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Lactobacillus rhamnosus Ability of Aflatoxin Detoxification

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Abstract

The present study was conducted to isolate and identify *Lactobacillus rhamnosus* from locally fermented dairy products collected from different markets in Irbid city, Jordan. Thereafter, the ability of *Lb. rhamnosus* to detoxify aflatoxins (AFs) was investigated *in vitro* after incubation on 37°C in MRS medium and in artificial intestine fluid (AIF). Three *Lb. rhamnosus* out of nine different species of Lactic Acid Bacteria (LAB) isolated from 15 fermented dairy products samples were identified. The isolates were characterized based on their morphological, microscopic, cultural and biochemical properties. The selection of isolates as probiotics depended on their abilities to grow in pH levels between 2 to 6 and their tolerance to grow at 1.0 % bile salts concentrations, Furthermore, *Lb. rhamnosus* was able to adhere to mucus onto the intestine surface at 54.7%. The ability of *Lb. rhamnosus* of AFs detoxification has significantly (p<0.05) increased with the increase in incubation periods, and the detoxification percentage after 72h incubation in each MRS medium and AIF, was 76% and 81.6%, respectively.

Keywords*: Lb. rhamnosus*, Aflatoxins, Probiotics, Detoxifications.

1. Introduction

Mycotoxins are secondary metabolites of molds that contaminate over than 25% of the human food (Moss, 2002). They have been found in homes, agricultural settings and food; they could be able to cause different human health problems, because they have wide toxic effects, ranging from short-term mucous membrane irritation to damaging the internal organ, depression of the immune system and cancer (Williams, 2004; Mohamad *et al*., 2015). Almost the diseases related to causes by mycotoxins were related to consuming contaminated food (Hussein *et al*., 2015). The most important kind of mycotoxin is the aflatoxins group which include aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) and the metabolites. These aflatoxins were well-characterized biologically and toxicologically (Wagacha and Muthomi, 2008). Aflatoxins are among the most potent mutagenic and carcinogenic substances. They were classified by the International Agency for Research on Cancer (IARC) as a Class 1 human carcinogen (IARC, 2002). They are associated with many chronic health risks, including the induction of cancer, immune suppression, digestive, blood and nerve defects (Bryden, 2007).

Aspergillus flavus and *A. parasiticus* are capable of colonizing a wide variety of food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit (Thalij *et al*., 2015). Ability of these fungi to produce aflatoxins depends on multiple climate factors, such as drought stress, rainfall, suitability of crop genotype, insect damage, and agricultural process (Mohammed *et al*., 2005; Wu and Khlangwiset, 2010). These foods were the main sources of human exposure to aflatoxin because they are so highly consumed worldwide and unfortunately they are also the most susceptible crops to aflatoxins contamination (Thalij *et al*., 2015).

Various physical and chemical methods have been developed to decrease the aflatoxins toxicity, but these methods have many limitations, such as loss of nutrition products, organoleptic qualities, undesirable health effects and high cost of equipment (Hussein *et al*., 2014). These disadvantages have stimulated recent prominence on biological methods of degradation of aflatoxins (Basappa and Shantha, 1996).

Lactobacillus is a broad genus from Lactic Acid Bacteria (LAB) characterized by formation of lactic acid as a major metabolites product of carbohydrate utilization. It is a genus of gram-positive, non-spore-forming, microaerophilic and some other characteristics (Satokari *et al*., 2003). LAB are common and usually being inhabitants of the GI and the vagina in the bodies of humans and animals (Hammes and Vogel, 1995).

Several publications have reported *in vitro* ability of binding by LAB and some species of yeast with mycotoxins, such as aflatoxin B1 (Hernandez-Mendoza *et al*., 2009; Hernandez-Mendoza *et al*., 2010). Some species of LAB were reported to be the strongest binder of aflatoxin (Fazeli *et al*., 2009). The interaction was influenced by the peptidoglycan structure and, more

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accurately, by its amino acid composition (Niderkorn *et al*., 2009). The LAB, which have been used as probiotics, were considered potential mycotoxin decontaminating microorganisms because of their ability to bind these toxic metabolites (Hernandez-Mendoza *et al*., 2010).

