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Mycotoxins adsorption properties of Kefir microorganisms

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Disse a flor para o pequeno príncipe:

é preciso que eu suporte duas ou três larvas se quiser conhecer as borboletas.

Antoine de Saint-Exupéry

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RESUMO

As micotoxinas são metabolitos secundários tóxicos produzidos por fungos, que ocorrem na alimentação humana e animal. Deste modo o controlo e a mitigação dos impactos das micotoxinas na saúde humana e animal são uma preocupação atual. O Kefir, leite fermentado produzido a partir de grãos de bactérias e leveduras que vivem numa associação simbiótica, provou ter propriedades de adsorção de micotoxinas e potencial para reduzir a biodisponibilidade destas. Assim, o objetivo deste projeto foi avaliar as propriedades de adsorção das micotoxinas pelo Kefir e pelos seus microrganismos. Para isso, a adsorção de aflatoxina B1 (AFB1), ocratoxina A (OTA) e zearalenona (ZEA) por grãos de Kefir, leite fermentado de Kefir e dois microrganismos isolados de Kefir (*Kazachstania servazzii* KFGY7 e *Lactobacillus kefiri* KFLM3) foi testada em condições que simulam o sistema gastrointestinal. Para além disso, neste estudo, a composição química dos polissacarídeos produzidos por Kefir foi avaliada por Cromatografia líquida de alta eficiência (HPLC) e Cromatografia em camada fina (TLC). Com base nos resultados obtidos, foi comprovado que o Kefir é capaz de adsorver AFB1, ZEA e OTA sob condições que simulam o sistema gastrointestinal. No entanto, foi observado um decréscimo acentuado na adsorção das micotoxinas nas fases oral e intestinal, devido ao efeito exercido pelo pH, enzimas e sais adicionados. Na fase intestinal, os grãos de Kefir ainda adsorveram 10,3 ± 2,8% de AFB1, 16,6 ± 2,4% de OTA e 30,4 ± 6,7% de ZEA, e o leite fermentado de Kefir, 4,0 ± 0,1% de AFB1, 7,2 ± 0,0% de OTA, e 23,5 ± 0,4% de ZEA. Nas condições testadas, *L. kefiri* KFLM3 e *K. servazzii* KFGY7 apresentaram as melhores capacidades de adsorção. Na fase intestinal, *L. kefiri* KFLM3 ainda adsorveu 38,1 ± 3,9% de AFB1, 33,6 ± 3,8% de OTA e 68,7 ± 1,2% de ZEA, e *K. servazzii* KFGY7, 25,6 ± 5,9% de AFB1, 27,6 ± 5,9% de OTA e 67,8 ± 0,4% de ZEA. Assim, os microrganismos que compõem o Kefir são os principais responsáveis pelas suas propriedades de adsorção de micotoxinas. A micotoxina que foi melhor adsorvida por todas as amostras testadas foi a ZEA. As análises por HPLC revelaram que os grãos de Kefir e o leite fermentado de Kefir contêm um heteropolissacarídeo (61% e 41%, respetivamente) que é composto por unidades de glucose e galactose numa proporção quase igual. A análise por TLC permitiu determinar que este heteropolissacarídeo é maioritariamente composto por dissacarídeos e trissacarídeos.

Em conclusão, este estudo prova que o Kefir é uma fonte de microrganismos que podem adsorver as micotoxinas sob condições que simulam o sistema gastrointestinal.

PALAVRAS-CHAVE: MICOTOXINAS, KEFIR, ADSORÇÃO, SIMULAÇÃO GASTROINTESTINAL

ABSTRACT

Mycotoxins are toxic secondary metabolites produced by fungi, which occurs in human food and animal feed. In this way, the control and mitigation of their impact on human and animal health are of current concern. Kefir, a fermented milk produced from grains made up of bacteria and yeast that live in a symbiotic association, proved to have mycotoxin-adsorbing properties and potential to lower the bioavailability of mycotoxins. Thus, the objective of this project was to evaluate the mycotoxins-adsorption properties of Kefir and their microorganisms. For that, aflatoxin B1 (AFB1), ochratoxin A (OTA) and zearalenone (ZEA) adsorption by Kefir grains, Kefir fermented milk and two microorganisms isolated from Kefir (*Kazachstania servazzii* KFGY7 and *Lactobacillus kefiri* KFLM3) was tested in conditions that simulate gastrointestinal tract. Furthermore, in this study, the chemical composition of polysaccharides produced by Kefir was evaluated by High performance liquid chromatography (HPLC) and Thin-layer chromatography (TLC). Based on the results obtained, it was proven that Kefir is capable of adsorbing AFB1, ZEA, and OTA under simulated gastrointestinal conditions. Nevertheless, it was observed a marked decrease in the adsorption of the mycotoxins in the oral and intestinal phases due to the effect exerted by the pH, enzymes and salts added. In the intestinal phase, Kefir grains were still adsorbing 10.3 ± 2.8% of AFB1, 16.6 ± 2.4% of OTA, and 30.4 ± 6.7% of ZEA, and Kefir fermented milk, 4.0 ± 0.1% of AFB1, 7.2 ± 0.0% of OTA, and 23.5 ± 0.4% of ZEA. In tested conditions, *L. kefiri* KFLM3 and *K. servazzii* KFGY7 showed the best adsorption capacities. In the intestinal phase, *L. kefiri* KFLM3 was still adsorbing 38.1 ± 3.9% of AFB1, 33.6 ± 3.8% of OTA, and 68.7 ± 1.2% of ZEA, and *K. servazzii* KFGY7, 25.6 ± 5.9% of AFB1, 27.6 ± 5.9% of OTA, and 67.8 ± 0.4% of ZEA. Thus, the microorganisms that compose Kefir are the main responsible for its mycotoxins-adsorption properties. The mycotoxin that was better adsorbed by all the samples tested was ZEA. The HPLC analyses revealed that Kefir grains and Kefir fermented milk contain heteropolysaccharides (61% and 41% (w/w) in total mass, respectively) composed of glucose and galactose units in an almost equal proportion. The TLC analysis allowed to determine that this heteropolysaccharide is mostly composed of disaccharides and trisaccharides.

In conclusion, this study proves that Kefir is a source of microorganisms that can adsorb mycotoxins under simulated gastrointestinal tract conditions.

KEYWORDS: MYCOTOXINS, KEFIR, ADSORPTION, SIMULATE GASTROINTESTINAL CONDITIONS

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List of Abbreviations

MRS	Man Rogosa Sharpe
YPD	Yeast Extract - Peptone – Dextrose
LAB	Lactic acid bacteria
AAB	Acetic acid bacteria
AFB1	Aflatoxin B1
ZEA	Zearalenone
OTA	Ochratoxin A
BEN	Bentonite
HPLC	High Performance Liquid Chromatography
TLC	Thin-Layer Chromatography
rpm	Revolutions per minute
rt	Retention time
ANOVA	Analysis of variance
p	*p-value*
DP	Degree of polymerization
GI tract	Gastrointestinal tract
CP	Polymer composition

CHAPTER 1

INTRODUCTION

1.1 Mycotoxins: an overview

Mycotoxins are secondary metabolites produced by filamentous fungi, belonging mainly to the genera *Penicillium, Fusarium, Aspergillus*, and *Alternaria,* which are characterised by their low molecular weight and toxicity (Bennett, 1987). Although genes that regulate mycotoxins production have been identified, the role of mycotoxins in the life of fungi is not yet fully understood as they are not essential for their survival (Fox and Howlett, 2008; Yu and Keller, 2005). Between 1960 and 1975 many scientists were involved in research on these toxic agents (Rocha et al., 2014). Currently, more than 500 types of mycotoxins have been identified, however, only about 10 to 15 are considered to be of public health interest (Stein and Bulboacă, 2017) with aflatoxins, deoxynivalenol, ergot alkaloids, fumonisins, ochratoxin A, patulin, and zearalenone being the most prominent due to their high incidence in foods (Bočarov-Stančić et al., 2011).

Studies have shown that mycotoxins present neurotoxic, nephrotoxic, carcinogenic, hepatotoxic, immunosuppressive and mutagenic characteristics (Rocha et al., 2014). The toxic effect of mycotoxins on animal and human health is known as mycotoxicosis. This disease is neither infectious nor contagious and only results from mycotoxins effects (Hussein and Brasel, 2001). Its severity depends on several factors, including the age and nutritional conditions of the infected person, the toxicity of the mycotoxin involved, the time of exposure as well as the amount ingested or inhaled (Peraica et al., 1999). Risks to human health are usually associated with direct consumption of food but may also occur through the dermal and inhalation routes. Mycotoxins can enter human food chain through two different pathways: directly through ingestion of food derived from plants or indirectly via contaminated animal food. Contamination of these foods can occur not only during production but also during processing, transportation and storage. In humans, ingestion of mycotoxins occurs mainly via consumption of corn, rice, wheat and other cereals, tree nuts, peanuts and spices (Rocha et al., 2014) (Figure 1).

Figure 1 | Foods with higher levels of mycotoxins contamination.

Meat, milk or eggs from animals fed with contaminated feed can also be a source of mycotoxins since some of these toxins can accumulate in the body of animals (Smith et al., 1995).

1.1.1 Aflatoxins

Aflatoxins, identified in the early 1960s, are one of the most toxic secondary metabolites for humans and animals (Wild and Gong, 2010). These mycotoxins present four natural forms: B1, B2, G1 and G2. Aflatoxin B1 (AFB1) is the most potent natural carcinogen known (Squire, 1981). They are difuranocoumarin derivatives produced through the polyketide pathway of fungal species such as *Aspergillus parasiticus*, *Aspergillus nomius* and *Aspergillus flavus* (Hussein and Brasel, 2001; Kumar et al., 2016).

AFB1 (Figure 2) may be present in a wide range of food commodities, particularly in crops such as nuts, corn, wheat, and peanuts (Jelinek et al., 1989; Severns et al., 2003). When aflatoxins enter into the body via ingestion, its absorption and subsequent transport through the circulatory system to the liver occurs rapidly. In the liver, AFB1 is biotransformed into aflatoxin M1 (AFM1) (Giovati et al., 2015). Then, it is converted into toxic reactive epoxides which are capable of binding covalently to intracellular macromolecules such as DNA, RNA and enzymes causing damage to liver cells (Boermans and Leung, 2007).

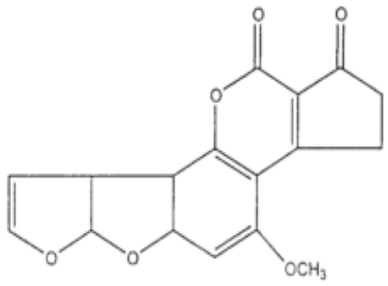

Figure 2 | Chemical structure of aflatoxin B1 (Bennett and Klich, 2003).

Although aflatoxins are well recognised for causing damage to liver cells or even liver cancer, they have other significant toxic effects. For example, the chronic exposure of animals to aflatoxins may compromise their immunity and interfere with their metabolism of essential micronutrients and proteins. Despite these effects have not been extensively studied in humans, available information indicates that some of them are at least observed in animals (Williams et al., 2004). Furthermore, they can over activate

the inflammatory response resulting from the respiratory, cutaneous or even mucosal routes (Romani, 2004) and cause teratogenicity (Kumar et al., 2016).

It is estimated that about 4.5 billion of the world's population is exposed to aflatoxins (Williams et al., 2004). Due to this incidence, there is a great concern and demand to develop appropriate methods in aflatoxins research that allow their detection and subsequent quantification to ensure the control and the health safety of consumers (Kumar et al., 2016).

1.1.2 Ochratoxins

Ochratoxins are mycotoxins resulting from the secondary metabolism of various species of the *Aspergillus* or *Penicillium* genera, which are characterised for being weak organic acids and a derivative of an isocoumarin (Bayman et al., 2002). They are a group of potent renal mycotoxins that contain various forms, the most well-known are ochratoxin A, B, C (Boermans and Leung, 2007; Malir et al., 2013).

In 1965, during a study of several fungal metabolites, where the goal was to discover new mycotoxins, ochratoxin A (OTA) was found as a metabolite of *Aspergillus ochraceus* (Bennett and Klich, 2003). OTA (Figure 3) is produced by several species of the genus Aspergillus, mainly Aspergillus westerdijkiae (previously known as *A. ochraceus*) but also by *Aspergillus carbonarius* and some strains of *Aspergillus niger* (el Khoury and Atoui, 2010). Two *Penicillium* species (*Penicillium verrucosum* and *Penicillium nordicum*) also produce OTA (Cabanes et al., 2010; Pitt, 1987). OTA occurs naturally in many plant products such as wheat, barley, coffee, beans, spices, cocoa and nuts, and is also detected in processed products such as breakfast cereals, wine, beer, grape juice and animal products such as meat, eggs and milk (Abrunhosa et al., 2010; Coronel et al., 2011).

OTA is composed of an isocoumarin known as ochratoxin α (OTα) and a molecule of L-β-phenylalanine, which are bound by an amide bond. The conversion of OTA into OTα via hydrolysis of the amide bond is the main route of detoxification of this mycotoxin, since OTα is non-toxic and has a biological half-life 10 times inferior to OTA (Thieu et al., 2008).

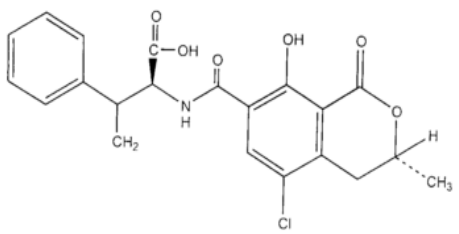

Figure 3 | Chemical structure of ochratoxin A (Bennett and Klich, 2003).

When food contaminated with OTA is ingested, the toxin enters rapidly into the bloodstream, where it can be detected (Dinis et al., 2007). OTA is also a cumulative toxic compound because it is easily absorbed by the stomach and small intestine, but the biliary and urinary tract present difficulties in its elimination, so it accumulates in the bloodstream, liver and kidneys, binding to serum proteins, mainly to albumin, thus limiting their transfer of blood to liver and kidney cells (Ringot et al., 2006).

This accumulation disturbs the protein synthesis and other physiological pathways occurring in the proximal tubular cells, which leads to interstitial fibrosis and degeneration of the proximal tubules (Boermans and Leung, 2007; Krogh, 1992). OTA also binds to DNA molecules and induces kidney tumours in animals, even though its carcinogenic mechanism remains unclear and controversial (Faucet et al., 2004; Mally et al., 2004). Additionally, OTA has been classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen but has other toxicological properties such as nephrotoxic, hepatotoxic, teratogenic and immunotoxic effects. Thus, as for aflatoxins, it is indispensable to control the exposure of the population to this mycotoxin, so the risks to human health can be reduced (Boermans and Leung, 2007).

1.1.3 Zearalenone

Zearalenone, also known as ZEA, (Figure 4) is a 6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone (Hussein and Brasel, 2001). This secondary metabolite is produced by fungi of the genus *Fusarium*; such as *Fusarium equiseti*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium semitectum*, but mainly by *Fusarium graminearum* and *Fusarium culmorum* (Kowalska et al., 2016). From the various cereal crops where they may propagate, corn is where the growth of these fungi is more frequent (Boermans and Leung, 2007). ZEA producing fungi can attack crops not only during their development in the field but also during their storage (Kowalska et al., 2016). Human exposure may occur directly through ingestion of contaminated food or indirectly through products derived from animals exposed to ZEA (Kowalska et al., 2016). In the human food chain, it can be found essentially in cereals and their derived products (Kriszt et al., 2012).

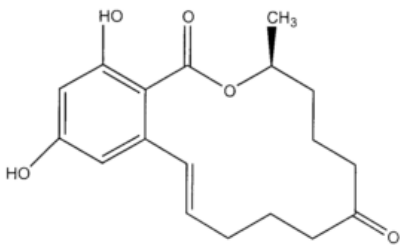

Figure 4 | Chemical structure of zearalenone (Bennett and Klich, 2003).

This mycotoxin exhibits structural similarities to estrogen and binds to cytosolic estrogen receptors on target cells forming the receptor-zearalenone complex (Riley and Norred, 1996). After its formation, the transcription of estrogen-sensitive genes is activated, and the translation of new proteins expressing estrogenic effects in the target cells occurs (Boermans and Leung, 2007). Thus, it is easy to realise that some of the signs of zearalenone poisoning are related to the hyperstimulation of estrogen-dependent tissues (Boermans and Leung, 2007). The various effects resulting from the action of ZEA include precocious puberty, change in serum progesterone and estradiol levels, breast cancer, decreased fertility, changes in thyroid gland weight as well as in the pituitary and adrenal glands (Zinedine et al., 2007).

1.2 Mycotoxins in Portugal

In general, the population of countries that have high incidence of malnutrition, poor food storage, processing and preservation facilities, and inadequate regulations are more subseptible to be exposed to mycotoxins. In developed countries, although this exposition still occurs, the mycotoxins levels and incidences in food are much lower. In these countries, competition between producers for product quality is stronger, resulting in stricter control of all producing steps, from raw material reception to final product consumption (Bennett and Klich, 2003). Another important factor is that people of developed areas benefit from a more varied diet, contrary to the population of developing countries, where food shortages result in a greater demand for food, regardless of their quality. Besides that, the issues of control and quality are not always respected in these areas (Shephard, 2008).

