PAPER

THE COMBINED EFFECT OF *THYMUS VULGARIS* EXTRACT AND PROBIOTIC BACTERIA (*LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM BIFIDUM)* ON AFLATOXIN M_1 CONCENTRATION IN KEFIR BEVERAGE

MOHAMMAD HOSSEIN MARHAMATIZADEH[1]* and SEDIGHEH RAMEZANIAN GOOSHEH[2]

[1]Department of Food Hygiene, Faculty of Veterinary Medicine, Kazerun Branch, Islamic Azad University, Kazerun, Iran
[2]Department of Microbiology, Kazerun Branch, Islamic Azad University, Kazerun, Iran
*Corresponding author. Tel.: +98 9177210645
E-mail address: drmarhamati@gmail.com

ABSTRACT

The current study was conducted to evaluate the effect of different doses of *Thymus* extracts (0, 2, 4 and 6) gr/L and various values of *lactobacillus acidophilus* and *bifidobacterium bifidum* on decreasing of fixed amount of aflatoxin in kefir beverage. The results showed that *Thyme* extracts reduced the amount of Aflatoxin M_1. *Thyme* extract in combination with probiotic bacteria decreased Aflatoxin M_1 more than *Thymus* alone. The most reduction of Aflatoxin M_1 levels was detected by using 4 gr/L *Thymus* extract and 1×10^8 *Lactobacillus acidophilus*. Moreover, results revealed that *Thymus* extracts and probiotic bacteria could reduce AFM_1 in kefir drink.

Keywords: aflatoxin M_1 reduction, *Bifiobacterium bifidum*, *Lactobacillus acidophilus*, Thymus extracts, Kefir

1. INTRODUCTION

Thymus is a small aromatic perennial herbaceous plant that cultivated in frequency due to their wide use in the food, cosmetic, and pharmaceutical industries (NABAVI *et al.*, 2015). The genus *Thymus* is a taxonomically complex group of aromatic plants traditionally used for medicinal purposes because of their antiseptic, antispasmodic and antitussive properties (PINA-VAZ *et al.*, 2004, NABAVI *et al.*, 2015). Several researchers demonstrated that extracts and essential oils of some *Thymus* spp. have antiviral, antibacterial and antifungal activities. (FAZELI, *et al.*, 2007, BEHNIA, 2008, SHARAFZADEH and ALIZADEH, 2012).

Probiotics are used in dairy products as well as in food supplements and in agriculture as feed additives because of their beneficial health effects (NAGPAL *et al.*, 2012). According to the currently international FAO/WHO definition (2001), probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Probiotics improve the health of both animals and humans through the improvement of its intestinal health by the regulation of microflora, stimulation and development of the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance and reducing the risk of certain other diseases (KUMAR *et al.*, 2011; NAGPAL *et al.*, 2012).

At present, probiotics are introduced as suitable replacing of antibiotics in order to confront with pathogens in animals and human being, and consumption of probiotic food products and medicines have considerable vogue. Several investigations suggested that using probiotic is associated with reducing the risk of antibiotic-associated diarrhea (32). The foods which contain probiotic bacteria are put in the group of special products, and according to the dairy products International Federation (IDF) recommendation, these probiotic products should have a minimum concentration of 106 CFU/g probiotic bacteria and consumer a total of some 108 to 109 probiotic microorganisms should be consumed daily for the suitable probiotic effects (15).

The most commonly important probiotics belong to *Lactobacillus* and *Bifidobacterium* genus. There are many well-characterized strains of *Lactobacilli* and *Bifidobacteria* available for human use (HUSSAIN *et al.*, 2009; KECHAGIA *et al.*, 2012). Some of the beneficial properties of probiotics are anticarcinogenic, immunologic enhancement, antiatherogenic, cholesterol-lowering, anti-obesity and antidiabetic characteristics (NAGPAL *et al.*, 2012).

