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PROTECTIVE EFFECTS OF PROBIOTICS IN THE MODEL OF CADMIUM TOXICITY IN RATS

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ZAŠTITNI EFEKAT PROBIOTIKA NA MODELU TOKSIČNOSTI KADMIJUMA KOD PACOVA

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PROTECTIVE EFFECTS OF PROBIOTICS IN THE MODEL OF CADMIUM TOXICITY IN RATS

Summary

Cadmium is a ubiquitous environmental toxicant that causes a variety of disturbances in biological systems, including renal dysfunction and liver tissue degeneration. On the other hand, it is supposed that beneficial properties of probiotic bacteria are related to their capacity to adhere or bind different targets, thus leading to improved intestinal microbial balance and other benefits to host. Bearing aforementioned in mind, the present study was undertaken to investigate the protective effect of probiotic supplementation against cadmium-induced toxicity in rat.

Male Wistar rats *Rattus norvegicus*, weighing 130±10 g, were acclimated to 22±2°C in metabolic cages (3 rats/cage) and maintained under a 12h light/12h dark cycle. Animals were divided into four groups: 1) controls; 2) probiotics treated; 3) $CdCl_2$ treated; and 4) probiotics + $CdCl_2$ treated.

The cadmium concentrations were obtained in the blood, liver, kidney, and feces, as well as the blood alanine aminotransferase (ALT), aspartate aminotransferase (AST), adrenaline and noradrenaline activities. Furthermore, histomorphological changes of liver and kidney were determined, as well as the level of total supeoxide dismutase (SOD) and catalase (CAT) activities, and zinc and cooper concentration. Finally, cadmium genotoxicity were determined both *in vitro* and *in vivo* by Comet test, as well as the alterations in the fecal microflora content.

Based on our results we can conclude that:

1. Exposure of rats to cadmium chloride (70ppm) alone resulted in significant decrease in body weight gain compared with the control group. The body weight in co-treated animals was increased compared with the cadmium treated group. Overall, there were not significant changes between all of the experimental groups in both food and water intake.
2. The present results revealed that probiotics in a mixture with cadmium acted beneficially to an organism, increasing the cadmium concentration in feces, and consequently decreasing its concentration in blood and both liver and kidney. On the other hand, administration of cadmium alone caused a significant increase of zinc concentrations in both organs, kidney and liver.
3. Administration of cadmium showed alterations in histopathological changes in liver. Parenchyma of liver was evidently changed after Cd-treatment. Sinusoids were dilated, especially in the proximity of the central vein, and hepatocytes often contain nuclei with compacted chromatin, while nucleoli were not visible. Kupffer cells showed morphological characteristics of activation. At the same time, in co-treated rats the degenerative changes of liver parenchyma are extenuated but still evident.
4. It was showed in this study that administration of cadmium increased the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum of the cadmium treated animals. On the other hand, adding probiotics simultaneously with cadmium chloride restored the altered values to the normal level.
5. The histopathological changes in kidneys of rats were also evaluated. In the cadmium treated group, proximal tubules epithelium consists of swollen or necrotic cells, necrosis of the whole tubules was reported, there is no visible pas-positive brush border, nor inducing glomerular shrinkage. At the same time, in co-treated rats the

degenerative changes of kidney are extenuated. There were no differences observed between the probiotics and the control group.

6. The activities of antioxidant enzymes such as total superoxide dismutase (SOD) and catalase (CAT) were measured in. The activity of CAT decreased in both liver and kidney in the cadmium treated animals, with no changes in the co-treated group compared to the control group. On the other hand, pattern of SOD activity changes differ between liver and kidney: in liver, cadmium causes SOD activity increase, while in kidney this enzyme activity decreases. However, in the co-treated animals SOD activities back to control level.
7. Adrenaline and noradrenaline were significantly increased in animals treated with $CdCl_2$ compared to the controls, while probiotics alone did not affect the amount of these catecholamines. At the same time, levels of both adrenaline and noradrenaline decreased in the co-treated animals.
8. The treatment by cadmium chloride resulted in significant increase of DNA damage in both exposures *in vitro* and *in vivo.* On the contrary, cadmium given simultaneously with probiotics produced a significant decrease in genotoxicity which was induced by cadmium.
9. Administration of cadmium resulted in significantly reduced numbers of all different types of microflora which were accounted for in this study. At the same time, adding probiotic to Cd resulted in increased only of lactobacilli comparing to Cd treated animals.
10. Overall, present results indicate that probiotics actively contribute in cadmium removal from an organism, probably by binding to their bacterial cell wall, as proposed earlier.

11. Further researches are needed to define specific probiotic combination that would be most effective in binding Cd^{2+} and other heavy metals, as well as different toxins and carcinogens.

Keywords: rat, probiotics, cadmium, Lactobacillus, Bifidobacterium, alanine aminotransferase, aspartate aminotransferase, superokside dismutase, catalase, adrenaline, noradrenaline

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ZAŠTITNI EFEKAT PROBIOTIKA NA MODELU TOKSIČNOSTI KADMIJUMA KOD PACOVA

Rezime

Kadmijum je široko prisutni toksikant životne sredine, koji izaziva različite poremećaje u biološkim sistemima, uključujući tu i bubrežnu disfunkciju i degeneraciju tkiva jetre. Sa druge strane, pretpostavlja se da su pozitivne osobine probiotskih bakterija u vezi sa njihovom sposobnošću da vezuju različite ligande, što dovodi do poboljšanja crevne mikrobijalne ravnoteže i drugih koristi za domaćina. Imajući u vidu pomenuta, ova studija je sprovedena da istraži zaštitni efekat probiotika protiv kadmijum-indukovanog toksičnosti kod pacova.

Muški pacovi *Rattus norvegicus* Wistar soja, težine 130±10 g, gajeni su u standardnim laboratorijskim uslovima (22±2°C, režim 12h svetlo/12h mrak) u metaboličkim kavezima (3 pacova/kavezu). Životinje su podeljene u četiri grupe: 1) kontrolne jedinke; 2) životinje tretirane probioticima; 3) životinje koje su primale kadmijum u formi $CdCl_2$; i 4) životinje tretirane probioticima i kadmijumom. U krvi je određivana koncentracija kadmijuma, aktivnost alanin aminotransferaze (ALT) i aspartat aminotransferaze (AST), kao i nivoi adrenalina i noradrenalina. U homogenatima tkiva jetre i bubrega određivane su koncentracije kadmijuma, cinka i bakra, kao i aktivnosti ukupne superoksid dismutaze (SOD) i katalaze (CAT). Takođe, ispitivan je i histomorfološki profil tkiva jetre i bubrega u svim grupama životinja. U fecesu eksperimentalnih životinja određivana je koncentracija kadmijuma i mikrobijalni profil odabranih vrsta bakterija crevne mikroflore. Na kraju, Comet testom je testirana genotoksičnost kadmijuma *in vitro* and *in vivo*.

Na osnovu naših rezultata mogu se izvesti sledeći zaključci:

1. Davanje kadmijuma eksperimentalnim životinjama u formi kadmijum hlorida i koncentraciji 70ppm u pijaćoj vodi izaziva značajno smanjenje u porastu telesne mase tokom 5 nedelja eksperimenta. Istovremeno, između eksperimentalnih grupa nema značajnih promena u količini popijene vode i pojedene hrane.
2. Istovremeno davanje probiotika zajedno sa kadmijumom dovodi do povećanja koncentracije kadmijuma u fecesu i pratećeg smanjenja njegove koncentracije u krvi, jetri i bubregu. Istovremeno, administracija kadmijuma dovodi do značajnog povećanja koncentracije cinka u jetri i bubregu.
3. Kadmijum izaziva značajne histopatološke promene tkiva jetre. Parenhim tkiva jetre je izmenjen, sinusoidi prošireni, naročito u proksimalnom delu centralne vene, a hepatocite često sadrđe nukleuse sa kondenzovanim hromatinom, bez prisustva nukleolusa. Kupferove ćelije pokazuju morfološke znake aktivacije. Davanje probiotika zajedno sa kadmijumom smanjuje intezitet nabrojanih promena, iako one ostaju evidentne.
4. Kadmijum povećava aktivnost ALT i AST u krvi eksperimentalnih životinja, što dodatno ukazuje na oštećenja jetre. Međutim, davanje probiotika zajedno sa kadmijumom vraća aktivnost oba enzima na kontrolni nivo.
5. Kadmijum izaziva značajne histopatološke promene i tkiva bubrega. U epitelu proksimalnih tubula uočavaju se nekrotične i nabubrele ćelije. Davanje probiotika zajedno sa kadmijumom smanjuje intezitet nabrojanih promena.
6. Pod delovanjem kadmijuma aktivnost katalaze je smanjena u oba tkiva, i jetri i bubregu Sa druge strane, obrazac promena aktivnosti SOD pokazuje tkivnu specifičnost: dok je pod delovanjem kadmijuma aktivnost ovog enzima u jetri

povećana, u bubregu je ona smanjena. Davanje probiotika zajedno sa kadmijumom dovodi do restitucije aktivnost ovih enzima blizu kontrolnog nivoa

7. Kadmijum značajno povećava koncentraciju adrenalina i noradrenalina u krvi ispitivanih životinja. Istovremeno, sami probiotici ne menjaju koncentraciju ni jednog niti drugog kateholamina. Davanje probiotika zajedno sa kadmijumom smanjuje kadmijumom izazvano povećanje oba kateholamina.
8. U kulturi hepatocita kontrolnih životinja (*in vitro*) i hepatocitima životinja tretiranih kadmijumom (*in vivo*) ovaj metal izaziva značajan porast oštećenja DNK. Davanje probiotika zajedno sa kadmijumom značajno smanjuje ovaj efekat kadmijuma.
9. Kadmijum smanjuje koncentraciju svih ispitivanih bakterija crevne mikroflore. Davanje probiotika zajedno sa kadmijumom značajno smanjuje ovaj efekat samo u slučaju laktobacila, povećavajući njihovu, kadmijumom smanjenu koncentraciju u fecesu.
10. Na osnovu svega može se zaključiti da probiotici aktivno učestvuju u uklanjanju kadmijuma iz organizma, verovatno ga vezujući proteinima ćelijskog zida.
11. Neophodna su dalja istraživanja u cilju određivanja specifične kombinacije probiotika koja bi bila najefikasnija u uklanjanju kadmijuma, ali i drugih teških metala i karcinogena.

Ključne reči: pacov, probiotici, kadmijum, laktobacili, bifidobakterije, alanin aminotransferaza, aspartat aminotransferaza, superoksid dismutaza, katalaza, adrenalin, noradrenalin

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1. Cadmium

1.1. General introduction

The word cadmium derives from the Latin *cadmia* (now known as "calamine") and the Greek *kadmeia* (Greenwood and Earnshaw, 1989). Cadmium is a chemical element, atomic number 48, and atomic weight 112.4, a soft, malleable, ductile and bluish-white metal. It has been identified as a distinct element in 1817 in Germany. It occurs in nature in association with zinc and lead (0.16 mg kg^{-1} in the earth's crust). It has been found in soil and rocks, including coal and mineral fertilizers, which contain cadmium at variable concentration levels (Greenwood and Earnshaw, 1989).

Cadmium is a potent environmental toxin and is found naturally in very limited quantities. Classified by the International Programme on Chemical Safety (IPCS) as a category 3 mutagen/carcinogen and a severe hazard by the American Department of Health, cadmium is a highly toxic element.

Cadmium enters the atmosphere via several ways (Hutton, 1983, JarupL., 2002, Jarup, 2003). Through natural activities, such as volcanic activity (both on land and in the deep sea), weathering and erosion, and river transport (World Health Organization 2010). As an alloy, in electroplating of other metals and as a pigment which causes contamination of air, water and land. The extensive use in the manufacture of alkaline batteries and plastics (Registry, 2008), and the major source of cadmium present in the rural regions is the result of human activities such as phosphate fertilizing, especially smelting of non-ferrous metal ores, fossil fuel combustion and municipal waste incineration (World Health Organization 2010).

The metal is well known to enter the food chain and can undergo bioaccumulation in the different animals and human tissues (Roccheri *et al.*, 2004). The usual sources of cadmium

for the general population are predominately smoking, inhalation of polluted air and consumption of contaminated seafood and water (Jarup *et al.*, 2000).

In humans and other mammals, Cadmium expressed toxicity on several systems, organs and tissues, such as respiratory, digestive, reproductive, skeletal system and some sensitive organs including liver and kidney - the two primary target organs (Zalups and Ahmad, 2003). Cd accumulates primarily in the kidney and is eliminated very slowly with a half-life of 15-20 years (Jarup, 2003). The chronic exposure to low Cd doses predominantly leads to renal proximal tubular metabolic acidosis and osteomalacia. With the rise of world-wide industrialization in combination with a longer life expectancy today, the level of environmental Cd has risen and Cd-induced human disease is of increasing public health concern.