The objectives of the present study are to investigate the ability of *Lb. rhamnosus* isolated from local fermented dairy products to degrade AFs produced by *A. parasiticus* after incubation in MRS medium and AIF.

2. Materials and Methods

2.1. Lactobacillus rhamnosus Isolation and Identification

Lactobacillus rhamnosus isolation was conducted from Locally Dairy Fermented (LDF) samples that were collected from different markets in Irbid city in Jordan. Fifty mL of each LDF sample were mixed with 10 mL of MRS broth medium (Oxoid, UK) and incubated statically under aerobic conditions at 37 ˚C for 48 h. The last two series of dilution mixture of each sample were spread on MRS agar plates supplemented with 1.1 mM bromocresol purple, and incubated anaerobically at 37˚C for up to 48 h, (Sujaya *et al*., 2001). Single yellow colonies were selected randomly from the MRS agar plates, then transferred into test tubes containing 10 ml of MRS broth, and incubated at 37˚˚C for 24 h under aerobic conditions. The pure colony isolates were streaked onto MRS agar plates. The isolated bacterium was examined by comparing its bacterial colony and cell morphology, gram staining properties, acid and gas production from different carbohydrates as carbon sources. Fermentation was observed after incubation for 24 and 48 h anaerobically at 30˚˚C and 37˚˚C. In addition, identification was completed using other biochemical profiles and combined with the descriptions contained in Bergey's Manual of Systematic Bacteriology (Kandler *et al*., 1986). The bacteria were maintained by routine subculture at 4 °C in slant tubes with MRS agar for further investigation (Kozaki *et al*., 1992).

2.2. Tolerance Ability of Lb. rhamnosus to Low pH Values

Five mL from MRS medium tubes were adjusted to pH levels at 2, 3, 4, 5 and 6 using optimal amount from artificial gastric juice. *Lb. rhamnosus* cells were pre-cultured in 5mL of MRS broth at 37˚C for 24 h under aerobic conditions, then a 1ml of aliquot of the culture broth was harvested by centrifugation at 15000 g for 5 min and washed twice with PBS. The bacterial cells were suspended in 100 µL of PBS and incubated with 5mL of MRS broth medium at various pHs levels at 37 ˚C for 3 h under aerobic conditions. After incubation, 50 µL of the culture broth were appropriately diluted with PBS then streaked on MRS agar plates. Viable cells were counted after anaerobic incubation at 37 ˚C for 48 h (Sultana *et al*., 2000).

2.3. Tolerance Ability of Lb. rhamnosus to Bile Salts

This test was performed by inoculating 100 µl of bacterial cells pre-cultured at 37 ˚C for 24 h in 5mL of MRS broth containing bile salts (BDH, UK) at 0.3, 0.5 and1% and then incubated at 37 ˚C for 4 h under aerobic conditions. Viable cells were counted as described by Deshpande *et al*. (2014).

2.4. Adhesion Properties of Lb. rhamnosus

The adhesion assay, to screen the ability of *Lb. rhamnosus* bacteria to adhere to cells, was performed using the Adhesion Index (AI) (Gratz *et al*., 2004). The assay procedure was completed according to Lee *et al*. (2003).