Kephir (kefir) is a viscous, highly acidic beverage produced from cow, goat, sheep or mare milks. The fermentation is initiated by "kefir grains" (clusters of yeast and bacteria), which are added to raw, pasteurized or UHT-treated milk (SARKAR, 2007; RIBEIRO and RIBEIRO, 2010). Kefir contains high contents of thiamine, riboflavin, pantothenic acid, vitamin C, protein and minerals; hence, kefir has both therapeutic and nutritional attributes (SARKAR, 2007). Kefir also has greater amounts of threonine, serine, alanine and lysine than milk (SARKAR, 2007). Kefir also has medical effects in order to treat hypertension, body skin fineness, stress and depression, cholesterolemia and arthritis (XIAO *et al.*, 2003; NINANE *el al*., 2005).

Aflatoxins are naturally occurring mycotoxins that are produced by some species of fungi like *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin M_1 (AFM_1) is one of the metabolites of Aflatoxin B_1 that is excreted into milk when lactating animals are given feed with aflatoxin contaminated food (10). Contamination of milk or milk products with AFM_1 is considered as a potential risk for public health (11, IARC, 2002).

International Agency for Research on Cancer (IRAC) classified AFM_1 as a group 2B agent (possibly carcinogenic to humans) and exposing to chronic aflatoxin causes acute liver problems, mutation and liver cancer (BEHFAR *et al.*, 2012). Aflatoxin M1 is relatively stable during pasteurization, sterilization and preparation of dairy products (FALLAH,

2010). Therefore, industries sustain irretrievable economic losses, if it is not controlled, and its potential risks for human health especially for children (ELKHOURY *et al.,* 2011). The aim of this research was to produce kefir and reviewing *Thymus* extract effects in combined with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on the AFM_1 concentration in kefir drink.

2. MATERIALS AND METHODS

2.1. Chemicals and instrumentation

Lyophilized *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (CHR Hansen Company, Denmark) were used as probiotic bacteria. The pasteurized low fat milk (1.5% fat) and kefir grain (Iran) were used to produce kefir drink. *Thymus* extracts (Zard band Company, Iran) (were prepared at 3 different concentrations including 2, 4, 6 g/L. AFM_1 was procured from Merk Company, Iran) and 200 ppb concentration was used to add the kefir drink as follow explanation.

2.2. Producing kefir drink

In order to produce kefir drink, 1 gram kefir grain was added to 1 litter pasteurized low fat milk (1.5% fat), and this was incubated at 38°C and acidity measurements were performed at different times until reaching 42°C (Standard and industrial search of Iran). At least this product was kept in refrigerator at 4°C, subsequently (MOATTER and SHAMS KASHANI, 1378; Iran standard and industry research institute, 1385).

2.3. Adding aflatoxin M_1 and *Thymus* extracts to produced kefir

Ten mL of produced kefir was poured equally in 4 tubes and 10 mL of contaminated milk with 200 ppb of AFM_1 was added to each tube. Finally, different doses of *Thymus* extract (2, 4, 6 g/L) were added to the samples (OTLES and CAGINDI, 2003; MARHAMATIZADEH *et al.*, 2011; MARHAMATIZADEH *et al.,* 2012).

2.4. Adding aflatoxin M_1, *Thymus* extract and *Lactobacillus acidophilus* or *Bifidobacterium bifidum* to kefir

Four g/L fixed *Thymus* with 1×10^8, 3×10^8 and 6×10^8 *Bifiodobacterium bifidum* or *Lactobacillus acidophilus* were poured in 4 tubes and 10 mL of contaminated milk by 200 ppb of AFM1 was added to them, subsequently (OTLES and CAGINDI, 2005; MARHAMATIZADEH *et al.,* 2011; MARHAMATIZADEH *et al.,* 2012). Finally, all samples were kept into the incubator for 24 hours at 24°C. After 24 hours, the coagulation was separated from liquid by using a cloth filter, and remained liquid was incubated at 14°C for 24 hours. It was kept for 48 hours in refrigerator at 4°C and all samples were analyzed with ELISA reader (Europroxima Company), subsequently.

2.5. ELISA test

Samples were centrifuged at 2,000 rpm for 10 min at 7°C and the upper oil layer was removed. Then, 100 mL of these samples were used for ELISA test. ELISA reader device in three repetitions and distinguished three optical densities for every sample was done. And every of three optical densities were put in Excel program (collected by Euro- Proxima

Company) and three concentrations were got that the average of these three shows AFM_1 reminder in every samples.