1.2. Transportation of cadmium into intracellular space and distribution

Absorption of cadmium by circulation system depends on the form of the patterns and particle size of cadmium .For instance, cadmium chloride is directly absorbed to the systemic circulation, because of its high degree of solubility in water (Registry, 2008).

The normal absorption of cadmium via the intestinal tract is relatively little, ranging from 0.5 to 12 %in various species of animals, with 4.7-7% in humans (Rahola *et al.*, 1972).

Liver and kidney have a strong affinity for cadmium comparing with other organs in the body (Satarug *et al.*, 2003). It was found that approximately 50% of the total body cadmium is located in these two tissues.

Because of fact that is a divalent cation, cadmium crosses the cell membrane via calcium channels, especially in the absence of external calcium. Furthermore, cadmium has ability to penetrate into cells by utilising the zinc transports. Additionally cadmium transport into cells might be connected with several organic compounds, for example cysteine and glutathione (Bridges and Zalups, 2005).

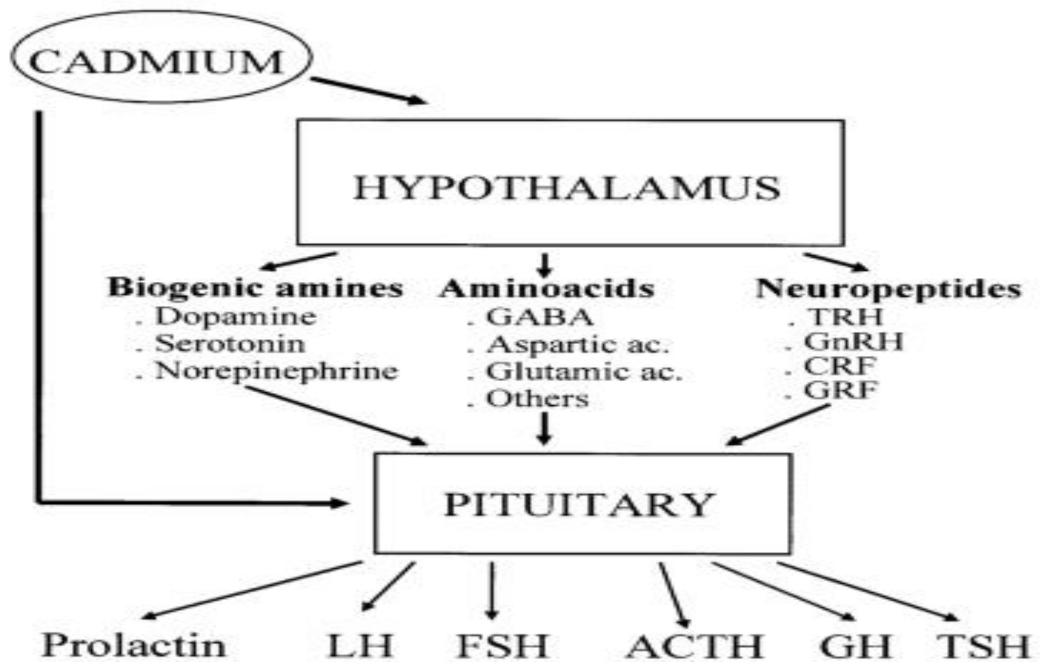
Administration of cadmium induces activity of metallothionein proteins. Mammalian metallothioneins contains a single polypeptide chain of sixty one amino acid residues, among them twenty cysteines providing the ligands for seven metal-binding sites. Native metallothioneins are usually heterogeneous in metal composition, with Zn, Cd, and Cu occurring in varying proportions (Nordberg, 1978).

The main function of metallothionein is the protection of the cells against the zinc toxicity; it may be possible also to give protection against cadmium because of the similarities in the chemical properties of zinc and cadmium. Inside the cell, cadmium has ability to substitute zinc bound to metallothionein. The exchange and competition between these metals for binding to metallothionein is usually common (Klaassen *et al.*, 1999).

1.3. Biological effects

Cadmium compounds have been shown to induce numerous cases of poisoning. Several of these have been cases of inhalation of very high concentrations. On the other hand, most other recorded cases have resulted in consumption of contaminated foods and drinking water and beverage (Registry, 2008). The toxicity of cadmium vary considerably depending on the chemical form, particle size, period of time and route of exposure. Cadmium (Cd) is a toxic heavy metal which can be accumulated in human body and environment long-term. The health risks of environmental Cd pollution arise all over the world since the “itai-itai” disease, caused by chronic Cd poisoning, appeared in Japan in 1950s.

It was documented that Cd can cause bone demineralization, either through direct bone damage or indirectly as a result of renal dysfunction (Staessen *et al.*,1999).



Picture 1. Cadmium can affect the hypothalamic–pituitary axis at the hypothalamic and/or pituitary level (Lafuente and Esquifino, 1999)

In addition to that, in the last few decades there have been a lot of evidence of the effects of cadmium on the endocrine system. As such, Cadmium might play a role in the aetiology of gonadal dysfunction (Clarkson *et al.*, 1985 , Li and Heindel, 1998). Furthermore, it has been reported that cadmium can affect the activity of the hypothalamic–pituitary–testicular axis by acting on the hypothalamus (Das *et al.*, 1993, Andersson *et al.*, 1997., Gutierrez Reyes and Albores, 1998), the pituitary (Lafuente and Esquifino, 1999), and testes (Laskey and Phelps, 1991).

Cadmium creates its toxic effects via oxidative damage to cellular organelles by inducing the generation of reactive oxygen species (ROS), consisting mainly of O_2, H_2O_2 and OH (Stohs *et al.*, 2000). The molecular mechanism that may be responsible for the cadmium induced oxidative stress are far from being understood, but reports have indicated that cadmium does this via an indirect phenomenon (Watkin *et al.*, 2003). Reactions of these ROS with cellular biomolecules have been shown to lead to lipid peroxidation, membrane protein

damage, altered antioxidant system, DNA damage, altered gene expression and apoptosis (Thijssen *et al.*, 2007, Stohs *et al.*, 2000). In addition to that, depletion of glutathione and other endogenous antioxidants may also contribute significantly to the development of cadmium-induced toxic oxidative stress (Bagchi *et al.*, 1996). If these ROS-mediated stress events are not balanced by repair processes, affected cells undergo apoptosis or necrosis (Thevenod, 2003).

1.4. Effect of cadmium on liver and kidney

Cadmium at birth in mammals in fact is absent but accumulates with time especially in the liver and kidney, so that up to 75% of the total body burden is found in these organs (Friberg L *et al.*, 1985., Bellinger D *et al.*, 2004). Renugadevi and Prabu, (2010) reported that cadmium causes liver damage and an increase in activities of serum hepatic marker enzymes such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT) and serum total bilirubin (TB). Increase in levels of aspartate transaminase and alanine transaminase is a crucial parameter to detect liver damage (Williamson *et al.*, 1996). Cadmium hepatotoxicity is probably affected in two ways: on one hand, by the occurrence of inflammatory state, and on the other hand by direct toxic action of cadmium on liver cells. In addition, chronic exposure to cadmium significantly decreases levels of antioxidant enzymatic (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S transferase (GST)), as well as of the non-enzymatic antioxidants (reduced glutathione (GSH)) (Renugadevi and Prabu, 2010). Furthermore, it has been reported that administration of cadmium via different routes can induce severe nephropathy in humans (Geyer, 1995), and animals (Brzoska *et al.*, 2003), or creatinine increase in plasma and urea increase in the rat serum (Al-Hashem *et al.*, 2009).

Several mechanisms have been reported to explain the toxic effect of cadmium on renal cells. Cadmium may cause nephrotoxicity by generating free radicals and/or by inducing necrosis and apoptosis (Dally and Harwing, 1997).

2. Probiotics

2.1. General Introduction

Probiotics are microorganisms introduced orally in the gastrointestinal tract that are able to contribute positively to the activity of intestinal microflora, and therefore to the health of its host. Most probiotic bacteria belong to the group of lactic acid bacteria (LAB). Among them, *Lactobacilli* and *Bifidobacteria* reportedly play a significant role in maintaining the intestinal ecosystem and stimulating the immune system of the host (Saarela *et al.*, 2002). The typical number of endogenous faecal lactobacilli per gram of feces ranged from 10^5 to 10^8 cfu (Kimura *et al.*, 1997, Holzapfel *et al.*, 1998).

Several *in vitro* properties of probiotics, like adhesion, resistance to pH, etc., are usually investigated to determine if a specific selected strain is suitable as a probiotic (Collins *et al.*, 1998).

It has been revealed that the functional and technical properties of probiotics are strain-specific (Collins *et al.*, 1998, Laiho *et al.*, 2002). These include general characteristics (e.g., origin, identity, resistance to mutations, resistance to environmental stress prevailing in the GIT such as low pH, bile acid and pancreatic juice), technical considerations (e.g., *in vitro* growth and processing properties), functional and beneficial properties such as adhesion and colonization to intestinal mucosa, competitiveness, antagonism against pathogens, and stimulation to immune response, and safety concerns such as non-invasive potential, non-transferable resistance against therapeutic antibiotics and non-virulence factors (Holzapfel and Schillinge, 2002, Holzapfel, 2005)

The exposure of probiotics to several stresses starts in the stomach, where pH values as low as 1.5 are naturally struggled (Lankaputhra and Shah, 1995). After passage through the stomach, probiotics expose to bile salts in the upper intestinal tract (Northfield and McColl, 1973). Following these relatively cruel conditions, the probiotics have to adhere with epithelia and grow in the lower intestinal tract (Conway *et al.*, 1987), as a result they are able to exert health benefits to the host for a long period.

The utilization of probiotics for the therapeutic treatment of many cases of digestive problems and infectious diseases (e.g., rotavirus diarrhea, influenza virus and *Helicobacter pylori)* has increased over the last few years (Goktepe *et al.*, 2006). It was reported that Probiotic strains such as *Lactobacillus rhamnosus* strain GG (ATCC 53103) and *L. rhamnosus* strain LC-705 (DSM 7061) can remove aflatoxin B from aqueous solution (Haskard *et al.*, 2001), as well as mutagen pollutants of food-stuff (Turbic *et al.*, 2002). Also, *Bifidobacterium longum* 46, *L. fermentum* ME3 and *B. lactis* Bb12 have the capacity to bind cadmium and lead from the water (Halttunen *et al.*, 2007). The almost common probiotic genera like *Lactobacilli* and *Bifidobacteria* play a vital role in maintaining the intestinal microflora and stimulating the immune system of its host (Saarela *et al.*, 2002.). On the other hand, the sufficient number of probiotics which are believed to produce a healthy balance between beneficial and potentially harmful microflora in the gastrointestinal tract should be around 10^7 cfu/g or mL. (Suskovic *et al.*, 2001).

2.2. Physiology of function food

Food is no longer considered by consumers only in terms of taste and immediate nutritional needs, but also in terms of its ability to provide specific health benefits beyond its basic nutritional value. Currently, the largest segment of the functional food market is provided by the food targeted towards improving the balance and activity of the intestinal microflora (Saarela *et al.*, 2002).

The concept that food could act as medicine was first regarded thousands of years ago by the Greek philosopher and father of medicine, Hippocrates, who once wrote: 'Let food be thy medicine, and let medicine be thy food'. However, nowadays, the concept of food having medicinal value has been reborn as 'functional foods'. A probiotic may also be a functional food (Chow, 2002). Functional foods are defined as: 'foods that contain some health-promoting component(s) beyond traditional nutrients'. Functional foods are also known as designer foods, medicinal foods, nutraceuticals, therapeutic foods, superfoods, foodiceuticals, and medifoods. In general, the term refers to food that has been modified in some way to become 'functional'. One way in which foods can be modified to become functional is by the addition of probiotics (Lindner *et al.*, 2010).

Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria in the intestinal tract. Such bacteria are called ‘Probiotics’ and have been predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, both of which have been extensively studied and established as valuable native inhabitants of the GIT (Salminen *et al.*, 1998a., Fuller, 1989, Capela *et al.*, 2006).Various microorganisms, particularly species of *Lactobacillus* and *Streptococcus*, have traditionally been used in fermented dairy products to promote human health as well as food functionality and flavour.

