GENOTOXICITY OF ETHIDIUM BROMIDE IN ALBINO MICE TREATED WITH BACTERIAL PROBIOTIC

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Ethidium bromide (EtBr) is a synthetic chemical molecule of heterocyclic aromatic nature that follows the phenanthridine family. This drug can bind to nucleic acids through the intercalation process, in which the aromatic molecule is inserted between adjacent base pairs (Watkins, 1952). Genotoxicity of EtBr has been addressed in several studies, which confirmed that EtBr is a genotoxic agent, meaning that this substance can destroy DNA and RNA molecules, and many aspects of genetic toxicity. Interestingly, EtBr inhibited gene expression in *Escherichia coli;* this action has comforted its ability to bind to nuclear acids and act as nucleic acid- polymerases inhibitor (Waring, 1965).

Green Screen Assay, one of the genotoxicity tests, is performed in yeast and described as a sensitive and inexpensive assay. Implementation of this assay on ethidium bromide showed that EtBr is a genotoxic drug, and it has other effects, such as chromosomal changes, despite not being able to prove or denied its carcinogenicity (Cahill *et al.,* 2004). The genotoxicity of EtBr was confirmed using the luciferase enzyme activity, which in turn depends on the *p*53 gene. This test showed that EtBr is a strong genotoxin (Ohno *et al.,* 2005). EtBr also can destroy human chromosomes; thus, it caused apparent structural chromosomal changes, but according to the used dose (Wu *et al.,* 2006). Several studies have been conducted to detect EtBr mutagenicity, which was strongly confirmed (Prabhu *et al.,* 2010). Also, EtBr carcinogenicity was proven and was classified as a direct or a primary carcinogen that does not require activation by metabolism, but its action is directed toward nucleic acids. Furthermore, EtBr can inhibit communication between cells by affecting gap–junctions and being a genotoxic and carcinogenic agent (Na *et al.,* 1995). In contrast, some studies concluded that EtBr was an unknown carcinogenic agent (Sakai *et al.* 2010).

Probiotic is living microorganism added to animal and human food, giving a healthy and robust influence. They positively affect humans and animals' health by improving the balance of bacteria in the intestines when given in sufficient quantities (Hill *et al.,* 2014). Probiotics are

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often bacterial organisms that affiliate to some well-known genera, such as *Lactobacillus*, *Escherichia*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, and *Bacillus spp*., but some yeast break this general rule due to their ability to work as probiotic in some cases (Lukjancenko *et al.,* 2012). Despite that the lack of clinical trials supports probiotic safety for humanity (Slashinski *et al*., 2012), there is a general agreement for its health benefits for mammals. Many studies have pointed to the active role of probiotics in the face of chronic and acute diseases that afflict humans and animals, such as digestive system diseases, a widespread phenomenon among humans, respiratory system infections, female reproductive tract infection, allergy, cholesterol, and cancer (Mohania *et al.,* 2013). The administration of lactic acid bacteria (LAB) has been shown to effectively reduce DNA damage induced by chemical carcinogens (Li and Li, 2003). These anticancer effects of probiotics are inhibiting intestinal bacterial enzymes that convert procarcinogens to more proximal carcinogens (Nagpal *et al*., 2012).

The present study aimed to test the EtBr seriousness effect on researchers' health and confront these expected risks using probiotics intake. The current study was conducted by treating mice with different EtBr doses parallel with daily LAB-supplement to mice feed for one month. Histological examination and molecular studies were applied to mice's liver after completing a month of the experiment.

MATERIALS AND METHODS

Materials

A total of 49 male Swiss albino mice, *Mus musculus*, with bodyweight (b. w.) at 25-30g, were obtained from the Faculty of Medicine, Ain Shams University, Cairo, Egypt. Ethidium bromide was purchased from Fluka Biochem Co. (Cat. No. 46065), while LAB was obtained from the Dept. of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University. Commercial powdered milk (Nido) was purchased from the local market to be used as a probiotics carrier. It was mixed with liquid media contains Lactic acid bacteria, then the mixture was incubated at 37° C/ 24 hours and finally stored at 4ºC. Probiotics were added to mice food at ($24x10^7$ CFU/g).

Methods

Experimental design

Mice were randomly divided into seven groups; each one contained seven animals. Mice of groups one, two, and three respectively received high (60 mg/kg b. w.), median lethal (50 mg/kg b. w.), and low dose (40 mg/kg b. w.) of EtBr as drinking solutions in addition to a unique dose (24x107 CFU/g) of bacterial probiotics mixed with original food. Mice of group four received regular food and drink, representing control group. Mice of groups five, six, and seven received EtBr only as drinking solutions with different concentrations, representing three different EtBr doses (high, median lethal and

low dose, respectively). The duration of this experiment was one month of different daily treatments. Controlled experimental conditions were applied, as regular light/dark cycles were every 12 hours. Optimum temperature was set at 20-22 ºC with adequate ventilation, cleanliness, and proper nutrition (water and commercial standard diet pellet ad libitum).