2.6. Statistical analyses

The data was analyzed by using Kruskal-Wallis test in a meaningful surface at $P< 0.05$ by SPSS software (SPSS for Windows, version 20, SPSS Inc, Chicago, IL, USA).

3. RESULTS

3.1. Evaluating primary milk contamination with AFM_1

The results showed that primary milk contains 32 ppb AFM_1 and then 200 ppb of AFM_1 were added to milk that totally AFM_1 becomes 232 ppb. Table 1 shows the changes of aflatoxin level in the present of *Thymus* extracts, *Bifidobacterium bifidum* and *Lactobacillus acidophilus* bacteria in various groups.

Table 1: Comparison of AFM_1 concentration in the presence of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* bacteria and *Thyme* extracts.

Sample tubes	Added Aflatoxin (ppb) AFM_1 value added in the form of (ppb)	Initial Aflatoxin (ppb) AFM_1 value primary	The remaining amount of aflatoxin (ppb) AFM_1 value	Reduced aflatoxin (ppb) AFM_1value Decreased (ppb)	Reduced aflatoxin (%) AFM_1value decreased (%)
40 g kefir	200	232	174.3	57.7	24.87
2 g/l *Thyme* extract	200	232	154.6	77.4	33.36
4 g/l *Thyme* extract	200	232	155.9	76.1	32.80
6 g/l *Thyme* extract	200	232	162	69.8	30
4 g *Thyme* extract and 1×10^8 *Bifidobacterium bifidum*	200	232	169.5	62.5	26.93
4 g/l *Thyme* extrac and 3×10^8 *Bifidobacterium bifidum*	200	232	175.3	56.7	24.43
4 g/l *Thyme* extract and 6×10^8 *Bifidobacterium bifidum*	200	232	148.3	83.7	36.07
4 g/l *Thymus* extract and 1×10^8 *Lactobacillus acidophilus*	200	232	125.8	106.2	45.77
4 g/l thymus extrac and 3×10^8 *Lactobacillus acidophilus*	200	232	186.1	45.9	19.78
4 g/l thymus extracts and 6×10^8 *Lactobacillus acidophilus*	200	232	169.1	63	27.15

3.2. Evaluating kefir containing *Thymus* in detecting AFM_1

The results indicated in Table 1 showed that *Thymus* extracts in kefir caused AFM_1 reduction largely. The reduction percent of AFM_1 in the kefir samples containing different concentrations of *Thymus* extracts 2, 4 and 6 gr/L were 33.36%, 32.80% and 30%,

respectively. The most reduction of AFM1 was detected by 2 gr of *Thymus* and *Thymus* extract with 6 g/L concentration showed the least percent of AFM_1 reduction.

3.3. Evaluating probiotic kefir containing *Thymus* and *Lactobacillus acidophilus* in AFM_1 reduction

The data in Table 1 shows that fixed quantity of *Thymus* (4 gr/L) with different amount of *Lactobacillus acidophilus* declined Aflatoxin. Reduction of AFM_1 in the presence of *Thymus* extract and different value of *Lactobacillus acidophilus* 1×10^8, 3×10^8 and 6×10^8 were 45.77%, 19.78% and 27.15, respectively. The most decrease of Aflatoxin was detected with 1×10^8 level of *Lactobacillus acidophilus* and the amount 3×10^8 of bacteria has the least reduction of aflatoxin.

3.4. Evaluating probiotic kefir containing *Thymus* and *Bifidobacterium bifidum* in deleting AFM_1

The results showed that *Bifidobacterium bifidum* bacteria decrease the amount of AFM_1 in the samples. The most decline percent of AFM_1 in the presence of 6×10^8 of *Bifidobacterium bifidum* occurred with 36.07%; and 1×10^8 and 3×10^8 of bacteria with 26.93% and 24.43%, respectively, had the least AFM_1 reduction.

