Chapter 13

Insights into the Interaction of Milk and Dairy Proteins with Aflatoxin M_1

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Additional information is available at the end of the chapter

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Abstract

In this chapter, up-to-date data regarding the nature of protein interaction with a contaminant such as aflatoxin M_1 (AFM_1) is detailed. Considering that AFM_1 is a relevant toxin present in milk and dairy products, it is important to understand such interaction. With this in mind, some specific features of protein chemistry and structure are discussed. AFM_1 presence and origin in milk and the latest approaches in AFM_1 chemical analysis with special attention to sample preparation techniques to eliminate milk protein–AFM_1 interaction will also be addressed. Emphasis will be given to the interaction of AFM_1 with whey proteins of which little has been described. In order to represent such interactions, recent scientific evidence is briefly discussed which describes the outcome, stability, and distribution of the toxin among the fractions, especially during the cheese-making process. An *in silico* model is presented in which some details of the AFM_1-protein interactions are described. Finally, two technological applications of proteins in the food industry which are affected negatively by AFM_1 contamination, are provided as an example of how the contaminant has a deep relationship in protein behaviour.

Keywords: aflatoxin M_1, whey, milk, dairy, products, protein interaction

1. Introduction

1.1. Milk structure

Milk is a rather complex biological fluid that includes components such as fats, proteins, and many other constituents; this means that multiple chemical equilibriums are at work within the liquid. Proteins throw additional complexities to the mix as they can exhibit amphoteric and

zwitterion behaviour. Each interaction established by or among proteins may be mediated by hydrophobic pockets, multiple ionic bridging, aromatic ring staking, and henceforth.

Casein, a major phosphoprotein found in milk is composed of several casein fractions that are distinguished by electrophoresis and are designated as α-, β-, and k-caseins (in order of decreasing mobility at pH 7.0). In bovine milk, casein composition, in mg mL^{-1}, consists approximately as follows: αs_1 (12–15); αs_2 (3–4); β (9–11); and k (2–4) [1, 2]. These fractions vary in molecular weight, isoelectric point, and level of phosphorylation. It is important to keep in mind these structural features since during milk clotting, k-casein is the fraction involved directly in enzymatic cleavage by chymosin during the cheese making process [3].

For the following discussion, the composition of major milk proteins will be fundamental. In cow milk, for example, the protein composition during seasons vary between 33.8–34.8, 31.2–32.6, 15.5–16.2, 2.0–2.1, 9.6–9.9, and 5.6–6.5 g protein/100 g total protein for α-, β-, and k-casein, α-lactalbumin, β-lactoglobuin, and other proteins (e.g. bovine serum albumin and lactoferrin) [4].

Casein micelles are spherical in shape, with a diameter ranging from 50 to 500 nm (average ca. 120 nm). The more recent model for casein micelle structure is the 'dual-bonding' model suggested by Horne [5–9]. This model proposes that micelle structure is governed by a balance of hydrophobic interactions and colloidal calcium phosphate-mediated cross-linking of hydrophilic regions. This micelle is stabilized by k-casein providing both steric and electrostatic repulsion.

The hydrofilic fraction of milk includes several components, mostly sugars (i. e. lactose, unique to milk/dairy products) and water-soluble proteins (whey proteins). Lactose is a disaccharide composed of a molecule of galactose and glucose connected through a $\beta(1\rightarrow4)$ glycosidic bond. Lactose is the primary osmotic component of the milk whose principal biological function is regulating milk secretion. Hence, lactose is the steadiest component in milk, averaging 4.6%, and is unique to this biological fluid [10]. Whey proteins include α-lactalbumin, β-lactoglobulin, blood serum albumin, and immunoglobulins and account for 17% of proteins. Milk proteins also contain fat globule membrane proteins and a large variety of enzymes and hormones [11]. An extensive analysis of the chemistry and biochemistry involved in dairy foods is in Fox and co-workers' book [12]. Additional structural information regarding milk protein is to be discussed later in the chapter.

1.2. Aflatoxin M_1 in dairy products

Aflatoxins are bis-furanocoumarin secondary metabolites produced by some species of filamentous fungi under specific conditions. Fungi that are capable of producing these contaminants are ubiquitous in the environment and have been identified in a wide array of foods (especially cereals and grains) including animal feeds (commodities in which aflatoxins are heavily regulated). Aflatoxins such as B_1 and G_1 are produced mainly by the *Aspergillus* species *A. parasiticus*, *A. flavus*, and *A. nomius* [13, 14] using a biosynthetic route shared with norsolorinic acid [15]. Production of these toxins is favoured in tropical and subtropical climates [16, 17] both before harvest and during storage. These toxins exert negative effects in

humans and animals including reproductive, immunological, and weight gain issues, among several others. It has already been established that these compounds are carcinogens and teratogenic [16, 18].