2.3. Historical perspective of probiotics

Escherich was the first to describe the microorganisms in gastrointestinal tract of an infant and suggested usefulness of their colonization, indigestion and consequently understanding the pathology and microbial therapy of intestinal diseases (Azizpourk *et al.*, 2009). At the same time, Doderlein postulated the beneficial association of vaginal bacteria in inhibiting the growth of pathogenic bacteria by producing lactic acid (Goktepe *et al.*, 2006).

Moro in 1900 and Beijerinck in 1901 reported that there is beneficial association of LAB within human hosts (Goktepe *et al.*, 2006).

The habit of consuming fermented milk has a long history going back hundreds of years. At the beginning of the 20th century Metchnikoff, the Nobel Prize winner in Medicine in 1908, at the Pasteur Institute first introduced the probiotic concept linked the health and longevity, by observing the long life of Bulgarian peasants who consumed fermented milk (Azizpourk *et al.*, 2009). He proposed that ingestion of lactobacilli might suppress the putrefactive effects of gastrointestinal metabolism and reversed the effects of the proteolytic organisms which caused autointoxication (Fuller, 1992, Lindner *et al.*, 2010). In addition, he suggested that the large intestine, useful to mammals in managing rough food composed of bulky vegetables, is useless in humans, and is the site of dangerous putrefactive processes which can be opposed by introducing lactobacilli into the body (Lindner *et al.*, 2010). *Bifidobacteria* was observed by Tissier in 1906 in the breast-fed infants and their vital role in maintaining health. Also he documented clinical benefits from modulating the microflora in infants with intestinal infections (Tissier, 1906). At the same time, there are a lot of researchers who were suspicious about the benefits of bacterial therapy and questioned in particular whether the yoghurt bacteria (*L. bulgaricus*) were able to survive intestinal transit, colonize and confer their benefits to the host.

After Metchnikoff's death in 1916, the center of research activities moved from Europe to North America. The workers in the USA started the use of *L. delbrueckii* subsp. *bulgaricus*. They reasoned that since the effect was being manifested in the gastrointestinal tract, it would be better to use an organism which originated from that site. At that time *L. acidophilus* was the lactic acid bacterium which was most commonly isolated from the gastrointestinal tract. When this was used in human feeding trials it gave encouraging results in treatment of

constipation. It was reported in the early 1920s, *L. acidophilus* milk have therapeutic effects on the metabolism digestion.

The concept of the health benefits of the microorganisms was spread. It was suggested that colonization and growth of these microorganisms in the digestive tract were essential for their efficacy, and therefore, the use of intestinal isolates was supported by many researches. In Japan in the early 1930s, Shirota focused his research on selecting the strains of intestinal bacteria that could survive passage through the gastrointestinal tract and on the use of such strains to develop fermented milk for distribution in his clinic. His first product containing *L. acidophilus* Shirota (subsequently named *L. casei* Shirota) was the basis for the establishment of the Yakult Honsha Company (Azizpourk *et al.*, 2009).

Unfortunately, the research in this area in the period between late 1930s and late 1950s was suppressed due to extraordinary conditions the world was facing at that time (depression, war) (Azizpourk *et al.*, 2009). At the end of the last century, it became clear that probiotic bacteria had many health benefits on the host including improved metabolic function, development and homeostasis of the immune system and improvement the ability of body to encounter pathogens (Del Piano *et al.*, 2006). Nowadays, it became obvious that there were many species of lactic acid bacteria other than *L. acidophilus* present in the gastrointestinal tract. Therefore, a lot of different species of the genera *Lactobacillus, Streptococcus* and *Bifidobacterium* were incorporated into probiotic preparations (Azizpourk *et al.*, 2009).

2.4. Definition of probiotics

The word probiotics comes from the Greek meaning “for life”. Probiotics were first defined by Kollath in 1953 to denote all organic and inorganic food complexes in contrast to harmful antibiotics. Lilly and Stillwell (1965) defined probiotics as “microorganisms promoting the growth of other microorganisms”. Although numerous definitions have been

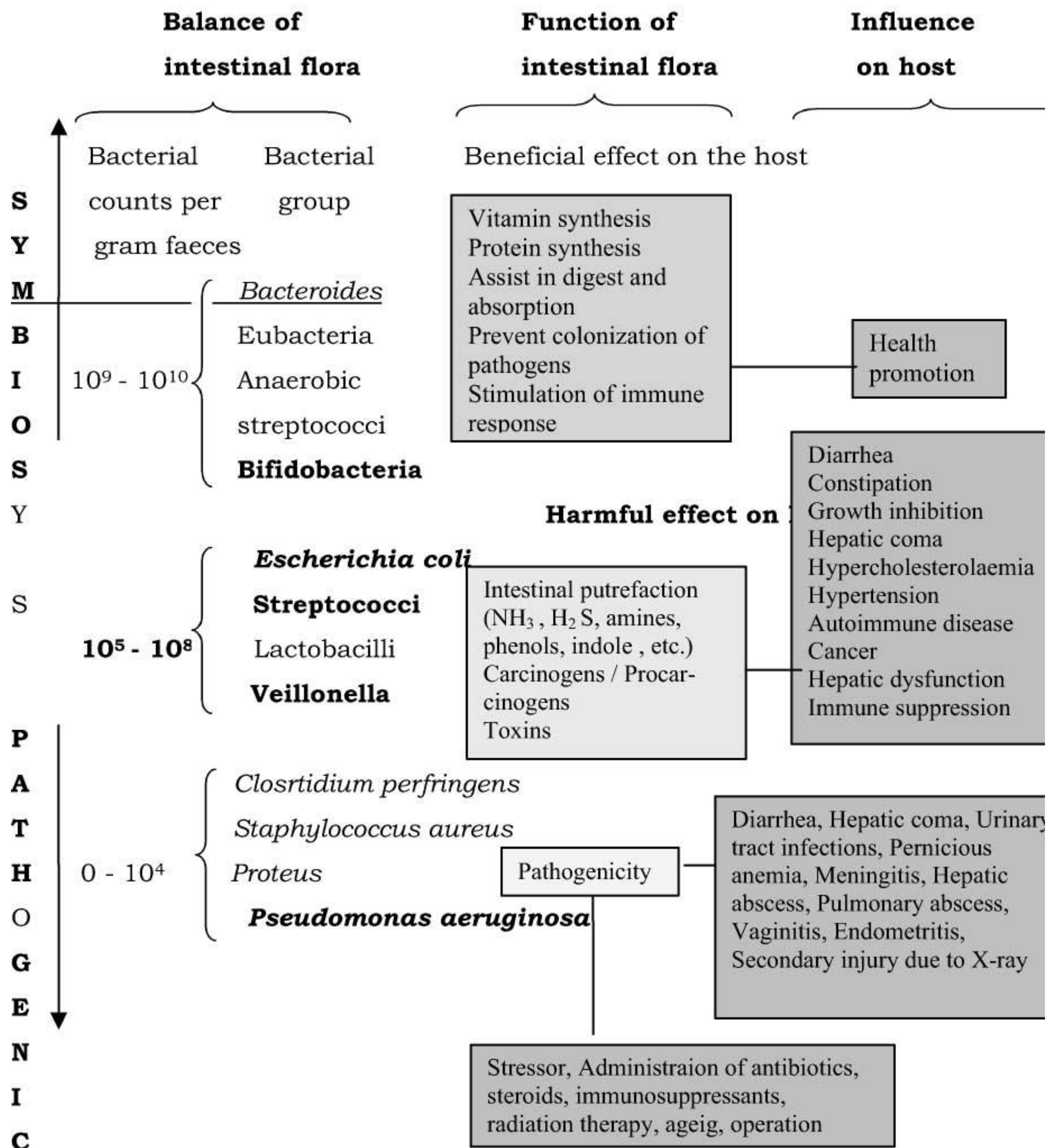
proposed since then, most have failed to be completely satisfactory because they lack statements such as “stabilization of the gut flora” (Goktepe *et al.*, 2006).

Havenaar and Veld (1992), have defined probiotics as “mono- or mixed cultures of live microorganisms which, when applied to animals or humans, beneficially affect the host by improving the properties of the indigenous microflora”. When these probiotic bacteria are present in yogurt and other fermented foods, they may beneficially alter the normal gut flora (Metchnikoff 1907). Probiotics were defined by Salminen *et al.* (1998b) as the 'food which contains live bacteria beneficial to health', whereas Marteau *et al.* (*2001*) defined them as 'microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being'. Probiotics have also been defined by the European Union (EU) Expert Group on Functional Foods in Europe (FUFOSE) to be “viable preparations in foods or dietary supplements to improve the health of humans and animals” (FUFOSE working group, 1999). More recently, probiotics have been referred to as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”.

2.5. The gastrointestinal ecosystem

The gastrointestinal tract contains complex societies of indigenous microbes with a various and concentrated microbial population that contribute in numerous interactions with the chemical environment (such as digestion), create some vitamins, adhesion and colonization in the gastrointestinal tract. The mucosal surface area increases by circular folding which contributes to about a 3-fold increase, through the production of villi, for a 7- to 10-fold increase, and by the formation of intestinal microvilli, which results in a 15- to 40-fold increase (Hooper *et al.*, 2001, Holzapfel *et al.*, 1998). Varying numbers of bacteria are found throughout the GIT, ranging from 10^1-10^3 cfu/mL or g in the stomach contents; 10 cfu/g in the terminal ileum and approximately 5 × 1011 cfu/g in the distal colon contents (Goktepe *et al.*, 2006). In

1987, Mitsuoka proposed a hypothetical scheme in which he illustrates the interrelationship between intestinal bacteria and human health (Fig. 2)



Picture 2. The interrelationship between intestinal bacteria and human health

The gastrointestinal tract is an obvious target for the development of functional foods, acting as the interface between a diet and the metabolic events which sustain life (Salminen *et al.*, 1998a). The intestinal microflora serves a vital role in several metabolic activities,

including complex carbohydrate digestion, lipid metabolism, and glucose homeostasis (Backhed F *et al.*, 2005). The intestinal epithelium plays a vital protective function, preventing passage of different detrimental substances and the gut plays an important role in several clinical infections (Wilmore *et al.*, 1988).

Major functions of the gut microflora include metabolic activities that result in salvage of energy and absorbable nutrients, important trophic effects on intestinal epithelia and on immune structure and function, and protection of the host against colonization by alien microbes. Gut flora might also be an essential factor in certain pathological disorders, including multisystem organ failure, colon cancer, and inflammatory bowel diseases. Nevertheless, bacteria are also useful in promotion of human health. Probiotics and prebiotics are known to have a role in prevention or treatment of some diseases (Guarner and Malagelada, 2003). Most bacterial infections in critically ill or immunecompromised patients are caused by the patients' own microflora, and many patients dying of sepsis or multiple system organ failure have enteric bacteremia for which no septic focus can be identified. The bacteria detected in feces reflect the bacteria present in the distal colon, thus studies of the human gastrointestinal tract microbiota usually involve analysis of faecal samples (Moore *et al.*, 1978).

The human gastrointestinal tract is a diverse and complex ecosystem containing on a huge number of bacteria: about 400 different species live and grow in men gut as symbionts (Simon and Gorbach, 1984). Several of these bacteria are potential pathogens and convert to infection and sepsis to the host, for example when the integrity of the bowel barrier is physically or functionally destroyed under some circumstances (Guarner and Malagelada, 2003). The lumen of the human stomach is essentially sterile due to a low gastric pH. However, micro-organisms are known to reside in the mucosal layer that overlies the gastric epithelium. This includes *Helicobacter pylori*, which has attracted a great deal of research interest. This organism uses its flagellae to invade the gastric mucus layer and thereafter adhere to epithelial

cells. In conjunction with a production of ammonia, this allows effective colonization of the stomach. The stomach, duodenum (0-1 0^4 bacteria/g of the luminal contents) and small intestine (1 0^5- 10^6 bacteria/g) contain smaller number of bacteria adhering to the epithelia and some other bacteria in transit.

Table 1. Common resident gut microflora

Table. Common resident gut microflora

Part of GIT	Some common resident bacteria
Mouth and oropharynx	*Streptococcus viridians, Streptococcus pneumoniae*, Beta-haemolytic streptococci, coagulase-negative Staphylococci, *Veillonella spp., Fusobacterium* spp., *Treponema* spp., *Porphyromonas* spp., *Prevotella* spp., *Neisseria* spp. and *Branhamella catarrhalis, Candida* spp., *Haemophilus* spp., *Diphtheroids, Actinomyces* spp., *Staphylococcus aureus, Eikenella corrodens*
Stomach	*Streptococcus, Staphylococcus, Lactobacillus, Peptostreptococcus*
Small intestines	*Lactobacillus* spp., *Bacteroides* spp., *Clostridium* spp., *Mycobacterium* spp., Enterococci, bacteria of Enterobacteriaceae,
Large intestines	*Bacteroides* spp., *Fusobacterium* spp., *Clostridium* spp., *Peptostreptococcus* spp., *Escherichia coli, Klebsiella* spp., *Proteus* spp., *Lactobacillus* spp., Enterococci, *Streptococcus* spp., *Pseudomonas* spp., *Acinetobacter* spp., coagulase-negative Staphylococci *Staphylococcus aureus, Mycobacterium* spp., *Actinomyces* spp., *Bifidobacterium bifidum, Enterobacter* spp., *Peptococcus* spp., *Methanogens* (Archaea), *Salmonella* spp.