In Portugal, mycotoxins also occure in food and animal feed. Between 2000 and 2007, Martins et al. (2008) analyzed samples of corn, soy, rice, barley, wheat, silage and gluten for the presence of AFB1, deoxynivalenol (DON) and fumonisin B1 (FB1) (Table 1). The same authors, also tested samples of mixed feed (for poultry, fish aquaculture, horses, cattle, swine, pet and laboratory rats) for AFB1, OTA, DON, ZEA and FB1 (Martins et al., 2008). The results of these analysis are described in Table 2.

Table 1 | Occurrence of aflatoxin B1 (AFB1), deoxynivalenol (DON) and fumonisin B1 (FB1) in raw materials (soy, rice, corn, wheat, barley, ensilage and gluten) in Portugal from 2000 to 2007 (Martins et al., 2008).

Matrix	AFB1		DON		FB1	
	n_+/N	µg/kg	n_+/N	µg/kg	n_+/N	µg/kg
Soy	2/66	1-10	0/26	-	0/26	-
Rice	9/17	5-20	3/17	100-200	1/17	15
Corn	30/248	1-45	15/74	100-500	12/58	10-300
Wheat	9/80	1-10	8/50	100-200	4/50	10-40
Barley	5/74	1-10	3/29	100-300	2/29	10
Ensilage	2/13	6-10	0/13	-	0/13	-
Gluten	6/15	1-15	2/15	100	0/15	-
Total	63/513	1-45	24/224	100-500	19/208	10-40

n_+ = positive sample; N=total of samples; (-) = not detected

Table 2 | Occurrence of aflatoxin B1 (AFB1), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEA) and fumonisin B1 (FB1) in animal's feed (dairy cattle, swine, poultry, horses, fish, laboratory rats and pet) in Portugal from 2000 to 2007 (Martins et al., 2008).

Feed	AFB1		OTA		DON		ZEA		FB1	
	n_+/N	µg/kg	n_+/N	µg/kg	n_+/N	µg/kg	n_+/N	µg/kg	n_+/N	µg/kg
Dairy cattle	374/1001	1-80	0/50	-	Nd		Nd		Nd	
Swine	27/261	1-21	1/50	2-4	9/291	100-1649	4/30	104-356	0/285	-
Poultry	35/161	1-21	0/50	-	Nd		Nd		3/52	24-34
Horses	0/50	-	30/50	2-5	15/50	100-320	0/50	-	Nd	
Fish	0/20	-	Nd		Nd		Nd		Nd	
Laboratory rats	0/31	-	Nd		Nd		Nd		Nd	
Pet	0/60	-	5/20	2-4	3/20	100-130	Nd		3/20	12-24
Total	436/1584	1-80	36/220	2-5	27/361	100-1649	4/80	104-356	6/357	12-34

n_+ = positive sample; N=total of samples; (-) = not detected; Nd = not determined

These studies demonstrated that the mycotoxin problem exists in Portugal, both in food and in feed, so it is important and necessary to find ways to combat these agents and their adverse effects.

1.3 Decontamination of mycotoxins by yeasts and bacteria

1.3.1 Yeasts

Several studies have reported the ability of yeasts to adsorb mycotoxins. For example, probiotic strains of *Saccharomyces cerevisiae* were able to bind AFB1 (Bueno et al., 2007; Pizzolitto et al., 2013; Yiannikouris et al., 2004) and also to resist to gastrointestinal conditions improving rumen fermentation and feed digestibility (Dogi et al., 2011). Also, Corassin et al. (2013) proved that AFM1 could be removed from dairy products by *S. cerevisiae* as a result of its adsorption to yeast cell walls.

The mechanisms responsible for the bond of mycotoxins by yeasts are still not fully understood. However, authors like Petruzzi et al. (2014) and Ringot et al. (2007) suggest that some macromolecules present in yeast cell walls play the major role. β-D-glucans (Fruhauf et al., 2011), glucomannans (Raju and Devegowda, 2002) and mannan-oligosaccharides (Parlat et al., 1999) have been pointed as the main responsible for such adsorbing properties. Pfliegler et al. (2015) corroborate this theory, because they observed that non-viable yeast and their cell wall derived-products retained the adsorption capacity of viable cells. Other authors also showed that non-viable cells (for example cells treated with heat or acid) have better adsorption efficiencies (Nunez et al., 2008; Shetty et al., 2007; Yue et al., 2011). Pfliegler et al. (2015) also concluded that the yeast cell wall composition varies from species to species and that is the main factor affecting their adsorption capacity. Other variables that affect the amount of toxin removed are the toxin and microorganism concentration (Gonzalez Pereyra et al., 2014), as well as the total amount of cell wall (Armando et al., 2012). Additionally, the content of β-D-glucan and its three-dimensional array on the cell wall also affects the binding process according to Yiannikouris et al. (2004).

1.3.2 Lactic acid bacteria

In addition to yeast studies, lactic acid bacteria (LAB) have also been evaluated for the adsorption of mycotoxins. For example, Phillips et al. (2002) found that dead cells of *Lactobacillus rhamnosus* were able to adsorb 75% of AFB1 and 62% of OTA, thus confirming that dead cells showed high efficiency. Bzducha-Wrobel et al. (2014) verified the same dynamics for OTA when comparing the decontamination efficiency of thermally inactivated biomass with that of living cells. The authors found that dead bacterial cells bound 46% to 53% of the initial toxin content, while live biomass bound only 15% to 16%, under the same conditions. With respect to ZEA, interactions between this mycotoxins, its α-zearalenol derivative (α-ZOL) and strains of *Lactobacillus* were reported by several researchers. Desheng et al. (2005)

demonstrated that, after co-incubation of ZEA and *Lactobacillus*, a considerable proportion (38% to 46%) of the mycotoxin was recovered from bacterial pellets. Similar results were also obtained by Deng et al. (2010), which showed that some strains of *Lactobacillus* achieved adsorption percentages between 46% and 64%. In general, according to several authors (Desheng et al., 2005; Faucet-Marquis et al., 2014; González-Arias, 2013; Yang et al., 2014), the acid or thermal inactivation of lactic acid bacteria increases the adsorption efficiency of AFB1 and AFM1, as well as, the one of ZEA (Desheng et al., 2005; Phillips, 1999).

In order to understand the mechanism involved in the adsorption process it is important to know the composition of LAB cell walls. LAB cell walls consists of a thick matrix of peptidoglycan, the major structural component of the cell wall enclosure, which is associated with other components such as teicoic and lipoteichoic acid, polysaccharides and proteins (Chapot-Chartier and Kulakauskas, 2014; Shetty and Jespersen, 2006). These bacteria have the ability to synthesize cytoplasmic storage polysaccharides such as glycogen and starch (Wilkinson, 1963), cell surface associated polysaccharides (peptidoglycan and lipopolysaccharides, lipooligosaccharides, teicoic acids, lipoteichoic acids) and other cell wall polysaccharides (Chapot-Chartier, 2014; Mistou et al., 2016; Schmid et al., 2015; Tytgat and Lebeer, 2014). Regarding the mechanism involved in the adsorption process of mycotoxins by LAB, it is still unknown. However, the mechanism used by LAB may be similar to that described for yeasts, since among the exocellular polysaccharides produced by LAB, it is possible to highlight the presence of an homopolysaccharide, β-(1,3)-d-glucans (Zeidan et al., 2017) and heteropolysaccharides (Zajšek et al., 2011), which may also be involved in the adsorption process. Studies by Zhang and Ohta (1991) suggest the involvement of peptidoglycan and polysaccharides in the binding of mycotoxins. On the other hand, Haskard et al. (2001) have indicated the dominant role of the theicolic acids in the binding mechanism of aflatoxin.

1.4 Kefir

1.4.1 Microbial composition of Kefir grains

Kefir, which is native from the Caucasus and Eastern European regions, is a traditional dairy drink produced by the direct addition of Kefir grains to milk (Leite et al., 2013). Kefir comes from the Turkish word “keyir” that meaning "good feeling" because of the sensation you get when you drink it (Leite et al., 2015). The Kefir grains (Figure 5) are white or yellow, having an irregular, gelatinous shape and a diameter ranging from 0.3-3.5 cm (Hamet et al., 2013).

Figure 5 | Kefir grains.

They are composed of several microbial genera and species (Table 3), which are in a symbiotic association and are enclosed in a protein matrix and a natural polysaccharide known as kefiran (Garofalo et al., 2015). Therefore, Kefir is a source of probiotics strains. Thus, the result of the acid-alcoholic fermentation of milk carried out by these microorganisms is a fermented milk product with unique properties, distinctive flavour and viscosity (Leite et al., 2013). Kefir can be a source of different nutritional constituents such as peptides, amino acids, bacteriocins, vitamins B1, B12 and K, acetaldehyde, ethanol, CO_2, exopolysaccharides, acetoin, diacetyl, folic acid, calcium, lactic acid and acetic acid (Garofalo et al., 2015).

Table 3 | Microbial composition of Kefir grains (Garofalo et al., 2015; Pogačić et al., 2013).

Lactic acid bacteria (LAB)	*Lactobacillus kefiranofaciens*
	Lactobacillus kefiri
	Lactobacillus kefirgranum
	Lactobacillus brevis
	Lactobacillus parakefiri
	Lactobacillus delbrueckii
	Lactobacillus acidophilus
	Lactobacillus helveticus
	Lactobacillus fermentum
	Lactobacillus casei
	Lactobacillus paracasei
	Lactobacillus gasseri
	Lactobacillus plantarum
Yeasts	*Kazachstania unispora*
	Kluyveromyces marxianus
	Kluyveromyces lactis
	Torulaspora delbrueckii
	Candida kefir
	Pichia fermentans
	Saccharomyces cerevisiae
	Kazachstania exigua
Acetic acid bacteria (AAB)	*Acetobacter fabarum*
	Acetobacter lovaniensis
	Acetobacter syzygii
	Acetobacter orientalis

The production of Kefir can be made from the whole, semi-skimmed or skimmed pasteurised sheep, cow, camel, goat or buffalo milk. However, the most common are the one produced with cow's milk. In Figure 6 they are described the steps for domestic production of Kefir.

Figure 6 | Domestic production of Kefir. 1. Separated Kefir grains, 2. Addition of milk to the Kefir grains at room temperature, 3. Fermentation for 10 to 24 h, 4. Filtration and separation of Kefir grains and 5. Possible addition of the Kefir grains to fresh milk to start a new fermentation. Kefir milk can be refrigerated (4 °C) and is adequate for consumption. Adapted from Rosa et al. (2017).

1.4.2 Benefits of Kefir

Due to its microbial composition, Kefir is considered a probiotic source (Nalbantoglu et al., 2014). Some studies suggest that Kefir consumers have a higher abundance of probiotic bacteria in the intestines, resulting in an improvement in human health (Prado et al., 2015). Farnworth (2005) and Rizk et al. (2009) have further demonstrated that Kefir increases the ability to digest lactose, relieving symptoms of lactose intolerance. Also, this product can lower cholesterol levels (Prado et al., 2015).

Kefir has increasingly captured the interest of the scientific community due to its beneficial properties as its antibacterial, antihypertensive and hypo-cholesterolaemic effect; anti-allergenic, anti-carcinogenic and antioxidant activity; improved digestion and tolerance to lactose; and control of plasma glucose (Rosa et al., 2017). The benefits associated with each constituent of Kefir are shown in Figure 7.

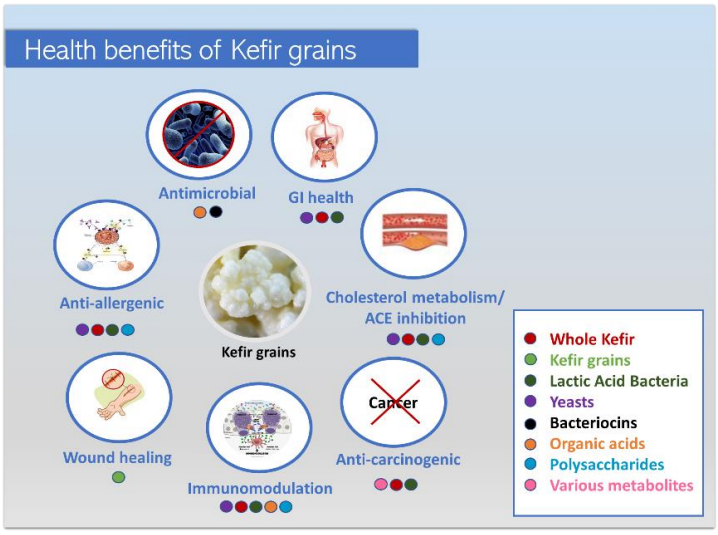

Figure 7 | Major health benefits associated with Kefir and its constituents: Kefir grains, lactic acid bacteria, yeasts, bacteriocins, organic acids, polysaccharides and other metabolites. GI: gastrointestinal immunity; ACE: angiotensin-converting enzyme. Adapted from Bourrie et al. (2016).

Kefir is a complex product, which benefits result from the different constituents that it contains. The potential beneficial effects of Kefir on human health and physiology are resumed in Figure 8.

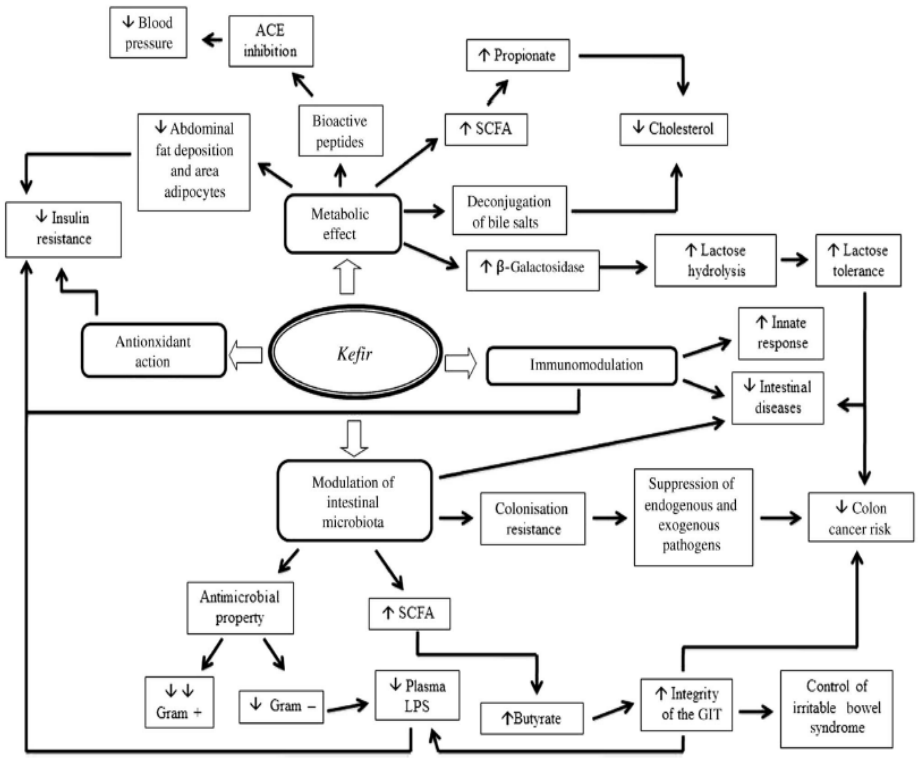

Figure 8 | Schematic diagram of the beneficial physiological effects of Kefir on human health. ACE, angiotensin-converting enzyme; GI, gastrointestinal tract; LPS, lipolysaccharides; SSFA, short-chain fatty acid. Adapted from Rosa et al. (2017).

1.5 Kefir - A potential source of probiotics to combat mycotoxins

In addition to the adsorption of mycotoxins by yeasts, lactic acid bacteria and other bacteria, recent studies suggest that Kefir also can counteract the harmful effect of mycotoxins because it holds mycotoxin-adsorbing properties (Isakhani et al., 2014 ; Taheur et al., 2017). Isakhani et al. (2014) reported the ability of Kefir grains to bind 92% of AFM1 (0.5 µg/L) in milk. Taheur et al. (2017) carried out adsorption experiments with a Kefir consortium in milk and found that 100% of ZEN, 94% of OTA and 82% of AFB1 added to milk was adsorbed by the microorganisms. Following, they isolated microorganisms from the consortium and showed that strains *Lactobacillus kefiri* KFLM3 *(L. kefiri* KFLM3), *Kazachstania servazii* KFGY7 (*K. servazzii* KFGY7), and *Acetobacter syzygy* KFGM1 were the most active in adsorbing these mycotoxins.

1.5.1 Kefiran and heteropolysaccharide from Kefir

As already mentioned, recent studies found that the Kefir consortium can adsorb high amounts of the mycotoxins ZEN, OTA and AFB1 (Taheur et al., 2017). It is now important to understand what components of Kefir are responsible for this adsorption.

Kefir is composed of numerous microorganisms. From its composition, it is possible to highlight the presence of LAB, which can synthesise hetero and homopolysaccharides (Badel et al., 2011; Van Hijum et al., 2006). The main heteropolysaccharide present in Kefir grains is kefiran (Zajšek et al., 2011). Nevertheless, it is important to note that Kefir produce predominantly homopolysaccharides, such as fructans or glucans (Prado et al., 2015). Although there are no studies regarding the direct action of Kefir polysaccharides on mycotoxins, kefiran may be involved in the adsorption of the different mycotoxins (Pop et al., 2016). This polysaccharide has been regarded as safe since Kefir has long been consumed as food (Micheli et al., 1999), and has been classified as a water-soluble glucogalactan (Prado et al., 2015).