In dairy products, the most relevant toxins are AFM_1 and M_2 which are hydroxylated metabolites of aflatoxin AFB_1 and B_2, respectively. For example, dairy cows upon ingestion of contaminated feed discard these metabolites (generated during cytochrome P_{450}-mediated detoxification) through urine, faeces, and milk [16, 19]. The toxin is secreted into milk, with an elapsed time of about 12 hours and a peak time of approximately 24 hours. Moreover, Veldman and co-workers [20] have demonstrated that B_1 to M_1 toxin carryover is proportional to the milk yield and high throughput cows (i.e. > 25 kg day^{-1}) and was estimated to be 2.66 ± 1.24%. However, a more recent analysis has estimated carryover as high as 6.2% [21].

AFM_1 is a relatively small molecule (328.3 g mol^{-1}) which exhibits slight affinity towards water (10–30 μg mL^{-1}); hence in milk, it is partitioned into the water and cream parts of milk. It also can be bound to milk proteins [22]. In the case of AFM_1 in milk, one may expect that no homogeneous distribution will be encountered. Considering the semipolar characteristics of AFM_1, a strong relationship between casein and the toxin may be expected. In fact, appraisals have estimated that 30% of AFM_1 is associated with milk non-fat solids. Milk processing usually has a dramatic effect on AFM_1 concentration. Enrichment of the non-fat solid portion with AFM_1 usually results from fat separation. For example, buttermilk usually retains higher concentrations of toxin when prepared from naturally contaminated cream, a similar situation occurs during skim-milk manufacturing. Though some reports may contradict one another, in general, no AFM_1 reduction has been found when preparing other dairy products like cheese and yogurt.

Cheese processing usually results in the accumulation of AFM_1 in the curd; this is especially the case for matured cheeses. It has been suggested that this phenomenon is due to AFM_1-protein interactions (e.g., with casein the major protein present in cheese [96 g casein/100 g cheese]). However, contrasting data has been offered towards the contamination of the whey fraction. This is relevant since whey products (previously considered just a by-product of cheese manufacturing) have found important applications in the food industry.

All these data hints toward a profound processing and technological effect on the structure and composition of dairy products (e.g. temperature may influence immunoglobulins, which are related to the behaviour of fat globules and casein micelles) and intricate the interactions between AFM_1 and other dairy components, including whey proteins, further still [23]. Structural modifications also occur to milk when industrial processing takes place here; milk fat globules are reduced in size (< 1 μm diameter; [24]) and are dispersed uniformly through the rest of the fluid, preventing creaming of the milk. Other relevant factors responsible for differential distribution are the degree of milk contamination, milk quality, and cheese-processing techniques, extraction technique, methodology, and expression of the results. These interactions are discussed below in more detail.

Toxin elimination from food and feed is problematic as these compounds have shown to be thermally stable [25, 26] and may prevail in foods for long periods of time [27, 28]. For example,

AFM_1 is not deactivated during unit operations common to milk processing such as pasteurization or UHT treatment [29] and it is found in dairy products and have been reported to concentrate in cheese [30].

2. Recent advances in aflatoxin M_1 determination in milk and dairy products

Due to its carcinogenic potential, contamination of milk and dairy products with AFM_1 may generate risk to humans and animals. Hence, it is considered as a public health concern. Considerable efforts have been made towards evaluating concentrations of AFM_1 in dairy products. Complex matrices (especially those with high protein and lipid concentrations) make mycotoxin screening methods difficult, and this matrix effect must be overcome. Method for AFM_1 quantification must be able to eliminate matrix interferences and determine AFM_1 accurately. Some standardized methods as ISO 14501:207 (IDF 171:2007) [31] and AOAC 2000.08 [32] have been issued using modern quantification techniques and considering several paramount aspects of matrix interference. To eliminate proteins and lipids during the usual chromatographic method developing process, analysts must resort to sample pre-treatment procedures [33]. Conventional methods to achieve adequate sample treatment include, among the most popular, high-speed centrifugation, liquid-liquid extraction, solid phase extraction, and immunoaffinity separation [34].

However, methods for other more complex milk products are still lacking. This void has been filled in recent years by research involving chemical approaches and improvements in sample pre-treatment techniques. Methods for the determination of AFM_1 in matrices other than milk such as butter, yogurt, cheese, and sour cream have also been developed (**Figure 1**) [30, 35, 36].