Histological analysis

The histological observations due to the treatment with EtBr only and EtBr combined with Probiotics were examined in liver and testes tissues according to Bancroft *et al*., (1996); the main steps were: Fresh samples taken from the liver and testes of mice, representing different groups were fixed in 10 % formalin for 24 h; fixed samples were washed in tap water; Serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration; Specimens were cleared in xylene and embed in paraffin at 56 ºC in a hot air oven for 24 h; Paraffin bees wax tissue blocks were prepared; The blocks were sectioned by slide microtome at 4 µ thickness; Obtained tissue sections were collected on deparaffinized glass slides and stained by hematoxylin & eosin stain and examined using a light microscope.

Molecular genetics analysis

DNA fragmentation

DNA fragmentation analysis was done using a hypotonic solution (6% potassium chloride) to extract high-density nucleic acids (unpublished data). The main steps were: 0.5 g frozen liver was disrupted in 1.5 ml Eppendorf tube; One ml 6% KCl fresh prepared was added; Samples were vortexed for 10 min (to homogenize the sample); Homogenate samples were incubated at 55 ºC / 2 hours; Incubated samples were centrifuged at 1000 rpm / 15 min; Resulted supernatant (contained the DNA) was transferred to new sterile and DNase-free Eppendorf tube; Five µl DNA + 5 µl loading dye were mixed and electrophoresed on 2 % agarose gel (Johansson, 1972), 6 µl Gel Pilot Mid-Rang ladder 100 (Qiagen, Germany) was loaded in a separate well to provide fragment sizes of 2000, 1000, 750, 500, 250, and, 100 bp; The run was performed for one hour at 100 volts using an Applex submarine unit (10 cm x 8 cm). The amplicons were revealed using 1x EtBr on UV- trans-illuminator and photographed by Gel documentation system (SYNGENE, UK).

Quantitative PCR analysis

RNA extraction

Pure total RNA was isolated from frozen liver tissue (under sterilization conditions). According to the manufacturer's recommendations, reagents of ISOLATE II RNA Mini Kit (Bioline, Germany) were used. Concentrations of all samples were measured on a spectrophotometer; then, we made different concentrations equal by adding sterile water to complete the following steps correctly.

Complementary DNA (cDNA)

Extracted RNA was converted to cDNA by using the Tetro cDNA synthesis kit (Bioline, Germany). A two-step reverse transcriptase-polymerase chain reaction was applied according to recommendations submitted by the manufacturer. All reagents and samples were mixed well and briefly centrifuged, then kept on ice before use.

Real-Time PCR run

The RT reaction was amplified for cDNA of interest, a gene-specific primer of *p*53 was used to detect the *p53* mRNA and relatively quantify its amount; its sequences were 5' CCC AAA CTG CTA GCT CCC AT 3' as a forward primer (*p53* Mm-F1291), and 5' ACT ACT CAG AGA GGG GGC TG 3' as a reverse one (*p53* Mm-R1567), which were synthesized through Invitrogen, the UK for amplification of desired gene (*p53*). These primers were newly designed and based on GenBank accession (NM_ 001127233.1) using Geneious 8.1 software. Primer3 online tool was used to test hairpin formation and self-annealing of the designed primers and perform in silico PCR of those primers in the University of California, Santa Cruz database (http://genome.ucsa.edu), on the mouse genome. The expected molecular size of the in-silico PCR was 277 bp.

Stratagene MX3000 P (Agilent Technologies) machine was used to apply qPCR on representative samples, which consisted of 10 µl of 2 x Sensi FAST SYBR® No-Rox kit (Bioline, Germany), 0.5 µl *p53* reverse primer (*p53*Mm-R1567), 0.5 µl *p53* forward primer (*p53*Mm-F1291), 1 µl cDNA template and nuclease-free water (up to 20 µl). The conditions of the used program were: initial denaturation at 92 ºC / 2min, repeated 40 cycles of 92 ºC / 5 sec for denaturation phase, 56 ºC / 15 sec for annealing phase, and 72°C / 26 sec for extension phase. A disassociation test was performed from 95 ºC to 50 ºC at 10 min intervals.

Real-time PCR data analysis

The qPCR CT (Cycle Threshold) values were used to compare and estimate the relative expression level of *p53* in comparison to control. Excel 2013 was used to estimate Δ CT, as Δ CT target = CT control – CT treatment, $E = 10^{-(1/slope)}$, and Ratio $_{(test/calibrator)} = E^{CT(calibrator) - CT(test)}$. As the amount of PCR product, was doubled each cycle of exponential amplification resulted in a 2-fold increase in the number of copies; thus E was set to 2.

RESULTS AND DISCUSSION

Several criteria for studying the impact synthetic chemicals or microorganisms under test should be considered, such as the method of exposure, the duration and frequency of exposure, target organ, and the impact of substance (Klaassen, 2013). It was reported that touching or inhaling ethidium bromide leads to numerous risks on the object's genetic material (Harisha, 2005 and Garbett *et al.*, 2007). However, the question was, what would happen if the material has already reached the digestive system? The mice's

body was exposed to EtBr through the digestive tract (drinking) while probiotics were added to their diet to answer this question. Probiotics were used as a protective agent against the side effect of EtBr due to the recorded health benefits (Patten and Laws, 2015). Indeed, materials entering through the mouth are processed in the liver, and then the process of detoxification may obscure the full emergence of the real impact of EtBr. A single dose of probiotics was used while three different EtBr doses were applied every day for one month before the autopsy.

