratio: 3.12) and animal feed (both marketed and self-cultivated) without aflatoxin binder (adjusted Odds Ratio: 4.67) as risk factors for AFM1 excretion in milk. Moreover, the increase in the age of the animal was observed as protective factor with adjusted Odds Ratio of 0.83 and the increase in parity was found to be non-significantly associated with AFM1 excretion (adjusted OR: 1.03; p-value 0.79).

The association of AFM1 excretion in milk of bovines with their indicators of rumen fermentation was analyzed by computation of the Pearson correlation coefficient (r). The analysis revealed that the pH (r = 0.384), MBRT (r = 0.429), SAT (r = 0.312) and ammonia nitrogen content (r = 0.337) of rumen liquor are positively correlated with AFM1 excretion in milk, in comparison to TVFA's content (r = -0.249), TBC (-0.429) and TPC (r = -0.137) of rumen liquor which were found to be negatively correlated.

Conclusions

- Overall, 56.22% (228/402) of samples exceeded the maximum permissible limit (MPL) set by the European Commission (EC), whereas, 13.43% (54/402) of samples exceeded the MPL set by Food Safety and Standards Authority of India (FSSAI).
- The AFM1 concentration in milk ranged from 0.005 to 6.832 ppb with the overall mean concentration ± standard deviation (SD) of 0.269±0.07 ppb.
- Of all the 22 districts of Punjab, Hoshiarpur (100%) and Moga (100%) districts of Punjab had the highest number of samples above EC-MPL for AFM1 in milk while Muktsar (64.7%) and Moga (65%) had the highest number of samples above FSSAI-MPL for AFM1 in milk. Milk from the Ludhiana district of Punjab had the highest mean concentration of AFM1 (0.89±1.47 ppb) and from Kapurthala, the lowest (0.01±0.009 ppb).

- Buffalo milk was found to be more contaminated with AFM1 than cattle milk both in terms of AFM1 prevalence and mean concentration.
- Milk from commercial dairy farms was found to be more contaminated with AFM1 than milk from household dairy establishments both in terms of AFM1 prevalence and mean concentration.
- On univariable analysis, high milk yield (i.e., >13 kg/day), below average age (i.e., ≤6 years), animals in ≤3rd parity and hygiene scores >1 were found to be risk factor at animal level, whereas, intensive dairy farming system and animal feed without aflatoxin binder were identified as farm level risk factors for AFM1 in milk. The analysis of cattle breeds in relation to AFM1 excretion in milk showed non-descript HF cross and HF breed to have higher excretion of AFM1 in milk in comparison to Sahiwal and Jersey breed.
- The multivariable analysis also identified the above average per day milk yield, higher animal hygiene scores (>1), intensive dairy farming system and animal feed without aflatoxin binder as risk factors for AFM1 concentration above EC-MPL in milk.
- The pH, MBRT, SAT and ammonia nitrogen content of rumen liquor increased while the TVFA's content, TBC and TPC of rumen liquor decreased with increasing concentration of AFM1 in milk of bovines, reflecting the negative impact aflatoxins have on rumen fermentation.

Recommendations

Taking into account the findings of the present study, the following recommendations were drawn:

- There is an urgent need to educate the dairy farmers through awareness campaigns about the sources and the risk factors related to presence of AFM1 in milk of bovines.
- A regular state-wide monitoring system is required for continuous detection of AFM1 in milk, so that high risk districts/areas can be appropriately addressed.
- Further extensive *in-vitro* and *in-vivo* studies are required for better understanding of complex interaction between rumen microflora and aflatoxins.

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APPENDIX

School of Public Health and Zoonoses

Guru Angad Dev Veterinary and Animal Sciences University

Performa for animal and farm related factors

Date of visit:						
District:						
Village:						
	Farm Id: **Sample ID:**					
Animal-specific factors	**Milk Yield** (kgs/day)					
	Age (in years)					
	Parity					
	Breed					
	Animal Hygiene Index* (details provided below)					
Farm-specific factors	**Type of farm** (Commercial/Household)					
	Source of feed (Marketed / self-cultivated feed with or without binder)					
	Farming System (Intensive/Semi-intensive/Extensive)					
	Feed Storage Conditions (Open/Covered)					

***Animal Hygiene Index:** The animal hygiene indexing was carried out as described by Cook (2002). The method charts the soiling and manure contamination in three main areas: lower leg (rear only), udder and the upper leg (rear only), and flank. All study animals were scored on a scale of 1 to 4 during sample collection, lower scores being indicative of cleaner body regions and vice-versa. The details of the hygiene scoring indexes have been provided below.

Details of hygiene score index

Animal Hygiene Index	Body regions		
	Lower leg	**Udder**	**Upper leg**
1	Little or no manure above the coronary band	No manure present	No manure present
2	Minor splashing of manure above the coronary band	Minor splashing of manure near the teats	Minor splashing of manure
3	Distinct plaques of manure above the coronary band	Distinct plaques of manure on the lower half of udder	Distinct plaques of manure with hair showing through
4	Solid plaque of manure extending high up the leg	Confluent plaques of manure encrusted on and around the teats	Confluent plaques of manure

Dyes used during experiments

1. **Lugol' iodine**

Iodine	5 gm
Potassium iodide	10 gm
Glycerol	30 ml
Formalin	10 ml
Distilled Water	to make 100 ml

2. **Nigrosin dye**

Nigrosin	5 gm
Distilled water	20 ml
Methanol	80 ml

VITA

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