4. CONCLUSIONS

The aim of the present study was the evaluation of the effect of *Thymus* extract and *Lactobacillus acidophilus* and *Bifidobacterium bifidum* bacteria on the reduction of Aflatoxin amount in kefir beverage. Furthermore, the possibility of producing a new probiotic food based on kefir and *Thymus* was assessed.

The results of present study indicated that *Thymus* extract has anti-aflatoxin activity and capability for aflatoxin reduction. The *Thymus* extract in combination with probiotic bacteria has the more ability for aflatoxin decline. The results of the current research showed that *Thymus* extract with 2 gr/L concentration in combination with 6×10^8 *Bifidobacterium bifidum* have the most effect in reduction of AFM1 and the strongest anti-aflatoxin activity was shown by the *Thymus* extract with 4 gr/L concentration in combination with 1×10^8 *Lactobacillus acidophilus.* Our results are agreed with other studies (OTLES and CAGINDI, 2003; LEE *et al.*, 2003; TRATNIK *et al.*, 2006).

Probiotic foods have been produced in order to treat intestinal infections as well as genital diseases. In three decades ago, commercial probiotic products have been supplied to the world market grew. Kefir is a fermented dairy product which originates from the Caucasus Mountains in Eastern Europe (TRATNIK *et al.*, 2006). Kefir is the most popular probiotic product in Europe. Kefir has beneficial effects in healing and homeostasis due to its vitamins, minerals and essential amino acids (OTLES and CAGINDI, 2003). The vitamin content of kefir affects both type of milk and microbiological flora (SARKAR, 2007, ARSLAN 2014).

Aflatoxins are a group of fungal secondary metabolites which are recognized as being of economic and health importance (10). AFB_1 is currently of great interest due to their toxic, carcinogenic and mutagenic nature on human and animal health (11). A number of studies reported that many micro-organisms, including bacteria, yeasts, moulds, actinomycetes and algae are able to remove or degrade small amounts of aflatoxin in foods and feeds (LEE *et al.*, 2003).

Several lactic acid bacteria strains have shown different capabilities for binding AFM_1 in solutions and in milk (HASKARD, *et al.*, 2001). The various studies have reported that certain *lactobacilli* and *Bifidobacteria* are capable of removing AFB_1 from liquid solutions by binding the toxin (PELTONEN *et al.*, 2001; HASKARD *et al.*, 2001). Some researchers have suggested that aflatoxin binds predominantly to polysaccharides and peptidoglycans of the bacterial cell wall (LAHTINEN *et al.*, 2004). LOPEZ *et al.*, (2003) showed that *Saccharomyces* yeast reduced 90% AFM_1 in the milk.

Powders and extracts of many herbs, plants and spices have been reported to inhibit the production of aflatoxin (PARANAGAMA *et al.*, 2003). A recent study showed that the essential oils of *T. daenensis* and *Thymus* spp. (Elam ecotype) flowers exhibited antibacterial activities against *L. monocytogenes* from chicken meat (GHASEMI PIRBALOUTI *et al.*, 2009). LIXANDRU *et al.*, (2010) evaluated antimicrobial activity of some plant essential oils against bacterial and fungal species. The results showed *Thymus*, *Coriander* and *Basil* oils proved the best antibacterial activity, while *Thymus* and *Spearmint* oils better inhibited the fungal species. *Thymus vulgaris* extracts with 0.01 and 1% concentration decreased the AFB_2 production by 83 and 91 % (FOUAD *et al.*, 2013). HAMZAWY *et al.*, (2012) reported that ethanolic and aqueous extracts of *Thymus vulgaris* has potential hepatorenoprotective effects against aflatoxins because of antioxidant properties and radical scavenging activity.

In conclusion, the results of the current study revealed that *Thymus* extracts and probiotic bacteria can reduce AFM_1 in kefir drink. Our results are agreed with other researches that showed probiotic bacteria and Thymus extract have anti-aflatoxin activity. Moreover; it may be stated that aflatoxins are not only a big problem at crop production level, but also it has become a global health topic due to the consequences following their consumption in animals and human being. So it is important to develop the useful protocols to eliminate aflatoxins from contaminated food.

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