Histological and molecular analyses were applied to determine the effect of EtBr on the digestive system and the possible protective action of probiotics to reduce such effects**.**

Histological effects screening

Liver examination

The liver was affected in all treatments compared to the control group. Generally, a wide range of tissue changes emerged; fatty and degenerative changes, inflammation, hepatic veins dilation, and focal hemorrhage were the common histological signs of the treatments (Table 1 and Fig. 1).

Hepatotoxicity is a satisfactory infects the liver due to exposure to the influence of harmful chemicals, toxins, drugs, or any other factor. The detoxification job of the liver makes it the most vulnerable organ to be affected. The histological change degree depends on the duration of exposure, used dose, and the exposed object. Although the liver toxicity phenomenon is single multi-faceted, it has many histological manifestations such as portal hypertension, fatty liver, inflammation, etc. Many hepatotoxicity mechanisms have been clarified in various studies, which focused mainly on mitochondrial failure which in turn results in disable many vital processes in the liver cells finished with mentioned symptoms of hepatotoxicity (Navarro and Senior, 2006). In this regard, histological examination of affected organs is a powerful method to determine cytotoxicity, especially chemical-induced toxicity.

Administration of EtBr at a low dose

This treatment led to dilation and congestion of portal and central hepatic veins (plate 1, D). Furthermore, focal bleeding in hepatic parenchymal cells (plate 1, E). L dose of EtBr could cause severe alterations in hepatic cellular. Those manifestations reflect the occurrence of portal hypertension, which is considered the gateway to cirrhosis of the liver and many of its complications (de Franchis, 2010). Therefore, the proposed interpretation of this case was due to the dosage of ethidium bromide, which impacted the liver and was on its way to cause cirrhosis. The dose may lead to erratic hepatitis blood pressure, which explains the liver suffering from the detoxification of the substance being tested, despite the need for other supportive studies to obtain more explanation.

Administration of EtBr at a medium lethal dose

This treatment led to fatty changes (plate 1, F), the portal vein's congestion, and inflammatory cell infiltration in the portal area (plate 1, G). The appearance of fatty changes is a result of various causes as drugs, toxins, rapid weight loss, malnutrition, Insulin resistance, viral infection, pregnancy, oxidative stress, and genetics (Adams *et al.*, 2005) but also, many studies have shown that fat accumulation in the liver relates to metabolic disorders (Fabbrini and Magkos, 2015).

It is worth mentioning that the mechanisms of fat accumulation in the liver vary from one reason to another, but do not get out of one or more of the following events: increased fatty acids synthesis decreased oxidation of fatty acids, and presence of fatty acids in large quantities to the liver and disruption of triglyceride cycle (Treinen-Moslen, 2001). So, any altered pathway of this process, from which lipid β-oxidation leads effectively in an imbalance in the presence of fat in the liver. However, the existence of many pathways of lipid β-oxidation as peroxisomal β-oxidation and mitochondria β-oxidation, but the latter remains the most important and most influential, despite the emergence of some devastating diseases result of altered peroxisome (Bartlett and Eaton, 2004). In this connection, it can be explaining the emergence of a change in liver fat as a result of a direct impact of ethidium bromide on the mitochondria of liver cells (Stewart *et al.*, 2010), leading to an imbalance in the performance and function of this organelle, from which β-oxidation for lipids either increase or decrease.

Other manifestations were expected to appear associated with fat deposits, according to the "two-hit" hypothesis (Mehta *et al.*, 2002 and Bigorgne *et al.*, 2008); fatty acids accumulation in the hepatocellular "first hit" led to inflammation "second hit". Other liver injury symptoms appear after those two hits as fibrosis, cirrhosis, and liver carcinoma (not found in our histological results). Both environment and genetics contributed in "second hit" that could be explained simply by the action of adipose tissue of secreting specific cytokines that stimulate other mediated factors through specific pathways and trigger circulating lymphocytes to the liver leading to inflammation. It cannot overlook other factors' role in this mechanism as gut-derived bacterial endotoxin and oxidative stress for reactive oxygen species (ROS). In this setting, the mitochondrion is one of ROS's most important sources (Mehta *et al.*, 2002). By contrast, not in all cases the "second hit" was the inflammation, which would occur first, followed by steatosis (Tiniakos *et al.*, 2010). The appearance of the portal vein congestion reflected that EtBr at LD_{50} could cause hypertension, as shown at the treatment of L dose of EtBr (de Franchis, 2010).

