Modulation of ochratoxin-induced oxidative stress, genotoxicity and spermatotoxic alterations by *Lactobacillus rhamnosus* GG in male Albino mice

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Abstract: The mycotoxin ochratoxin A (OTA) is a widespread contaminant in human food and animal feed. It is a carcinogenic, genotoxic, teratogenic, immunotoxic, and hepatonephrotoxic agent. Therefore, the present study was designed to assess the possible protective effect of *Lactobacillus rhamnosus* GG (LGG) against OTA-induced toxicity in mice. Four groups of 30 mice each were used: control group, LGG-treated group (1×10^{10} CFU), OTA-treated group (1.8 mg/kg b.w.) and a group of mice given LGG two hours before OTA gavage. The levels of malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) activity were measured in of liver and kidney. Bone marrow micronucleus test and chromosomal aberrations in spermatocytes, as well as mitotic and meiotic activities were performed to assess the genotoxicity; besides sperm parameters were evaluated. Results showed that OTA significantly decreased the body weight. OTA significantly elevated the tissue levels of MDA, whereas the levels of GSH as well as SOD activity were significantly decreased in both liver and kidney. OTA increased statistically the frequencies of MNPCEs in bone marrow and structural and numerical aberrations in spermatocytes. In addition, mitotic and meiotic activities of somatic and germ cells were declined significantly. Also, OTA caused a high significant reduction in cauda epididymal sperm count, sperm motility and increased sperm abnormalities, as compared to control. In mice received LGG before OTA gavage, a significant amelioration in LPO in liver and kidney, by increasing the contents of GSH and SOD activity, have been occurred. Cytogenetic analyses revealed that LGG administration before OTA gavage significantly reduced frequencies of MNPCEs in bone marrow and chromosomal aberrations in spermatocytes, and recovered mitotic and meiotic activities as well. Moreover, gavage LGG before OTA intoxication caused significant recovery in all sperm parameters studied. In conclusion, LGG was found to be safe and successful agent counteracting the oxidative stress and protected against the genotoxicity induced by OTA, in addition to reduction in spermatotoxic alterations.

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1 Introduction

Ochratoxin A (OTA) is a ubiquitous secondary metabolite produced by a number of mold genera, including *Penicillium* and *Aspergillus* (Castella et al., 2002; Pardo et al., 2005). OTA is found in a wide range of foodstuffs including wheat, corn, oats, beans, nuts and coffee beans, and in dry foods such soybeans, garbanzo beans, nuts and dried fruit, also in grapes, coffee, and pork (Bennet and Klich, 2003; Sage et al. 2004). OTA is hepatonephrotoxic, neurotoxic, immunosuppressive, cytotoxic, genotoxic, mutagenic, teratogenic, and carcinogenic agent (IARC, 1993; IPCS 2001). OTA was classified by the International Agency for Research on Cancer, as a possible human carcinogen (2B) (IARC, 1993). Its toxicity has been associated with inhibition of protein synthesis, DNA and RNA synthesis, mitochondrial dysfunction, formation of DNA adducts, disruption of calcium homeostasis, and the generation of reactive oxygen species (Gekle et al., 2005; Ringot et al., 2006; Marin-Kuan et al., 2006; Rached et al., 2007). Chronic exposure to OTA in humans found to be associated with a high incidence of progressive nephropathy and urinary tract tumors (Pfohl-Leszkowicz and Manderville, 2007). Schwartz (2002) hypothesized that OTA is a causative agent for testicular cancer. OTA induced lipid peroxidation (LPO), formation of reactive oxygen species (ROS) and consequent oxidative DNA damage, and decreasing intracellular reduced glutathione (GSH) level. Schaaf et al. (2002) observed an elevation in ROS levels, depletion of

GSH levels and an increase in oxidative DNA damage in rat proximal tubular cells and in LLC-PK1 cells treated by OTA. Furthermore, *in vitro* gene expression data obtained in HK-2 cells suggested an induction of mitochondrial ROS production due to OTA exposure. LPO levels were significantly increased in rat serum and in liver and kidney exposed to OTA, whereas, the glutathione level and antioxidant enzyme activities (SOD, CAT, GPX and GR) were significantly decreased (Meki and Hussein, 2001). Moreover, gene expression data showed that OTA alters a battery of genes, in F344 rat's kidney, that are involved in antioxidant defense and detoxification (Marin-Kuan et al., 2006); these results suggested a reduction of antioxidant defence as mechanism of OTA nephrocarcinogenicity (Cavin et al., 2007). Furthermore, DNA-oxidative damage was detected in HK-2 cells, at cytotoxic concentrations (Arbillaga et al., 2007). Also, oxidative damage to DNA was detected in target (kidney) and non-target (liver) tissues in male F344 rats (Kamp et al., 2005; Mally et al., 2005). Chromosomal aberrations have been induced by OTA *in vivo* in mouse cells (Bose and Sinha, 1994) and *in vitro* in human-derived hepatoma cells (Ehrlich et al., 2002). Dose dependent increases in the frequency of DNA single-strand breaks and alkali-labile sites, as measured by the Comet assay, and in micronuclei frequency were obtained in primary kidney cells from both male rats and human of both genders treated with OTA (Robbiano et al., 2004). OTA induced micronuclei (MN), in a dose-dependent manner, in Syrian hamster fibroblasts (Dopp et al., 1999), in human hepatic (HepG2) cells (Ehrlich et al., 2002) and in V79 Chinese hamster fibroblast cells and in primary cultures of porcine urothelial bladder epithelial cells (Föllmann, et al., 2007). OTA induced, also, MN in cytokinesis-blocked lymphocytes and led to a clear decrease in the percentage of binucleated cells in human lymphocytes (Dönmez-Altuntas et al., 2003). It also induce DNA-ploidy in kidney after chronic exposure (Brown, et al., 2007), and causes increase in endoreduplicated cells (Mosesso, et al., 2008). OTA inhibits the catalytic activity of topoisomerase II and may interfere with chromosome distribution during cell division (Cosimi, et al., 2009). Concerning reproductive toxicity, OTA was found to be a reproductive toxicant and prolong exposure to it caused a significant decrease in sperm count and increased abnormalities in sperm morphology (Bose and Sinha, 1994; Biró et al., 2003). In a recent study, male albino mice were treated orally with OTA (50 and 100 μg/day) for 45 days; alterations in various reproductive parameters were observed (sperm count, sperm motility, sperm viability and fertility rate), in a dose-dependent way (Chakraborty and Verma, 2009).