GIT - Gastro intestinal tract

The microflora is mainly comprised of facultative anaerobes and obligate anaerobes. Approximately 95% of the intestinal bacterial inhabitants in humans is comprised of obligate anaerobes, including *Bifidobacterium, Clostridium, Eubacterium, Fusobacterium, Peptococcus*, *Peptostreptococcus* and *Bacteroides*. In contrast, around 1% to 10% of the intestinal inhabitants is comprised of facultative anaerobes, including *Lactobacillus, Escherichia coli, Klebsiella, Streptococcus, Staphylococcus* and *Bacillus.* Aerobic organisms are not present in the intestinal tract of healthy individuals with the exclusion of *Pseudomonas*, which is present in a small very amount. Most of the bacteria are present in the colon where the luminal contents range between 10^{11} to 10^{12} bacteria/g[3]. A constant interaction between the host and the microbes provide important health benefits to the human host (Salminen *et al.*,

1998a). Some common resident bacteria found at different locations in the GIT are listed in the Table (1).

It occurs within a few days after birth when the different species of microbiota start growth and colonization in the gastrointestinal tract of new born infants and this procedure continues during the whole life. The ecological factor has a main role in determining the extent and type of colonization, for instance, differences exist between people living in developed countries and those in developing countries (Salminen *et al.*, 1998a, Adlerberth *et al.*, 1991). The type of delivery (passage through the birth canal versus caesarean section) and the type of food (breast versus formula feeding) might also affect the colonization pattern in the first week after delivery (Long and Swenson, 1977). Some bacteria can modulate expression of genes in host epithelial cells (Hooper *et al.*, 2001), thus creating a favourable habitat for themselves by preventing growth of other bacteria introduced later. The initial colonization is therefore very relevant to the final composition of the permanent flora in adults.

It has been shown that anaerobic bacteria outnumber aerobic bacteria by a factor of 100–1000 (Simon and Gorbach, 1984). The predominant genera in human beings are *Bactero ides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus,* and *Ruminococcus*, followed by aerobes (facultative anaerobes) such as *Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus,* and *Proteus* (Simon and Gorbach, 1984, Salminen *et al.*, 1998a). Every individual has several hundreds of species, with a particular combination that is distinct from that found in other individuals (Simon and Gorbach, 1984, Moore and Moore, 1995).

2.6. Probiotics strains of human origin

In the last few decades there was an increased number of researches on gut microbial ecology, although, only a small number of about 400 species of different genera have been

cultivated and studied with regard to their physiology, metabolic interactions, and taxonomy (Goktepe *et al.*, 2006).

Table (1) presents the LAB found likely to be associated with the human host (Goktepe *et al.*, 2006). The large intestine is heavily colonized by *Bacteriodes* and the Gram-positive, anaerobic genera *Eubacterium* and *Bifidobacterium,* while lactobacilli are the main species in the vagina and are also naturally present in the oral cavity (10^4-10^8cfu/g), the ileum (10^{3}-10^7 cfu/g), and colon (10^{5}-10^8 cfu/g), where they play an important role in maintenance of a stable gut mucosa (Lidbeck and Nord, 1993).

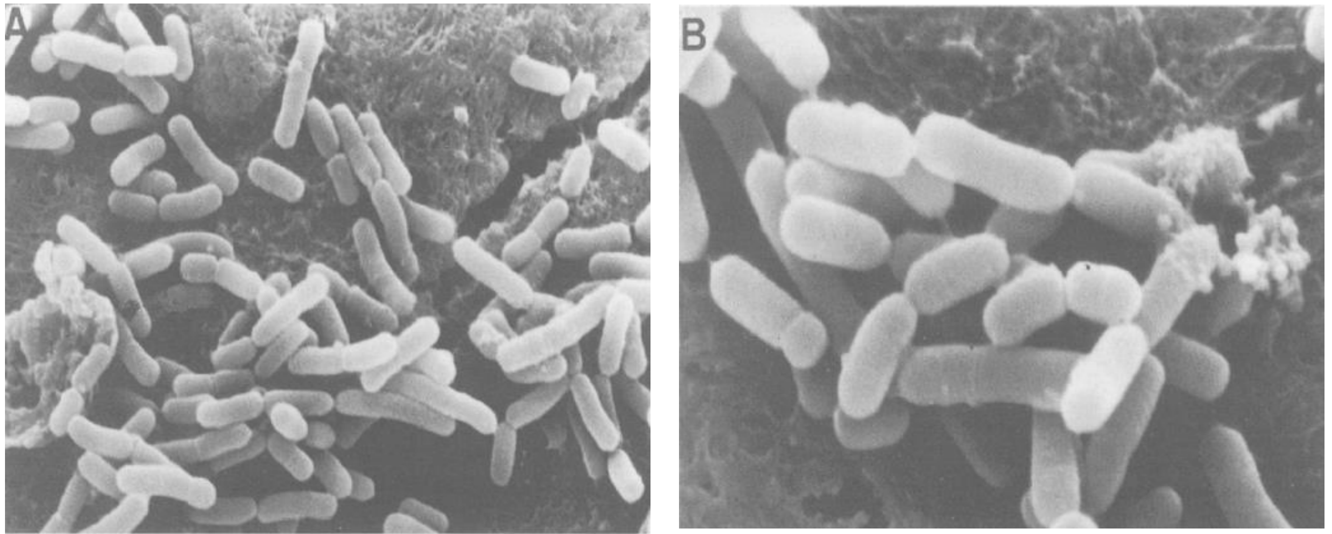
It was demonstrated that bifidobacteria and lactobacilli are common bacterial species in the faecal of breast-fed infants (Long and Swenson, 1977). There is a lack of research evidence that has demonstrated a single dominant species in the human GIT. However, *L. acidophilus* has been recovered in relatively high numbers from the GIT. Strains of *acidophilus* have been isolated from the intestinal tract of humans, as well as animals such as rodents and birds.

2.6-1. Lactobacilli

Bacillus acidophilus were first isolated and described in 1990 by Moro. It was reported that 56 species of the genus *Lactobacillus* have been recognized (Lindner *et al.*, 2010).

Lactobacilli are in general characterized as gram-positive, non-spore forming rods and non-flagellated rods or coccobacilli, usually non-motile, that are catalase negative, and do not reduce nitrate (Bernet *et al.*, 1994, Fooks and Gibson, 2002, Lindner *et al.*, 2010). They are fastidious, acid-tolerant, and strictly fermentative (either homo- or hetero): lactic acid is the major end product of sugar fermentation (Axelsson, 1998). They are generally accepted as safe to ingest, and have been, together with *Bifidobacterium,* granted with GRAS status (Salminen

et al., 1998) in production of fermented dairy products and supplements to food and pharmaceutical prepares (Goktepe *et al.*, 2006, O'Sullivan *et al.*, 1992).



Picture 3. Scanning electron microscopy of Lactobacillus acidophilus strain (Bernet *et al.*, 1994).

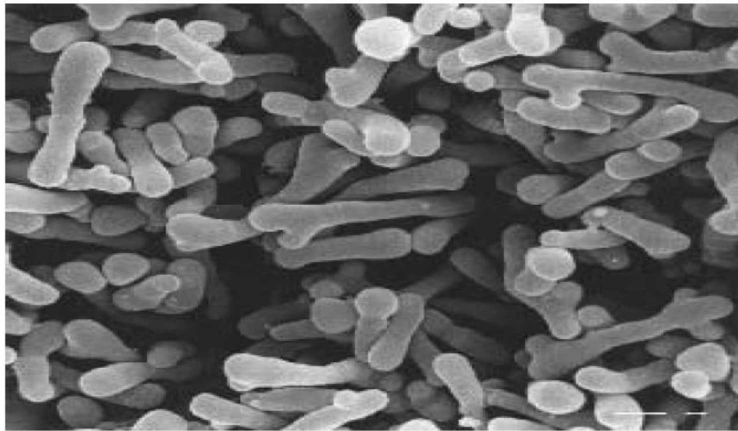
By producing lactic acid and bacteriocins which decrease grow of numerous microorganisms, *Lactobacilli* are capable to regulate gut micro flora (Marteau *et al.*, 2004). *Lactobacilli* are found in a variety parts throughout the gut and genital tract and present an important part of the indigenous microbiota in humans and higher mammals (Salminen *et al.*, 1996).

2.6-2. Bifidobacterium

In 1899, Tissier was the first researcher who isolated, described and typified *Bacillus bifidus*, describing it as rod-shape, non-gas producing, anaerobic microorganisms. *Bifidobacterium* is in general characterized as gram positive, non-spore forming, non-motile and catalase negative anaerobe (Lindner *et al.*, 2010, Gomes and Malcata, 1999).

Bifidobacterium is distributed in various ecological niches throughout the gastrointestinal and genital tracts and constitutes an important part of the indigenous microflora of men and higher animals. Gomes and Malcata (*1999*) reported that thirty species of the genus

Lactobacillus have been recognized, ten of which are from human sources (dental caries, feces and vagina), seventeen from animal intestinal tracts or rumen, two from wastewater and one from fermented milk. Laine (2003) isolated thirteen *Bifidobacterium* strains from the feces of healthy elderly subjects.



Picture 4. Scanning electron microscopy micrographs of a Bifidobacterium sp. strain

The exact prevalence of lactobacillus and bifidobacterial spp. in the intestinal tract of humans is still accurately unknown. *Lactobacillus crispatus, L. gasseri, L. salivarius,* and *L. reuteri* have been reported as the major species of the *Lactobacillus* microflora (Fujisawa *et al.*, 1990). Whereas, *Lactobacillus johnsonii, Lactobacillus ruminis, Lactobacillus casei,* and *Lactobacillus brevis* have been detected occasionally, *Bifidobacterium longum* has been found predomonently in adult human GIT, and *Bifidobacterium bifidum* was detected occasionally. In contrast, *Bifidobacterium infantis* and *Bifidobacterium breve* were detected predominantly in infant feces, while *B. longum* and *B. bifidum* detected occasionally.

2.7. Production of antimicrobial compounds by probiotics

Probiotic bacteria such as LAB produce many antimicrobial substances which have several advantages in comparison with pathogens and other harmful bacteria (Drider *et al.*, 2006, Soomro *et al.*, 2002). These compounds include fatty acids, organic acids, hydrogen

peroxide, and diacetyl, acetoin and the best studied small, heat-stable inhibitory peptides named 'bacteriocins'(Simova *et al.*, 2009). There are three types of Bacteriocins which are created by LAB, type: 1- lantibiotics; 2- small hydrophobic heat-stable peptides, and 3 - large heat-labile proteins (Drider *et al.*, 2006).