Administration of EtBr at high dose

This treatment led to fatty change, and other degenerations appeared in the hepatocytes all over the hepatic paren-

chyma (plate 1, H) associated with focal hemorrhage (plate 1, I). Dilated and congested portal vein appeared with inflammatory cell infiltration in the portal area surrounding the dilated bile ducts (plate 1, J). According to the mentioned "two hits" theory, fat deposition "first hit" followed by inflammation "second hit" which was one of the causes of venous congestion (Kakar *et al.*, 2004). Thus, the existence of fat deposits in the hepatocytes led to a defect in its job performance, and then those symptoms occur, where a strong correlation was found between liver fat and abnormal manifestations of hepatic veins (Northup *et al.*, 2008).

Focal hemorrhage is a change in the hemostatic system attributed to a disruption in platelet production regulated by the liver. The platelets are responsible for keeping blood loss (Lisman *et al.*, 2002). Other studies have shown a relationship between those changes and liver failure in the performance of its functions because of chronic or acute diseases (Lisman *et al.*, 2010).

Inflammation is a defensive condition carried out by the Immune system when there is a detrimental inducer to a specific site in the body of an organism; immune cells of different types are blown special Kupffer cells sills around the injury site to secrete specific toxins to eliminate the injured and abnormal cells, but these toxins harmful to other healthy cells (Hall, 1996). The inflammations continued for many diseases that affect the liver special liver steatosis (Yin *et al*., 2007), so deposits of fats are often accompanied by inflammation.

The mechanism by which hepatic veins became abnormal may be due to alteration in blood flow regulated by ant thrombin III, protein S and protein C, and anticoagulant proteins produced by the liver. Any change in those anticoagulant proteins by an increase or decrease reflected directly on the veins and the case of passing blood, liver fat has not been lost in the events of change in these proteins' level, especially, Protein C (Northup *et al.*, 2008). Nevertheless, the question remains; why did specific changes associate in some doses of ethidium bromide without appearing in others? Moreover, why did other tissue changes be general with each dose? The answer needs many of the illustrations studies.

Administration of EtBr at low dose combined with probiotic bacteria

This treatment led to a degenerative change in the hepatocytes surrounding the dilated central vein (plate 1, K). Interestingly, liver tumors appeared by morphological examination (data not shown). The L+P group's liver appeared differently from the rest of previous transactions; lack of liver abnormality was detected. However, there were some adverse effects, such as degenerative change and dilated central vein. This means that new manifestations appeared while others' disappearances were already related to L treatment without probiotics.

Degeneration is an abnormal shape of liver cells, which increase in size with

almost completely lost vital functions, making it closer to die, so hepatocellular degeneration is the first step on the way to necrosis. This abnormal cellular manifestation occurred when the cell membrane was altered; then, permeability was affected. Subsequently, ion pumps were affected, followed by an influx of sodium and water, leading to ballooning degeneration. Another cause of degeneration was the toxic effects of bile acids on cytoplasmic components, leading to the gradual death of the cells and their degeneration (Dancygier and Schirmacher, 2010) that may explain the emergence of dilation in the biliary duct.

The appearance of liver tumors reflected the low gene expression of the *p53* gene known to be tumor suppressor gene (Isobe *et al.*, 1986), thus when functions of *p53* were affected and disappeared, tumors were accordingly formed (Desilet *et al.*, 2010). So probiotics were thought to inhibit *p53*, inducing tumors' appearance due to EtBr treatment, although probiotics are potent cancer treatment tools (James *et al.*, 2016). Appeared tumors represented a piece of evidence for EtBr carcinogenicity, by contrast to what was proven about unknown carcinogenicity of EtBr (Sakai *et al.*, 2010).

Administration of EtBr at median lethal dose combined with probiotic bacteria

This treatment led to inflammatory cell infiltration surrounding the adjacent hepatocytes' dilated central vein wall (plate 1, L). Furthermore, liver tumors appeared by morphological examination (data not shown). This means that not all manifestations appeared in this treatment than its counterpart (LD_{50}), while the new one appeared (tumors). The absence of some manifestations recorded in LD_{50} reflects probiotics' relative ability to coop with the EtBr effect but at a limited level. While the appearance of tumor referred to probiotic effect on gene expression of *p53* as discussed previously.

Administration of EtBr at high dose combined with probiotic bacteria

This treatment led to dilation in the portal vein (plate 1, M)**, i**n addition to the observation of liver tumors that appeared by morphological examination (data not shown). These results confirmed the previous interpretation; thus, dilation of portal vein reflected that EtBr could do its action although the presence of probiotics, which means that used probiotics could not completely coop with EtBr effect. Interestingly, tumors appearance resulted from *p53* inhibition, which may be caused by probiotics.

Probiotic bacteria cannot prove complete efficient in curbing the histological effects of EtBr, unlike expected, where all symptoms related to different used doses of EtBr did not disappear completely, but even new symptoms appeared in the L+P group (degeneration changes). This means that the cytotoxic effect of EtBr did not end at one hundred percent. However, it remains to a lesser extent, which means that used dose of LAB was unable to completely counter EtBr influence, this is supported by the incomplete

lack of the ability of fermented dairy products to resist colorectal cancer in mammals (Capurso *et al*., 2006), suggesting a relative ability of the resistance. However, they need confirmatory studies to rely on those microbes in formal treatment.