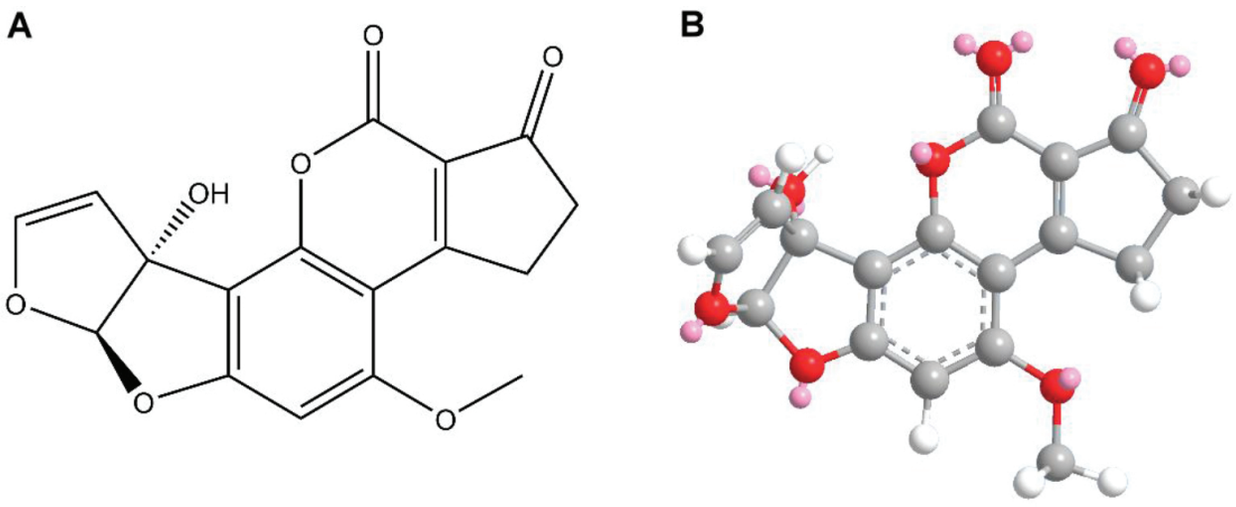

Figure 1. A 2D and B 3D rendering of the aflatoxin M_1 structure. 3D structure minimized energy using MM2 calculations (total energy of 46.7868 kcal mol^{-1}); pink-coloured beads represent non-bonding electron pairs.

2.1. Magnetic nanoparticles

Recently, modified magnetic nanoparticles coated with 3-(trimethoxysilyl)-1-propanthiol and modified with ethylene glycol bis(mercaptoacetate) were used as sorbent [37]. In this method very good results were reported which resulted in a novel, very specific, sensitive method for the determination of AFM_1 in liquid milk. Since authors Hashemi and Taherimaslaka reported that magnetic particles may be reused, and considering that is a relative inexpensive sample preparation treatment, a modified version of this approach may very well, in the future, be suited as a food technology to remove AFM_1 from milk in bulk. However, practical costs may hinder a future application.

In this case, the sorbent includes two thiol and carbonyl groups [37]. Thus, it can be concluded that electrostatic interactions through S and O atoms (respectively) are responsible for the interaction with the lactone ring and –OH group in furan ring of AFM_1. This type of interaction has also been reported to be responsible for the adsorption of AFB_1 by clays. Said interactions seem to be sufficiently strong to disrupt AFM_1-matrix interactions. Evidence from the interactions that result from AFM_1 and the sorbent ligands may be used as clues to help elucidate possible interactions of amino acid residues and AFM_1 within proteins. For example, one may hypothesize that residues such as Trp/Phe, Cys/CY2, Met, and Asp/Asn or Glu/Gln, through an aromatic ring, sulfur groups, and carboxylic acids, respectively, are responsible for interacting with AFM_1.

2.2. Chemically-based sorbents

A recent report has described an automated analysis of aflatoxin M_1 in milk and other dairy products using tandem mass detection. In this case, two liquid-liquid sequential extractions followed by a solid phase extraction were applied before HPLC analysis. Acetonitrile and salt were used both as a solvent system and protein denaturing/precipitation agents. In this case, Campone and coworkers [38] use an additional cleaning step using 10 mL/100 mL aqueous methanol before introducing the sample in C18 cartridges. Organic phase dilution is accomplished by adding water to the extract containing the analyte; this is a very common practice especially when using SPE/IAC columns. This practice usually results in particle agglutination in the solution when the water is added; this hinders the extraction process as the SPE/IAC pores are obstructed. The extraction steps carried out, achieve signals with no significant matrix interference and limits of detection that are below those required by the EU in milk and dairy products (EU Regulation 1881/2006) [38].

Another recent assay used Oasis® HLB hydrophilic balance, to extract AFM_1 with excellent recoveries (92.8%; [39]). Previously, similar results were obtained for powdered milk but using fluorescent analysis instead of mass detection [40]. These specific types of sorbents are based on a copolymer which exploits both in the π-π interaction of divinylbenzene and hydrophilic characteristics of *N*-vinylpyrrolidone, is a water-wettable polymeric sorbent stable at pH from 1 to 14 (http://www.waters.com. Accessed 09 Jan 2016). This sorbent has found a widespread application for biological sample pre-treatment because it prevents access to matrix components, such as proteins. However, in order to obtain good results, pH and extraction solution composition must be carefully adjusted. Unequivocally, pH plays a pivotal

role here as protein tridimensional structure is dramatically affected by the hydronium ion activity in specific media. In this particular research, the results indicated that the best signal was obtained at pH 5.0 of crude extraction solution with 100% methanol as the eluting solution [39]. Structural differences and principles between several sorbents based technologies may be found in the paper by Boonjob and co-workers [41].