Relatively to the molecular structure of kefiran, this is still not well understood, but Rimada and Abraham (2001) and Zajšek et al. (2011) sustains that this polysaccharide is a hexa- or heptasaccharide branched with repeating units of D-glucose and D-galactose in almost equal proportion. According to Kooiman (1968), each unit is constituted by a regular pentasaccharide, to which one or two residues of polysaccharides are randomly connected. This heteropolysaccharide has several beneficial properties for health, such as anti-inflammatory, antifungal, antitumor and antibacterial properties, antioxidant activity, and epithelium protection (Micheli et al., 1999; Prado et al., 2015).

Kefiran is used in cosmetics, pharmaceutical industries and can also be used as a food grade additive for fermented products because it improves the rheological properties of chemically acidified skim milk, forms viscous solutions at low concentrations, has excellent stability in a wide range of temperatures and pH (Pop et al., 2016), maintain the properties of gel and avoids the loss of water during storage (Prado et al., 2015).

1.5.2 Glucans from Kefir

On the other hand, the homopolysaccharides synthesised by Kefir strains (for example, fructans or glucans) are molecules that are composed only of a single type of monosaccharides - fructose or glucose, respectively (Badel et al., 2011; Van Hijum et al., 2006). The production of fructans and β-(1,3)-d-glucans without branching result from the metabolism of *Lactobacillus* spp. (McIntosh et al., 2005; Prado et al., 2015). Glucans are also present in yeasts, being the main components of their cell walls, since they represent about 50 to 60% of the wall dry weight (Fruhauf et al., 2011; Salazar and Asenjo, 2007).

Glucans produced by yeasts are β-(1,3)-d-glucans that contain long β-1,6 side branches, and that are associated with other elements, such as chitin and mannoproteins (Aimanianda et al., 2009; Volman et al., 2008).

Concerning mycotoxins, there are some studies that sustain that these glucans can play a crucial role in their adsorption by yeasts. Jouany et al. (2005) demonstrated that β-D-glucans present in yeast cell walls are responsible for the adsorption of ZEA and that its 3D structure affects the adsorption process, since the existence of electrostatic and hydrophobic interactions between units of glucose in single helix of β-D-glucans and the mycotoxin are important key factors for the adsorption efficiency. Yiannikouris et al. (2006) found that β-d-glucans, and specifically β-(1,3)-d-glucans moderately branched with β-1,6-d-glucans chains from the cell wall of *S. cerevisiae* had affinity for mycotoxins. Consequently, the adsorption capacity of yeast cell wall products depend on a suitable extraction and preparation of the cell wall material before it use as a mycotoxin binder (Fruhauf et al., 2011).

According to some manufacturers of yeast cell wall derived products, preparations that contain yeast glucans, in addition to promoting growth and to add nutritional value, they can bind mycotoxins *in vitro* and *in vivo,* especially ZEA, without altering the nutritional value (Fruhauf et al., 2011). Nonetheless, glucans activity may be affected by parameters such as the primary structure of the homopolysaccharide, its solubility, molecular weight as well as the degree of branching (Zekovic et al., 2005).

1.6 *In vivo* digestion

The digestion process is an important parameter to take into account in the evaluation of novel mycotoxin adsorbing materials. This is a complex process, which transforms the food we eat into smaller components and energy, and that occurs in the gastrointestinal tract (GI tract), a long, connected tubular structure that begins with the mouth and ends with the anus (Jard et al., 2011). The food is propelled forward within the system, altered by enzymes into usable particles and then absorbed along the way by the bloodstream and distributed throughout the body (González-Arias et al., 2013).

The digestion is a sequential process and the mouth is the entry point for food. As soon as the first piece of food enters the mouth, the saliva is released by the salivary glands in the oral cavity and chewing begins, with food being broken into smaller particles that can be more easily attacked by the enzymes present in saliva (amylases), resulting in the decomposition of some polysaccharides (González-Arias et al., 2013). Then, from the glands lining the stomach, gastric acids and enzymes (pepsin and some gastric lipases) are secreted and, with the aid of the stomach muscles, the process of chemical

and mechanical decomposition of food continues, occurring mainly the degradation of proteins and peptides, although some lipolysis may also occur (Decker and Corby, 1980). After the food is emptied from the stomach, it will move into the small intestine (made up of three segments, the duodenum, jejunum and ileum). Thus, the duodenum is largely responsible for continuing the process of food decomposition, because the presence of lipids in this segment stimulates the secretion of the pancreatic fluid (containing lipase/pancreatic colipase) and bile from liver (composed by bile salts, phosphatidylcholine and cholesterol). At this stage, peristalsis (contractions) also helps the movement of food and its mixture with digestive secretions. On the other hand, the jejunum and ileum are the main responsible for the absorption of nutrients and their delivery into the bloodstream. In the large intestine, it occurs the reabsorption of minerals, water and some vitamins such as vitamin K and biotin that are produced by some bacteria that inhabit the intestinal flora. The material that has not been digested forms the feces, which accumulate in the rectum and are eliminated from the anus (Ramos et al., 1996).

1.7 *In vitro* digestion model

1.7.1 The process and main physiological components

Many *in vitro* digestion models based on animal or human physiology have been developed to study the bioaccessibility of nutrients, pharmaceuticals, neutraceuticals, dietary supplements, food ingredients, as well as to evaluate anti-mycotoxin additives. These models may vary in their mode of operation, differing in the number and type of steps included in the simulation of the digestion process (mouth, stomach, small intestine and large intestine), mechanical stresses (e.g. stirring) and composition of digestive fluids used in each step, for example, enzymes, buffers and salts (Doll et al., 2004).

In general, the models used to simulate GI are models that simulate the sequential (compartmentalized) exposure of food from the mouth to the stomach and finally the small intestine. These models are easy to perform, allow fast processing of a large number of samples, are inexpensive and have no ethical constraints (González-Arias et al., 2013; Minekus et al., 2014). However, these models do not simulate some physiological processes that occur during digestion, such as peristalsis or the existence of intestinal microbiota (González-Arias et al., 2013). Also, several factors such as sample characteristics, ionic composition, enzymatic activity, applied mechanical stress and digestion time, significantly influence the results of *in vitro* digestion methods (Marroquin-Cardona et al., 2011). Therefore, although *in vivo* conditions can never be completely and adequately simulated under *in vitro*

conditions, it is necessary to maximize *in vivo* digestion conditions, whereby parameters such as temperature, time in each compartment, gastric/intestinal juice composition, enzymatic activity and pH changes should also be considered (Bueno et al., 2005). Nevertheless, *in vitro* models can provide valuable information, which can help in the planning of subsequent *in vivo* studies in order to avoid the unnecessary sacrifice of experimental animals.

Focusing on the model used in this study, the model proposed by Minekus et al. (2014) (Figure 9) takes into account what occurs in the first three compartments of the GI tract (mouth, stomach and small intestine). According to Doll et al. (2004), most of the *in vitro* digestion models use a digestion temperature of 37 °C, which allows simulating human body temperature. The procedure described by Minekus et al. (2014) uses a water bath set for this temperature and an integrated stirrer to simulate mixing/stirring of the digestion process. This protocol intends to simulate the oral, gastric and intestinal phases, using components that simulate oral (SSF), gastric (SGF) and intestinal (SIF) fluids.

In the oral phase, the use of α-amylase, calcium chloride, SSF and water is intended to mimic the saliva composition. The enzyme α-amylase is mainly responsible for the conversion of starches to oligosaccharides and monosaccharides such as glucose (Doll et al., 2004). SSF is used to simulate the wetting and lubrication of solid food masses by salivation. The duration of the incubation times of the samples in the various simulated digestive fluids should mimic the digestion times reported in humans. So, at this stage the samples remain incubated for 2 minutes, simulating the chewing and swallowing process.

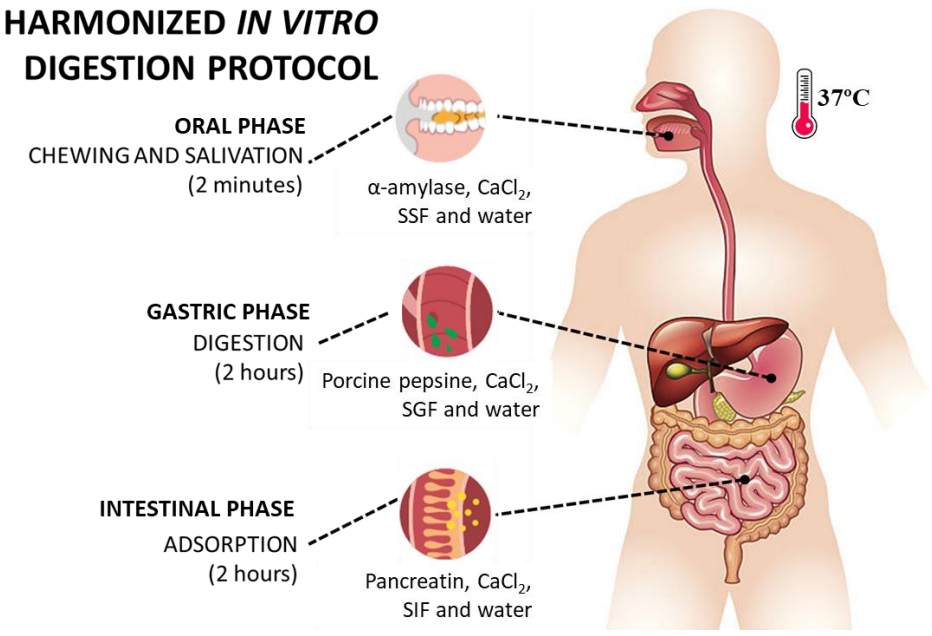

Figure 9 | Harmonized *in vitro* digestion protocol (oral, gastric and intestinal phase). Adapted from (Minekus et al., 2014).

In the next step of the digestion, the gastric phase, SGF is used to mimic the action of gastric juice which is secreted by the gastric glands of the stomach (González-Arias et al., 2013). Regarding the action of gastric juice, due to the secretion of hydrochloric acid, a decrease of the pH in the stomach occurs. Gastric pH values were reported between 1 and 3 (Avantaggiato et al., 2003, 2004; Galvano et al., 2001). Thus, to simulate the decrease of pH in this phase, the pH is set to 3. According to González-Arias et al. (2013), pH decrease by gastric juice is important for the stabilization of pepsin which has optimal proteolytic activity in the same pH range (i.e., pH 1-3) and is responsible for breaking down proteins and peptides into smaller peptides and amino acids (Doll et al., 2004).

Finally, in the intestinal phase, the use of SGF allows to simulate the action of components such as bile and pancreatin that significantly affect the digestion process, since they help in the process of digestion through the breakdown of foods into parts that can be more easily absorbed (Hur et al., 2011). According to González-Arias et al. (2013), pH values of *in vitro* intestinal fluids employed in experiments to test mycotoxins ranged from 6 to 8.2. In this model the pH is set to 7.

CHAPTER 2

MATERIALS AND METHODS

2.1 Biological material

The microorganisms used in these experiments are *L. kefiri* KFLM3 and *K. servazii* KFGY7. These strains were previously isolated from Kefir grains, as described by Taheur et al. (2017). Stock cultures of *L. kefiri* KFLM3 and *K. servazii* KFGY7 were stored at -20 °C in 1 mL eppendorf tubes containing liquid Man-Rogosa-Sharpe culture medium (MRS broth, Oxoid), liquid Yeast Extract-Peptone-Dextrose medium (YPD), and 20% (v/v) of glycerol. In this study, Kefir grains were also used. They were obtained locally.

2.1.1 Culture of *L. kefiri* KFLM3 and *K. servazzii* KFGY7

L. kefiri KFLM3 was revived in 10 mL of MRS supplemented with 20% tomato juice (Fluka) and 1 g/L L-cysteine (Fisher Chemical). *K. servazzii* KFGY7 was revived in 10 mL of YPD, which is composed of 20 g/L glucose (Fisher), 20 g/L peptone (Himedia) and 10 g/L yeast extract (Difco). The microorganisms were incubated at 30 °C for 2 days for inocula preparation. Then, flasks with 100 mL of MRS broth and 100 mL of YPD, previously sterilized at 121 °C for 15 min in an autoclave, were inoculated under aseptic conditions with 4 mL of inoculum and incubated at 25 °C for 5 days with agitation (150 rpm). *L. kefiri* KFLM3 and *K. servazzii* KFGY7 were also cultivated in 100 mL of UHT milk at 30 °C for 4 days in static conditions. After the incubation period, the fermented media were centrifuged (8600 ×g, 25 °C, 10 min) and the obtained pellets were lyophilized, grounded in a mortar and stored at room temperature.

2.1.2 Production of Kefir grains

To obtain suficient material for this study, it was necessary to reactivate and propagate the Kefir grains. The Kefir was produced using the traditional production method (Rosa et al., 2017), which involved the cultivation of Kefir grains in 100 mL of milk at room temperature during 24 h. After the fermentation, the Kefir grains were separated from fermented milk. The process was repeated several times. Finally, Kefir grains were collected and stored at 4 °C. A portion of the Kefir grains and the fermented milk was lyophilized, grounded and stored at room temperature.

2.2 Adsorption of mycotoxins in buffer solutions

2.2.1 Preparation of buffer solutions with mycotoxins

To evaluate the adsorption of mycotoxins at different pH values it was prepared the following buffer solutions: 0.1 M KCl/HCl buffer at pH2, 0.1 M citrate/phosphate buffer at pH 5 and 0.1 M phosphate buffer at pH 7, according to Stoll and Blanchard (1990) Next, mycotoxins AFB1 (Sigma), OTA (Biochemics) and ZEN (Sigma) (1 μg/mL each) were added to the buffers.

2.2.2 Adsorption tests in buffer solutions

The adsorption experiments were performed as described by Fernandes (2016) with some modifications. Samples tested were the lyophilizates obtained from Kefir grains, Kefir fermented milk, *L. kefiri* KFLM3 cultivated in MRS/milk and *K. servazzii* KFGY7 cultivated in YPD/milk. Each lyophilizate was tested at three different pH (pH 2, 5 and 7) in triplicate. First, 20 mg of each lyophilizate were weighed into microtubes of 2 mL. Then, 1 mL of buffer containing the mycotoxin mixture was added. Tubes were vortex agitated and incubated at 37 °C under rotary shaking for 1 hour. After the incubation, they were centrifuged (12000 ×g, 15 min) and 0.8 mL of the supernatants were collected to clean microtubes containing 0.8 mL of acetonitrile/methanol/acetic acid (78/20/2, v/v/v). Those samples were then vortex agitated and filtered to clean 2 mL amber vials using a syringe filter (Nylon, 0.45 μm). Samples were preserved at -20 °C until analyzed by High Performance Liquid Chromatography (HPLC), as described in section 2.3.1.

2.3 Adsorption of mycotoxins under gastrointestinal conditions

The gastrointestinal tract conditions were simulated following the procedure described by Minekus et al. (2014). Samples tested were the lyophilizates obtained from Kefir grains, Kefir fermented milk, *L. kefiri* KFLM3 cultivated in MRS/milk and *K. servazzii* KFGY7 cultivated in YPD/milk. Briefly, 1 g of each lyophilizate was dissolved in 3 mL of distillate water with vortex agitation. Then, a 3 mL solution containing 10 μg/mL of each mycotoxin (AFB1, OTA and ZEA) was added. A 1 mL sample, representative of the initial conditions, was collected for further analysis of mycotoxins concentration by HPLC. Samples collected were preserved in ice until processed. Further, the solution was exposed to the conditions that simulate the first stage (the oral phase) by adding 60 μL of amylase, 4 mL of simulated salivary fluid (SSF) (15.1 M KCl, 3.7 M KH_2PO_4, 13.6 M $NaHCO_3$, 0.15 M $MgCl_2.(H_2O)_6$, 0.06 M $(NH_4)_2.CO_3$, and 1.1 M

HCl), 0.025 mL of $CaCl_2.(H_2O)_2$ (to obtain 1.5 M in the fluid) and 0.475 mL purified water. Since human α-amylase was not available, it was used an amylase from Megazyme, which activity (17260 U/mL) was previously quantified according to α-amylase enzymatic assay described by Minekus et al. (2014) (Annex I – Figure 21). The tubes were then incubated for 2 min at 37 °C in a shaking bath. After the incubation period a sample of 1 mL was collected and maintained in ice until processed.

Following, the second stage (the gastric phase) was simulated by adding 1 mL of a solution of porcine pepsin (2000 U/mL in purified water), 7.2 mL of simulated gastric fluid (SGF) (6.9 M KCl, 0.9 M KH_2PO_4, 25 M $NaHCO_3$, 47.2 M NaCl, 0.1 M $MgCl_2(H_2O)_6$, 0.5 M $(NH_4)_2CO_3$ and 15.6 M HCl), and 0.0045 mL of $CaCl_2(H_2O)_2$ (to obtain 0.15 M in the fluid). After this addition, the pH was adjusted to 3.0 by adding 1 M HCl and purified water (volume required to dilute the SGF stock solution) and the tubes were again incubated in a shaking bath at 37 °C for 2 h. After the incubation period a sample of 1 mL was also collected, and ice cooled.