The general belief that probiotics are safe and their regular intake leads to general improving mammalian health, particularly resistance to a wide range of cancers (James *et al.,* 2016). However, some old evidence of probiotic ability to cause harm to some organisms, in particular some strains of LAB was reported (Mackay *et al*., 1999),

Control group of mice

This treatment led to the liver's regular appearance; thus, regular histological features were cleared in all hepatic cellular components. This normality resulted from receiving typical food and drinks. The aim of conducting this treatment was to obtain healthy animals used for comparison with treated animals, and then histological alteration, induced by tested material, became apparent.

Molecular studies analysis

Gene expression is a process that turns genotype to phenotype, so it is considered the secret key that must be studied well at its different levels when any change appears on the organism, particularly diseases. This brings us to the concept of Gene expression analysis, the study of the functional product of a gene in specific cell /tissue, is applied using various ways. A qPCR is a powerful tool in this regard, so it was used in the present study to measure the gene expression of *p53* gene.

DNA fragmentation analysis

Applying the DNA fragmentation procedure resulted in high-density nucleic acids. Electrophoresis of genomic DNA obtained by this technique appeared normal in all samples (Fig. 2). The photo illustrated the lack of direct effects of ethidium bromide and bacterial probiotics on the genetic material; therefore, there was no DNA fragmentation related to all treatments compared to the control group.

As for EtBr, these results contrasted with the expected; thus, the DNA intercalation process can destabilize the genetic material and negatively affect the chromosomal structure. So EtBr, an intercalator, can be regarded as a significantly contributed agent to cells' toxicity. In other words, it is well known that most intercalating agents have an inhibitory effect on DNA topoisomerases, which control the topography of the nucleic acid, leading to obstructing the correct pathway of gene expression in addition to causing abnormal chromosomal packaging. These effects can be completed by the occurrence of programmed cell death represented in DNA fragmentation (Wang *et al.,* 1997; Singer *et al*., 1999). That did not happen, where it did not produce any fragmentation in the genetic material by ethidium bromide. These discrepancies can be explained by the occurrence of another kind

of response, such as cell cycle arrest or DNA repair system enhancement (Woods and Vousden, 2001; Oren *et al.,* 2002). Otherwise, maybe more time was needed.

As for probiotics, DNA fragmentation analysis results were expected because it is well known that probiotics, especially LAB, have the anti-carcinogenic property (Li and Li, 2003). It was also proven that some strains of LAB could cope with chemical-induced genotoxicity in rats (Pool-Zobel *et al.,* 1996) and other mammals (Burns and Rowland, 2000). Many other studies have dealt with probiotics as living beneficent microbes play an active role in the resistance to the genetic material's negative changes (Madrigal-Santillán *et al.,* 2006; Corsetti *et al.,* 2008; Verdenelli *et al.,* 2010; Dominici *et al.,* 2011). Also, probiotic administration safety in a human was recorded, which indicates the absence of any undesirable effects on health; thus, genetic material was not affected (Van den Nieuwboer *et al.,* 2015).

Although not all studies showed probiotic force in responding to the health dangers, there are failures made by those microbes in this regard, such as the study in which products of fermented milk failed to prevent colorectal cancer (CRC) ultimately, where they succeeded in tests without the other (Capurso *et al.,* 2006). It is worth mentioning that the species but the strain of used probiotic showed highly specialized therapeutic. The experiment's dosage and conditions can control many of the obtained results in a clinical trial (Whorwell *et al.,* 2006). It is imperative to make entirely sure that each experiment has particular circumstances that control obtained results. However, there are essential criteria that must be met to achieve the desired goal of probiotics usage as probiotics can survive in the gastrointestinal tract and its inability to work as a pathogenic or toxic organism (Shalke, 2013).

Real-time PCR

After obtaining cDNA and applying q-PCR using a real-time PCR machine, obtained CT values were used to estimate ΔCT and Ratio of the *p53* gene expression, using subtractive equations. CT values of EtBr treatment groups were lower than CT values of EtBr with probiotics treatments, so all values (ΔCT and Ratio) estimated using CT values were different in the same way (Table 2 and Fig. 3).

Obtained CT values showed that all treatments led to alteration of *p53* gene expression, but at different levels than the control group. This means that EtBr, whether alone or with probiotic, could make an effective change even entered the cell, particularly hepatocellular. However, all doses of EtBr strongly affected gene expression of *p*53, but the high dose of EtBr was the most dose that resulted in high gene expression of *p*53; this means that this dose must be taken into account due to its significant influence on the health of mice because the high gene expression of *p53* means that the hepatic cell tried to coop with a form of cancer. There

was a relationship between the EtBr dose and the level of *p*53 expression; this was cleared from calculated Ratios.

Since alteration of *p53* gene expression is one of genotoxicity signs (Kirsch-Volders *et al.*, 2003), any agent that can cause induction of *p53* is considered genotoxin. This means that EtBr is a genotoxic agent due to obtained results, which reflected the induction of *p53* due to treatment with EtBr. Genotoxicity of EtBr may be due to its structure; thus, it contains a cationic compound (bromide), which has a high potential genotoxicity effect due to its covalent interaction with the negatively charged nucleic acids (Snyder and Arnone, 2002).