2.3. Molecular imprinted polymers

Lately, another methodological novelty in using molecularly imprinted polymers has emerged; some toxins (e.g. EASIMIP™ Patulin, R-Biopharm, Darmstadt, Germany) are already commercially extracted through molecularly imprinted polymers as column sorbents. These sorbents are tailor-made structures with an encoded selectivity toward a given analyte [42]. Using AFB_1 as a template and a methacrylate moiety as an electron acceptor, Wei and coworkers suggested that the extraction mechanism involved all the oxygen atoms in the AFM_1 molecule [43]. This evidence is another possible mechanism of interaction. Despite AFM_1 being a relatively simple molecule, it exhibits multiple interaction sites; this is further aided by the fact that a slight torsion is expected in the furan ring when considering a spatial 3D/MM2 minimized structure model. Moreover, Wei and coworkers also indicated that matrix compound removal was more efficient using the sorbent that has immunoaffinity-based counterpart. Despite the advantages in terms of analyte specificity of immunoaffinity clean-up based approach for quantification of AFM_1 in dairy products, this technology is not without some drawbacks. Toxin adsorption may be limited due to antibody interferences of other matrix compounds, or extraction may be hindered by the interaction of the toxin with matrix components [44]. In fact, chemical based solid phase extraction sorbents may have an advantage over immunoaffinity column in terms of interacting with matrix components due to steric hindrance due to the space occupied by the antibodies used to prepare the latter.

Hence, the interaction of AFM_1 with proteins and other matrix components has demonstrated to have analytical consequences. More recently, other methods have used a more direct approach to circumvent poor analytical performance parameters (e.g. improve unsuitable limits of detection, screen interfering matrix co-eluents, reduce or eliminate undesirable interactions with matrix components, and mend methods with low recoveries) to unequivocally identify and determine the exact concentrations of this contaminant which is found in every level in milk products.

2.4. Enzymatic-based approaches

When using a commercial immunoaffinity column during a diagnosis for the determination of AFM_1 in three dairy products, relatively low recoveries have been observed when analyzing fresh cheese samples and sour cream [45]. Interestingly, no such effect was seen in ready-to-consume fluidized milk, and this may respond to a technological advancement in immunological sorbents (toxin-specific antibodies coupled to gel particles). Considering that recoveries in cheese and sour cream were lower than expected using a direct extraction with methanol approach and in an effort to reduce the amounts of organic solvents (specially chlorinated solvents) used during the toxin extraction process, our research group hypothe-

sized that dairy proteins and fats played a relevant role in sequestering AFM_1, hence limiting its contact with the extraction solvent. This is supported by the fact that analytical recoveries increase dramatically when proteolytic and lipolytic enzymes are added as part of sample treatment previous to the extraction mixture [45]. Later on, Pietri and coworkers [46] used pepsin digestion in a similar fashion, corroborating Chavarría and coworkers recent findings. In some degree these results also support, at least indirectly, that proteins derived from dairy products, such as α-lactalbumin/β-lactoglobulin and casein, indeed interact with AFM_1.

2.5. Outlooks

Methodological approaches when determining AFM_1 are of relevance since matrix components interfere with the extraction and recovery of the toxin, in particular for fluid milk (and foods derived thereof) since it is a rather complex biological fluid. Considering the nature of the samples tested using the methods stated before, is not surprising that analytical extraction must require stronger conditions when treating other dairy products other than milk. In this regard, for example, Holstein and Jersey's milk (*Bos taurus*) contain, in average, 3.5 to 5.5 and 3.1 to 3.9 fat and protein, respectively. However, when considering the cheese product, even a tenfold increase in concentration may be expected both in fat and protein, in the case of sour cream, up to a fivefold increase in fat may be expected, retaining similar concentrations in protein. Methods which rely on techniques that involve direct surface interaction with the toxin during sample preparation steps (or during measurement) interaction with the toxin, may find hindrance in matrix components of milk and other dairy products during analysis. For example, caseins are known as surface blocking agents. This may be especially important in methods using biosensors (e.g. the recently applied immuno-chip technology [47] or electrochemical immunosensor [48], direct spore adsorption [49] or lateral flow immunoassay [50]). An excellent review by Adami and co-workers [51] considers sample preparation techniques prior to in depth analysis.