Finally, the third stage (intestinal phase) was simulated by adding 8.6 mL of intestinal fluid (SIF) (6.8 M KCl, 0.8 M KH_2PO_4, 85 M $NaHCO_3$, 38.4 M NaCl, 0.33 M $MgCl_2{\cdot}(H_2O)_6$ and 8.4 M HCl), $CaCl_2{\cdot}(H_2O)_2$ (to obtain 0.6 M in the fluid), 0.67 g of pancreatin suspension diluted in 5,98 mL of SIF, 182,3 mg bile solution diluted in 2.28 mL of SIF, 1 M NaOH to adjust the pH to 7.0 and purified water (volume needed to dilute the SIF). Then, the samples were incubated at 37 °C for 2 h and, at the end, one sample of 1 mL was also collected and cooled. The enzymatic activity of collected samples was interrupted with the addition of 30 μL of 1 M NaOH (volume required for the pH to be greater than 8). Regarding the intestinal phase, 10 μl of an enzyme inhibitor pefabloc SC (1 M) were used to interrupt the enzymes' activity. Thereafter, the samples were centrifuged (12000 ×g, 30 min) and transferred to vials for further analysis of the adsorption in HPLC. All samples were tested in duplicate and a negative control (water) and positive controls (activated charcoal and bentonite) were also performed. The control assays were performed using 1 mL of water, 1 g of bentonite and 1 g of activated carbon in substitution of lyophilizates and following the same procedure described above for them.

In order to evaluate the effect of amylase on the mycotoxins' adsorption, an assay with the Kefir grains and Kefir fermented milk was carried out without the addition of this enzyme.

Pancreatin, porcine pepsin, bile extract porcine, HCl, $NaHCO_3$, $(NH_4)_2{\cdot}CO_3$ and pefabloc SC were purchased from Sigma-Aldrich. NaOH, KCl, $CaCl_2{\cdot}(H_2O)_2$, KH_2PO_4 and NaCl were obtained from Panreac and $MgCl_2{\cdot}(H_2O)_6$ was purchased from Merck.

2.3.1. Mycotoxins quantification by HPLC

Mycotoxins were quantified by HPLC according to Fernandes (2016). The mobile phase used in the determination of AFB1 was a mixture of H2Od/methanol/acetonitrile (3:1:1, v/v/v), and the one used in the determination of OTA and ZEA, a mixture of H2Od/methanol/acetic acid (65:35:1, v/v/v). The mobile phases were filtered (GHP, Pall) and degassed on ultrasound. The flow rate was set to 1 mL/min. The column used was a C18 reversed-phase YMC-Pack ODS-AQ column (250 × 4.6 mm I.D., 5 μm) attached to a pre-column with the same stationary phase. The parameters of detection of AFB1 were: excitation = 365 nm, emission = 435 nm and Gain = 100. For OTA and ZEN, the parameters were: excitation = 280 nm, emission = 460 nm and Gain = 1000. A calibration curve was done for the different mycotoxins, using different standards concentration between 0.05 - 1 μg/mL (Annex I – Figure 22, 23 and 24). The HPLC system was equipped with a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector and a Jones Chromatography 7971 column heater that was maintained at 35 °C for AFB1, and at room temperature for OTA and ZEA determination. The instrument and chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively.

2.4 Isolation and identification of polysaccharides produced by Kefir strains

To evaluate the chemical composition of Kefir grains and Kefir fermented milk, their polysaccharides were treated by acid hydrolysis. The monomers resulting from the hydrolysis of polysaccharides were analyzed by HPLC (section 2.4.3).

2.4.1 Polysaccharides extraction from Kefir grains structure

Kefir grains hold polysaccharides inside of their structure but also contain polysaccharides adsorbed into the grains surface. To characterize the totality of polysaccharides, they were previously extracted from the grains before the treatment by acid hydrolysis. The extraction of polysaccharides from Kefir grains followed the methods reported by Lin and Chien (2007), Rimada and Abraham (2006) and Zekovic et al. (2005) with the modifications introduced by Zajšek et al. (2011). Briefly, 2 g of lyophilized Kefir grains (in triplicate) were treated with 150 mL of boiling water, with continuous stirring at 200 rpm for 3 h. Then, 30 mg of trichloroacetic acid (20% in water) was added to the cooled sample and left overnight to precipitate proteins and microbial cells. To remove the precipitate, the sample was centrifuged (10000 ×g, 20 min). After that, the supernatant was collected, the polysaccharides precipitated by adding an equal volume of cold acetone (> 99.5%) and stored in the refrigerator overnight.

Finally, after a centrifugation at 10000 ×g for 20 min at 4 °C, the sample pellet was washed with cold acetone, dried for 48 h at 42 °C and stored at room temperature for further use in the acid hydrolysis assay.

2.4.2 Acid hydrolysis of Kefir polysaccharides

To determine the chemical composition of lyophilized Kefir grains and Kefir fermented milk, it was necessary to evaluate the initial chemical composition of the sample (without treatment) as well as its moisture. For this, 40 mg of lyophilized Kefir grains (triplicate) were diluted in 1 mL of distilled water. Samples were filtered (nylon, 0.22 µm) and analyzed by HPLC for the determination of the saccharides, as described in the section 2.4.3. The moisture of each sample was evaluated using the Moisture Analyser mac (Radwag).

Acid hydrolysis was performed according to the standard Laboratory Analytical Procedures (LAPs) for biomass analysis provided by the US National Renewable Energy Laboratory (Sluiter et al., 2008). A mass of 0.3 g lyophilized Kefir grains after extraction were weighed into hydrolysis tube (in triplicate). A volume of 3 mL of 72% sulphuric acid was added to each tube and then placed in a 30 °C water bath for 1 hour. Samples were homogenized during hydrolysis with a glass rod. After hydrolysis, samples were transferred to glass bottle and then diluted to a concentration of 4% by adding 84 mL of deionized water. The samples were weighed and autoclaved at 121 °C for 1 hour. After the autoclave cycle, the hydrolysates were cooled and weighed. Finally, 1 mL of each sample was filtered (nylon, 0.22 µm) and frozen until further analysis by HPLC.

This assay was also carried out for the Kefir fermented milk and lyophilized grains of Kefir without the previous extraction treatment to quantify only free polysaccharides.

2.4.3 Polysaccharides quantification by HPLC

Hydrolyzed samples of the polysaccharides were quantified by HPLC (Jasco, Tokyo, Japan), using an Aminex HPX-87H column (Bio-Rad). The monomers resulting from the hydrolysis of polysaccharides were measured with a refractive index (RI) detector (Jasco, Tokyo, Japan). The elution was conducted at 0.6 mL/min flow rate and at 60 °C. The mobile phase was 0.005 M H_2SO_4 in ultrapure water filtered through a 0.2 µm nylon filter (Millipore) and degassed. Standards of cellobiose, glucose, galactose, xylose and arabinose were used and the calibration curves were plotted (Annex I – Table 12).

2.4.4 Determination of the chemical composition of Kefir

To quantify the compounds existing in the hydrolyzed samples (polysaccharides extracted from Kefir grains, intact Kefir grains and Kefir fermented milk) it was necessary to consider some parameters that are summarized in the following formula:

$$\boldsymbol{CP = F \times c \times \frac{[A]}{d} \times \frac{(P+PMH\times H)}{PMH\times(1-H)} \times (100 - CE) \times FC} \qquad (1)$$

Where:

CP: polymer composition

F: factor that corrects the degradation of sugars

C: stoichiometric correction factor

d: density of the analyzed solutions (1025 g/L)

P: weight of water added (84 g)

PMH: total weight of material (0.3 g)

CE: content of extracts (0)

H: moisture of the material (g of water/g wet material)

[A]: monomer concentration (g/L)

FC: correction factor - weight of the glass bottle after autoclaving/initial weight

The tabulated parameters used to quantify the compounds of the hydrolyzed samples are found in the Table 4.

Table 4 | Factor that corrects the degradation of glucan, xylan, arabinan, acetyl groups (F) and stoichiometric correction factor (C) for the calculation of the polymer (CP) composition in the different samples tested (polysaccharides extracted from Kefir grains, intact Kefir grains, Kefir fermented milk).

	Glucan	Xylan/Arabian	Acetyl groups
F	1.04	1.088	1
C	0.9	0.88	0.72

The HPLC analysis allowed to identify the peaks corresponding to each monosaccharide through their retention time (min). Each monosaccharide was quantified using the area (mV/min) of the peak and the respective calibration curve. To calculate the composition of polysaccharides (CP) it was necessary to previously determine the concentration of each monosaccharide. For this, the equation of each calibration curve (Annex I – Table 12) was used. Substituting (y) in the equations by the area of each

peak it was possible to determine the concentration of monosaccharides present in each sample (x). Once the concentrations have been obtained, the mass of each free monosaccharide (mg) was calculated considering the dilution factors. The correction factor (FC) was also determined by dividing the weight of the glass bottle after autoclaving by its initial weight. Thus, with the concentration and the calculated FC, with the humidity obtained and with the remaining tabulated values (Table 4), it was possible to determine the composition according to the equation (1). Finally, the CP value in percentage was converted in mass (mg), the value of free initial mass was discounted to the CP of each polysaccharide and the result was converted to a mass percentage.

2.5 Evaluation of the chemical composition by TLC

The Thin-layer Chromatography (TLC) was another method used to evaluate the chemical composition of the different samples. TLC analysis was performed according to Reiffova and Nemcova (2006) with some modifications.

2.5.1 Preparation of standards solutions and TLC reagents

Standard solutions of glucose, fructose, sucrose (5 g/L), fructooligosaccharides – FOS (1-kestose, nystose and 1F-fructofuranosylnystose), and frutalose® OFP (Sensus) were prepared in distillate water. Samples (freeze-dried Kefir grains, freeze-dried Kefir fermented milk, non-lyophilized grains and Kefir fermented milk and *L. kefiri* KFLM3) were diluted in 1 mL of distilled water.

The detection reagent was prepared as following: 1 g of diphenylamine and 1 mL of aniline were dissolved in 80 mL acetone; then, 11.8 mL of phosphoric acid was carefully added; the final solution was diluted up to 100 mL with acetone.

2.5.2 Chromatography and detection

Separation of monosaccharides from the solutions was carried out by TLC on silica gel plates (20x20 cm). For this, 2 μL of each sample was applied at the base of the plate. The mobile phase used to run the TLC was a mixture of butanol-ethanol-water (5:3:2, v/v/v). The applied analytes were separated by the mobile phase contained in the TLC chamber, for about 3 hours, at room temperature. After the plates development, the mobile phase present in the plate was evaporated in a fume hood for 15 min. Finally, to detect the spots, the TLC plate was sprayed with a mixture of diphenylamine-aniline-phosphoric acid in acetone and dried for 5 min at 120 °C.

2.6 Statistical analysis

The data obtained were analyzed using GraphPad Prism version 7.00 for Windows (La Jolla California, USA) at a significance level of 5%. Results were expressed as mean values ± standard deviation. Two-way ANOVA, Tukey's multiple comparisons test and Sidak's multiple comparisons test were performed to test significant differences.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Adsorption of mycotoxins in buffer solutions

These preliminary experiments aimed at understanding the influence of pH in the mycotoxin adsorbing properties of Kefir. Lyophilizates of Kefir grains, Kefir fermented milk and two microorganisms isolated from Kefir grains (*K. servazzii* KFGY7 and *L. kefiri* KFLM3) were tested in buffer solutions at pH 2, 5 and 7, containing a mixture of three mycotoxins (AFB1, OTA and ZEA). Figure 10 shows the results obtained in these experiments. For ZEA and AFB1, most of adsorption percentages at pH 2 and pH 5 were similar. For OTA, there was an average decrease of 8% from pH 2 to pH 5. On the other hand, when comparing the adsorption at pH 7 to that obtained at pH 5, it was verified that there was a high loss of adsorption, in average, 33.6% for AFB1, 14.0% for ZEA and 53.1% for OTA. This decrease was more pronounced for AFB1 and OTA than for ZEA. The results obtained for AFB1 agree with (Faucet-Marquis et al., 2014) which verified that, when testing yeast cell walls at pH 5 and 7, the adsorption of AFB1 was 45% and 2%, respectively. Regarding OTA and ZEA, Faucet et al. (2004) tested yeast-based products as an adsorbent, and also observed decreases in adsorption at pH 7.0, verifying that ZEA was better adsorbed at pH 5 (75%) than at 7 (60%). Regarding OTA, this mycotoxin was only significantly adsorbed at pH 3 (50%).

To better analyze the results obtained, it is necessary understanding the adsorption process and how it occurs. During this process, different types of intermolecular interactions are involved, which affect the adsorption capacity significantly and which are often a reversible phenomenon. According to Di Gregorio et al. (2014), the adsorption forces of *Van der Waals* and electrostatic attraction or repulsion (including polarization, dipole and quadrupole interactions) are the main factors responsible for the adsorption process. Adsorption is a complex process where the electrostatic attraction occurs between an ionized molecule and an adsorbent agent (Di Gregorio et al., 2014). The electrostatic or hydrophobic connections can be affected by pH, since the molecule and the adsorbent can change their ionic form according to pH, and the electrostatic repulsion will oppose the hydrophobic binding that would be occurring at a lower pH (Ventom and Asenjo, 1991). Eventually, in this experiment, the increase in pH buffer solutions may result in changes in adsorbent charge, since it may create an environment of lower electrostatic attraction and consequently disrupt the mycotoxin-adsorbent complex.

Concerning OTA, the decrease in the adsorption capacity at pH 7 can be justified by the fact that according to Huwig et al. (2001), this mycotoxin assumes a monoanionic form (OTA^{-1}) at pH> 4.0, a dianionic form (OTA^{-2}) at pH> 7.0 and an open lactone form (OP-OTA) under alkaline conditions. Consequently, at pH 7, OTA takes an ionic form which makes the interaction with the adsorbent weaker, resulting in its release and justifies the noticeable decrease that occurred in these experiments.

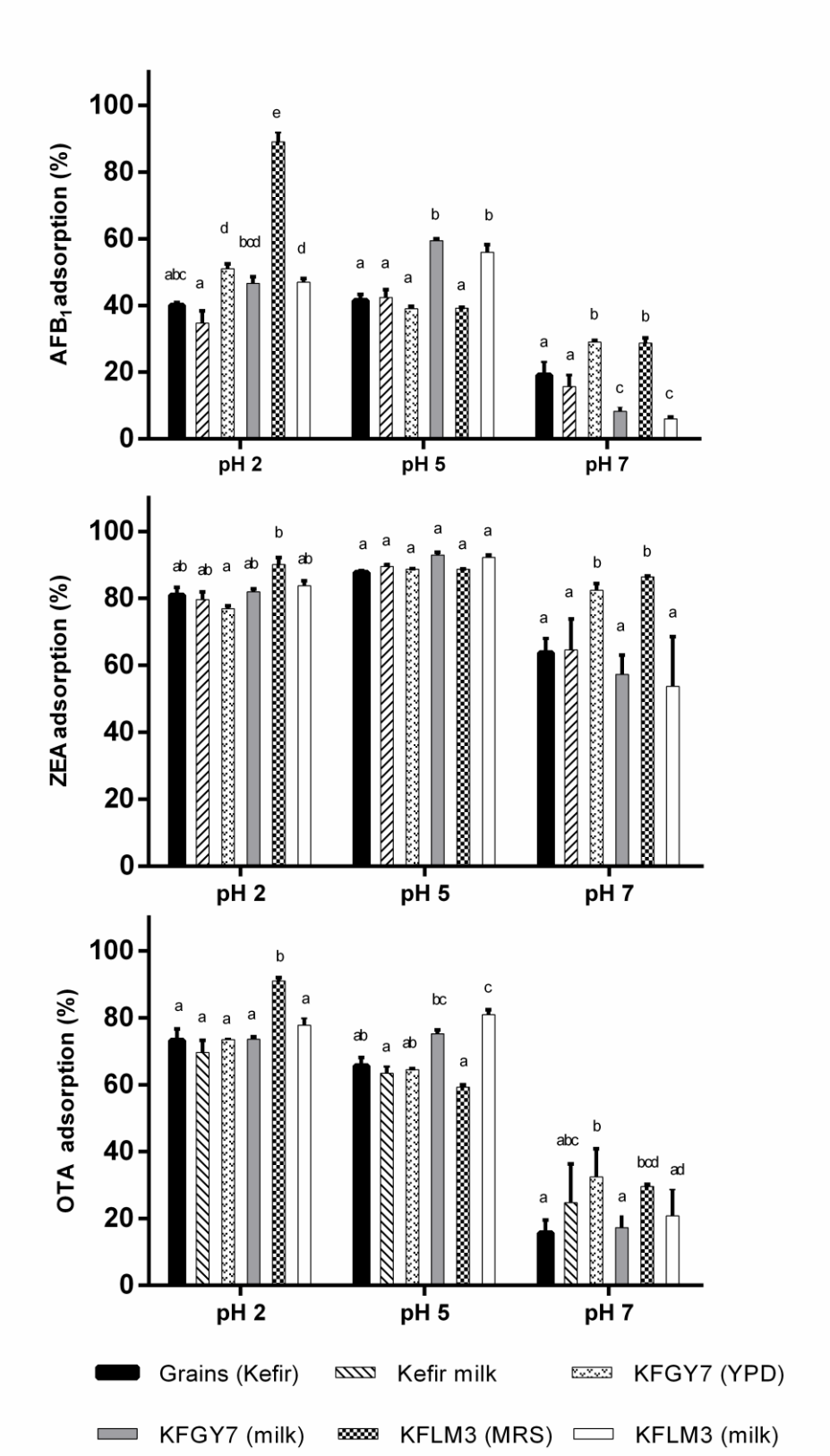

Figure 10 | Percentage of AFB1, ZEA and OTA (%) adsorbed by Kefir grains, Kefir fermented milk, *K. servazzii* KFGY7 in YPD/milk and *L. kefiri* KFLM3 in MRS/milk at pH 2, 5 and 7. Results are expressed as the average of three replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the different samples within each pH.