Many studies have an agreement with EtBr genotoxicity; thus, it was proven that EtBr adversely affected the transcription process of specific genes. It could directly interact with genetic material asserting its genotoxic effect (Waring, 1965). Another study showed EtBr genotoxicity depending on the presence of chromosomal aberrations induced with EtBr or not; using mammalian cells treated with EtBr, the obtained results showed that a few hours of treatment was sufficient to negatively affect the chromosomes (McGill *et al.,* 1974). Genotoxicity induced by chemicals has been indirectly measured in human cells using a wide range of tested chemicals *in vitro*. The activity of luciferase was appreciated; this enzyme was associated with *p*53*R2* response to genotoxins. EtBr could give a positive result in this assay (Ohno *et al.,* 2005). Like what was obtained about the EtBr ability to affect the centromere more heavily than chromosomes' arms (Wu *et al.,* 2006).

In contrast, EtBr cannot significantly induce *p53* after nearly three hours of treatment. So EtBr was regarded as an intercalating agent with no genotoxic effect, represented by the inability to induce *p53* (Nelson and Kastan, 1994). Since the actual act of *p53* is curbing tumors and cancers, it was more active in swelling. Increasing *p53* gene expression in the presence of EtBr meant that this chemical has a carcinogenic effect. However, this conclusion was the opposite of what was proven of unknown carcinogenicity of EtBr (Sakai *et al.*, 2010).

EtBr can stimulate *p53* in the treatments with EtBr only; otherwise, this effect was relatively decreased in the treatments of EtBr combined with LAB. This result means that used probiotic could slightly make a reverse effect, cleared by the obtained CT values. In other words, probiotic bacteria's presence led to a relative reduction of gene expression of *p53* compared to induced form in EtBr treatments. This observation can be returned to the anticarcinogenic of lactic acid bacteria, which may resist the harmful influence of EtBr, so less need for high production of *p*53. This hypothesis is supported by the disappearance of some of the histopathological symptoms resulting from EtBr. Several studies confirmed the antigenotoxic and anticarcinogenic effect of probiotics, especially LAB, which can reduce DNA damage caused by chemical

carcinogens in rats (Pool-Zobel *et al.,* 1996). LAB can decrease the level of specific bacterial enzymes (nitroreductase, glycosidase, azoreductase, and B-glucuronidase) and transform the pre-carcinogens into high active carcinogens (Li and Li, 2003). Interestingly, the anti-genotoxicity of probiotics was proven in human cells (Burns and Rowland, 2004).

The effect of yeast was tested in mice treated with aflatoxin. It was able to reduce micro-nucleated normochromic erythrocytes, in addition to its ability to change and modify the aflatoxin compound (Madrigal-Santillán *et al.,* 2006). While *Lactobacillus* extracted from cheese was able to discourage genotoxins (Corsetti *et al.,* 2008). Another evidence of probiotic efficacy was *Lactobacilli*'s ability to reduce the genotoxic effects of 4-NQO and turn it into a less toxic compound (Verdenelli *et al.,* 2010). On the other hand, the effect of four species of bifid bacteria has been tested in mice treated with some chemical genotoxins; the result showed the superior ability of these bacteria to reduce genotoxicity of tested chemicals (Dominici *et al.,* 2011).

Not all studies showed probiotic force in responding to the health dangers, but there are failures made by those microbes in this regard, such as the study in which products of fermented milk failed to prevent colorectal cancer (CRC) ultimately, where they succeeded in tests without the other (Capurso *et al.,* 2006). It is worth noting the participation of many factors in their influence on the results of such clinical trials, such as probiotic strain, dosage, and other trial conditions (Whorwell *et al.,* 2006). So another hypothesis is introduced, in which the low level of *p53* gene expression returns to the direct effect of probiotics; thus, LAB may inhibit *p53* itself that allows tumors to be formed (as described). However, it is imperative to ensure that each experiment has particular circumstances that control the obtained results. However, there are essential criteria that must be met to achieve the desired goal of probiotics usage as probiotics can survive in the gastrointestinal tract and its inability to work as a pathogenic or toxic organism (Shalke, 2013).

CONCLUSION

In the presence of EtBr, whether alone or with probiotic bacteria, *p53* was induced, reinforcing the idea that these microbes' presence did not confront the damage leading to the induction of gene. The lower gene expression in the presence of probiotics may be back to the direct effect of LAB on *p53*. The absence of fragmented DNA in all treatments indicates that EtBr has no genotoxic effect either alone or combined with LAB, although this interpretation is questionable due to *p53* induction. In contrast, histological examination results supported the cellular toxicity of EtBr and the incomplete ability of LAB in the face of that toxicity. The weak effort of probiotic bacteria may be due to the experiment's special conditions, so more supportive measures and confirmatory studies are required.