3. Evidence of AFM_1 association with milk proteins

3.1. Experimental indications

Though the results of several investigations have showed a close relationship of AFM_1 to casein [28, 52], a lot of research reinforce the fact that a significant part of toxin concentration lose out through whey, a fact which is especially interesting considering the poor solubility of AFM_1 in water [22]. Hence, its retention or transport along the cheese/whey fraction may be assisted by other molecules, such as proteins, using non-covalent interactions.

The earlier report revolves around the interaction of AFM_1 with dairy proteins such as casein was performed by Brackett and Marth [53]. Therein, a simulated milk ultrafiltrate with casein micelles or containing AFM_1, was dialyzed against each other, and more AFM_1 was found in the casein-containing solution. Furthermore, AFM_1 contaminated milk treated with proteolytic enzymes demonstrated that higher concentrations of the toxin were found after the

enzymatic treatment when compared with the control, which in turn suggested AFM_1 binding by milk proteins. The affinity of AFM_1 towards dairy protein fractions was firstly investigated in depth by Govaris and co-workers [54] which described the distribution of AFM_1 between whey and curd during cheese manufacturing [54]. Their results showed that 40–60% of the total AFM_1 amount was retained in the whey fraction. In this particular case, Govaris and co-workers studied Teleme cheese which is a soft cheese with 50.3–57.2 moisture, 15.2–17.7 protein, and 42–43 fat-in-dry matter, in g/100 g. To be able to compare such results with data obtained for other types of cheese, manufacturing and processing of Teleme cheese must be accounted for, a detailed report can be found in a chapter written by Pappa and Zerfiridis [55]. Other researchers since then have corroborated these results obtaining similar data [54, 56, 57–60].

Other results demonstrated that the highest concentration of the toxin is found in the curd [57–59, 61, 62]. According to some authors [57, 62], ca. 60% of the AFM_1 is found in the curd. Kamkar et al. found an increase of threefold of the content of AFM_1 in curd versus that found in whey. Since usually higher concentration of AFM_1 are found in cheese [56, 61], concentrations of the contaminant can be considered as cheese is produced from several litres of milk.

Recently obtained data, gathered during the manufacture of fresh Turrialba cheese (a very distinct and recognizable Costa Rican cheese with < 55 moisture, ≥ 14,5 protein, ≥ 18,5 fat-in-dry matter, acidity 0.1-0.3 and 1.5-2 salt, in g/100 g), indicate that whey proteins have a much more affinity towards AFM_1 than casein. An *in vitro* assay using exact quantities of both AFM_1 and proteins showed that solutions of α-lactalbumin and casein require concentrations of 2.5 g/100 mL and 7.5 g/100 mL, respectively, to obtain trivial concentrations of AFM_1 on the supernatant [63]. Added evidence demonstrated that not only do some whey proteins exhibit an association capacity towards AFM_1 similar to that of casein but also that each protein is bound to AFM_1 at different ratio. Furthermore, we have also demonstrated a similar behaviour among casein fractions. In decreasing order of association or affinity towards AFM_1: $alpha_s$ (100%), beta (54.5%), and kappa casein (21.4%) [63]. This last result is interesting since the major biochemical modification suffered by casein during cheese clotting during its processing is an enzymatic hydrolysis of the kappa fraction. On the other hand, highly unspecific proteolytic enzymes (e.g., pepsin) can reduce AFM_1-protein interactions. A protease such as the one used during our survey [45] can hydrolyse ≥ 70% of casein to amino acids and has demonstrated a more efficient cleavage of casein than other proteases such as trypsin or chymotrypsin. Such data may explain Barbiroli and co-workers [64] results which indicated that no changes in AFM_1 concentration were observed during the enzymatic hydrolysis that occurred during the cheese-making process.

Evidence of AFM_1 binding suggests another application of purified whey proteins as modulator of toxicity toward animals and may hold potential for protecting animals against AFM_1toxigenic potential and to minimize the possibility of this or other toxic metabolites to reach the human food supply. When naturally contaminated milk is treated to obtain processed foods, a significant fraction of the whey may be contaminated with AFM_1. Later, it is important to keep in mind that whey and related components (due to known nutritional value and

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health benefits related to consumption) already have several commercial uses as a dietary in-feed supplement, nutritional supplement, and whey-based beverages.

In the same regard, Cattaneo and coworkers recently demonstrated that during ricotta cheese (a whey based product) production, AFM_1 retained ca. 6% of its initial concentration and the remainder is lost in the liquid portion [65]. The same authors showed some insights toward interaction of AFM_1 and whey proteins, supporting our findings [63]. For example, the authors demonstrate that during a simple ultrafiltration step, at least 60–80% of the toxin is lost in permeate and that technological processes such as spray-drying do reduce AFM_1 contamination levels in pulverized milk solids despite substantial loss of water.