Currently there is considerable interest in the potential antigenotoxic and anti-carcinogenic effects of probiotics. Lactic acid producing bacteria (LAB), particularly *lactobacilli* and *bifidobacteria* are considered as the most probable agents responsible for these effects. Probiotics have been proved to exert health-promoting influences in human and animals (Ouwehand et al., 2002; Saxelin et al., 2005). *Lactobacillus rhamnosus GG (LGG)* is one of the best-studied probiotic bacteria in clinical trials for treating and/or preventing several intestinal disorders, including inflammatory bowel diseases and diarrhea (Yan and Polk, 2002, 2006). Furthermore *LGG (*ATCC 53013*)* efficiently binds, *in vitro,* several mycotoxins, including aflatoxin B1 and aflatoxin M1 (Pierides et al., 2000; El-Nezami et al., 2002). It had been reported that some strains of lactobacillus could protect against toxins contained in foods such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, mycotoxins and reactive oxygen species (Knasmüller et al., 2001; Stidl et al., 2007). Probiotic *lactobacilli* demonstrated antimutagenic activity against 4-nitroquinoline-1-oxide and N-methyl-NV-nitro-N-nitrosoguanidine by microbial and mammalian cell-based tests (Caldini et al., 2005). Protection against *in vivo* genotoxicity had been observed after co-administration of LAB with N-methyl-N-nitro-N-nitrosoguanidine ((Pool-Zobel et al., 1993). It had been claimed that lactic acid bacteria which are contained in fermented foods and are part of the intestinal microflora may protect human against colon cancer (Wollowski et al., 2001; Rafter, 2004; McGarr et al., 2005).

Strategies for minimizing the possible deleterious effects resulting from human and animals exposure to genotoxic and/or carcinogenic agents in our environment are of utmost need. The aim of the present study was to evaluate the *in vivo* antioxidant, antigenotoxic and antispermatotoxic effects of lactic acid bacteria (*Lactobacillus rhamnosus GG)* against the well-known mycotoxin ochratoxin A in male albino mice.

2-MATERIALS AND METHODS

2.1.Materials:.

2.1.1.Chemicals, reagents, and reagent kits,

Were purchased from Riedel-de Haën, Germany and Biodiagnostic, Cairo, Egypt. Ochratoxin A was obtained from Food Toxicology and Contaminants Dept., National Research Centre, Egypt as a crude mycotoxin.

2.1.2. Bacterial strain and culture preparation:

Lactobacillus rhamnosus strain GG (ATCC 53013) was a kind gift provided by Food Toxicology and Contaminants Dept., National Research Center, Egypt as lyophilized powder and stored at -80°C. LGG cultures were prepared according to the procedure of El-Nezami et al. (2002). In which, bacterial cultures of LGG were obtained by incubating 0.1 g of lyophilized bacteria in 10 ml of deMan-Rogosa-Sharpe (MRS) broth under aerobic conditions at 37°C for 24 h. The number of lactic acid bacteria cells was enumerated by serial dilution in peptone water (0.1 % w/v) and plate counts on deMan-Rogosa-Sharpe agar (MRSA) medium.

2.2.Methods:

2.2.1 Experimental Animals:

Male Swiss Albino mice (*Mus musculus*) three months old weighing 25-30 grams were obtained from the animal house colony, National Research Centre (Giza, Egypt). The animals were maintained on standard casein diet and water *ad libitum* and housed individually in a temperature-controlled and artificially illuminated room free from any source of chemical contamination.

2.2.2.. Experimental design

Mice were randomly divided into four groups each consisting of 30 mice, each group was divided into three subgroups (10 mice for each). Animals were treated orally for successive 7 days as follows: (1) untreated control given $NaHCO_3$ and MRS broth daily, (2) treated with OTA (1.8 mg/kg b.w.) in 0.4ml $NaHCO_3$, (3) treated with LGG (1×10^{10} CFU) in MRS broth and (4) treated with the LGG (1×10^{10} CFU) 2 hours before OTA gavage (1.8 mg/kg b.w.). On the 8th day of the study, the 1st subgroup was killed and femoral bones were removed, stripped and cleaned from extraneous tissues. Also, liver and kidney samples were dissected out and washed immediately with ice-cold saline to remove as much blood as possible, and then stored immediately at -80º C until analysis. On the 15th day of the study, the 2nd subgroup was killed and both testes removed and washed in warm citrate saline. At the end of the experiment (35th day), cauda epididymis, of the 3rd subgroup, were quickly isolated, blotted free of blood and utilized for the analysis of various reproductive parameters.