In addition, it is necessary to take into account that enzymes such as amylase, catalase, peroxidase and others may be present, since all lyophilizates have microbiological origin. That way, these enzymes can hydrolyze the binding centers or affect the involved mechanisms in other ways, reducing the adsorption capacity of tested samples. The efficacy of the adsorbents and the effect that pH exerts depends on various factors such as the composition and physicochemical structure of the adsorbent and mycotoxin (Ghofrani Tabari et al., 2018; Ringot et al., 2007; Sabater-Vilar et al., 2007). The most important role is assumed by the physical structure of the adsorbent (total charge, charge distribution, pore size and accessible surface area). Furthermore, the properties of the adsorbed mycotoxins, such as polarity, solubility, shape and charge distribution, also play an important role. In general, the binding capacity increases with the surface area and chemical affinities between the adsorbent and mycotoxin (Huwig et al., 2001; Kabak et al., 2006; Zárate et al., 2000).

After analyzing how the adsorption process occurs and how it is affected by the pH, it is interesting to interpret the variations between the lyophilizates in order to evaluate which has the highest adsorption capacity. As it can be observed in Figure 10, *L. kefiri* KFLM3 (MRS) presents the best performance at pH 2, adsorbing 89.1 ± 3.0% of AFB1, 90.1 ± 2.1% of ZEA and 91.0 ± 1.0% of OTA. At pH 5, the microorganisms grown in milk showed higher efficiency in the adsorption of the mycotoxins, and there were no significant differences between *L. kefiri* KFLM3 and *K. servazzii* KFGY7 in AFB1 and OTA adsorption. For ZEA, there were no significant differences between any of the samples tested. Finally, at pH 7 there are no significant differences between the tested microorganisms grown in MRS and YPD ($p<0.05$) and the best results were obtained for the ZEA, with *K. servazzii* KFGY7 (YPD) and *L. kefiri* KFLM3 (MRS) adsorbing 82.4 ± 2.0% and 86.4 ± 0.3% of this mycotoxin, respectively. For OTA, an adsorption of 32.4 ± 8.4% was found by *K. servazzii* KFGY7 and 29.6 ± 0.6% by *L. kefiri* KFLM3. Finally, *K. servazzi* KFGY7 and *L. kefiri* KFLM3 were able to adsorb 28.7 ± 1.6% and 29.0 ± 1.0% of AFB1, respectively.

In resume, it can be highlighted that an important criterion for the evaluation of mycotoxin adsorbents is their efficiency at different pH levels (acidic and neutral). The results obtained allowed a preliminary understanding of Kefir adsorbing properties. It could be verified that Kefir grains and Kefir fermented milk, as well as the yeast and bacteria isolated from the grains presented high adsorption efficiency of AFB1, ZEA and OTA. In addition, according to the results obtained, adsorption may be disturbed by neutral pH, since the increase of pH may affect the adsorbent-mycotoxin complex by altering the ionic form/charge of the mycotoxins or the surrounding environment, which may create a lower electrostatic attraction. However, in order for the adsorbents tested to be applied efficiently, they must be

efficient throughout the gastrointestinal tract and the mycotoxin adsorbent complex remain stable, ensuring that desorption of the toxin during digestion does not occur (Hur et al., 2011). Accordingly, the next step was to perform adsorption assays under gastrointestinal conditions.

3.2 Adsorption of mycotoxins under simulated gastrointestinal conditions

3.2.1 Adsorption performances of activated carbon and bentonite

Activated carbon is a non-soluble powder, formed by the pyrolysis of organic materials (Jard et al., 2011), that has high porosity and surface to mass ratio (500-3500 m^2/g), which gives it an excellent capacity of adsorption (Huwig et al., 2001). There are several applications of activated carbon in the industry, such as analytical chemistry, environment, agriculture, fuel purification, gas, alcoholic beverages and medicine, and it has been used as a treatment for severe poisoning since the 19th century (Ramos et al., 1996). In addition to these applications, there are studies that prove that activated carbon can adsorb the major mycotoxins in aqueous environments (Huwig et al., 2001). Bentonites (BEN) are designated as clays with a layered crystalline microstructure of variable composition (Di Gregorio et al., 2014). They are often referred to as smectites, since this is the dominant clay mineral. The adsorption efficiency of BEN depends basically on its content of montmorillonite and interchangeable cations (Kolosova, 2011; Marroquin-Cardona et al., 2011). Due to its high surface area and the high cation exchange capacity of the smectite group, it can adsorb organic substances through the penetration of cations and polar molecules (Bočarov-Stančić et al., 2011; Di Gregorio et al., 2014).

In this experiment, the adsorption capacity of activated carbon and BEN under conditions that simulate the gastrointestinal tract were tested so they could be used as positive controls for comparison purposes with Kefir lyophilizates. The results obtained are shown in Figure 11. As it can be seen in Figure 11, the adsorbing capacity of activated carbon remained constant over the different GI phases and maintained a high efficiency for all mycotoxins. The percentages of adsorption in intestinal phase reach 97.8 ± 0.1% for AFB1, and 100% for ZEA and OTA. These results show that the adsorption capacity of the activated carbon is not affected by the pH, nor by the enzymes or salts added during the digestion process. These results are in agreement with Decker and Corby (1980), who verified that activated carbon adsorb AFB1 efficiently *in vitro* at a neutral pH. In addition, it has been shown that the activated carbon may be suitable in ZEA and DON binding (Bueno et al., 2005; Doll et al., 2004; Sabater-Vilar et al., 2007). Avantaggiato et al. (2007) also performed an *in vitro* study to analyze the efficacy of a

carbon/aluminosilicate-based product (2%) and an adsorption of 88% AFB1, 44% ZEA and 29% FB1 was observed.

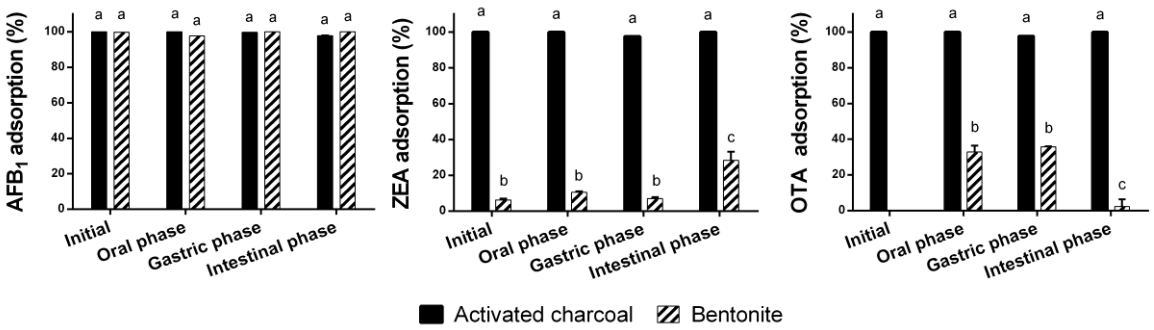

Figure 11 | Percentage of AFB1, ZEA and OTA (%) adsorbed by activated charcoal and bentonite under gastrointestinal conditions (oral, gastric and intestinal phase). Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between each phase for each adsorbent and mycotoxin.

In Figure 11, it can also be observed that BEN adsorbed 100% of AFB1 in all phases, performing similarly to activated charcoal. However, the same did not occur for ZEA and OTA, since the percentages of adsorption remained substantially low throughout the different phases. At the end of the process, in the intestinal phase, the percentage of ZEA and OTA adsorbed by BEN were of only 28.4 ± 4.8% and 2.3 ± 4.1%, respectively. According to Santos et al. (2011) and Thieu et al. (2008) BEN was initially used to bind aflatoxins and found to be less effective as a binding agent for other mycotoxins. Huwig et al. (2001) also concluded that BEN has a more pronounced ability to adsorb aflatoxins (90-95%) than ZEA and OTA. So, the results obtained are in accordance with the expected ones. With regard to AFB1 binding by BEN, some studies suggested that aflatoxins may interact at multiple sites, especially at the interlayer region, but also at the edges and basal surfaces of these clays (Deng et al., 2010; Phillips, 1999; Phillips et al., 2002).

The evaluation of these two products allow their use as positives controls to validate the GI model and compare performances with the Kefir lyophilizates. Briefly, activated carbon presented greater versatility and applicability since it proved to be an efficient binding agent for AFB1, ZEA and OTA, while BEN only showed high efficiency in the adsorption of AFB1.

3.2.2 Adsorption performances of Kefir samples

Another objective of this work was to compare the mycotoxin adsorption capacity of the Kefir grains with that of Kefir fermented milk, to evaluate if the grains structure and composition have influence in the adsorption of mycotoxins. The adsorption of AFB1, ZEA and OTA by Kefir grains and Kefir fermented milk is shown in Figure 12. In general, for both lyophilizates, it can be observed a decrease in the adsorption of the mycotoxins throughout the simulated digestion process. However, the Kefir fermented milk presented lower percentage of adsorption when compared to the grains. These results demonstrate that although grains and milk microbial composition is similar, grains structure as well as its three-dimensional arrangement seems to confer protection not only against the enzymes added during the simulated GI process, but also against the physical conditions established (i.e. pH, temperature, time and agitation). However, this protective effect is maintained only until the gastric phase, since in the intestinal phase it was observed a considerable decrease of the adsorption percentages for all samples and mycotoxins tested.

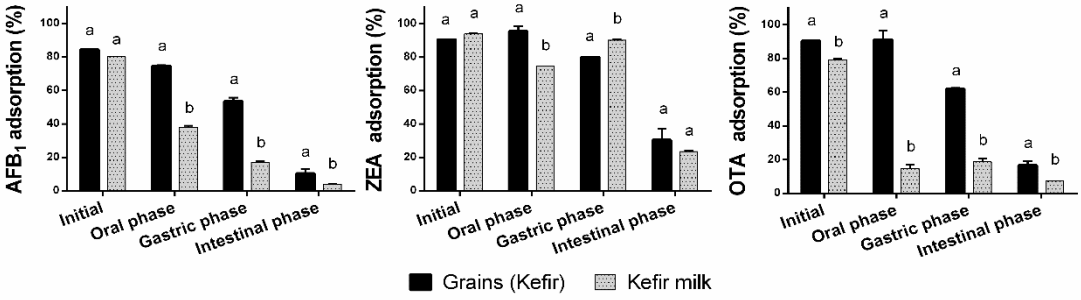

Figure 12 | Evaluation of the adsorption of AFB1, ZEA and OTA (%) by Kefir grains and Kefir fermented milk under gastrointestinal conditions (oral, gastric and intestinal phase). Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the lyophilizates in each phase and each mycotoxin.

In the preliminary tests (Figure 10), it was verified that there were no significant differences between the Kefir grains and Kefir fermented milk adsorption. Thus, the pH seems to similarly affect the adsorption capacity of these samples. But on the other hand, when exposed to conditions that simulate the gastrointestinal system (Figure 12) the grains presented greater resistance and maintained higher percentages of adsorption. However, in the intestinal phase, the grains structure is not sufficient to resist to the action of pancreatic enzymes and bile for 2 hours at pH 7, since low adsorption percentages are obtained, as it can be observed in Figure 13. In this phase, although adsorption percentages between Kefir grains and fermented milk are still statically different they are more similar between them, as compared to the other phases.

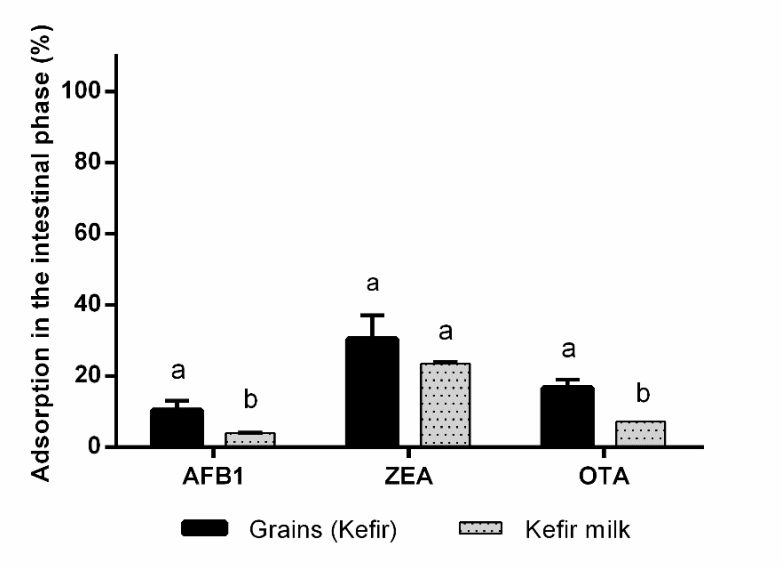

Figure 13 | Adsorption of AFB1, ZEA and OTA (%) by Kefir grains and Kefir fermented milk in the intestinal phase. Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the lyophilizates for each mycotoxin tested.

The adsorption obtained for AFB1, ZEA and OTA into the grains, in the intestinal phase, was of only 10.3 ± 2.8%, 30.4 ± 6.7% and 16.6 ± 2.4%, respectively. At this stage, the combination of pancreatin, bile and pH 7 seems to destabilize the mycotoxin-adsorbent binding sites, causing the release of the mycotoxins. As already mentioned, the pancreatin used in this study is a porcine pancreatin that contains enzymatic components such as trypsin, amylase, lipase, ribonuclease and proteases. Thus, the enzymes existing in this solution may have acted on the surface of microorganisms, causing the lysis of cell wall proteins, mannans and β-glucans, and consequently the loss of the three-dimensional form of cell walls (Adamitsch et al., 2003; Bzducha-Wrobel et al., 2014; Ventom and Asenjo, 1991). According to Coleman et al. (1980) and Heuman et al. (1996) bile salts at high concentrations can also rapidly dissolve membrane lipids and cause dissociation of membrane proteins resulting in the leakage of cell contents and cell death. Thus, bile can eventually act in the same way in the binding centers of mycotoxins, changing its structure and causing their release, resulting in the observed lower percentage of adsorption in the intestinal phase.

In general, this assay proved that the Kefir grain structure confers protection against the harsh digestion conditions until gastric phase and allowed to confirm that ZEA is the best adsorbed mycotoxin, as verified in the preliminary tests (Figure 10). In addition, it is possible to state that Kefir fermented milk has some detoxifying properties against AFB1, ZEA and OTA, thus increasing the number of advantages already described for this fermented product (Bourrie et al., 2016; Prado et al., 2015; Rosa et al., 2017).

In this way, it was possible to confirm that Kefir users, by drinking this beverage, are indirectly protecting their organisms against mycotoxins. Thus, this consumption would be beneficial for human health, since mycotoxins are present in most foods that humans consume.

3.2.3 Influence of amylase in the adsorption performances of Kefir samples

In order to evaluate the influence of amylase on the adsorption of Kefir grains and fermented milk, an assay was performed without the addition of this enzyme in the oral phase.

In the oral phase (Figure 14), α-amylase and simulated salivary fluid (SSF) were added and the sample remained in contact with the mycotoxins for 2 minutes at 37 °C, under agitation. During this step the salivary α-amylase acts as an endoglycosidase, hydrolyzes starch and related α-(1,4)-linked polysaccharides, and initiates starch digestion and transformation into oligo- and monosaccharides (González-Arias, 2013). Therefore, the optimal conditions for amylase activity are similar to those used in the oral phase (pH 7 and 37 °C) (Yang et al., 2014). As it can be observed in Figure 14, the amylase shows no influence on the mycotoxin adsorbing properties of Kefir lyophilizates. The only exception was for OTA adsorption by Kefir grains lyophilizates, where significant differences can be observed ($p<0.05$). In the following phases, there was no improvement over the results obtained with the addition of amylase (data not shown).

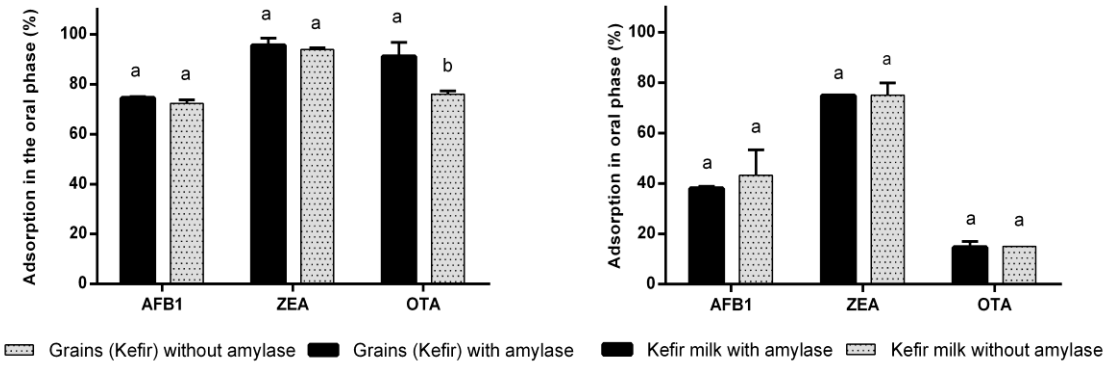

Figure 14 | Adsorption of AFB1, ZEA and OTA (%) by Kefir grains and Kefir fermented milk in the oral phase with or without amylase. Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the different samples and for each mycotoxin tested.

This analysis (Figure 14) leads to the conclusion that the decrease of the mycotoxins adsorption by Kefir fermented milk, which was previously observed in the oral phase (Figure 12), is essentially due to pH. These results also agree with the results obtained in the preliminary tests (Figure 10), where it can be observed the negative effect of pH 7 in the adsorption of mycotoxins.