3.2. An *in silico* analysis to estimate AFM_1-protein interaction

In addition to the direct evidence of the AFM_1 molecular interactions that occur during its association with proteins, an *in silico* analysis shows several interesting features regarding this phenomenon. When a MM2 energy optimized molecule of AFM_1 is docked (cluster typed as small molecule-protein, with a clustering RMSD of 4.0) with β-lactoglobulin (1BEB) and α-lactalbumin (1CJ5), both from *Bos taurus*, the result is several simulations with high scores. Such models demonstrate that a considerable number of protein sites are capable — at least theoretically— to bind ound AFM_1. Hence, a single protein molecule could bind a large number of AFM_1 molecules; this seems to be in agreement with the macroscopic behavior of AFM_1 when associated with dairy proteins. For example, the highest score prediction for α-lactalbumin (a calcium-binding lysozyme-like protein, [66]) occurs in the pocket amongst two β turns and a β sheet, that are, 161 His and 155 Gln/158 Glu coil and the 61 Trp and 151 Phe β strands (**Figure 2**).

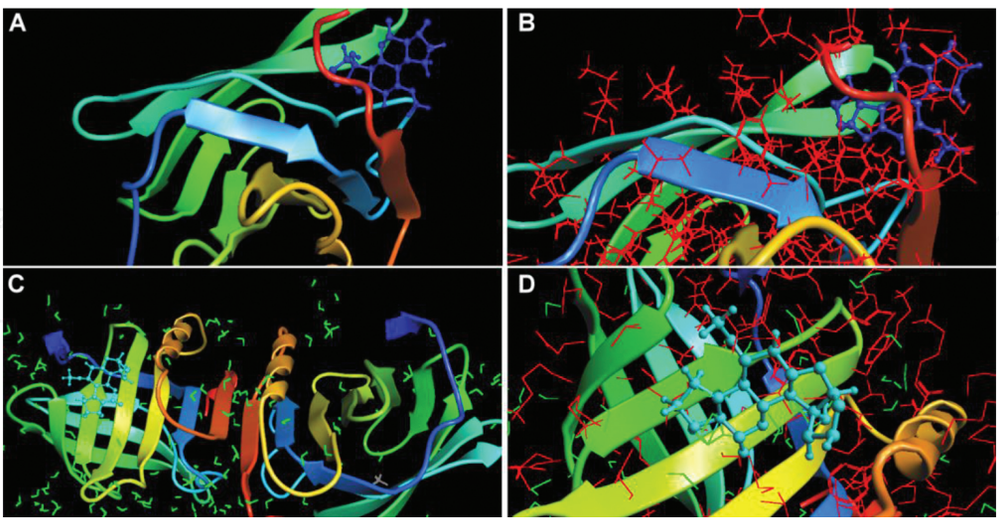

Figure 2. Depiction of possible interactions between AFM_1 and A,B α-lactalbumin or C,D β-lactoglobulin, only the with the highest score is shown. Panels A and C show the spatial interaction of the molecule with the protein (ribbon cartoon representation). Panels B and D demonstrate amino acid residues, represented in red. In all panels, AFM_1 is illustrated in dark blue/cyan using ball and stick models. In panels C and D water molecules are represented using green wireframes [67].

The case of β-lactoglobulin — composed of two subunits under physiological conditions — is more complicated. β-Lactoglobulin is a major whey protein of ruminant species and has been found in milk from other species. Its amino acid sequence and three-dimensional structure show that it is a lipocalin. The more energy stable prediction situates the AFM_1 molecule between the 5 Gln, 6 Thr, 177 Leu β strands and the 144 Pro coil of the A chain. But other interactions sites are possible. For example, another prediction may occur in the hydrophobic crevasse between the two α-loops. A detailed look on the tridimensional structure of β-lactoglobulin may be found in a work written by Sawyer [68].

Needless to say, this is a rough prediction considering the complexity of the interactions that may occur during AFM_1-protein interaction within a complex matrix such as milk. For example, casein is found in a micellar structure behaviour which, besides considering calcium ions and hydrophobic interactions that held together, such micelles must bear in mind ligand competition (dominated by K_f) and other substrates (e.g. citric acid). Protein-protein and protein-water interactions also play a distinct role in the association with AFM_1. Interestingly, the binding capabilities of β-lactoglobulin have been described previously with other ligands (i.e. SDS and lipophilic molecules such as retinol, cholesterol, palmitate, and vitamin D_2) through a central binding cavity [69]. One relevant feature that distinguishes β-lactoglobulin from α-lactalbumin is that, in pure form, the latter will not form gels upon denaturation because no free thiol groups are available as starting-point for a covalent aggregation reaction. Noteworthy, α-Lactalbumin surpass β-lactoglobulin in affinity towards AFM_1 [63], such structural differences may explain the dissimilarities described here-in.