2.5. Aflatoxin Production

The aflatoxin was produced from *Aspergillus parasiticus* NRRL 2999 which was obtained from College of Agricultural Tikrit University, Laboratory of Food Science Department. Then, an assurance of mold strains was done on the basis of morphological characteristics using Scotch tape preparation and cultural characteristics after cultivation on malt extract agar and potato dextrose agar, according to (Sammson *et al*., 1992). Thereafter, a fermentation of rice was done by the method of Boller and Schroeder (1973). Successfully fermented rice was then steamed to kill the fungus, dried and ground to a fine powder. The aflatoxin content in rice powder was measured by ELISA as follows:

2.6. Aflatoxin Assay

Aflatoxin extraction was performed according to Kawamura *et al*. (1988). An aliquot of each rice fermented at 2 g was shaken for 10 min at 150 rpm with 10 mL methanol: water (70:30, v/v). The crude extract was then filtered through Whatman No. 1 and diluted in PBST (PBS + 0.05% Tween 20) for intracellular (ic-ELISA) determination.

Aflatoxins were determined by a monoclonal antibody-based ic-ELISA using Aflatoxin ELISA Test Kits (Shenzhen Lvshiyuan Biotechnology Co., Ltd. Guangdong, China) sensitivity: 0.1ppb and as the product protocol procedure. This test kit was based on the competitive enzyme immunoassay for the qualitative-quantitative detection of Aflatoxins in the rice. The coupling antigen was pre-coated on the micro well stripes. The AFs in the sample and the coupling antigens pre-coated on the micro-well stripes compete for anti-Aflatoxin antibodies. After the addition of the enzyme conjugate, the TMB substrate was added for coloration. The Optical Density (OD) value of the testing sample has a negative correlation with the AF concentration in the sample. This value was compared to the standard curve and the AF concentration was subsequently obtained. The average absorbance was calculated from the individual absorbance obtained from triplicate wells and the results were expressed as percentage of binding. This ELISA procedure was completed according the description guide from the manufacturing company (Shenzhen Lvshiyuan Biotechnology Co., Ltd. Guangdong, China).

2.7. Assessing the Ability of Lb. rhamnosus to Detoxify AFs

Lb. rhamnosus was activated in MRS broth at 37°C for 24 h., and viable counts (approximately 1.5×10^8cfu/ml) were calculated by McFarland procedure (Winn *et al*., 2006). One ml of activated culture was inoculated into 100 mL of fresh MRS broth. Aflatoxin was added to make the mixtures containing 2.5µg/ml of aflatoxin. MRS broth containing aflatoxins was used as a control and was not inoculated with activated culture. Each mixture was incubated at 37°C for 72 h., with shaking at 150 r/min. At 0, 24, 48, and 72 h of fermentation, 5 ml of fermented

broth was taken out and centrifuged (14,000×g, 10 min, at 4°C) with ultra-centrifuge (Sigma-Aldrich), supernatant fluid was filtered through 0.22-µm filter twice and kept at 4°C before it was analyzed for aflatoxins content (Niderkorn *et al*., 2006).

2.8. Assessment of Lb. rhamnosus Ability to Detoxify AFs in Artificial Intestinal Fluids

The ability of *Lb. rhamnosus* to detoxify AFs against simulated intestinal fluids was tested as described by Fernandez *et al*. (2003), with some modifications. One ml of 24 h., culture-broth was harvested by centrifugation at 14000 g for 5 min at 4 °C, washed with sterilized PBS, and suspended in 100 mL of PBS. The cell suspension was added to 900 ml of AIF (RICCA CHEMICAL COMPANY, USA). The bacterial suspensions were incubated at 37 °C for up to 72 h. with agitation at 160 rpm. Fifty mL of the Aliquots of the mixture were taken at each 0, 12, 24, 36, 48, 60 and 72 h., of incubation, and were used for an appropriate dilution then they were streaked on MRS agar plates (in triplicates) and were incubated at 37 °C for 48 h., under anaerobic conditions, followed by counting of viable cells.

Another amount of 50 ml from suspension, at different incubation periods, was used to detect the AFs contents after extraction according to Kawamura *et al*. (1988). The suspension was shaken for 10 min at 150 rpm with 10 ml methanol: Water (70:30,v/v). The crude extract was then filtered through Whatman No.1 and was diluted in PBST (PBS+0.05% Tween 20) for ic-ELISA determination. Aflatoxins were determined by a monoclonal antibody-based ic-ELISA using Aflatoxin ELISA Test Kits (Shenzhen Lvshiyuan Biotechnology Co.,Ltd. Guangdong, China) and the procedures were completed according to the same steps mentioned above.