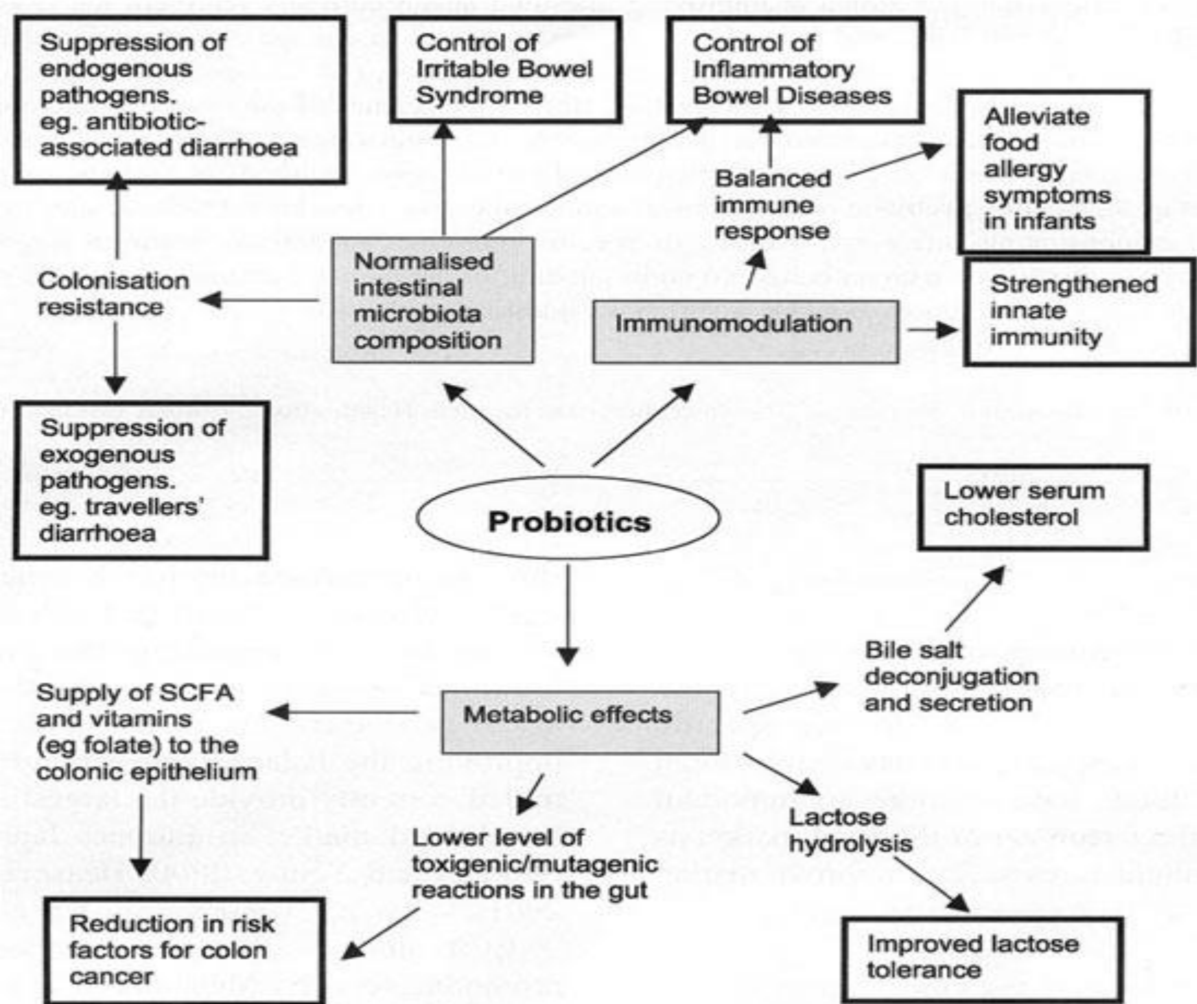
It was reported that the majority of common *Lactobacillus* spp. create antimicrobials, for instance bacteriocins, those are *Lactobacillus sakei* and *Lactobacillus curvatus. Lactobacillus sakei* has been shown to have a capacity to produce bacteriocins sakacin A, M, P, 674, K, and T, all antimicrobial substance against *Listeria monocytogenes* (Schillinger and Lucke, 1989).

2.8. Benefits of probiotics

Probiotics are components of food that produce useful physiological effects through their interrelationships with the gut. Beneficial effects of the practical use of probiotics for human health go back at least as far as 1908 when Metchnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (Danone, 2001, Parvez *et al.*, 2006).

Nowadays, scientists have been focused on the health-promoting effect of functional food. Probiotics have been used as functional food in human and animal nutrition for the past few years. The beneficial effects of probiotic will depend on a number of factors including the strain chosen, level of consumption, duration and frequency of exposure, and the physiological condition of the individual (Kopp-Hoolihan, 2001). It was documented that the probiotic bacteria has numerous healthy effects on the consumers such as prevention of urogenital diseases, alleviation of constipation, protection against traveler's diarrhea, reduction of hypercholesterolemia, protection against colon and bladder cancer, prevention of osteoporosis and food allergy (Lourens-Hattingh and Viljoen, 2001), prevention of genital and urinary tract

infections (Matofari *et al.*, 2011, Redondo-Lopez *et al.*, 1990, Martin *et al.*, 1999), immunostimulatory effects (Aattouri *et al.*, 2001).



Picture 5. A possible effect of probiotics on human health (Mach, 2006)

Gill *et al.* (2000) reported that supplementation of the diet with probiotic strains *Lactobacillus rhamnosus* (HN001, DR20), *L. acidophilus* (HN017) and *B. lactis* (HN019, DR10), both *in vivo* and *in vitro*, enhance immunity in healthy mice. Oral ingestion of probiotics modulates immunity of blood leukocytes in humans (Schiffrin *et al.*, 1997). Also it

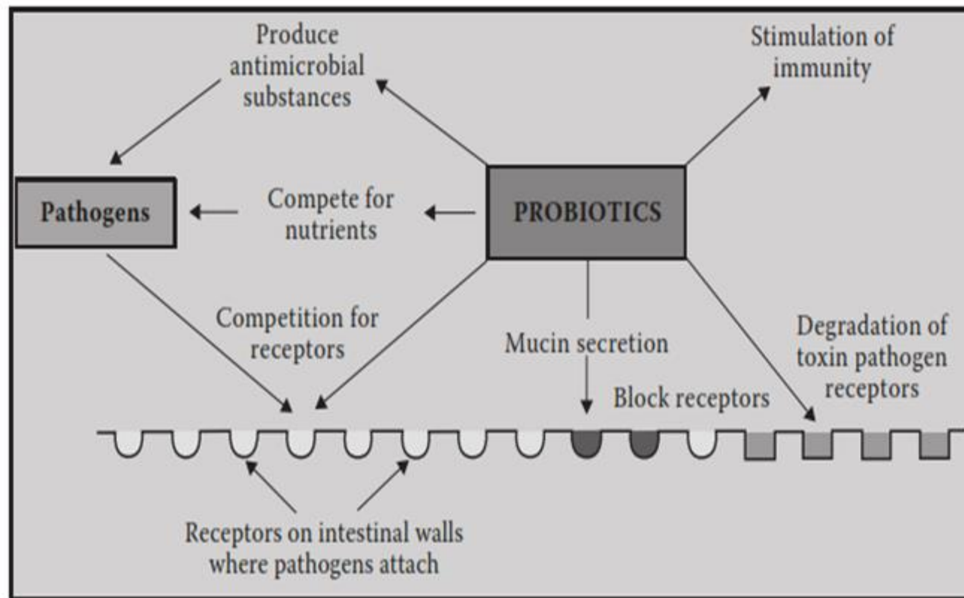
was reported that oral administration of LAB to rats increases lymphocyte proliferation and interferon production (Aattouri *et al.*, 2001).

Some bacterial species, for example *L. casei* ssp. *Rhamnosus* are recognized for their capacity to prevent the colonization of gastrointestinal tract by pathogenic bacteria such as enteropathogenic *E. coli*, enterotoxigenic *E. coli*, and *Klebsiella pneumoniae* using *in vitro* model with Caco-2 cell line (Forestier *et al.*, 2001).

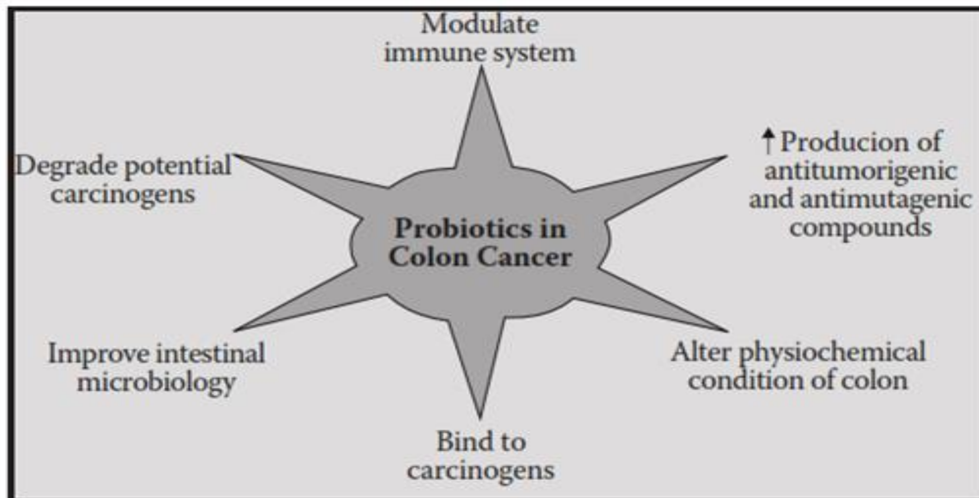
2.9. Mechanisms of probiotic action

The mechanisms by which probiotics exert biological useful benifites healthy to a host are still unclear, but there are some theories to explain their mode of action (Fig. 6): a) the production of inhibitory/antimicrobial substances such as are organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids; b) acting as competitive antagonists, i.e. competition for adhesion sites and nutrients in the gastrointestinal tract (Scarpellini *et al.*, 2008); c) receptor competition, where probiotics compete with microbial pathogens for limited number of receptors present on the surface of the intestinal epithelium (Marotta *et al.*, 2007) and d) stabilization of intestinal permeability barrier, thus restricting colonization by pathogens and eliminating foreign antigens which have penetrated the mucosa, and stimulating the immune system. (Fasano and Shea-Donohue, 2005).

Several different studies *in vitro* and *in vivo* have also suggested that probiotics could have positive potential to exert anticarcinogenic effects (Goldin and Gorbach, 1980, Goldin *et al.*, 1996). Furthermore, some studies have suggested that administration of probiotics could suppress the proliferation of tumor cells (Lee *et al.*, 2004). Some of possible mechanisms by which probiotics may regulate cancer as seen in Fig 7.



Picture 6. A possible mechanism of probiotic action on receptors on the intestinal wall where pathogens attach (Cho and Finocchiaro, 2010)



Picture 7. Modulatory effect of probiotics on colon cancer (Cho and Finocchiaro, 2010)

On the other hand, Oelschlaeger (2010), documented that probiotics may regulate colorectal cancer by the following three possible mechanisms:

(*i*) Probiotics might be able to modulate the host's defenses including the innate as well as the acquired immune system. This mode of action is most likely important for the prevention

and therapy of infectious diseases but also for the treatment of (chronic) inflammation of the digestive tract or parts of it. In addition, this probiotic action could be important for the eradication of neoplastic host cells;

(*ii*) Probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones. This principle is in many cases of importance for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut;

(*iii*) Finally, probiotics effects may be based on actions affecting microbial products like toxins and host products, *e.g.* bile salts and food ingredients. Such actions may result in inactivation of toxins and detoxification of the host and food components in the gut.

2.10. Capacity of probiotics to bind toxic substance

It is known that lactic acid bacteria are able to bind aflatoxin B1 *in vitro* and *in vivo* (Kankaanpaa *et al.*, 2000), but this property seems to depend on bacterial strain. Compared to *L. plantarum* and *L. fermentum*, *L. casei* was reported to be the strongest binder of aflatoxin (Abdel-Tawab *et al.*, 1999). Also, microorganisms such as *Saccharomyces cerevisiae* demonstrated good ability to bind this aflatoxin (Victor *et al.*, 1993, Prathapkumar *et al.*, 2007). In the study of Gratz *et al.* (2006) rats received doses of aflatoxin B1 and were fed with oral gavage containing *Lactobacillus rhamnosus* strain GG (ATCC 53013). After administration, an increase in the aflatoxin B1 in feacal excretion was observed due to bacterial binding.

Microcystins are toxins produced by freshwater cyanobacteria which can cause acute hepatoxicity and act as tumour promoters (Meriluoto *et al.*, 2005a). The probiotics *Lactobacillus rhamnosus* strain GG and *Bifidobacterium lactis* strain Bb12 have demonstrated the ability to bind to microcystin-LR, the most common and most toxic variant of microcystins. A higher removal of microcystin-LR was observed when *Lactobacillus rhamnosus* strain GG was heat-treated (46 %) (Meriluoto *et al.*, 2005b).

3. *Aims of the study*

There has been no specific treatment available for acute or chronic metal poisoning. Usually, cadmium toxicity has been often treated with commercially available chelating agents including ethylene-diamine-tetraacetic acid (EDTA), dimercaprol and mesomercaptosuccinic acid. On the other hand, there is histopathological evidence for increased toxicity in animals when these agents are utilized.