The interaction abilities of proteins with small molecules, such as AFM_1, must be influenced by several factors such as water absorption, protein concentration, pH, ionic strength, temperature, and the presence of other components of feed or food (e.g. saccharides, lipids and salts, rate and length of heat treatment, and conditions of storage). For example, whey proteins are usually more stable towards pH changes but sensitive to heat.

4. Aflatoxin distribution dependence in dairy processing

4.1. Evidence through cheese manufacturing

The interaction between AFM_1 and casein has been described more in detail above. However, evidence indicates that, during cheese production, part of the AFM_1 concentration is exuded with whey. Nevertheless, up to this date, little information is available regarding the possible interaction of AFM1 with the proteins that remain in this aqueous fraction. Results of several investigations on the stability of AFM_1 throughout cheese making and cheese ripening reported increase in AFM_1 concentration in cheese, as a function of cheese type, technologies applied, and the amount of water eliminated during processing [30].

Some authors showed high AFM_1 concentration in curd regardless of the cheese-making procedures employed [59, 62]. However, other evidence suggests that the retained AFM_1 fraction by casein or other dairy proteins is highly dependent on the manufacturing process

of the cheese, for example, Cavallarin and coworkers demonstrated a direct correlation between AFM_1 found in milk and the levels later found in cheese [70]. A particular relationship was attained for each cheese which reinforced that manufacturing process and chemical composition of each cheese impacts the retention of AFM_1. In their report, Cavallarin and co-workers discussed three different type of cheese (i.e. Robiola and Primosale, two kinds of fresh cheese, and Maccagno, a matured cheese); the distribution and fate of AFM_1 was investigated preparing these cheese type using naturally and artificially contaminated milk at three different concentration levels. The authors reported that concentration factors for fresh and matured cheese were of 1.42/2.20 and 6.71, respectively. Our data regarding a survey [45] in milk and fresh cheese indicate that contrary to other cases, Turrialba cheese exhibited consistently lower average concentrations of AFM_1 when compared with those found in milk. Such data suggest that fresh or whey-based cheese (which sustains much less treatment than matured ones) is less prone in concentrating AFM_1.

In protein chemistry, physicochemical factors such as pH, temperature, and ion concentration play a significant role in protein behavior. The collective action of thermal processing and pH can denature dairy proteins to such extent that they may be able to lose AFM_1 binding capacity [64], agreeing with our current results [63]. Hence, lower pH in the water portion or the final product may explain why in certain cheese types a differential partitioning is observed. For example, lower AFM_1 concentrations may be found in whey fraction when comparing different processing treatments [70].

In a similar fashion, Piera Cattaneo and coworkers [65] demonstrated that AFM_1 retention and distribution in Ricotta cheese is also dependent on processing and manufacturing. For example, ultrafiltration and spray-drying can contribute to reducing, in a significant manner, AFM_1 concentrations from contaminated whey. The latter fact contributes to our understanding of AFM_1 regarding technological processes and unit operations especially since the toxin is thermally stable.

In 2013, Motawee contended several points concerning Domiati cheese manufacturing. First, confirming other data, pasteurization reduced < 10% of the AFM_1; second, salting seems to affect the retention of AFM_1 in curd; third after a 90-day preservation of cheese, the initial concentration of $AFM_1$was reduced significantly [71]. Concordantly, findings in fresh Costa Rican cheese evidenced as high as 88.3% of AFM_1 reduction during storage in a 28-day period. However, even at 4°C, Turrialba cheese exhibited signs of spoilage after one month storage [63]. In contrast, Deveci [57] already demonstrated using White Pickled cheese that AFM_1 remains for up to three months of storage in brine, and that approximately 31% of the AFM_1 passed to whey, while only about 3% distributes to the brine during ripening. Comparing this last result to other similar data seems to suggest that salting may prevent the subsequent loss during cheese storage possibly due to an increase in ionic strength and disruption of the interaction between toxin and protein.

In the same regard, Fernandes and co-workers [72] found that during Minas Frescal cheese manufacturing, there was no effect of storage time on AFM_1. They also stated that starter culture in cheese did not influence the concentration or stability of AFM_1.

On the other hand, other dairy products have been researched, for example, Iha and coworkers [73] found that yogurt processing and storage had a marginal effect on AFM_1 concentration and that the total AFM_1 content in cheese and whey decreased approximately 25%. The authors found an increase of AFM_1 in cheese of 1.9-fold, but a decrement of 0.4-fold in whey, based on the initial aflatoxin present in the milk used for cheese manufacturing. This is an interesting result, since other research groups had established that lactic acid bacteria in dairy products has the capability of AFM_1 decontamination [74]. A very thorough review of other methods of milk detoxification has been performed recently by Giovatti and coworkers [75].