2.2.3.. Body weight

Mice were weighed at the beginning of the study, at the 8th day, 15th day and the 35th day from the beginning of the study. The percentage of weight gain or loss was then calculated.

2.2.4.. Biochemical analyses

2.2.4.1. Measurement of lipid peroxidation:

Liver and kidney tissues were homogenized individually in 20 mm Tris–HCl (pH 7.4). Homogenates were centrifuged at 6000 g for 30 min. MDA levels in the supernatants were determined using a spectrophotometric assay kit according to the manufacturer's instructions. Briefly, thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product the absorbance of the resultant pink product can be measured at 534 nm (Ohkawa et al., 1979). The lipid peroxidation values are expressed as nm MDA/mg tissue.

2.2.4.2. Reduced Glutathione (GSH) content

GSH levels were measured using a spectrophotometric assay kit according to the manufacturer's instructions. 5,5` dithiobis-2-nitrobenzoic acid (DTNB) is reduced by glutathione (GSH) to produce a yellow compound .The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm (Beutler et al., 1963). GSH values are expressed as mmol/g tissue.

2.2.4.3. Superoxide dismutase (SOD) activity

Liver and kidney homogenates were prepared in cold Tris–HCl (5 mmol/L, containing 2 mmol/L EDTA, pH 7.4) using a homogenizer. The unbroken cells and cell debris were removed by centrifugation at 10,000g for 10 min at 4º C. The supernatant was used immediately for the assays for SOD. 100µl of supernatants were added to 2.8 ml tris HCL buffer containing 25µl pyrogallol and 20µl catalase (Marklund and Marklund, 1974). The activities of all of these enzymes were determined. The SOD activities were expressed as units per mg of tissue.

2.2.5 Micronucleus test

Bone marrow slides were prepared according to the method described by (Krishna and Hayashi, 2000). The bone marrow was washed with 1 ml of fetal calf serum and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 min, followed by staining in May-Grünwald-Giemsa for 5 minutes then washed in distilled water and mounted.

For each animal, 2000 polychromatic erythrocytes (PCEs) were examined for the presence of micronuclei. In order to evaluate bone marrow cytotoxicity, we scored 1000 erythrocytes per animal and the rate of polychromatic erythrocytes (PE) relative to the number of normochromatic erythrocytes was calculated.

2.2.5. Chromosomal aberrations examination

Metaphases for analysis of chromosome aberrations in spermatocytes were prepared according to the method of Evans et al. (1964) and recommendations by Russo (2000) were considered. Structural aberrations analysis was studied in metaphase I (MI): MI with only 20 bivalents was scored; the presence of univalents, chromosome breaks, fragments and chain or ring multivalents, which are classified as reciprocal translocations were considered. For aneuploidy assay, metaphase II (MII) was studied: MII with 18<n>22 chromosomes were recorded, and polyploidy was considered as 2n, 3n or 4n. Fifty metaphase spreads were analyzed per animal. For Meiotic activity of spermatocytes; meiotic index was calculated as the frequency of MII/MI, normal ratio should be equal 2.

2.2.6.. Sperm parameters:

Sperm parameters were prepared and analyzed according to the protocols of Wyrobek and Bruce (1975).

2.2.6.1. *Collection of Epididymal sperm*:

Epididymal sperm were collected by cutting the cauda epididymis and perfusing the cauda with normal saline (0.9%) at 37◦ C. The epididymal perfusate was centrifuged at 225×g for 10 min. The pellet was re-suspended in 1.0 ml of normal saline. An aliquot of sperm suspension was used for the sperm examination.

2.2.6.2. Epididymal sperm counts and sperm motility:

Epididymal sperm counts and evaluation of the motility were performed visually using counting chamber. The count was repeated three times for each sample to minimize error, and calculated as 10^6 per sperm dilution. Sperm motility was determined by counting both motile and non-motile sperms in at least 16 separate and randomly selected fields. These results were expressed as percent motility.

2.2.6.3. Epididymal sperm morphology

A drop of sperm suspension was smeared onto a slide, left to dry; then stained with Eosin A, the slides were washed in water and air dried again. The smears were microscopically analyzed at a magnification of ×1000 for observation of abnormalities.

2.2.7. Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Tuckey's test or by Two-way ANOVA followed by Bonferroni's test comparing all groups. Analysis was conducted with GraphPad Prism software V.5.0.3 (Inc., San Diego, CA; USA).

3. Results

3.1. Change in body weight:

The current results indicated that no mortalities were recorded among any treated groups; no specific symptoms occurred within all groups. At the 8th day of the study, OTA-treated mice showed a significant reduction in body weight gain in comparison with the control group at $p<0.001$ (Fig. 1). Furthermore, at the 15th day, OTA-treated mice showed significant weight loss which was significant (6.10 % at $p< 0.001$), this weight loss reached 9.74 % at the 35th day and it was highly significant compared to all other groups at $p < 0.001$. On the other hand, mice treated with LGG before OTA showed an insignificant decrease in body weight gain compared with the control ($p<0.05$). This group showed a very significant weight recovery when compared with OTA group for all time points. Mice given LGG alone showed an insignificant increase in body weight gain compared with control at $p<0.05$.