The present investigation intended to evaluate therapeutic potential of mixture probiotic bacteria against cadmium toxicity *in vitro* and *in vivo* as following:

1. To determinate toxic effects of cadmium in rats given to drink solution of it in form of $CdCl_2$ (analytical grade, Fisher Scientific, UK) in concentration of 70 ppm in tap water, by measuring:
    a. water and food consumption, as well as the body weight gain in the group of animals treated with cadmium throughout the experiment;
    b. the alterations in liver, kidney, blood and feces cadmium concentration;
    c. histological changes in the liver and kidney in animals treated with cadmium;
    d. activities of serum hepatic marker enzymes aspartate transaminase (AST) and alanine transaminase (ALT);
    e. oxidative damage by comet assay analysis in hepatocytes of cadmium treated animas (*in vivo* determination) and hepatocytes of control animals subsequently treated with cadmium in cell culture (*in vitro* determination);
    f. levels of antioxidant enzymes total superoxide dismutase (SOD) and catalase (CAT) in the liver and kidney in animals treated with cadmium;
    g. the compositions of micro flora in feces of cadmium treated rats by counting the *E. coli*, enterobacteria and bifidobacteria concentration

2. To determinate effects of probiotic bacteria supplementation (commercial preparation PROBIOTIC®, Ivančić i sinovi d.o.o., Belgrade, Serbia), containing $5x10^9$ lyophilized cells of *Lactobacillus rhamnosus Rosell-11*, *Lactobacillus acidophilus Rosell-52* and *Bifidobacterium longum Rosell-175* strains on all above mentioned parameters;
3. To compare observed results in cadmium and probiotics treated animals with control group (intact animals);
4. To determinate possible protective effects of probiotic supplementation against cadmium toxicity in rats given to drink $CdCl_2$ solution together with probiotic supplementation, by comparing observed results with control, cadmium and probiotic group ones.

4. Animals

Male Wistar rats *Rattus norvegicus*, weighing 130±10 g, were acclimated to 22±2°C in metabolic cages (3 rats/cage) and maintained under a 12h light/dark cycle (with lights on at 06:00h and off at 18:00h. Rats were fed with commercial rat food (Veterinary Institute, Subotica, Serbia) and drank tap water *ad libitum*.

5. Cadmium and probiotic bacteria

Cadmium chloride (CdCl2, analytical grade) was by Fisher Scientific UK.

Probiotic used in this study was the commercial preparation PROBIOTIC®, Ivančić i sinovi d.o.o., Belgrade, Serbia. The capsules are declared to contain $5x10^9$ lyophilized cells of *Lactobacillus rhamnosus Rosell-11*, *Lactobacillus acidophilus Rosell-52* and *Bifidobacterium longum Rosell-175* strains.

6. Experimental design

The experiments were performed in compliance with the Serbian animal protection law and approved by the Ethical Committee of the University of Belgrade, Faculty of Biology.

The rats were randomly divided into four groups, 6 males each group. All groups were treated for five weeks as follows: Group 1 (control group) was given food and tap water *ad libitum*. Group 2 was given probiotic mixed with food at a dose of 5×10^8 colony forming units (cfu)/g of food. Group 3 received $CdCl_2$ at a dose of 70 ppm in the drinking water. Group 4 received both $CdCl_2$ and probiotic.

7. Animals sacrifice and sampling

At the end of treatment, the feces were collected and frozen at –80ºC until further analysis. Animals were decapitated with a guillotine (Harvard-Apparatus, Holliston, MA,

USA) without anesthesia, and blood was collected and divided into 2 sets of tubes: one with EDTA in order to obtain plasma, and second without EDTA in order to obtain serum. Tubes were centrifuged (1000 rpm for 10 min), and plasma was used for hormones assays, while serum was used for several biochemical analyses. In order to avoid day–night variations in the concentrations of the measured hormones, all sacrifices were performed between 8:00–10:00h.

After opening the abdominal cavity, the kidneys, adrenal glands and the most prominent frontal lobe of the liver were excised. Kidney and liver were divided into two parts: one was immediately frozen at –80ºC until biochemical analyses, while another was put in 10% formalin. In addition, one part of livers were processed to obtain single-cell suspensions in order to perform the Comet test to assess DNA damage.

Serum, plasma and feces were frozen at –80ºC until further analysis.

8. Sample Cd, Zn and Cu content analysis

8.1. Sample preparation of feces

Feces was dried at 105±5°C until the constant mass was archived, grinded in a mortar, and sieved trough 1mm sieve. Between 0.5 and 1.0 g of dry feces was measured and transferred into Teflon tubes, with addition of 7 ml of concentrated nitric acid and 1 ml of hydrogen-peroxide. The tubes were closed and digested by microwave (heated during 15 minutes from the room temperature to the 200°C and then for another 20 minutes at this temperature). The temperature was monitored on a display of a heat sensor inserted in tubes. At the end of digestion samples were cooled down to the room temperature, the tubes were opened, and solution filtered because of some undissolved silicates remains. Filtrates were transferred to the volumetric flasks of 25 ml and deionized water was added to the volume.

8.2. Sample preparation of kidney, liver and blood

Kidney, liver and blood were analyzed as a wet samples in the same manner as the feces (without prior drying), and the concentration of Cd was recalculated to the mass of a dry sample (determined by prior drying of the part of samples at a 60°C to a constant mass).

8.3. Determinations of metals

Metal concentrations of Cd, Zn and Cu were determined according to the US EPA SW-846 7000B method for the FAAS (flame atomic absorption spectroscopy) analysis with Varian, SpectraAA atomic absorption spectrophotometer.

Merck 1000 mg/L cadmium solution (1.19777.0500) was used for the preparation of standard series, with 50 ppm as the first standard, and from it all others standards for calibration curve.

Samples were measured during 3 seconds, with 1s of pre read delay at the wavelength of 228.8 nm for Cd, 324.8 nm for Cu and 213.9 nm for Zn, and each measurement was repeated 3 times. Absorption of the measured metals was determined and compared with the standard solutions. Masses of the metals in digestion solutions were divided by the mass of the dry samples and concentrations were reported in ppm units.

9. Determination of Microbiology parameters

9.1. Chemicals, media, and culture conditions

Cadmium chloride (CdCl2, analytical grade) was from Fisher Scientific UK. Lactobacilli were cultured on MRS agar (ScharlauChemie S.A., Spain) for 48h at 37°C. Bifidobacteria were plated on Bifidobacterium agar (HiMedia, India) in anaerobic conditions for 24h at 37°C. Enterobacteria and *E. coli* were monitored on MacConkey agar (MacConkey agar base Difco, USA) and ECC agar (HiMedia, India), respectively. Peptone water "Torlak",

Belgrade, contained 10 g pepton-4 “Torlak”, 5 g NaCl and 0.01 g Fuchsin S per liter of distilled water.

9.2. Identification of probiotic bacteria

Probiotic used in this study was the commercial preparation PROBIOTIC®, Ivančić i sinovi d.o.o., Belgrade, Serbia. The capsules are declared to contain $5x10^9$ lyophilized cells of *Lactobacillus rhamnosus Rosell-11*, *Lactobacillus acidophilus Rosell-52* and *Bifidobacterium longum Rosell-175* strains. In order to identify probiotic bacteria, MRS broth was inoculated with the content of one capsule of „PROBIOTIC®” and incubated at 30°C. After 48h the culture was streak-inoculated on MRS agar and Bifidobacterium agar and incubated for 48h at 30°C aerobically and for 24h at 37°C anaerobically, respectively. Using Gram staining and API 50 CH identification kit (bioMerieux, Lot. No. 842019201) we identified small grey colonies on MRS agar as *Lactobacillus rhamnosus*, big white colonies on the same medium as *Lactobacillus acidophilus* and colonies on Bifidobacterium agar as *Bifidobacterium longum*.

9.3. Microbiological analysis of feces

Samples of feces from each of the four treatment groups were collected and immediately transported to the laboratory. A feces specimen, 4 g from each group, was suspended in 36 ml of peptone water and homogenised. Samples were vigorously vortexed for 1 min and then centrifuged at 65g for 5 min to deposit any remaining solid matter. The number of lactobacilli and bifidobacteria was determined by spreading appropriate dilutions onto MRS agar and Bifidobacterium agar plates, respectively. The number of enterobacteria and *E. coli* was determined by plating appropriate dilutions onto MacConkey agar and ECC agar respectively. Experiments were performed twice, each with duplicate samples.

10. Estimations of Physiological parameters

10.1. Procedures for tissues homogenization

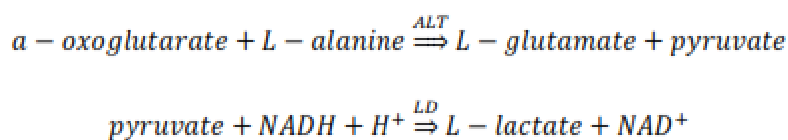
Tissue samples were homogenized in PBS buffer, in the tissue/buffer ratio 1/20 in the case of liver and kidney, or 1/50 ratio in case of adrenal glands.

The homogenates were centrifuged 20 min/12000rpm at 4°C in semi preparative centrifuge (Sorvall Super T21), and supernatants were collected.

10.2. Determination of alanine aminotransferase (ALT) activity

Alanine aminotransferase activity was measured in serum by Randox kit.

Reaction is based on the next principle:



$$a - oxoglutarate + L - alanine \overset{ALT}{\Longrightarrow} L - glutamate + pyruvate$$

$$pyruvate + NADH + H^{+} \overset{LD}{\Rightarrow} L - lactate + NAD^{+}$$

where ALT is serum alanine aminotransferase, and LD is lactate dehydrogenase.

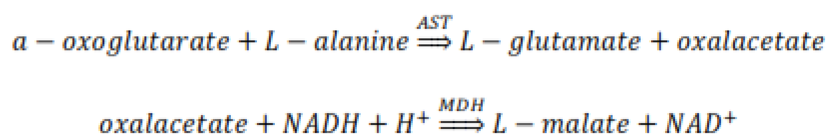
The oxidation of NADH to NAD^{+} is accompanied by a decrease in absorbance at 340nm, with ALT activity calculated as:

$$\frac{Units}{l} = 1746 \times \Delta A_{340nm/min}$$

where ΔA 340/min is a mean absorbance decrease between first and second, and second and third minute.

10.3. Determination of aspartate aminotransferase (AST) activity

Aspartate aminotransferase activity was measured in serum by Randox kit. Reaction is based on the next principle:



where AST is serum aspartate aminotransferase, and MDH is malate dehydrogenase.

The oxidation of NADH to NADH $^{+}$ is accompanied by a decrease in absorbance at 340nm, with AST activity calculated as:

$$\frac{Units}{l} = 1746 \times \Delta A_{340nm/min}$$

where ΔA 340/min is mean absorbance decrease between first and second, and second and third minute.

10.4. Determination of total superoxide dismutase (SOD) activity

Total SOD activity was performed on the base of the original method described by Misra and Fridovica (Misra and Fridovich, 1972). Upon adding a tissue homogenate sample, in presence of superoxide anion radicale the adrenalin spontaneously auto oxidized in adrenohrom, with absorbance change at a wavelength of 480 nm.

The absorbance change was monitored between 3 and 4 minutes because during this period the absorbance varied linearly. Total SOD activity was calculated by the following formula:

$$\frac{\text{The unit of enzymeactivity}}{\text{mg tkiva}} = \frac{2 \cdot (\Delta K - \Delta A) \cdot R}{V \cdot T \cdot \Delta K}$$

where:

ΔK — absorbance changes in adrenaline between the third and fourth minutes

ΔA — change in the absorbance of adrenaline with the sample between the third and fourth minutes

R — dilution

V — volume of the sample in ml

T — mg of tissue in a given sample

10.5. Determination of catalase (CAT) activity

Catalase activity was measured spectrophotometrically by the method of Beutler (1982). The principle of the method is based on the rate of hydrogen peroxide degradation by the action of CAT contained in the examined samples.

The absorbance change was monitored at 230nm every 30 seconds, starting from 30 seconds to 3 minutes (5 measurements). CAT activity was calculated by the following formula:

$$\frac{\text{The unit of enzyme activity} - \text{tissue}}{mg\ tkiva} = \frac{\Delta \mathrm{A}}{\mathrm{v.T.0.071}}$$

where:

Δ A — mean change in absorbance measured every 30 seconds

V — Volume of sample in ML

T — mg tissue in a given sample.

11. Adrenaline and noradrenaline concentration measurement

Adrenaline and noradrenaline are extracted by using a cisdiolspecific affinity gel, acylated and then derivatized enzymatically. The competitive ELISA kit was used, with antigen bounded to the solid phase of the microtiter plate. The derivatized standards, controls, samples and the solid phase bound analytes compete for a fixed number of antiserum binding sites. After the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm.

Determination of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standard concentrations.