4.2. AFM_1 interaction with probiotic structures

As organic solvents have the ability to release aflatoxin from binding to bacterial cell walls and peptidoglycan structural components, Haskard and coworkers have suggested that hydrophobic interactions play a significant role in the binding mechanism [76]. Reversible and irreversible binding are phenomena governing aflatoxin union to bacterial and yeast binding. Reversible unions seem to suggest non-covalent interactions between aflatoxin and hydrophobic pockets on the bacterial surface [77]. Further studies demonstrated that other different lactic acid strains can bind and remove AFB_1 in liquid media. Furthermore, said interaction behaves in a concentration-dependent manner [78, 79]. The mechanism for probiotic structure-AFM_1 interaction may also help understand the process in which such toxins interact with macro/biomolecules.

Though the exact mechanism is still unclear, our data seems to reinforce other publications which have found decontamination capabilities specifically in cheese [80] as well in other matrices [73, 80, 81]. Previous reports [79] have already documented the interaction between teichoic acids and other bacterial cell wall components by probiotic bacteria and yeasts [82]. Interestingly, an empirical observation also seems to indicate that as the lactic acid bacteria counts increases with time, more difficulties in cheese extract/digest are going past through the immunoaffinity columns, are common, probably due to higher cellular debris. Detoxification of probiotics has been demonstrated in other milk products as well [83].

In this regard, Bovo and coworkers [80] have already established that non-viable cell counts affect toxin sequestration. Hence, one might argue that despite pasteurization of milk, bacterial structures may remain and, as such, milk non-viable microorganisms may still play a role in the results observed. One additional aspect to consider as well is that during cheese ripening, biochemical phenomena such as lipolysis and proteolysis occur which in turn may affect toxin binding capabilities [84]. As time progresses, pH changes as a function of time due to biochemical processes that cheese suffers, and in this scenario, protein-toxin interactions may transform as the electrostatic and hydrophobic forces mutate, as hydronium ion activity/concentration changes. Microbial sorption has been explored recently as a viable way to reduce AFM_1 levels in milk.

Finally, the binding of AFM_1 by microbial cells has been reported as a rapid process [85, 86], based on our current data in which just an incubation periods of minutes are sufficient to bind completely AFM_1 to casein and whey proteins, these seems to be the issue for AFM_1-protein

interaction as well. However, as with proteins, evidence suggests that various strains of probiotics and different membrane components bind AFM_1 differentially [79].

5. Other implications of AFM_1 association with proteins

Despite being considered a by-product of cheese manufacturing, whey still preserves a rather complex composition that include proteins such as α-lactalbumin, β-lactoglobulin, serum albumin, lactoferrin and lactoperoxidase, glycomacropeptides (produced by rennets from k-casein), protease-peptones (generated by plasmin, mainly from β-casein) phosphopeptides, and other enzymes and oligopeptides as the result of hydrolysis [12], as such it retains nutritional value. Also, they are known to form gels capable of keeping different substrates (e.g. water and lipids) and providing texture properties desired for several foods [87], as such, a substantial number of commercially viable options (e.g., whey-based beverages or even whey based fitness supplements) have been developed [88]. Hence, the evidence up to date supports that there is in fact an implied risk of contaminated products to reach the customer. Another example lies in β-lactoglobulin fibrils. These structures are aggregates formed by incubation of β-lactoglobulin in various solvents with protein-denaturing capabilities, (usually worm-like ca. 7 nm in width and < 500 nm in length), with a "bead string" form [89], and are nowadays very prevalent in the food industry to increase viscosity and encapsulation and transport other compounds of interest [90]. Recently, Mazaheri and co-workers have demonstrated that β-lactoglobulin fibrils exhibit neurotoxicity in cell culture and are capable of causing free radical formation, and that the presence of AFM_1 increases this potential by favoring reactive oxygen species and causes non-trivial modifications in the protein structure [91].

6. Perspectives and conclusions

AFM_1 has the capability to interact with macromolecules, including proteins. These interactions are dependent on many factors which also affect the already complex protein chemistry. Milk and dairy products which are complex biological fluids usually bear considerable concentrations of proteins. Recent evidence points toward a broad binding capability of milk proteins including casein and whey proteins. These interactions are affected by unit operations and technological processing. Based on the latest advances in molecule discrimination such as X-ray crystallography, NMR or atomic force microscopy could eventually help us collect more direct evidence on the occurring interactions between AFM_1 and proteins at a molecular level.

Finally, AFM_1 distribution, outcome, and interaction with different dairy ingredients are not to be ignored, particularly considering the recent findings regarding the behaviour of AFM_1 in milk products and by-products. This is especially important for products which once were considered process wastes (such as whey and related products) since now they are known to be used in animal feeding, as nutritional supplements destined for human consumption and

even in the snack food industry. Therefore, whey AFM_1 contaminated products may serve a way to incorporate toxins within the food chain. Evidence supports the fact that dairy processing does influence AFM_1 concentrations.

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