As future work, it would be also important to test the influence of amylase on the yeast and bacteria lyophilizates, in order to verify if they are also unaffected by this enzyme.

3.2.4 Adsorption performances of Kefir microorganisms

This study aimed to evaluate the effect of the substrate on the adsorption of mycotoxins by the two microorganisms isolated from Kefir. For this, microorganisms isolated from Kefir grains were grown in rich laboratory culture media (MRS and YPD) and in milk. As it can be observed in Figure 15, in the initial step, the lyophilizates of the microorganisms grown in milk adsorbed better the mycotoxins than those grown in culture media, except for ZEA, where there were no significant differences between the two fermentation strategies ($p<0.05$). Nonetheless, in the oral phase, the lyophilizates from milk lost totally the capacity to adsorb the mycotoxins since those were totally released to the liquid fraction. Thus, it can be considered that the mycotoxin-adsorbent complexes of milk samples are not stable at the conditions of the oral phase. On the one hand, this behavior may be an indication that milk lyophilizates contain less biomass than the lyophilizates obtained with the culture media. On the other hand, this may be the result of adjusting the pH of oral phase to 7, since it may have destabilized the binding sites and dissociated the mycotoxin-adsorbent complexes. In the gastric phase, the pH was adjusted to 3 and the adsorption of milk lyophilizates recovered partially, suggesting that the acidic pH is more favorable to the binding of the mycotoxins. In the intestinal phase, the pH was adjusted again to 7 and, again, a substantial reduction of the adsorption percentages was observed.

In general, the behavior observed in the preliminary tests (Figure 10) is maintained, so that at acidic pH there is a good adsorption and when the pH is neutral the adsorption is weaker. Regarding the gastric and intestinal phases, it can be observed in Figure 15, that the use of MRS and YPD to obtain the microorganisms is advantageous in relation to milk since the mycotoxins-binding capacity of the culture media lyophilizates were significantly higher ($p<0.05$) then those of milk. The differences can be explained by the fact that the culture media promotes higher biomass grow of the microorganisms as compared to milk. If, as considered by Petruzzi et al. (2014) and Ringot et al. (2007), the main adsorption agents are the macromolecules present in yeasts and *Lactobacillus* cell walls, the amount of microorganisms will significantly affect the amount of existing binding sites, and consequently the adsorption of mycotoxins. Concerning ZEA, in the gastric phase no significant differences between the fermentation substrates were detected (Figure 15).

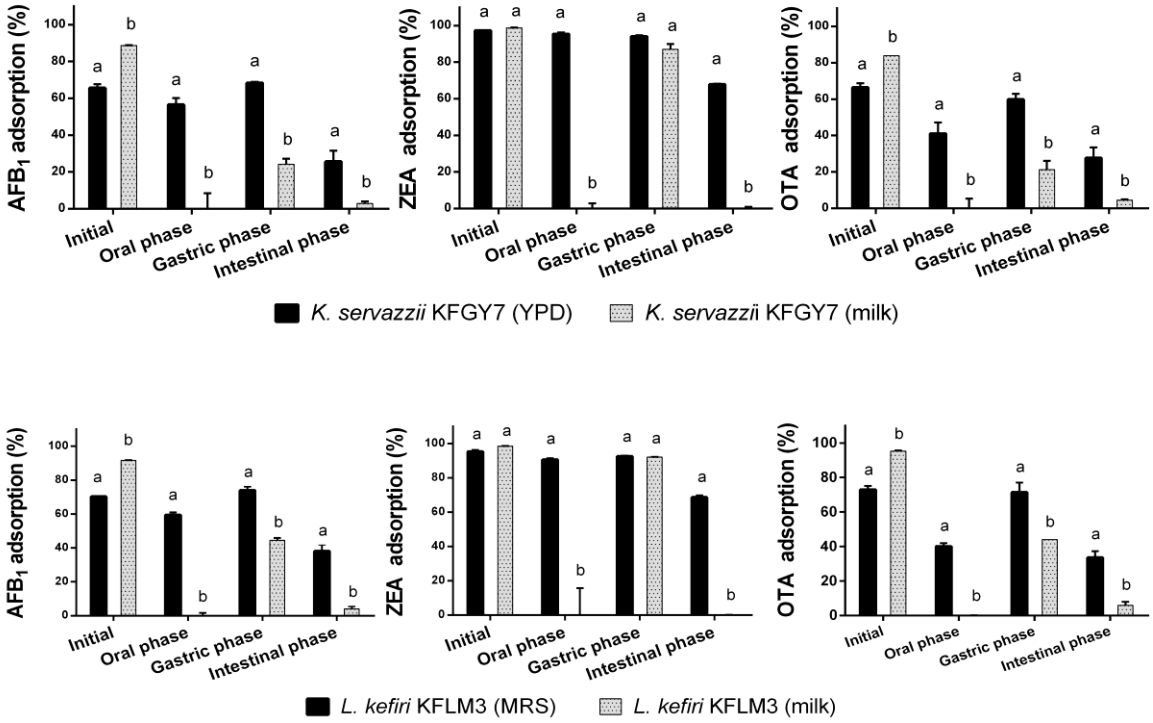

Figure 15 | Evaluation of the adsorption of AFB1, ZEA and OTA (%) by *K. servazzii* KFGY7 in YPD/milk and *L. kefiri* KFLM3 in YPD/milk under gastrointestinal conditions (oral, gastric and intestinal phase). Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the different samples in each phase and for each mycotoxin tested.

In general, when comparing the adsorption capacity in the gastric phase with the results obtained in the intestinal phase it was verified a marked decrease in all the samples tested. For ZEA, when the milk is used as the fermentation medium, the full release of the mycotoxin occurred. Furthermore, this analysis allowed to confirm again the strong influence of pancreatin and bile on the adsorption, as well as pH 7 in the intestinal phase. In order to confirm the effect of the enzymes added in the intestinal phase on the adsorption capacity of mycotoxins, it would be important to carry out assays without the addition of pancreatin and bile.

After the evaluation of the influence of the culture media on the adsorption of the microorganisms (Figure 15), is still important to compare the adsorption capacity of *L. kefiri* KFLM3 and *K. servazzii* KFGY7 (Figure 16).

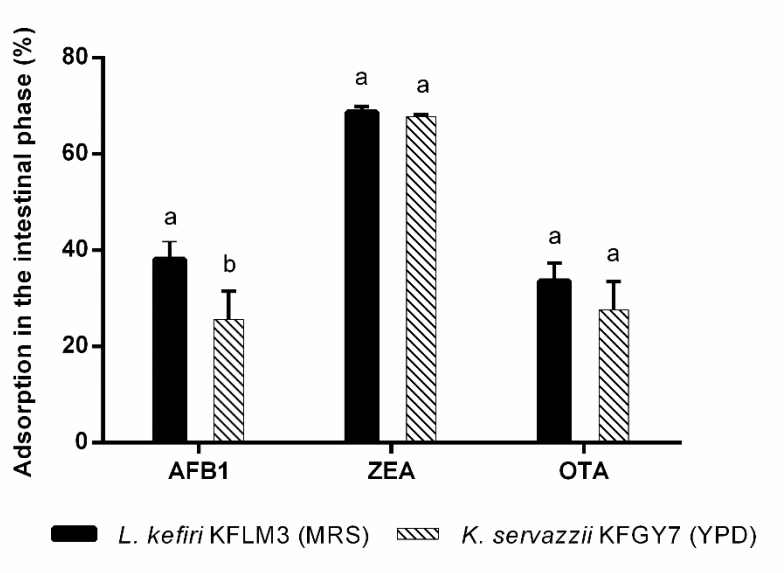

Figure 16 | Adsorption of AFB1, ZEA and OTA (%) by *L. kefiri* KFLM3 in MRS and *K. servazzii* KFGY7 in YPD in the intestinal phase. Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the different samples and for each mycotoxin.

By analyzing the above results (Figure 16), it can be verified that the two microorganisms showed similar adsorption rates for ZEA and OTA, but not for AFB1. *L. kefiri* KFLM3 adsorbed 38.1 ± 3.9% of AFB1, 68.7 ± 1.2% of ZEA and 22.6 ± 3.8% of OTA while *K. servazii* KFGY7 adsorbed to 25.6 ± 5.9% of AFB1, 67.8 ± 0.4% of ZEA and 27.6 ± 5.9% of OTA. Thus, it is possible to conclude that the results are quite satisfactory and that the two microorganisms tested are efficient in the adsorption of AFB1, ZEA and OTA. Once again, as in the preliminary tests (Figure 10), ZEA was the mycotoxin better adsorbed in all phase. Additionally, it was verified that the adsorption process is dependent of the fermentation substrate in which the microorganisms were grown, being favored when the culture media MRS and YPD were used.

3.2.5 Adsorption performances of Kefir consortium compared to Kefir microorganisms

In this experiment, the adsorption capacity of the Kefir grains (where the entire Kefir consortium is present) was compared with that of *L. kefiri* KFLM3 and *K. servazzii* KFGY7, the lactic acid bacteria and yeast isolated from Kefir grains (Figure 17). According to the histograms obtained for AFB1 and OTA, it was verified in the oral phase that grains are more resistant to pH 7 than the individual microorganisms.

The most marked differences in adsorption capacity were observed in mycotoxin OTA, since grains adsorbed 91.1 ± 5.7% of this mycotoxin while *L. kefiri* KFLM3 adsorbed 40.1 ± 1.9% and *K. servazzii* KFGY7 41.0 ± 6.2%. For ZEA no significant differences were verified in the oral phase between the grains and the microorganisms tested ($p<0.05$).

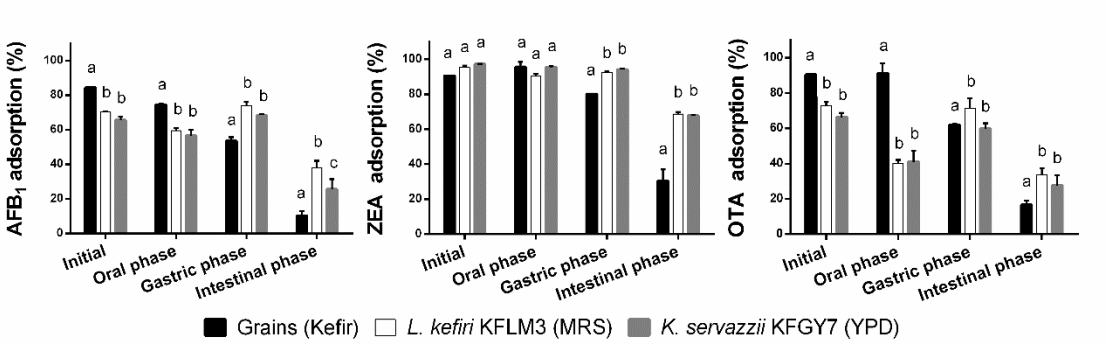

Figure 17 | Evaluation of the adsorption of AFB1, ZEA and OTA (%) by Kefir grains, *L. kefiri* KFLM3 in MRS and *K. servazzii* KFGY7 in YPD under gastrointestinal conditions (oral, gastric and intestinal phase). Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the different samples within the same phase for each mycotoxin.

In the gastric phase, the microorganisms presented better resistance to the acid conditions, resulting in an increase in AFB1 and OTA adsorption percentage. For OTA there was an increase of 31.3% for *L. kefiri* KFLM3 and 18.8% for *K. servazzii* KFGY7. For AFB1, *L. kefiri* KFLM3 and *K. servazzii* KFGY7 were able to increase the adsorption capacity by 15.5% and 11.8%, respectively. It is important to note that during this phase the pH is maintained at 3 and as verified in the preliminary tests (Figure 10), the acidic pH is more favorable to the adsorption of the mycotoxins. Concerning Kefir grains, as can be seen in Figure 17, their adsorption was slightly affected since there was a decrease in the adsorption percentages in the gastric phase when comparing with the oral one. In gastric phase, there were no significant differences of adsorption ($p<0.05$) between the two microorganisms. That way, the pepsin added in this phase does not seem to have affected the microorganisms adsorbing properties. However, in order to check whether or not pepsin affects the adsorption capacity of the microorganisms, as well as the grains, it would be interesting to carry out an assay without this enzyme as done for the amylase.

In the intestinal phase there was a marked decrease in the percentage of adsorption in all samples. For grains, it was observed a decrease of 43.3%, 49.7% and 45.3% in AFB1, ZEA and OTA adsorption, respectively, as compared to the gastric phase. Concerning the microorganisms, the adsorption of AFB1, ZEA and OTA by *L. kefiri* KFLM3 decreased by 35.8%, 23.9% and 37.8%, while the adsorption by *K. servazzii* KFGY7 decreased by 42.7%, 26.3% and 32.2%.

When assessing the mycotoxins adsorption capacity, it is necessary to take into account that, those responsible for the adsorption process, (probably proteins and glucans) produce adsorption sites and bind through various mechanisms such as hydrogen bonds and selective or hydrophobic reactions (Ghofrani Tabari et al., 2018). Thus, the enzymes added in the intestinal phase (pancreatin and bile) and the neutral pH, as observed in previous assays (sections 3.2.2, 3.2.4 and 3.2.5), seem to destabilize these binding centers, releasing part of the mycotoxins already adsorbed in the gastric phase, which explains the decrease adsorption observed in Figure 17.

In general, it is observed that microorganisms are more efficient in the adsorption process than the grains. Thus, the adsorption capacity of the grains is probably due microorganisms that are entrapped in the polysaccharide matrix, which corroborate with the previously described theories that indicate that the responsible for adsorption are cellular components of yeasts and bacteria (Petruzzi et al., 2014; Ringot et al., 2007).

In order to clarify if the components of yeasts and bacteria (glucans and kefiran) are actually responsible for the process of adsorption of mycotoxins it would be necessary to isolate the constituents and perform additional assays in simulated gastrointestinal conditions.

When comparing both microorganisms tested, in the intestinal phase (Figure 18) it is verified differences in the adsorption of AFB1, with *L. kefiri* KFLM3 adsorbing 38.1 ± 3.9%, and *K. servazzii* KFGY7 25.6 ± 2.9% of this mycotoxin.

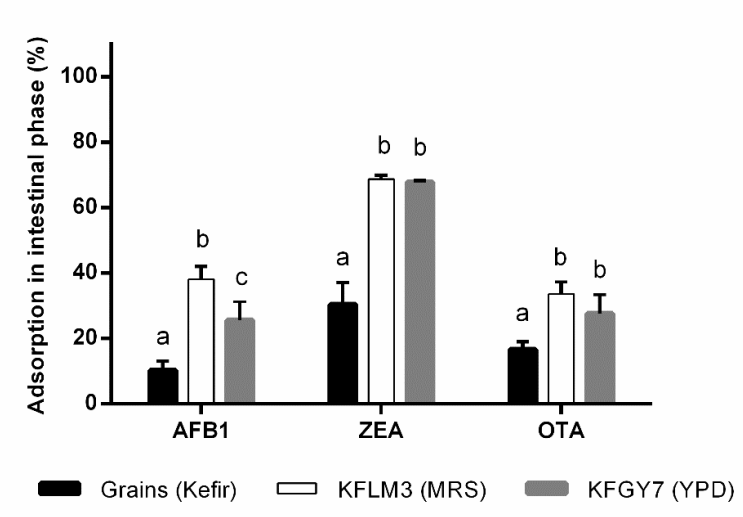

Figure 18 | Adsorption of AFB1, ZEA and OTA (%) by Kefir grains and *L. kefiri* KFLM3 in MRS and *K. servazzii* KFGY7 in YPD in the intestinal phase. Results are expressed as the average of two replicates and the error bars indicate standard deviation. Different letters represent significant differences ($p<0.05$) between the different samples and for the same mycotoxin tested.

Concerning ZEA, *L. kefiri* KFLM3 and *K. servazzii* KFGY7, adsorbed 68.7 ± 1.2% and 67.8 ± 0.4%, respectively. The results obtained for OTA were 33.6 ± 3.8% for *L. kefiri* KFLM3 and 27.6 ± 5.9% for *K. servazzii* KFGY7.

A recent *in vitro* study showed that the cell walls of *S. cerevisiae* and *L. rhamnosus* adsorbed 73.0% and 75.3% of AFB1 at pH 6. Regarding the adsorption of OTA, the lactic acid bacteria presented higher efficiency (61.7%) than the yeast (45.5%) (Ghofrani Tabari et al., 2018). When comparing these values with those obtained after simulating gastrointestinal conditions (Figure 17), it is easy to see the importance of this type of study, since the cell walls of the yeast and lactic acid bacteria presented lower adsorption percentages in the intestinal phase (Figure 18). Concerning the use of yeasts and lactic acid bacteria, most of the studies found in the literature report the adsorption of mycotoxins in buffer solutions, which, as we have seen, does not prove the real adsorption capacity of the materials, because under simulated GI conditions the adsorption dynamics change.

Armando et al. (2012) evaluated the binding of OTA and ZEA to *S. cerevisiae* in GI conditions. According with this author, OTA binding was tested under GI simulated with pH 2 and bile salts. For ZEA binding, a simulated gastrointestinal passage was performed, ressuspending the yeast cells in simulated gastric juice at pH 3 and after centrifugation, cells were added to artificial intestinal fluid at pH 8. The results obtained for *L. kefiri* KFLM3 and *K. servazzii* KFGY7 contradicts the study of this author, which found that GI conditions improve OTA and ZEA adsorption by *S. cerevisiae* and do not decrease interactions between adsorbent and mycotoxins.