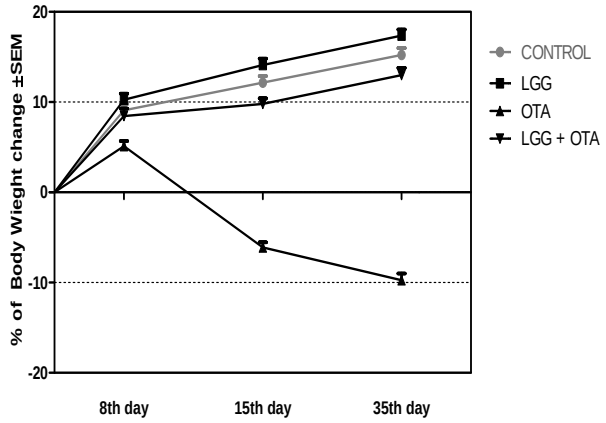

Figure 1: Effects of LGG on ochratoxin-induced mice body weight change.

3.2. Biochemical study

3.2.1. Effect on MDA levels

In OTA-treated mice, MDA level showed a high significant increase ($p<0.01$) in liver and kidney tissues as compared to that of control (table 1). LGG gavage before OTA-intoxication normalized MDA levels in liver tissues to that of control group, which was non significant in comparison with control, while it was still significantly higher in case of kidney tissue compared to control group ($p<0.01$). Whereas, the reduction in MDA in both liver and kidney in this group was statistically significant at $p<0.01$, when compared to the OTA group. In mice

receiving LGG alone, no significant differences were found in MDA level in kidney tissue when compared with control, while data showed a significant decrease in liver MDA level when compared with control at $p < 0.01$.

Table 1: Effects of LGG on MDA, GSH levels and SOD activity in liver and kidney of mice treated with OTA.

Experimental Groups	Parameters					
	MDA		GSH		SOD	
	LIVER	KIDNEY	LIVER	KIDNEY	LIVER	KIDNEY
Control (Broth/NaH CO_3)	339.0± 12.8^{a}	258.0± 6.60^{a}	13.1± 0.40^{a}	17.3 ± 0.31^{a}	29.1± 1.76^{a}	70.7± 3.11^{a}
Ochratoxin (1.8 mg/kg b.w.)	751.0± 15.0^{c}	798.0± 13.3^{c}	4.28± 0.39^{c}	8.12± 0.62^{c}	15.9± 1.56^{c}	26.8± 2.82^{c}
LGG (1×10^{10})	273.0± 8.70^{b}	214.0± 5.80^{a}	13.9± 0.28^{a}	19.4± 0.54 a	37.6± 1.22^{b}	78.4± 2.66^{a}
LGG plus Ochratoxin	352.0± 15.4^{a}	390.0± 14.3^{b}	10.6± 0.46^{b}	14.5± 0.37 b	27.6± 1.10^{a}	53.2± 1.85^{b}

Means with different superscript letters (a, b, c) are -significantly different ($P < 0.01$).
-All data are expressed as means ± SEM.

3.2.2 Effect on the reduced glutathione (GSH level)

Reduced glutathione content in both liver and kidney decreased significantly in OTA-treated group as compared to the control or LGG groups at $p < 0.01$. Mice received LGG before OTA intoxication showed a significant increase in GSH level when compared with the OTA-treated group at $p<0.01$. This increase was significantly below the GSH level of control and LGG groups at $p<0.01$. Mice given LGG alone exhibited increase in GSH content as compared to control, which was insignificant in liver and significant in kidney tissue at $p < 0.01$.

3.2.3 Effect on Superoxide dismutase (SOD) activity

OTA administered group showed high significant decrease in superoxide dismutase activity (in both liver and kidney) as compared to other groups ($p < 0.01$). However, the activity of SOD in the group received LGG before OTA was significantly increased as compared to the OTA-treated group in liver and kidney tissues (Table 1) at $p < 0.01$. This enhancement reached the value of controls in both liver and kidney, which was statistically nonsignificant ($p<0.01$) compared to control. Mice received LGG alone showed an insignificant increase in SOD activity in kidney tissue, while it was statistically significant in liver tissue, as compared with the control group at $p < 0.01$.

3.3. Cytogenetic studies:

3.3.1. Effects of LGG on OTA genotoxicity in bone marrow cells

Results for the MNPE rate are indicated in Table 2, mice treated with OTA showed a high significant increase in MNPCEs (with mean value of 32.8 at $P< 0.01$) when compared with other groups; where insignificant differences can be seen between the control and the LGG treated mice (a mean of 3.0 and of 2.4 per 2000 MNPCEs at $P< 0.05$, respectively). Mice received LGG before OTA gavage revealed a significant reduction in MN value (mean 11.0) with respect to the OTA-treated group, but this reduction was not enough to lower the MN to the basal level of control group, where it remained significant high. The mitotic index values were shown in table 2. OTA intoxication caused a high significant reduction in mitotic division compared with control ($p<0.01$). Whereas, administration of LGG before OTA treatment restored the division ability of bone marrow cells close to that of control, which was statistically significant when compared to the OTA-treated group at $p<0.01$. LGG given group exhibited no statistically differences in mitotic division, when compared to control or LGG plus OTA groups at $p<0.01$.

Table (2): Mean values of the frequencies of Micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow cells of mice administred ochratoxin and/or LGG.