SUMMARY

Ethidium bromide (EtBr) is a nucleic acid intercalating agent used extensively as a fluorescent dye in molecular genetics laboratories. The current study aimed to determine the potential histo- and genotoxicity of EtBr and investigate the antigenotoxic effect of probiotic bacteria (Lactic Acid bacteria; LAB) on mammalian tissue (albino mice). Mice were randomly divided into seven groups, with seven different treatments. Different EtBr doses were used individually as drinking solutions with and without probiotic bacteria, which was introduced as a single dose as a food additive. After one month of the treatments, the liver was tested using histological assay, DNA fragmentation analysis, and quantitative RT-PCR technique. No significant genotoxic effect for EtBr on the liver was observed on histological examination and DNA fragmentation analysis. However, a considerable increase in the expression of the *p53* gene and correlated with the dosages increase. However, the *p*53 expression was altered upon applying the probiotics, while some histological changes were detected, but no DNA fragmentation was detected. The seriousness of EtBr on the organism's health is conditional with the applied doses. Probiotics were not luckier than EtBr; it did not provide expected health benefits. This unexpected action of probiotics may be due to the used dose and how to use it. Further analysis of both histological and molecular aspects using multiple controls and integrative experiments is required to explain probiotics' paradox effect versus the EtBr effect on mammalian cells and tissues.

Keywords: EtBr, Probiotic bacteria, genotoxicity, q-PCR, DNA fragmentation.

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Table (1): Histological signs appeared in different treatment and their relative degrees shown as presence (+, ++) or absent (-).

Treatment / histopathological signs	Congested hepatic veins	Dilated hepatic veins	Fatty Change	Degeneration change	Inflammatory infiltration	Hemorrhage	Tumor observation
L	+	+	-	-	+	+	-
$L.D_{50}$	+	-	+	-	+	-	-
H	+	+	++	+	++	++	-
L+P	-	+	-	++	-	-	+
$L.D_{50}$+P	-	+	-	-	+	-	+
H+P	-	+	-	-	-	-	+

L: low dose of EtBr, $L.D_{50}$: the median lethal dose of EtBr, H: high dose of EtBr, L+P: low dose of EtBr combined with LAB, $L.D_{50}$+P: the median lethal dose of EtBr combined with LAB, and H+P: high dose of EtBr combined with LAB

Table (2): The Ct values of Samples amplified with Real-time PCR.

Treatment	CT value	Δct	Ratio
L+P	28.34	-1.06	0.479
H+P	27.95	-0.67	0.628
$L.D_{50}$+P	27.15	0.13	1.094
C	27.28	0	1
H	25.56	1.72	3.294
L. D_{50}	26.58	0.7	1.624
L	26.26	1.02	2.028

L: low dose of EtBr, $L.D_{50}$: the median lethal dose of EtBr, H: high dose of EtBr, L+P: low dose of EtBr combined with LAB, $L.D_{50}$+P: the median lethal dose of EtBr combined with LAB, and H+P: high dose of EtBr combined with LAB.

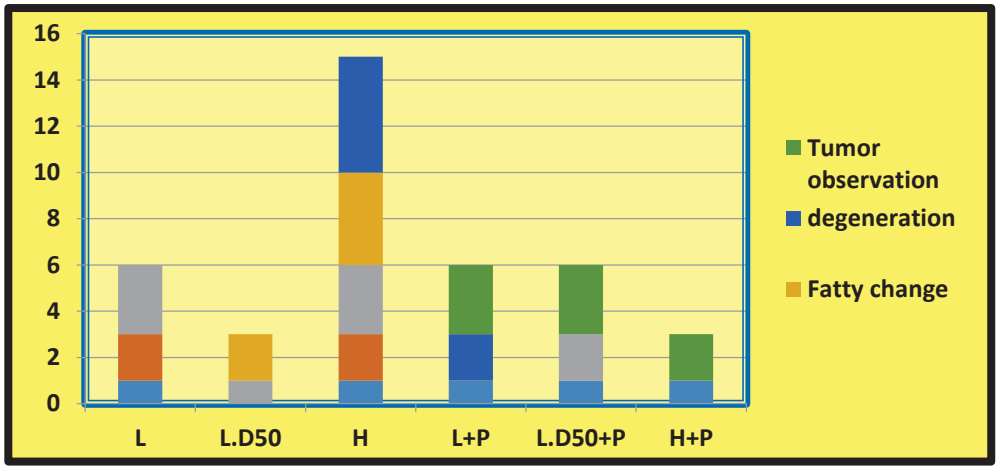
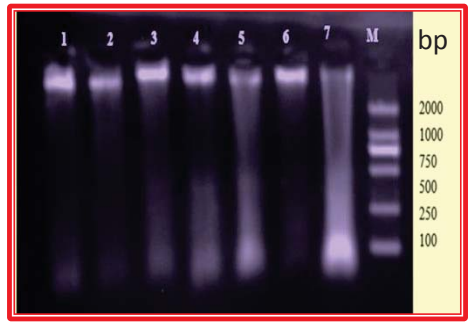


Fig. (1): Histogram showed different liver histological effects related to different doses of EtBr, whether alone (L, LD_{50}, and H) or combined with probiotics (L+P, LD_{50}+P, and H+P).