2.9. Statistical Analysis

Data were analyzed by the ANOVA analysis, using the general linear model of the Statistical Analysis System (SAS Institute, 2001). Significant treatment differences were evaluated using Duncan's multiple-range test (Duncan, 1955). All statements of significance are based on the 0.05 level of probability.

3. Results and Discussion

3.1. Isolation and Identification of Lb. rhamnosus

Isolation of the *Lb. rhamnosus* from the fermented dairy products was carried out using the morphological characteristics, after cultivation on MRS media. The growth on these media has been observed because it contained all nutrients needed to grow well. The colony appeared as restricted and in a pale yellow color in central of pellucid zone for each species.

Nine pure isolates were primarily assigned as different lactobacilli species. Since they appeared as Gram-positive, rods shapes were straight and they were cultivated on MRS- $CaCO_3$ in an aerobic environment and showed ability to utilize the $CaCO_3$. Moreover, they were catalase negative and unable to produce NH_3 from arginine (Gilliland, 1990).

The assurance diagnosis process for species level was completed with biochemical test after obtaining subcultures of pure colonies from each isolate on MRS media. Three isolates out of nine appeared as hetero-fermentative and gas producing; they were tentatively identified as *Lb. rhamnosus* (Tables 1). Also, *Lb. rhamnosus* differed from some other *lactobacillus* spp. in its capability to grow in pH range from 2 to 6, and at 25 to 45 °C while it was not able to grow at 10 °C. In addition, the isolates were capable of fermenting all carbohydrates when used as carbon sources, excepted D-arabinose and D-xylose. On the other hand, the other *Lactobacillus* spp. had a different fermentation action.

These results of biochemical tests of *Lb. rhamnosus* were in agreement with accurate data found in Berge's Manual Guide at Holt *et al*. (1994).

Table 1. Characteristics tests of *Lb. rhamnosus*

Phenotypic, cultures and biochemical tests characteristics		*Lb. rhamnosus*
shape of colony		appearance on MRS agar is pale yellow
Shape under microscope		Rods, usually straight
Gram stain reaction		+
Catalase activity		-
CO_2 from glucose		-
NH_3 from arginine		-
Growth at pH	2.0	+
	3.0	+
	4.0	+
	5.0	+
	6.0	+
Growth at Temp. °C	10	-
	35	+
	40	+
	45	+
Sugar fermentation	D-arabinose	-
	D-ribose	+
	D-xylose	±
	D-galactose	+
	D-mannose	+
	D-maltose	+
	D-lactose	+
	D-glucose	+
	D-sorbose	+
	L-rhamnose	+
	D-turanose	+

+, positive; —, negative; ±, undetermined

3.2. Parameters for Probiotics Characteristics

Optimal bacterial species, which were selected as probiotics, should have many characteristics, such as the ability to grow in stomach acidity, the resistance to bile salts and the capability to adhere to intestine epithelial cells.

The tolerance of *Lb. rhamnosus* to different pH levels after cultivation on MRS medium at 37 °C for 48 h is illustrated in Table 2. The *Lb. rhamnosus* showed an ability to grow in pH levels between 2 to 6. These results were in agreement with another study by Ali (2011) who found the same results for some lactic acid bacteria. The LAB tolerance to the acidic environment may indicate that they contain lipoteichoic acid and hydrophobic amino acids in S-layer proteins of cell wall of these bacteria (Frece *et al*., 2005). The tolerance of *Lb. rhamnosus* to different bile salts concentrations appeared important to

evaluate the bacterial species to be used as probiotics especially in cases of oral intake by the organisms.