	Untreated Control	OTA	LGG	LGG plus OTA
MNPCEs/2000 cells	3.0± 0.32^{a}	32.8± 1.65^{c}	2.4± 0.24^{a}	11.0± 0.71^{b}
% PCE/NCE	0.49± 0.005^{ab}	0.32± 0.011^{c}	0.52± 0.010^{b}	0.46± 0.10^{a}

Means with different superscript letters (a, b, c) are -significantly different ($P < 0.001$).
- All data are expressed as means ± SEM.

3.3.2. Effects of LGG on OTA genotoxicity in germ cells (spermatocytes MI, MII)

The results of our study revealed that oral treatment with ochratoxin induced structural and numerical chromosomal aberrations in germ cells of

male mice (Table 3). The administration of OTA caused a high increase in X-Y univalents and autosomal univalent which was highly significant at p<0.01 compared with control or LGG groups. The total structure aberrations (x-y and autosomal univalents) increased significantly (P < 0.01) in OTA-treated mice compared to other groups. In mice given LGG before OTA, structural aberrations were decreased significantly compared to the OTA-treated animals at p < 0.01, but still revealed a significant difference compared with the control group. The LGG only treated group showed no significant differences in structure aberrations in respect to the control. Periploidy, polyploidy and the total numerical aberrations observed in spermatocytes were significant elevated (p<0.1) in the OTA treated group compared to all other groups. The LGG plus OTA group showed a significant reduction in numerical aberrations compared to the OTA-treated group and no significant differences compared to the control and LGG groups, except for the total numerical aberration when compared with the LGG group. Meanwhile, the LGG only treated group showed no significant differences in numerical aberrations in respect to the control.

The meiotic index (Table 3) revealed a significant meiotic delay in mice treated with OTA with respect to all other groups (p < 0.01). In the LGG and LGG plus OTA groups, there were no significant differences observed compared with them or the control group.

Table 3: Mean values of different types of chromosomal aberrations in spermatocyte of male mice OTA-treated with or without LGG

Experimental Groups	Structural aberrations (MI)			Numerical aberrations (MII)			Meiotic Index
	X-Y univalents	Autosomal univalents	Total	Aeuploidy	Polyploidy	Total	
Untreated Control	1.2 ± 0.20^{ab}	1.0 ± 0.32^{a}	2.20 ± 0.32^{ab}	$0.8\pm.37^{a}$	1.40 ± 0.25^{a}	2.40 ± 0.25^{ab}	1.99 ± 0.028^{a}
OTA	5.8 ± 0.37^{c}	6.0 ± 0.71^{b}	11.80 ± 0.37^{d}	4.80 ± 0.37^{b}	6.40 ± 0.51^{b}	11.60 ± 0.68^{c}	1.22 ± 0.028^{b}
LGG	0.8 ± 0.20^{b}	0.60 ± 0.25^{a}	1.40 ± 0.40^{b}	0.60 ± 0.26^{a}	1.20 ± 0.20^{a}	2.00 ± 0.31^{b}	2.08 ± 0.053^{a}
LGG plus OTA	2.4 ± 0.26^{a}	1.8 ± 0.20^{a}	4.20 ± 0.37^{c}	1.40 ± 0.25^{a}	2.20 ± 0.20^{a}	3.60 ± 0.25^{a}	1.87 ± 0.072^{a}

Means with different superscript letters (a, b, c, d) are significantly different (P <0.05).

-All data are expressed as means ± SEM.

3.4. Spermatological parameters:

3.4.1. Sperm count and motility

Data on sperm concentration, motility and morphology are presented in Table 4. There was a highly significant change in the sperm concentration of mice treated with OTA showing drastic reduction in sperm concentration (6.8×10^6). Administration of LGG before OTA intoxication caused a significant increase in sperm count respect to the OTA-treated group (18.4×10^6); this increase was significantly below that of control and LGG groups at p < 0.01. In mice given LGG alone, a significant increase in sperm count was observed compared to the control at p<0.05.

The motility of the sperm was affected dramatically in OTA-intoxicated mice which was 26.0 %, this reduction was statistically highly significant at p <0.01. In LGG plus OTA group, there was a significant enhancement in sperm concentration (63.8%) when compared to the OTA-treated group. Again, this increase is still below the basal count of the control. LGG group showed no significant increase in sperm count in respect to that of the control. LGG group showed non-significant enhancement in sperm concentration, compared with control at p<0.05.

3.4.2. Sperm Morphology:

OTA induced a high significant increase in sperm abnormalities in comparing with control at p<0.001 (Table 4). The various head abnormalities were existed, specially head without hook, unusual head shapes, big head and decapitation (Plate 1 B, D). The mid-piece abnormalities consisted of hair-pin, folded, and disrupted neck (Plate 1 E). The tail abnormalities essentially consisted of angular and bi- or coiled tail (Plate 1 F, J). In OTA-treated mice, 10.6 % of sperm head was detached from the flagellum, which was significant compared to control at p<0.01. In addition, OTA caused a fairly high percentage of sperm (13.4 %) that had sticky flagellum (agglutination), where several sperms remained fused in various numbers over short to long distances (Plate 1H, 1G), it was statistically significant at p<0.01.The retention of cytoplasmic droplet (CD) by the cauda epididymal sperm of control as well as OTA-treated mice was observed (Plate1 K). The retention of CD by the cauda epididymal sperm was 10.6% in control mice whereas it was 44.6 % in the OTA-treated mice, this difference was statistically highly significant. In mice receiving LGG before OTA intoxication, different sperm abnormalities significantly reduced in comparing with OTA-treated group, this enhancement showed significant differences with respect with either control or LGG groups at p <

0.05. Meanwhile, LGG group, mice showed a significant reduction in CD retention when compared with the control group ($p<0.01$).