12. Histology

12.1. Fixation, dehydration and embedding of tissue specimens

The samples of left kidney (sagitally sectioned halves) and liver (left lobe) were isolated from the animals, thoroughly rinsed in ice-cold saline (0.9% NaCl) and immersed in 10% neutral-buffered formalin fixative (AnalR NORMAPUR®, VWR, USA). The fixation lasted for seven days.

The organs were then transferred in ascending series of ethanol (50%, 70%, 95%, 100%), two hours each). After dehydration, tissue samples were cleared in xylene (two changes, 15 min each) and immersed in Bioplast paraffin (56-58°C melting point, two changes: first 4-5 hours, second overnight) according to standard histological procedure.

12.2. Sectioning and staining

Organ samples were sliced in 5 μm thick sections on rotary microtome Spencer (No. 820, American Optical Company, Buffalo, NY, USA) and mounted on gelatinized microscopic slides. For staining, tissue sections were subjected to periodic acid-Shiff (PAS) reaction using commercial detection kit (04-130802, Bio Optica, Milano, Italy). This staining method is used to demonstrate normal and pathologic components on basement membrane of histological sections.

Specimens were examined and photographed using Leica DMLB (Wetzlar, Germany) light microscope and all alterations from the normal kidney and liver histological structure were registered.

13. Comet assay

13.1. Preparation of single-cell suspensions

Single-cell suspensions of liver tissue were prepared using a method adapted from Wilson *et al.* (1998). Liver was excised and chopped separately 10 times in 0.2 ml of HBSS using two fresh scalpel blades in a scissor-like movement on a Petri dish, washed off gently into a 15 ml centrifuge tube with a further 2.8 ml HBSS and 0.03 ml of 0.5% trypsin. The suspension was gently rocked for 10 min at room temperature, after which 10 ml of HBSS was added and the suspension passed through a 40 μm sieve to remove any large fragments that remained. After centrifugation (800g for 5 min), the supernatant was discarded and the pellet carefully re-suspended in 1 ml of HBSS. Cell viability was measured by trypan blue dye exclusion method and cell density was adjusted to 3×10^5 cell/ml.

13.2. In vitro exposure

Single-cell suspension obtained from the untreated rat liver (control group) was used to prepare comet slides embedded in agarose as described below. Slides were exposed to $CdCl_2$ (70 ppm), probiotic (1.28 mg/ml) and a combination of $CdCl_2$ and probiotic for 15 min at 22°C in the dark. Slides exposed to PBS only served as controls. The concentrations of Cd and probiotic bacteria corresponded to the concentrations for *in vivo* treatment. After exposure, slides were rinsed with PBS to remove residues and submitted to comet assay procedure as described below.

13.3. Procedure

The assay was performed as described by Tice *et al.* (2000).Cell suspension (30 μl) was mixed with 70 μl of 1% LMP (low melting point) agarose and added to slides (previously precoated1% NMP) that had been covered with a layer of 1% NMP (normal melting point) agarose. The slides were lysed (2.5 M NaOH, 0.1 M EDTA, 0.01 M Tris and TritonX-100, pH

10) for 1 h at 4 °C, transferred into electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min to allow DNA unwinding, and electrophoresed for 20 min at 25 V and 300 mA. Finally, the slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with 20 μl ethidium bromide (5 μg/ml) and analyzed using fluorescence microscope (Leica) and image analysis software (Comet IV, Perceptive Instruments). Fifty nuclei were analyzed per experimental point (in triplicate), and the percentage of the fluorescence in the comet tail was scored as a reflection of DNA damage.

14. Statistical analysis

Data for bacterial enumeration were statistically evaluated by two way analysis of variance (ANOVA), using SigmaStat (Version 3.1) software. Holm-Sidak comparisons were performed when ANOVA was significant. The level of significance was set at $p<0.05$. For the results of the comet assay, one-way analysis of variance (non-parametric ANOVA, Kruskal–Wallis test) was used to analyze differences between the treatments within each experiment. Dunncan Post Hoc test was used to compare median values of the percent of fluorescence in comet tails for all treatments; $p<0.05$ was considered as statistically significant.

RESULTS

1. Effect of applied treatment on body weight gain, food and water intake in rats

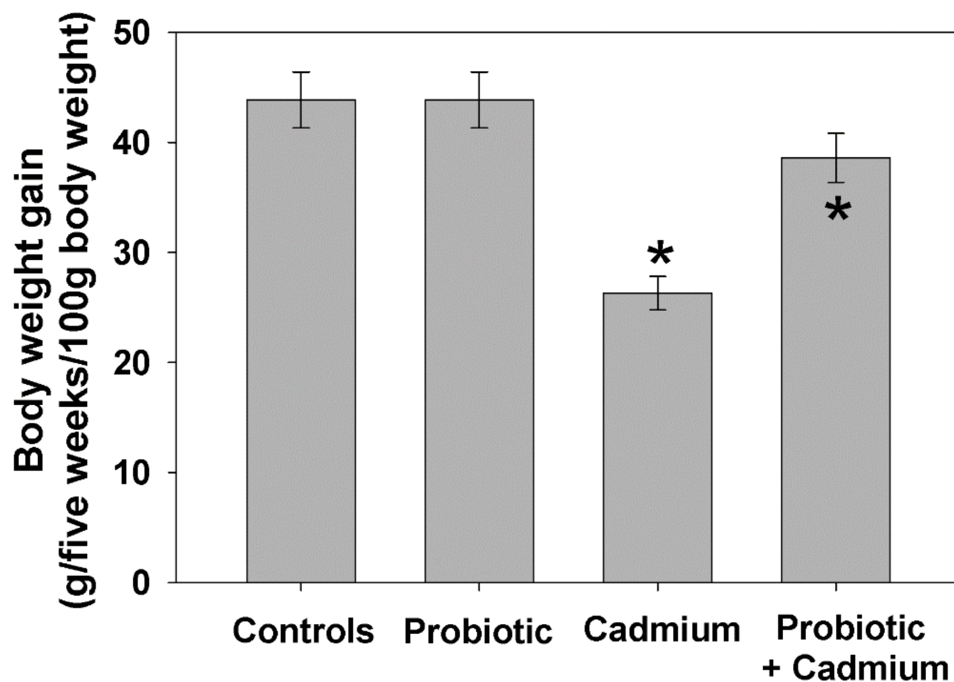


Figure 1A. Body weight gain in a period from the beginning to the end of treatment (five weeks) of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

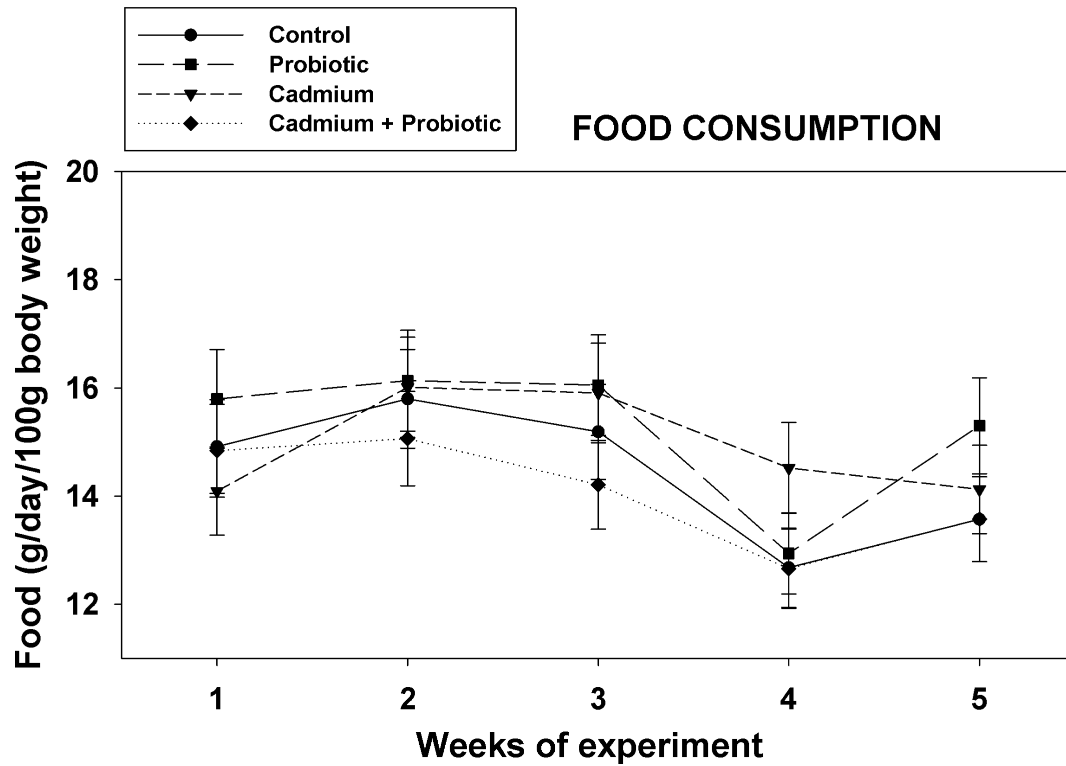


Figure 1B. Food consumption during a period from the beginning to the end of treatment (five weeks) of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

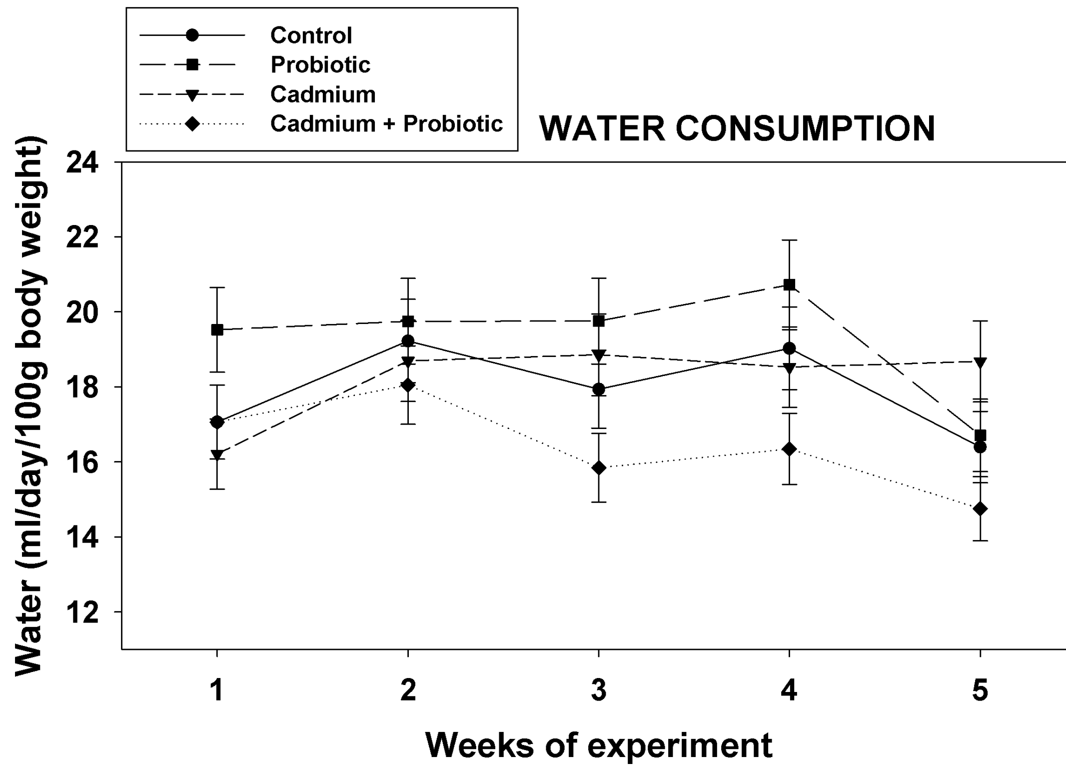


Figure 1C. Water intake during a period from the beginning to the end of treatment (five weeks) of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

The body weight gain, food and water intake of the rats in the experimental groups are presented in Figs.1. A, B, C. The results obtained indicated that body weight gain of rats exposed to cadmium chloride alone significantly decreased comparing to controls and probiotic treated animals. On the other hand, there were no significant changes recorded in the body weight gain of rats fed with probiotic bacteria strains simultaneously with cadmium chloride as compared to control group and probiotic treated animals. Regarding the both food and water intake, they were decreased (although not significantly) in cadmium treated animals if comparing to the control group. In general, food and water intake were not significantly alerted among all the experimental groups.