González-Arias (2013) reported the use of models that simulate gastrointestinal conditions in several studies in order to evaluate the bioavailability of AFB1, OTA and ZEA. For example, regarding AFB1, it is possible to highlight a study of Kabak et al. (2009), which found the bioaccessibility of AFB1 could be reduced by 37% using a probiotic bacteria (*Lactobacillus acidophilus* NCC12) in a RIVM *in vitro* model. Petruzzi et al. (2016) evaluated OTA binding by 3 wine strains of *S. cerevisiae* (W13, W28, and the commercial isolate BM45) after exposure to conditions simulating the passage through human gastrointestinal tract. Strain W13 showed better performances after exposure to gastric and intestinal conditions, adsorbing 30% of OTA.

Furthermore, there are already some studies on contaminated food and feed with mycotoxins, in which the adsorption capacity of yeasts and lactic acid bacteria is evaluated, simulating the GI conditions. Kabak et al. (2009) evaluated the effect of probiotic bacteria (*L. acidophilus* NCC 12, *L. acidophilus* NCC 68, *L. casei*, *Bifidobacterium bifidum* Bb13 and *Bifidobacterium bifidum* NCC 381) on the bioaccessibility of AFB1 and OTA in pistachio nuts, low spiked infant food and high spiked infant food. In this study they

used 4.5 g of the contaminated matrix with bacteria and simulated the conditions of the mouth (6 mL saliva, 5 min), stomach (12 mL stimulated gastric juice, 2 h, pH 2.5) and intestine compartment (12 mL stimulated duodenal juice + 6 mL stimulated bile and 2 mL $NaHCO_3$, 2 h, pH 6.5). *L. acidophilus* NCC 68 was able to reduce OTA bioaccessibility in naturally contaminated buckwheat and low spiked infant food. Relatively to AFB1, the highest reduction in its bioavailability resulted from addition of *L. acidophilus* NCC 12 and *L. casei*.

There are also some *in vivo* studies about the mycotoxins adsorption by yeasts. According to Madrigal-Santillan et al. (2006), Shetty and Jespersen (2006) and Firmin et al. (2011), *S. cerevisiae* has beneficial effects against AFB1exposure in the poultry industry. In addition, in animal feeding experiments with the whole yeast and its cell wall the reduction of AFB1 toxicity was observed, indicating the possible stability of the yeast-mycotoxin complex through the gastrointestinal (Celyk et al., 2003; Santin et al., 2003). According to the studies available for *in vivo* experiments, the observed effects depend on the level and type of mycotoxin, as well as the type and dose of adsorbent, duration of exposure and species/physiological condition of the animal. the combination of different adsorbents (mineral and organic) seems to be more effective in counteracting the adverse effects produced by the exposure of several mycotoxins (Vila-Donat et al., 2018). In addition, according to Vila-Donat et al. (2018) the combination of different adsorbents (mineral and organic) seems to present better results against the adverse effects resulting from exposure to contaminated feed.

This study allowed to verify that the microorganisms isolated from the Kefir grains (*L. kefiri* KFLM3 and *K. servazzii* KFGY7) present satisfactory results in the adsorption of AFB1, OTA and ZEA. Thus, it would be interesting to use these microorganisms in the production of probiotic products with mycotoxins adsorbing properties.

3.3 Isolation and identification of polysaccharides produced by Kefir strains

The next task consisted in the isolation and identification of the polysaccharides produced by Kefir. For this, freeze-dried samples of Kefir grains and Kefir fermented milk were treated by acid hydrolysis and analyzed by HPLC.

3.3.1 Kefir grains polysaccharides characterization and quantification by HPLC

The Kefir grains contain free polysaccharides, however they may also hold polysaccharides inside of their structure or adsorbed into the grains. Thus, in order to fully quantify the polysaccharides present in the samples, the polysaccharides in the structure of grains or adsorbed were extracted. After extraction, the samples were treated by acid hydrolysis (a) and then analyzed by HPLC. This analysis provided the peaks obtained for each monosaccharide present, with specific area (mV/min) and retention time associated (min). The results obtained are shown in Table 5 and Table 6. The retention times allowed to identify the monosaccharides present in the Kefir grains. When comparing the retention times of standards used (cellobiose, glucose, galactose, xylose and arabinose) with those of the samples, it was verified the presence of a peak in the same retention times for the following monosaccharides: glucose and galactose. For the retention time of the other standards no peaks were obtained, so it is possible to conclude that Kefir grains do not present in their composition cellobiose, xylose and arabinose. After identifying the monosaccharides present in the sample, their concentration was determined (g/L). Lastly, the polymer composition (CP) of each polysaccharide was calculated according to the equation **(1)** and converted to a mass percentage.

Table 5 | Parameters used for the determination of polymer composition (CP) in the Kefir grains after extraction and treatment by acid hydrolysis: moisture, initial weight of the glass bottle (Wi) (g), weight of the glass bottle after autoclaving (Wf) (g), correction factor (FC) and moisture of the material (H).

Moisture	Wi (g)	Wf (g)	FC	H
15.68 ± 0.91	249.72 ± 1.04	246.15 ± 0.95	0.99 ± 0.0005	0.16

Table 6 | Polymer mass (%) of Kefir grains after extraction and treatment by acid hydrolysis (a) and respective parameters for its determination: polymer composition (CP), free monosaccharides mass (g) and determined total polymer mass (g).

Compound	CP (%)	Free monosaccharides mass (g)	Determined total polymer mass (g)	Polymer mass	
				(g)	(%)
Glucose/ Glucan	30.64 ± 3.85	0 ± 0	91.91 ± 11.54	91.91 ± 11.54	50.10 ± 6.3
Galactose/ Galactan	30.51 ± 5.15	0 ± 0	91.53 ± 15.46	91.53 ± 15.46	49.90 ± 8.4

After the calculation, it was verified that Kefir grains (0.3 mg) contain 61.1 ± 9.0% of polysaccharides and that those are composed by 50.1 ± 6.3% of glucan and 49.9 ± 8.4% of galactan.

3.3.2 Analysis of free Kefir grains polysaccharides by HPLC

To evaluate the free polysaccharides present in the Kefir grains, another assay was carried out with freeze-dried samples of Kefir grains. In this assay, the polysaccharides were treated by acid hydrolysis without previous extraction (b). Firstly, the concentration and the initial mass of the monosaccharides were calculated. Then, the determination of the chemical composition of the Kefir grains (Table 7 and Table 8) was performed according to the procedure described for assay (a), obtaining 42.1 ± 7.8% of free polysaccharides with 48.3 ± 8.4% of glucan and 47.0 ± 9.2% of galactan.

Table 7 | Parameters used for the determination of polymer composition (CP) in the Kefir grains after acid hydrolysis: moisture, the initial weight of the glass bottle (Wi) (g), the weight of the glass bottle after autoclaving (Wf) (g), correction factor (FC) and the moisture of the material (H).

Moisture	Wi (g)	Wf (g)	FC	H
8.39 ± 0.22	445.00 ± 3.44	441.83 ± 3.48	0.99 ± 0.0002	0.08

Table 8 | Polymer mass (%) of Kefir grains after acid hydrolysis (b) and respective parameters for its determination: polymer composition (CP), free monosaccharides mass (g) and determined total polymer mass (g).

Compound	CP (%)	Free monosaccharides mass (g)	Determined total polymer mass (g)	Polymer mass	
				(g)	(%)
Glucose/ Glucan	21.40 ± 3.72	3.59 ± 0.0	64.20 ± 11.16	60.61 ± 11.16	48.26 ± 8.39
Galactose/ Galactan	20.71 ± 4.10	3.09 ± 0.07	62.12 ± 12.31	59.03 ± 12.28	47.00 ± 9.23
Arabinose/ Arabinan	2.23 ± 0.00	0.73 ± 0.04	6.68 ± 0.0	5.96 ± 0.04	4.74 ± 0.03

In this analysis (Table 8) the presence of arabinan (4.7 ± 0.03%) was also verified. However, since the previous analysis (Table 6) did not verify its presence and since no study reports arabinose as one of the constituents of Kefir grains polysaccharides, it would be necessary to carry out new analyses to characterize more precisely the polysaccharides present in the Kefir grains. In addition, it is important to note that the concentration obtained for arabinose (0.37 ± 0.06 g/L) is below the limit at which HPLC can quantify with assertiveness (0.5 g/L). For the other standards no peaks were obtained, so it is also possible to conclude that the Kefir grains do not present in their composition cellobiose and xylose, as verified in the previous analysis (Table 6).

Analyzed the results obtained by HPLC for the characterization of polysaccharides present in Kefir grains, it is possible to conclude that the polysaccharides present in the Kefir grains are kefiran, as described by Rodrigues et al. (2005), Rimada and Abraham (2006), Piermaria et al. (2009), Zajšek et al. (2011), Pop et al. (2016) and Montesanto et al. (2016). When comparing the obtained results by HPLC in the two assays (a) and (b), it was verified that Kefir is composed of 61.1 ± 9.0% of kefiran (40.9 ± 0.4% in free form and 20,1% in grains structure/adsorbed).

3.3.3 Analysis of free Kefir milk polysaccharides by HPLC

In this study, the chemical composition of Kefir fermented milk was also evaluated (c). Identified the monosaccharides present in Kefir fermented milk, the concentration and mass of the monosaccharides present in the milk was calculated. Then, the determination of the chemical composition of the Kefir fermented milk (Table 9 and Table 10) was performed according to the equation (1).

Table 9 | Parameters used for the determination of polymer composition (CP) in the Kefir fermented milk grains after acid hydrolysis (c): moisture, initial weight of the glass bottle (Wi) (g), weight of the glass bottle after autoclaving (Wf) (g), correction factor (FC) and moisture of the material (H).

Moisture	Wi (g)	Wf (g)	FC	H
7.74 ± 0.57	340.9 ± 20.3	328.2 ± 20.0	1 ± 0.0	0.08

Table 10 | Polymer mass (%) Kefir milk after acid hydrolysis (c) and respective parameters for its determination: polymer composition (CP), free monosaccharides mass (g) and determined total polymer mass (g).

Compound	CP (%)	Free monosaccharides mass (g)	Determined total polymer mass (g)	Polymer mass	
				(g)	(%)
Glucose/ Glucan	18.78 ± 0.18	21.78 ± 6.02	56.34 ± 0.53	34.56 ± 5.68	42.3 ± 7.1
Galactose/ Galactan	22.21 ± 0.18	32.55 ± 7.05	66.64 ± 0.55	34.09 ± 6.52	42.4 ± 8.1%
Arabinose/ Arabinan	4.27 ± 0.19	1.03 ± 0.12	12.84 ± 0.57	11.81 ± 0.52	14.7 ± 0.7%

The CP of the polysaccharide as well as the calculated mass percentage are shown in the Table 10 In the Kefir fermented milk it was verified that the sample presents 40.9 ± 0.4% of kefiran and that the polysaccharide is composed of 42.3 ± 7.1% glucan and 42.4 ± 8.1% galactan. No xylose and cellobiose were found in Kefir fermented milk.

In this sample it was also verified the presence of 14.7 ± 0.7% of arabinan in the kefiran, a value higher than that obtained in the kefiran present in grains in free form (4.7 ± 0.03%).

After analysis by HPLC and the confirmation that the polysaccharides present in the Kefir grains and Kefir fermented milk are the kefiran, the results also demonstrated that the kefiran isolated is a heteropolysaccharide which contains glucose and galactose units. The glucose/galactose ratios obtained for the samples of Kefir grains and Kefir fermented milk are shown in the following Table.

Table 11 | Ratios (glucose/galactose/arabinose) obtained in the samples analyzed (Kefir grains and Kefir fermented milk). (a) extraction and acid hydrolysis, *(b)* and (c) acid hydrolysis.

Samples tested	Ratio (glucose/galactose/arabinose)
Kefir grains: assay (a)	1:1:0
Kefir grains: assay (b)	1:1:0.1
Kefir milk (c)	1:1:0.3

When analyzing Table 11, a very similar proportion of glucan and galactan is found in Kefir grains and Kefir fermented milk. Regarding composition of milk kefiran no studies were found in literature. For Kefir grains, the same proportion glucan and galactan was already described by several authors (Kooiman, 1968; Mukai et al., 1990; Pop et al., 2015; Rimada and Abraham, 2006; Zajšek et al., 2011). According to Kooiman (1968), the purified kefiran of Kefir grains contained approximately 50% of galactose and 50% of glucose residues (ratio of 1:1). Mukai et al. (1990) verified that the polysaccharide was composed of only D-glucose and D-galactose in the molar ratio of 0.9:1. Pop et al. (2015) evaluated the influence of temperature and time on the kefiran composition, testing four different conditions: 70 °C/100 min, 80 °C/30 min and 90 °C/20 min and 100 °C/5 min. These authors verified the presence of glucose and galactose units in the polysaccharide and also found that the level of monosaccharides in tested samples are dependent on the kefiran extraction conditions. The highest level of monosaccharides was found in sample treated at 80 °C/30 min. Regarding the composition of kefiran isolated from Kefir grains Pop et al. (2015) obtained a glucose/galactose ratio of 0.94:1.1. In a more recent study, Zajšek et al. (2011) also characterized the kefiran polysaccharide present in Kefir grains and found the presence of 59.8% glucose and 40.2% galactose (ratio 1: 0.67).

With regard to the content in kefiran, it was possible to verify that the Kefir grains present more quantity of this polysaccharide (61.1 ± 9.0%) than the Kefir fermented milk (40.9 ± 0.4%). This result agrees with the expected since the Kefir grains are a consortium of microorganism presenting in its composition the LAB responsible for the production of kefiran.

3.4 Evaluation of the chemical composition by TLC

TLC was another method used to evaluate the chemical composition of the different samples, since the coupling of HPLC with TLC allows a more accurate analysis and consequently a more complete characterization of the chemical composition/structure. Thus, while HPLC analysis allowed the identification of the polysaccharides present in the samples (glucan and galactan), TLC allows a comparative qualitative analysis between samples, allowing to analyze the monomers that form the previously identified polysaccharides.

In first step standard solutions of fructose (F), glucose (G), fructooligosaccharides – FOS (1-kestose, nystose and 1F-fructofuranosylnystose) and Frutalose®OFP (Sensus), have been analyzed. Chromatograms obtained by analysis of standard solutions and samples Kefir grains (1), Kefir fermented milk grains (2), freeze-dried Kefir grains (3) and freeze-dried Kefir fermented milk (4) and *L. kefiri* KFLM3 (5) on thin layer are shown in Figure 19. When analyzing Figure 19, and concerning to the standard solutions analyzed, it is verified that the components are separated by their molecular mass. So, the first position on chromatograms in line belongs to monosaccharides G or F with the lowest molecular masses. TLC analysis provides information on the degree of polymerization of each sample (DP), allowing to identify the number of monomeric units in the samples tested. Thus, since glucose and fructose are constituted by only one monomer, these molecules are found in DP1. This analysis also allowed to verify that fructose spots presents a pink coloration while the glucose spots are blue.

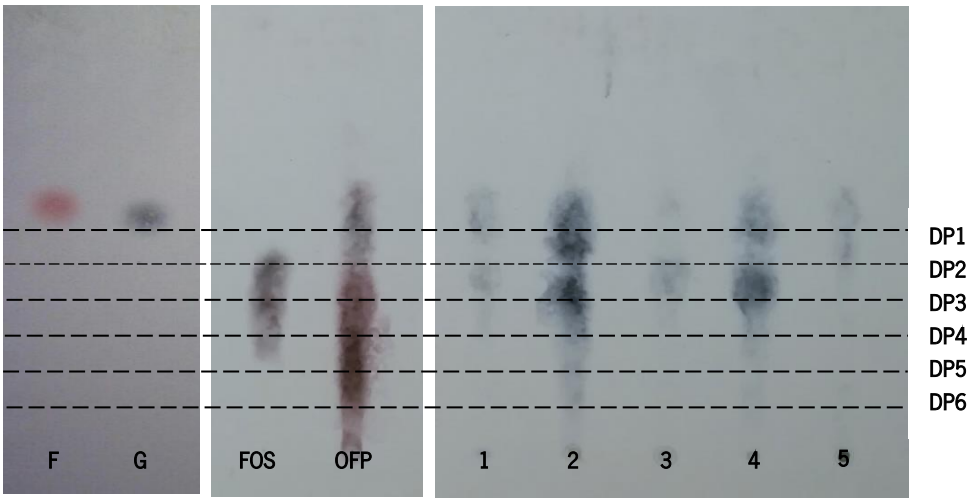

Figure 19 | TLC chromatogram of analyzed standard solutions fructose (F), glucose (G), fructooligosaccharides (FO), Frutalose (OFP) and of samples: Kefir grains (1), Kefir fermented milk (2), freeze-dried Kefir grains (3) and freeze-dried Kefir fermented milk (4) and *L. kefiri* KFLM3 (5). Mobile phase was butanol–ethanol– water (5:3:2, v/v); detection reagent: aniline–diphenylamine–phosphoric acid in acetone; volume of sample 2 µL. DP - degree of polymerization.

The third position on chromatographs in line belongs to FOS that, as confirmed by the color of the spots, presents in its composition, polysaccharides composed of F and G more specifically, trisaccharides (1-kestose (GF2) - DP3), tetrasaccharides (nystose (GF3) - DP4) and pentasaccharides (1F-fructofuranosylnystose (GF4) - DP5). Finally, the polysaccharide polymer chain with higher molecular mass was Frutalose®OFP (Sensus), a FOS commercial product hydrolyzed from inulin. When analyzing the color of the spots of this polysaccharide (more pink than blue) it is verified that Frutalose is composed mainly by Fructose. It is further verified that Frutalose®OFP presents molecules in DP1 to DP6, presenting mainly glucose in DP2 and fructose in the remaining DP. The results obtained in the TLC are in accordance with the datasheet of this product which indicates that it is composed of oligofructose (92% [F3 (DP3) + F4 (DP4) + F5 (DP5) + F6 (DP6)] and 8% [F1 + G1(DP1) and G2 + F2 (DP2)]).