Table 4: Effect of LGG on ochratoxin-induced changes in sperm parameters in male mice.

Experimental Groups	Sperm Count ($\times 10^6$)	% Sperm Motility	Sperm Morphology %						
			Head Abnormality	Mid-piece Abnormality	Tail Abnormality	Decapitation	Agglutination	Total Abnormality	Cytoplasmic Droplets
Untreated Control	22.6 ± 1.18^a	85.0± 1.58^a	2.8 ± 0.24^a	3.40 ± 0.24^a	2.40 ± 0.25^a	3.20 ± 3.7^a	0.00 ± 0.00^a	12.2 ± 0.74^a	10.6 ± 0.50^a
OTA	8.6 ± 0.98^c	26.0± 1.87^c	24.2 ± 1.16^d	19.6 ± 0.68^d	11. 0 ± 0.70^d	10.6 ± 0.75^d	13.4 ± 0.81^d	78.8 ± 1.28^d	44.6 ± 1.03^d
LGG	26.8 ± 1.21^a	89.0± 1.00^a	2.4 ± 0.25^a	2.80 ± 0.37^a	2.20 ± 0.20^a	3.2 ± 0.37^a	0.00 ± 0.00^a	10.6 ± 0.51^a	5.0 ± 0.32^b
LGG then OTA	18.4± 0.71^b	63.8± 1.87^b	10.0 ± 0.71^c	7.60 ± 0.40^c	3.80 ± 0.37^a	8.0 ± 0.55^c	5.20 ± 0.58^c	34.6 ± 1.10^c	19.6 ± 0.93^c

Means with different superscript letters (a, b, c, d) are significantly different ($P < 0.01$).

- All data are expressed as means ± SEM.
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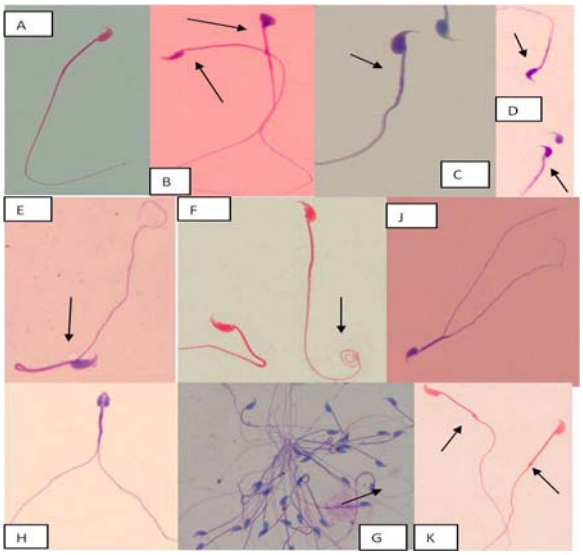

Plate 1: Giemsa-Eosin-stained sperm of mouse. (A) Control mouse. The various abnormalities of the sperm of OTA-treated mice (B–D) abnormal head shape; e.g, amorphous head, big head, banana-like; (E), hair-pin; (F-J) tail angulation bi- and coiled tails; (G), sperm agglutination ; (K), sperm retained CD

4. Discussion

Chronic exposure to OTA is believed to be an important factor for several diseases in human and animals, including renal tumors (Abdel-Wahhab et al., 2005; Brown et al., 2007). Therefore, many intervention strategies are set to prevent or alleviate OTA-induced disorders; probiotics are one of them, but still needed to be evaluated as protective agents for human health. In current study, the role of LGG on the OTA-toxicity was investigated in male albino mice. The present results clearly indicated that OTA treatment resulted in a significant reduction in body weight gain. These results are consistent with study in rats where the reduction in body weight was attributed to OTA and not to reduced food intake (Abdel-Wahhab et al., 2005). In the present study, this reduction in body weight may explained by the ability of OTA to generate free radicals (Pfohl-Leszkowicz et al., 1993), which may lead to DNA breakage, inhibition of protein biosynthesis and gluconeogenesis, lipid peroxidation, disruption of oxidative phosphorylation in mitochondria, inhibition of blood clotting and apoptosis (Hohler, 1998; Petzinger and Ziegler, 2000). LGG gavage alone resulted in insignificant increase in body weight gain; on the other hand, the pretreatment with LGG before OTA intoxication resulted in a significant improvement in body weight gain, comparable with the intoxicated group. This might be attributed to the alleviation of overall oxidative status, improved antioxidant defenses and decrease in genotoxic and cytotoxic effects (Bruno-Barcena et al., 2004; Klinder et al., 2004; Chen et al., 2005; Koller et al., 2008).