Lb. rhamnosus showed an ability to survive at certain bile salts concentrations (Morelli, 2000). The results in Table 2 indicate that the *Lb. rhamnosus* were able to grow at 0.3 to 1.0% bile salts concentrations.

This result was in harmony with that of Shi *et al*. (2012) who found that *Lb. rhamnosus* was able to grow in 0.3 to 1.0% of bile salts. Generally, the LAB, which were capable of growing with bile acids, were found to contain the bile salts hydrolase which function by stimulating the fraction of bile salts conjugated with glycine or taurine amino acids for carrying out the non-conjugated bile salts, which in turn is described as less dissolving and exerting with feces, and replacing with other new bile salts through manufacturing in liver from cholesterol. These results were in agreement with Aries and Hill (1970).

The ability of *Lb. rhamnosus* cells to adhere to rats' intestine mucus surface was illustrated in Figure 1. The results indicated that the *Lb. rhamnosus* was able to adhere at 54.7%. The adhesion ability was the essential characteristic for using *Lb. rhamnosus* as probiotics. The capability of adhesion refers to the S-layer protein in the cell wall, the protein percentage in this layer was 10 to 15% from total proteins the cell contains (Bezkorovaing, 2001).

Table 2. Tolerances Ability of *Lb. rhamnosus* to different levels of pH and bile salts

LAB Species	pH levels				
	2.0	3.0	4.0	5.0	6.0
Lb. rhamnosus	-	+	+	+	+
	Bile salts concentrations (%)				
	0.3	0.5	0.75	1.0	
	+	+	+	+	

+: *positive reactions* - : *negative reaction*

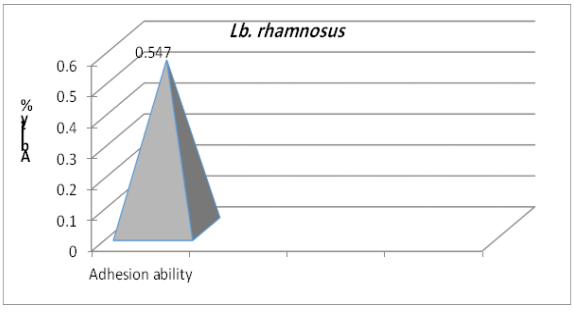

Figure 1. Adhesion Ability of Lb. rhamnosus with intestine cells

3.3 Lb. rhamnosus Ability of AFs Detoxification

The *Lb. rhamnosus* Ability of AFs Detoxification after 72 h. of incubation in MRS media was summarized in Table 3. The results showed that adding AFs to MRS media without inoculation of *Lb. rhamnosus* caused an insignificant change (p<0.05) in AFs concentrations through the incubation periods from 0 to 72 h. On the other hand, the incubation of MRS medium that contains AFs with *Lb. rhamnosus* caused a significant decrease in the AFs concentration, this happened with 0, 24, 48 and 72 h., at 2.5, 1.9, 1.2 and 0.6µg/mL, respectively, and the detoxification percentage was at 0.0, 25, 52 and 76%, respectively.

The results showed that the *Lb. rhamnosus* have the ability to degrade AFs contents in medium, and this degradable activity increased with the increase of incubation periods of *Lb. rhamnosus* with AFs.

Table 3. *Lb. rhamnosus* Ability of AFs Detoxification

Incubation periods (hours)	AF Concentration (µg/ml) in		Detoxification %
	AFs in MRS	AFs + *Lb.rhamnosus*	
0	2.5^{a} ±0.04	2.5^{a} ±0.04	0^{d} ±0.00
24	2.3^{a} ±0.06	1.9^{b}±0.06	25^{c}±2.14
48	2.5^{a} ±0.06	1.2^{c}±0.06	52^{b} ±2.51
72	2.4^{a}±0.02	0.6^{d} ±0.02	76^{a} ±4.37

a-d: Values within columns followed by different letters for different significance levels at 0.05.