Regarding the samples tested, it is verified that all spots are blue, confirming the absence of fructose and the presence of glucose in the kefiran composition. These results are in agreement with those obtained by HPLC (section 3.3) where the presence of glucose and galactose (Gal) in the kefiran was observed. Concerning Gal, although a standard solution for this monomer has not been used in the TLC, it is possible to conclude that the color of its spots may be blue.

Analyzing the Kefir fermented milk sample, it is verified that kefiran is essentially composed of monosaccharides (G and/or Gal), disaccharides (GG and/or GGal) and trisaccharides (GGG, GGalG and/or GGGal) although it also presents residues of G and Gal in DP4, DP5 and DP6.

In general, concerning the samples analyzed it was observed a similar profile in DP, in the TLC chromatogram. However, the samples differ in the level of concentration. The quantitative differences may be due to the fact that the dissolution process of the solid sample of Kefir grains and of the lyophilizates of the other samples has not been successful. On the other hand, it is still necessary to consider that the concentration in Kefir may actually be distinct among the samples tested, however, as noted above, TLC allows a qualitative analysis and indicates approximately, the amount present in the samples tested. Thus, in order to accurately compare the concentration of kefiran in the different samples, it would be necessary to evaluate them through a quantification method like HPLC, as it was done for the Kefir grains and Kefir fermented milk. In this study, as discussed above it has been found that Kefir grains have higher amounts of kefiran (61.1 ± 9.0%) than Kefir fermented milk 40.9 ± 0.4%.

Regarding the molecular structure of kefiran, it is still not well understood and there are few studies on this topic. Kooiman (1968) and Mukai et al. (1990) elucidated the chemical structure of kefiran from Kefir grains and from isolated Lactobacillus species. The structure proposed is a branched hexa- or

heptasaccharide repeating unit that is itself composed of a regular pentasaccharide unit to which one or two sugar residues are randomly linked (Figure 20).

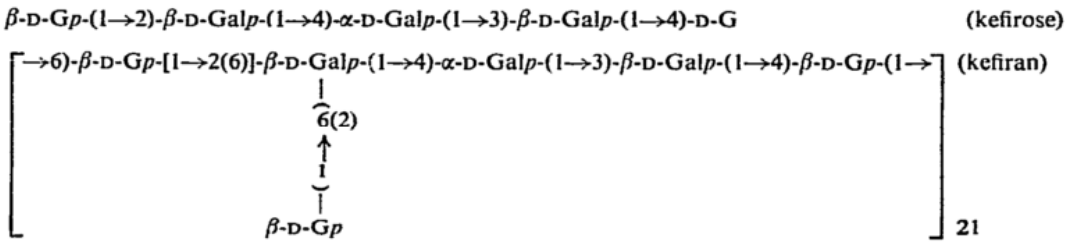

Figure 20 | Kefiran structure (Kooiman, 1968).

The results obtained by HPLC for the chemical characterization of kefiran present in Kefir grains and Kefir fermented milk indicate that the kefiran isolated is a heteropolysaccharide which contains units of glucose and galactose in almost equal proportion. Results obtained by the TLC analysis are different from the ones found into the literature, in the Kefir produced in this study, it was verified that polysaccharides of the kefiran are essentially composed of disaccharides (DP2) and trisaccharides (DP3), with residual amounts of DP4, DP5 and DP6 sugars.

CHAPTER 4

CONCLUSION

In summary, the main objective of this study was to evaluate the mycotoxins adsorption properties of Kefir microorganisms. Firstly, to understand the influence of pH on the adsorption properties of AFB1, ZEA and OTA by Kefir, preliminary experiments were carried out in buffer solutions (pH 3, 5 and 7). With this analysis, it was verified that the adsorption capacity of the Kefir grains, Kefir fermented milk and microorganisms isolated from Kefir grains (*L. kefiri* KFLM3 and *K. servazzii* KFGY7) is strongly affected at pH 7. Therefore, according to the results obtained, AFB1, ZEA and OTA adsorption is not favored by neutral pH.

After assessing the influence of pH, it was necessary to take into account that for the adsorbents to be applied efficiently, they must be efficient throughout the GI tract and the mycotoxin adsorbent complex remains stable, ensuring that the desorption of the toxin during digestion does not occur. Therefore, it is extremely important to carry out studies like this that evaluated the adsorption capacity of mycotoxins under conditions that simulate the GI tract. After this analysis, it was verified in all samples, that in the oral phase the adsorption capacity of the samples decreased. Thus, to understand the decrease in adsorption, the effect of amylase addition was evaluated, verifying that it does not influence the adsorption capacity of the Kefir grains and Kefir fermented milk. After this analysis, it was confirmed that the responsible for the decrease of the percentage of adsorption in the oral phase was the pH 7, which is in agreement with the results obtained in the preliminary tests in buffer solutions. In general, it was further verified that the intestinal phase is the step phase where the mycotoxin-adsorbent complex is less stable since it was observed a substantial release of AFB1, OTA and ZEA. The combination of pancreatin, bile and pH 7 seems to destabilize the mycotoxin-adsorbent binding sites, causing the release of the mycotoxins. In order to confirm the effect of the enzymes added in the intestinal phase on the adsorption capacity of mycotoxins, it would be important to carry out assays without the addition of pancreatin and bile, as it was done for amylase. It would also be interesting to evaluate the effect of pepsin in the gastric phase.

When comparing the adsorption capacity of the Kefir grains with the Kefir fermented milk, it was verified that the fermented milk showed lower adsorption. Thus, it was confirmed that the Kefir grains structure and its three-dimensional arrangement was an important factor that affects the adsorption of mycotoxins since it provides protection not only against enzymes added during the simulated gastrointestinal process but also against established physical conditions. This protective effect acts until the gastric phase, since in the intestinal phase a considerable decrease of the adsorption occurs.

In addition, it was also evaluated the effect of the fermentation substrate on the adsorption of Kefir microorganisms. After evaluating the results obtained, it was verified that the culture media or milk

used to grow the microorganisms have shown to influence the adsorption of mycotoxins. AFB1, ZEA and OTA adsorption was favored by the growth of the microorganisms in rich laboratory culture media (MRS and YPD), since the amount of microorganisms is consequently affected.

This study also allowed to compare the capacity of Kefir grains (consortium) with that of *L. kefiri* KFLM3 (MRS) and *K. servazzii* KFGY7 (YPD). In general, it was observed that microorganisms are more efficient in the adsorption process than the grains. Thus, the adsorption capacity of the grains appears to be due to their composition in lactic acid bacteria and yeasts, which corroborate with the previously described theories that indicate that the responsible for adsorption are cellular components of yeasts and bacteria (Petruzzi et al., 2014; Ringot et al., 2007). In order to clarify if the components of yeasts and bacteria (β-D-glucans, glucomannans, mannan-oligosaccharides and/or kefiran) are actually responsible for the process of adsorption of mycotoxins it would be necessary to isolate the constituents and perform new assays under simulated gastrointestinal conditions.

Although the intestinal phase strongly affected the adsorption capacity of the samples tested, the final percentages of microorganism's adsorption are satisfactory. In the intestinal phase, the adsorption of AFB1, *L. kefiri* KFLM3 (MRS) showed high efficiency (38.1 ± 3.9%). The results obtained for OTA were 33.6 ± 3.8% for *L. kefiri* KFLM3 (MRS) and 27.6 ± 5.9% for *K. servazzii* KFGY7 (YPD). Generally, ZEA was the mycotoxin that was better adsorbed by the bacterium (68.7 ± 1.2%), by the yeast (67.8 ± 0.4%) and by Kefir grains and Kefir fermented milk presenting promising results. The percentages obtained for the other mycotoxins by the microorganisms and by the other samples, although lower, they are positive results since the percentage that they can adsorb may be the difference between having or not a health problem, associated to the mycotoxicosis.

Concerning the results obtained by HPLC for the chemical characterization of kefiran present in Kefir grains and Kefir fermented milk, it was verified that the kefiran isolated is a heteropolysaccharide which contains units of glucose and galactose in almost equal proportion. The TLC analysis allowed to conclude that kefiran is a polysaccharide essentially composed of disaccharides (DP2) and trisaccharides (DP3).

In general, this study demonstrates that Kefir grains/strains and its fermented milk is capable of adsorbing AFB1, ZEA and OTA. So, it is important to continue this study continuing to evaluate *L. kefiri* KFLM3 and *K. servazzii* KFGY7 properties and composition, as well as isolating their constituents (β-glucans and kefiran) and test their adsorption capacity under GI tract conditions, in order to clarify if they are the main responsible for the adsorption. At the future level it would also be interesting to evaluate whether the dynamics of adsorption by Kefir or its constituents is maintained when tested with food or

feed. Additionally, it is important to note that the concentrations of AFB1, ZEA and OTA used in these tests are well above the levels stipulated by the regulations, since this was the only way to ensure reliable determinations by HPLC. So, the future studies should be done with mycotoxins concentrations at levels within EU regulations.

Another important point would be to find a way of making Kefir microorganisms more resistant to the digestion process, protecting the microorganism's cells with an entrapment based on polysaccharides in order to ensure greater adsorption in the intestinal phase, as it happened when using inorganic adsorbents as the activated carbon, which adsorbed 97.8 ± 0.1% of AFB1, and 100% of ZEA and OTA.

In general, it is important to conclude that studies such as the present one, are of high importance. As we know, several studies have shown that mycotoxins present neurotoxic, nephrotoxic, carcinogenic, hepatotoxic, immunosuppressive and mutagenic characteristics. Thus, it becomes necessary to investigate ways of controlling and mitigate mycotoxin impacts on human and animal health.

CHAPTER 5

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CHAPTER 6

ANNEXES

Annex I – Calibrations curves used in experiments

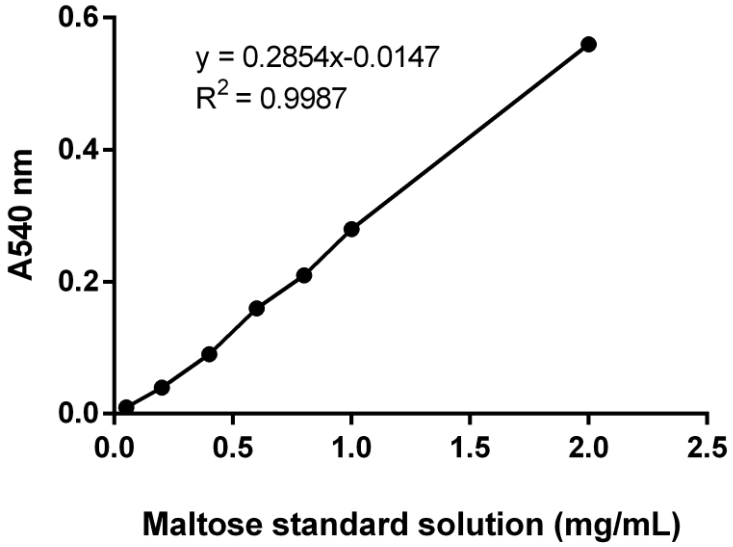

Figure 21 | Calibration curve for quantification of amylase enzyme (Megazyme) activity. Maltose standard solution (mg/mL) were analyzed in a microplate reader (Citation™ 3 from Biotek) in dark 96 wells plate at 540 nm. The results were fitted to a linear regression (R^2 = 0.9987) to obtain the equation (y = 0.2854x – 0.0147).

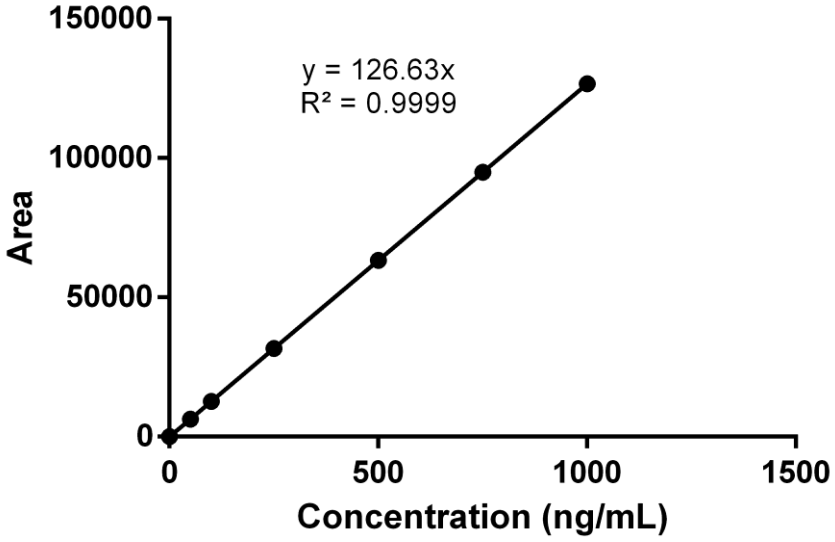

Figure 22 | AFB1 calibration curve. AFB1 standards with concentrations 0.5, 1, 2, 4, 6, 8 and 10 g/L were analyzed by HPLC-FL. The results were fitted to a linear regression (R^2 = 0.9999) to obtain the equation (y = 126.63x).

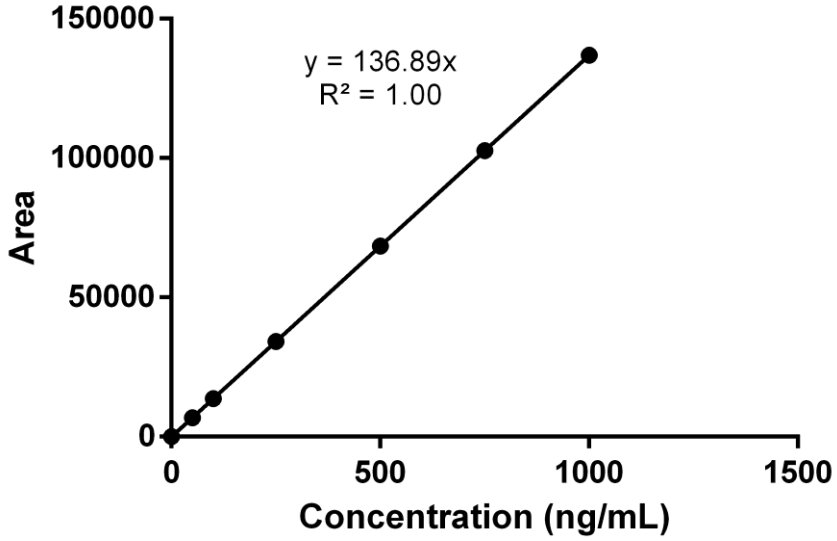

Figure 23 | OTA calibration curve. OTA standards with concentrations 0.5, 1, 2, 4, 6, 8 and 10 g/L by HPLC-FL. The results were fitted to a linear regression (R^2 = 1.00) to obtain the equation (y = 136.89x).

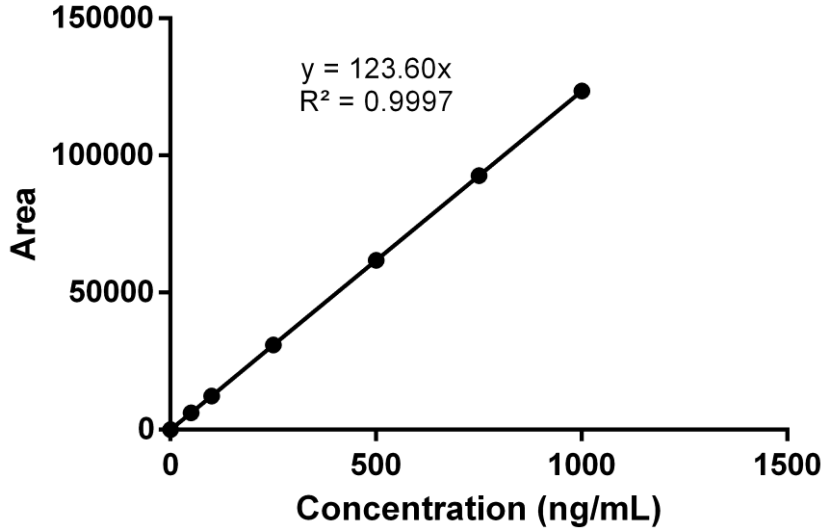

Figure 24 | ZEA calibration curve. ZEA standards with concentrations 0.5, 1, 2, 4, 6, 8 and 10 g/L were analyzed by HPLC-FL. The results were fitted to a linear regression (R^2 = 0.9997) to obtain the equation (y = 123.60x).

Table 12 | Equations of standards calibrations curves (Galactose: 0.375, 0.75, 1.5, 3, 6 g/L and Cellobiose, Glucose, Xylose and Arabinose: 0.25, 0.50, 1, 2, 3, 4 and 5.

Standards	Equations of calibration curve
Galactose	y = - 0.130 ± 8.786; R^2=1.0000
Cellobiose	y = - 0.378 ± 9,104; R^2 = 0.9960
Glucose	y = - 0.465 ± 8.980; R^2 = 0.9976
Xylose	y = - 0.608 ± 8.585; R^2 = 0.9977
Arabinose	y = - 0.677 ± 8.613; R^2 = 0.9978