The results of the experiment showed that OTA treatment elevated MDA concentration in liver and kidney tissues, which indicated to the increase of lipid peroxidation. MDA is an end product of lipid peroxidation, and this may be considered a late biomarker of oxidative stress and cellular damage (Vaca et al., 1998). The results of this study confirm and extend previous data which have demonstrated that OTA induces a significant increase in LPO in liver and kidney induced by OTA as increasing in malondialdehyde (MDA) production (Petrik et al., 2003; Abdel-Wahhab et al., 2005, 2008). Previously, Gautier et al. (2001) stated that OTA did evoke oxidative stress, which might contribute, at least in part, to OTA renal toxicity and carcinogenicity in rats during long-term exposure. To assess the balance of reactive oxygen species (ROS) production in liver and kidney, levels of non-enzymatic antioxidants GSH and enzymatic antioxidant (SOD) activity were measured. Current results showed that OTA caused significant decrease in the levels of GSH, along with decrease in the activity of SOD; this decrease indicated the cell damage of liver and kidney tissues (Doorten et al., 2004). Soyöz et al. (2004) stated that intracellular GSH status appears to be a sensitive

indicator of cell's overall health and its ability to toxic challenges, whereas, the decrease in SOD activity will increase the level of superoxide radicals, leading to an increase in oxidative stress enhancing early cell death, probably by apoptotic mechanisms. Our finding of decrease in the activities of SOD and GSH corroborates with that of previous studies (Ozcelik et al., 2004; Abdel-Wahhab et al., 2008). The results of the experiment with LGG showed that treatment with LGG before OTA gavage ameliorated the biochemical parameters in liver and kidney, where MDA level decreased and SOD activity increased, along with an increase in GSH contents. Many *in vitro* studies, reported that LAB strains possess antioxidant properties and inactivate ROS via enzymatic mechanisms, e.g. by a coupled NADH oxidase/ peroxidase system, superoxide dismutase and catalase (Kullisaar et al., 2003; Bruno-Barcena et al., 2004; Lee et al., 2005). *Bifidobacterium longum ATCC 15708* and to a lesser *extent L. acidophilus ATCC 4356* inhibited linoleic acid peroxidation and scavenged free radicals (Lin and Chang, 2000). Also, it was found in human and animal studies that some LAB strains, which inactivate ROS, decrease biochemical parameters of oxidative stress (Han, 2004; Songisepp et al., 2005; Han et al., 2006). In a recent study, Koller et al. (2008) investigated the prevention of oxidative DNA damage in human derived colon (HT29) cells by 55 strains of lactic acid bacteria, they indicated that the reduction of oxidative damage was only seen with viable bacteria but not with heat inactivated cells and that it took place when the colon cells were separated from the LAB by permeable filter membranes indicating that the bacteria release ROS protective factors into the medium.

Considering the genotoxicity, the present study indicated that OTA caused high significant increase in DNA damage and cytotoxic effects in both cell types; we have investigated the OTA genotoxicity by induction of micronuclei (MN) in somatic cells (bone marrow), as an endpoint suitable to detect both aneugenic and clastogenic effects and chromosomal aberrations in germ cells (spermatocytes). The present data revealed that OTA induced a very high significant increase in MN in bone marrow cells, and increased structural and numerical aberrations in spermatocytes. Moreover, mitotic and meiotic activities had declined in a significant way. These findings are in agreement with the previous studies; OTA induced micronuclei in ovine seminal vesicle cells (Degen, 1997), in Syrian hamster fibroblasts (Dopp et al., 1999) and in human hepatic (HepG2) cells (Ehrlich et al., 2002). Significant dose-dependent increases in the frequency of micronucleated cells were also obtained in primary kidney cells from both male rats and humans of both genders with OTA (Robbiano et al., 2004). A statistical increase of structural chromosomal aberrations and sister chromatid exchanges associated with a decrease of the mitotic index was observed in bovine lymphocytes (Lioi et al., 2004). *In vivo*, oxidative damage to DNA was detected in target (kidney) and non-target (liver) tissues in male F344 rats (Kamp et al., 2005; Mally et al., 2005). Also, OTA induced structural and numerical chromosomal aberrations in bone marrow and germ cells of male mice (Ezz El-Arab et al., 2006). As shown in table (2, 3), data showed that OTA reduced the mitotic and meiotic ability significantly in somatic and germ cells; these results are in agreement with many studies which had demonstrated that even at very low concentration, ochratoxin was able to induce apoptosis in kidney cells in rats (Soyoz et al., 2004), and inhibit cell cycle progression by arresting cells at G2/M phase (Palma, et al., 2007). In addition OTA modulates key regulators of chromosome segregation and progression through mitosis (Adler et al., 2009). OTA found to inhibit the catalytic activity of topoisomerase II and might interfere with chromosome distribution during cell division (Cosimi, et al., 2009). Pfohl-Leszkowicz et al. (1993) reported that the ability of OTA to generate free radicals and to enhance lipid peroxidation has been linked to the genotoxicity expressed by DNA adduct. Free radicals may lead to DNA breakage, inhibition of protein biosynthesis and gluconeogenesis, lipid peroxidation, disruption of oxidative phosphorylation in mitochondria, inhibition of blood dotting and apoptosis (Petzinger and Ziegler, 2000). Pfohl-Leszkowicz and Manderville (2007) proposed that OTA genotoxicity might be caused by direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action. Gautier et al. (2001) and Baldi et al. (2004) reported that oxidative stress is an important factor in OTA cytotoxicity. Arbillaga et al. (2006) suggest that oxidative stress precedes cytotoxicity and genotoxicity and plays an important role regarding the mechanisms involved in OTA nephrotoxicity and carcinogenicity. Also, El Golli-Bennour et al. (2010) found that OTA induced genotoxic and cytotoxic effects in cultured monkey kidney Vero cells. On the other hand, results showed that the administration of LGG before OTA-intoxication reduced the OTA-induced genotoxicity (somatic and germ cells by around three folds) and cytotoxicity in both cell types. These data are consistent with other experimental studies which had evidenced the ability of *lactobacilli* and *bifidobacteria* to decrease the genotoxic activity of some chemical compounds (Tavan et al., 2002;