The ability of degradation of AFs concentrations at 2.5 µg/mL in artificial intestine fluid during incubation with *Lb. rhamnosus* at 37°C with different period times from zero to 72 h are shown in Table 4. The *Lb. rhamnosus* counts have significantly (p<0.05) increased with the increase in the incubation periods to 72 h and the maximum count was at 48 h of incubation. The AFs concentration in the artificial intestine fluid was significantly removed by the *Lb. rhamnosus* fermentation in artificial intestine fluid and with the 0, 12, 24, 36, 48, 60 and 72 h of incubation and at 2.46, 2.24, 1.86, 1.27, 1.02, 0.74 and 0.46 ηg/mL, respectively. The AFs detoxification percentage at 72 h of incubation was 81.6%. The Mechanism of the AFs removal is shown in Tables 3 and 4. The AFs were bond by *Lb. rhamnosus* in each liquid medium and artificial intestine fluid was assayed at toxin concentration. The mechanism of aflatoxins detoxification by *Lb. rhamnosus* occurs by the interaction between ingredients of its cell wall with aflatoxins. The nature of binding is poorly understood till this moment; it also differs according to the types of ingredients. The binding between aflatoxins and bacterial cell wall ingredients modifies aflatoxins structures and gets a new structure form. The binding of *Lb. rhamnosus* and some other LAB species with all types of aflatoxins were conducted by same mechanism, because the activity of all types of aflatoxins depends on the same active groups, such as double bonds, OH, CH_3, etc. (Huang *et al*., 2017).

The results showed that *Lb. rhamnosus* was able to bind the AFs and the level of binding varied between the incubation times, the AFs concentration decreased in the MRS medium and in fluid with the increase of incubation time. These results were in agreement with Hernandez-Mendoza *et al*. (2009) who found LAB species' ability to bind the AFB1.

These results confirm the role of a cell wall-related physical phenomenon as opposed to a metabolic degradation reaction, and are consistent with the results reported by Haskard *et al*. (2000). The cell wall peptidoglycans of LAB was found by Teniola *et al*. (2005) as responsible for AFs removal. On the other hand, Niderkorn *et al*. (2009) reported that treatments affecting bacterial wall polysaccharides, lipids and proteins caused an increase in the binding with AFs, while those degrading peptidoglycan partially decreased it.

Table 4. *Lb. rhamnosus* Ability of AFs Detoxification in artificial intestine fluid.

Test types	Incubation periods (h.)						
	0	12	24	36	48	60	72
Lb. rhamnosus accounts (CFU/mL)	8.17^{e}±0.72	8.96^{d}±0.49	9.32^{c}±0.85	9.83^{b}±0.76	10.51^{a}±0.74	9.41^{c}±0.81	8.29^{e}±0.57
AFs concentration (ηg/mL)	2.46^{a}±0.05	2.24^{a}±0.06	1.86^{b}±0.03	1.27^{c}±0.01	1.02^{c}±0.02	0.74^{d}±0.07	0.46^{d}±0.02
AFs detoxification (%)	1.6^{g}±0.08	10.4^{f}±0.93	25.6^{e}±2.22	50.8^{d}±3.64	60.2^{c}±3.26	71.4^{b}±4.26	81.6^{a}±4.44

a-d: Values within rows followed by different letters for different significance levels at 0.05.

4. Conclusion

The present study investigated the ability of *Lactobacillus rhamnosus* to detoxify aflatoxins. It was isolated from locally fermented dairy products and identified by morphological, microscopic, cultural and biochemical characteristics. The results indicated the high ability of *Lactobacillus rhamnosus* in aflatoxins detoxification. This ability increased with the increase of incubation periods. These findings are important to food industry and public health; thus, aflatoxin is believed to possess high toxicity among various types of secondary metabolites produced by a larger number of *Aspergillus* spp. Many foods, such as grains (corn, sorghum, and millet), peanuts, beans, and nuts (almonds, pistachios, etc.), may support the growth of *Aspergillus*, and may be contaminated with aflatoxins.

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