Fig. (2): Gel photo of DNA electrophoresis of samples obtained from treated and controlled mice, detecting DNA fragmentation. 1= high dose of EtBr (H), 2= median lethal dose of EtBr (LD_{50}), 3= Low dose of EtBr (L), 4= Negative control (C), 5= H+ Probiotic (H+P), 6= LD_{50}+Probotic (LD_{50}+P), 7= L+Probiotic (L+P).



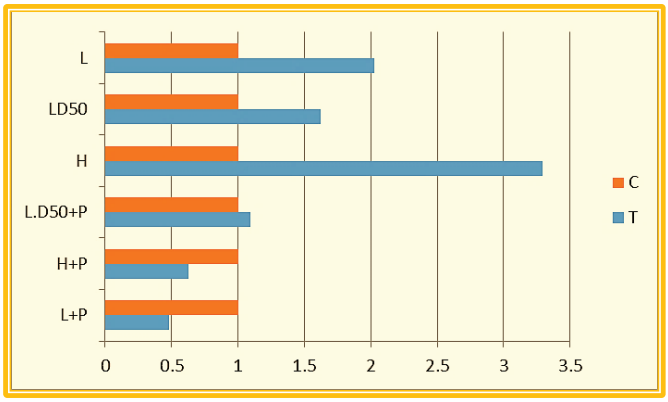


Fig. (3): Histogram showed the ratio of *p53* amplification obtained by real-time PCR.

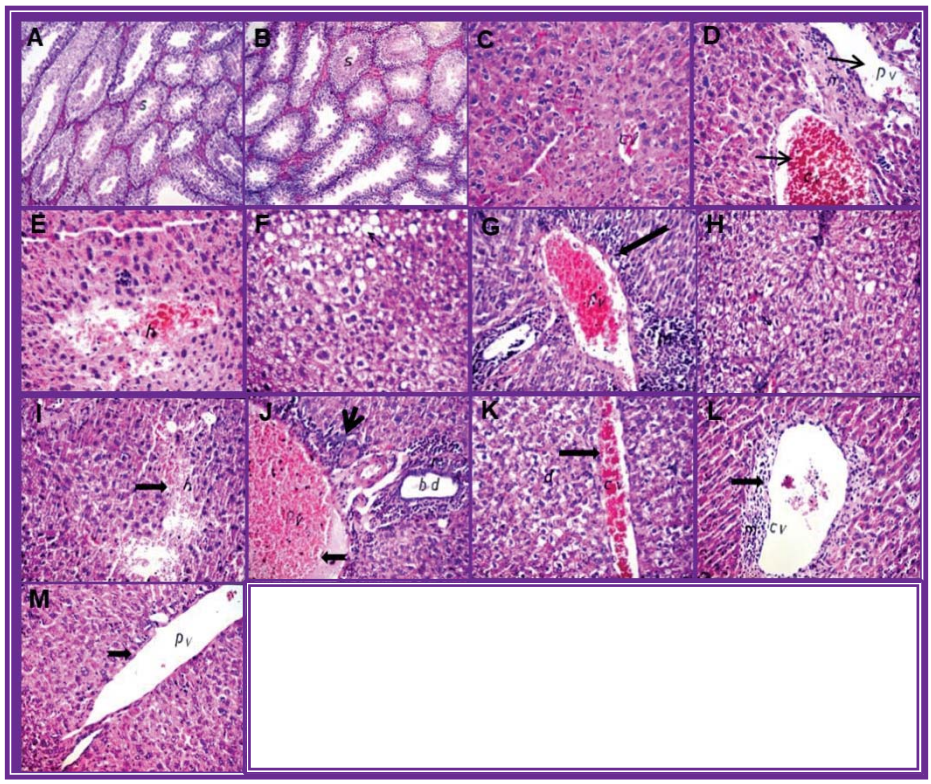


Plate (1): (A-B) Sections in tests of mice. (A) the negative control, showing the typical structure of testicles and spermatogonia (s). **(B)** a representative of the testicular tissue for all treatments, showing a typical structure of testicles and spermatogonia (s). **(C-M)** represents sections in the liver of mice (H & E X 400). **(C)** a section in the liver of negative control mice, **(D)** and **(E)** represent the low dose of EtBr. Arrows show dilation in hepatic veins, while (h) means hemorrhage. **(F)** and **(G)** median lethal dose of EtBr. In **(F)**, the arrow shows fatty change, while the arrow in **(G)** refers to the inflammatory cell's infiltration in the portal area. **(H), (I)** and **(J)** high dose of EtBr. **(H)** shows the fatty change and other degenerations in hepatocytes over the hepatic parenchyma**.** Arrow in **(I)** shows focal hemorrhage, while arrows in **(J)** show severe dilatation, congestion in the portal vein, and inflammatory cell infiltration in the portal area surrounding the dilated bile ducts. **(K)** the low dose of EtBr combined with a unique dose of probiotic bacteria, arrow shows dilated central vein. **(L)** the median lethal dose of EtBr combined with probiotic bacteria; arrow shows inflammatory cells infiltration surrounding the wall of the dilated central vein. **(M)** high dose of EtBr combined with probiotic bacteria; arrow shows dilatation in the portal vein.