Burns and Rowland, 2004; Caldini et al., 2008). Also, our results revealed that LGG gavage before OTA treatment, enhanced the mitotic and meiotic activities of bone marrow cells and spermatocytes in OTA-treated mice to nearly to the basal level of control animals; this finding is going along with the mechanistic studies by Yan et al. (2007) who found that LGG prevent cytokine-induced apoptosis in intestinal epithelial cells through secreting two soluble proteins (p75 and p40).

Regarding the reproductive toxicity, the present study clearly indicated that oral administration of OTA caused adverse effects on male reproductive parameters in mice (Table 4). In OTA-treated mice cauda epididymal sperm count was reduced significantly (6.8 $x10^6$), along with a decrease in motility (26.0%) and a dramatically increase in sperm abnormalities (78.8 %). These findings are in agreement with some authors who reported similar kind of observations in different animals emphasizing ochratoxin as a reproductive toxicant; OTA induced chromosomal abnormalities and a decrease in spermatogenic numbers in mice (Bose and Sinha, 1994), decreased motility and longevity of breeding boar semen (Solti et al.,1999), impaired spermatogenesis and caused accumulation of premeiotic germinal cells (Fenske and Fink-Gremmels, 1990), inhibited testosterone secretion in isolated testicular interstitial cells of gerbils in *in vitro* condition (Gharbi et al., 1993). In human, sperm motility reduction might be due to mitochondrial disruption and/ or an increase in lipid peroxidation (Lodish et al., 2003). The lipid peroxidation of unsaturated fatty acids in sperm membranes is one of the most important effects from ROS-induced cell damage (Hsieh et al., 2006), and might impair sperm motility (Saradha et al., 2006; Hsieh et al., 2006). Chitra et al. (2003) observed that increased levels of lipid peroxidation caused the reduction of sperm count and viability. Moreover, the results of the present study showed that OTA intoxication rendered a significantly higher percentage of the cauda epididymal sperm (44.6 %) to retain cytoplasmic droplets (CD) than in the control mice. The residual cytoplasm contains high concentration of certain cytoplasmic enzymes (G_6PDH, SOD), and are also a source of ROS (Gomez et al., 1996). ROS damages phosphatides of cell membrane by peroxidized metabolites of fatty acids, whereby damaging the sperm function and morphology (Alvarez et al., 1987). Our data substantiated these claims where midpiece and tail of OTA-treated mice showed a very high percentage of malformation (hair-pin, disruption, folding, tail angulation, tail coiling), which might caused by peroxidation of cell components and disrupted the cytoskeletal proteins (44.0 %). The sticky flagellum (13.4 %) observed in this study might formed by fusing of two or more spermatozoa, where two or more axonemes are in a common cytoplasm ((Agnes and Akbarsha, 2003). These data reflected the aberrant spermatogenesis and/ or spermiogenesis caused by OTA treatment.

On the other hand, pre-treatment with LGG significantly mitigates OTA-induced alterations in reproductive parameters in mice, where sperm count elevated to reach 18.4 x 10^6 and the motility recovered to 63.8 %. Also, sperm morphology showed a significant enhancement (44.2 %). Moreover, LGG reduced CD retention by about 2.3 folds with respect to the OTA-treated group, which might due to the reduction in ROS.

Some authors attributed the protective effect of these bacteria to different mechanisms such as binding of OTA *in vitro* (Del Prete et al., 2007; Fuchs et al., 2008; Mateo et al., 2010). Previous work with more than 250 strains of lactic acid bacteria showed that *Lactobacillus rhamnosus* strains, LGG and LC705 were the most efficient strains in binding a range of mycotoxins, including aflatoxins (El-Nezami et al., 1998). Piotrowska and Zakowska (2005) verified that *L. acidophilus* and *L. rhamnosus* caused OTA reductions of 70% and 87% of 1 mg OTA/L after five days at 37 °C, and that significant levels of the OTA were present in the centrifuged bacteria cells. Gratz et al. (2006) suggested that LGG treatment reduced the hepatotoxic effects caused by a high dose of AFB_1, by increasing the excretion of orally dosed aflatoxin via the fecal route and suggested that LGG was able to retain additional AFB_1 and AFM_1 inside the intestinal lumens of rats. Nevertheless, Fuchs et al. (2008) consider that metabolism may also be involved, where viable cells of *L. acidophilus* removed OTA more efficiently than unviable. In addition, LAB found to cause reduction of the formation of secondary bile acids (Mirasoli et al., 2002) and enhancement of the immune system (Wallace et al., 2003; Schultz et al., 2003; Bengmark and Martindale, 2005).

In conclusion, the overall data indicate that the LGG have a broad range of biomodulatory properties; alleviates the OTA-oxidative stress (by decreasing in LPO and enhancing the activity of antioxidant enzymes and glutathione content) and protects against OTA-genotoxicity; as well as mitigates the spermatotoxic effects induced by OTA. However, further studies are needed to better understand the *in vivo* possible mechanism(s) by which LGG may reduce OTA toxicity.

5. References

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