2. *Light microscopy examination of rat liver*

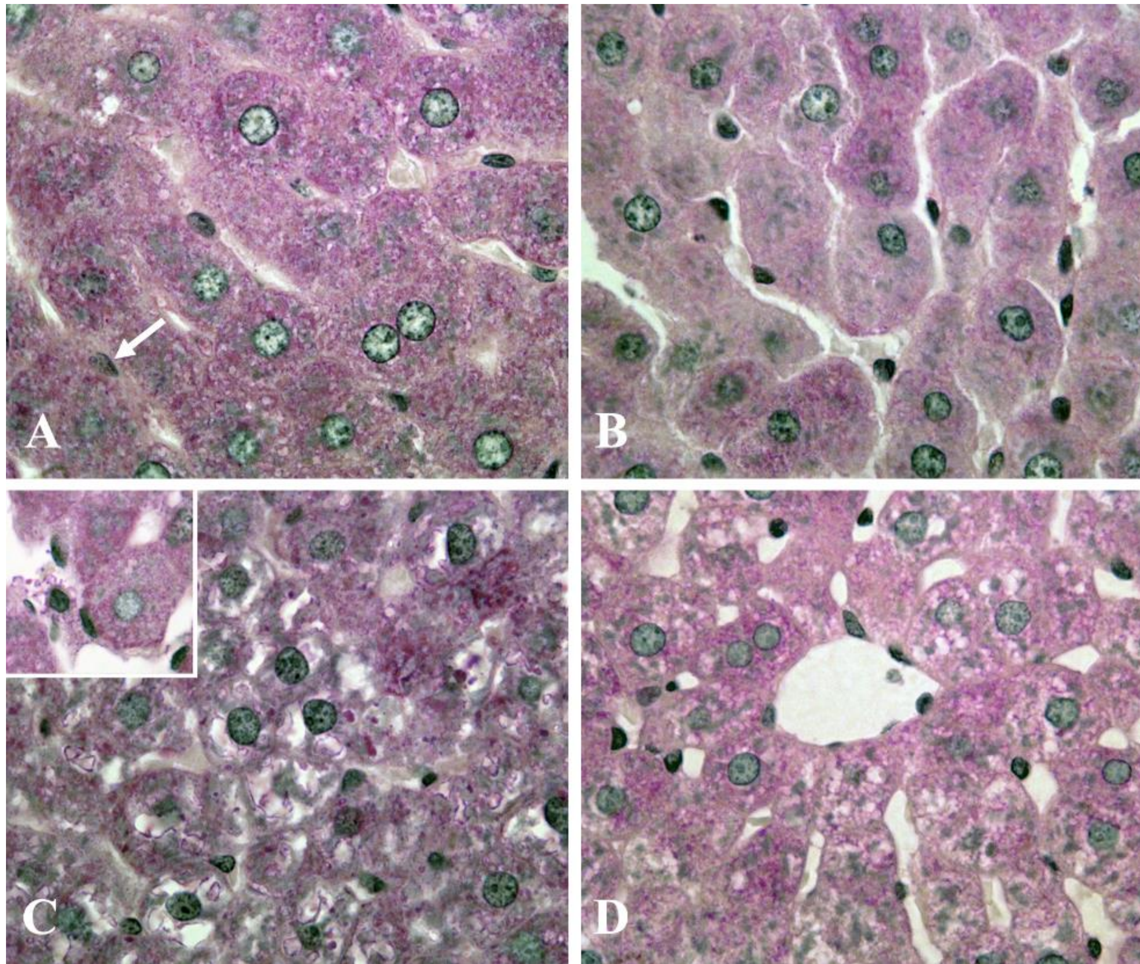


Figure 2A-D. Light microscopy of liver from control (A), probiotic-treated (B), cadmium-treated (C) and cadmium and probiotic-treated rats (D). Inset on C shows activated Kupffer cell. Magnification 100x, orig.

Liver and kidney, as main target organs of cadmium-induced toxicity, were subjected to histopathological analysis. Organs from control and rats consumed probiotic alone had normal macroscopic appearance and general histological structure (Figs. 2A and 2B) which was, however, affected by the intake of cadmium (Fig. 2C). Light microscopy analysis of liver revealed large areas of vacuolated hepatocytes and scattered hepatocellular necrotic foci throughout the tissue sections. Focal necrosis was manifested by blurred structure of hepatic cords and loss of nuclei (Fig. 5C). In the vicinity of terminal hepatic venule some hepatocytes with pycnotic nuclei were seen bordering enlarged sinusoids. Sinusoidal spaces were spanned by numerous activated Kupffer cells (Fig. 5C, inset). In animals co-treated with CdCl2 and probiotic, all histopathological changes were less pronounced and necrotic fields were absent (Fig. 5D).

3. Light microscopy examination of rat kidneys

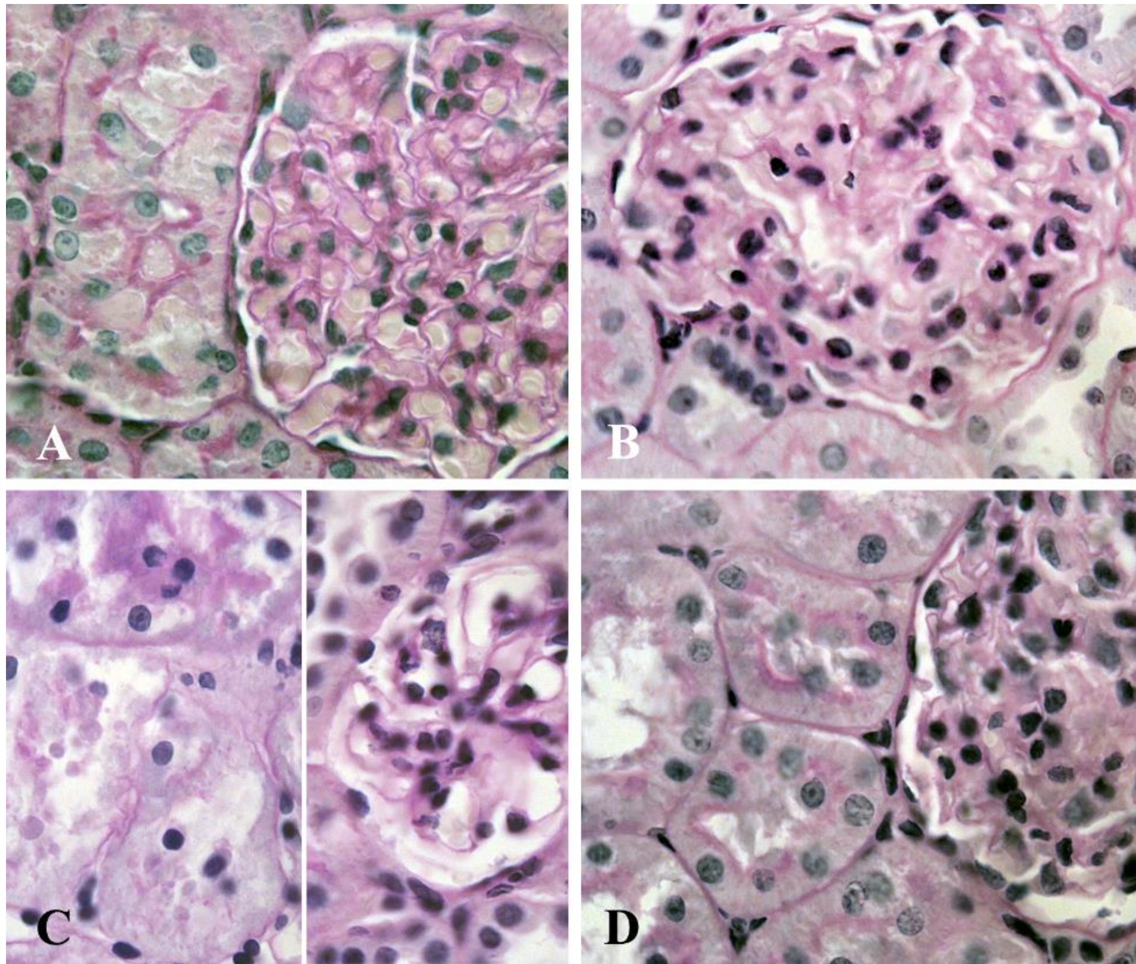


Figure 3A-D. Light microscopy of of kidney from control (A), probiotic-treated (B), cadmium-treated (C) and cadmium and probiotic-treated rats (D). On C, left, proximal nephrocytes vacuolization and necrosis are shown. C, right, demonstrates shrunken glomerulus with enlarged capillaries and reduction of cells. Magnification 100x, orig.

In kidney, organs from control and rats consumed probiotic alone had normal macroscopic appearance and general histological structure (Figs. 3A and 3B). However, renal corpuscles and proximal tubular epithelium showed remarkable signs of injury after CdCl2 treatment. Histopathological alterations of glomeruli (Fig. 3C, right) prograded from hypercellularity, through capillary enlargement and tuft hypersegmentation to terminal glomerular atrophy and widening of urinary space. Proximal nephrocytes were typically vacuolated, while brush border was mostly degenerated. Desquamated nephrocytes and/or nephrocyte nuclei often might be seen inside tubular lumen. In places, tubular necrosis was noted (Fig. 3C, left). Ingestion of probiotic attenuated CdCl2 induced glomerular and proximal tubular lesions ((Fig 3D). Although brush border of proximal nephrocytes was slightly irregular in comparison to control, most cells seemed to be well preserved. Glomeruli showed hypercellularity, glomerular segmentation was very rare, while more advanced alterations were not observed.

4. Effects of applied treatment on levels of AST and ALT in serum

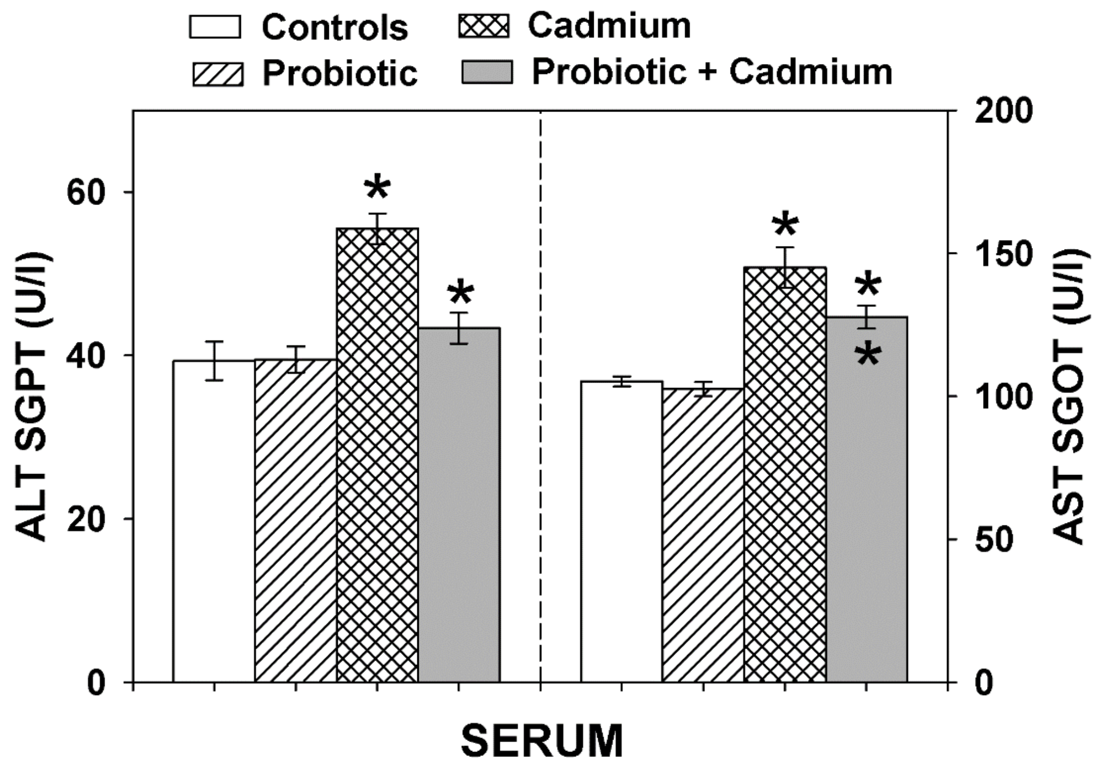


Figure 4. Serum ALT and AST activity (U/l) in controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

Fig. 4. illustrates that administration of cadmium chloride resulted in significant increase in levels of serum ALT and AST in comparison to both control and probiotic treated animals. In combination with probiotic, $CdCl_2$ significantly increased AST activity, but not that of ALT, compared to the control. However, the same treatment, if compared to $CdCl_2$ only treated rats, caused decrease in ALT and AST levels.

5. Liver changes in cadmium, copper and zinc levels

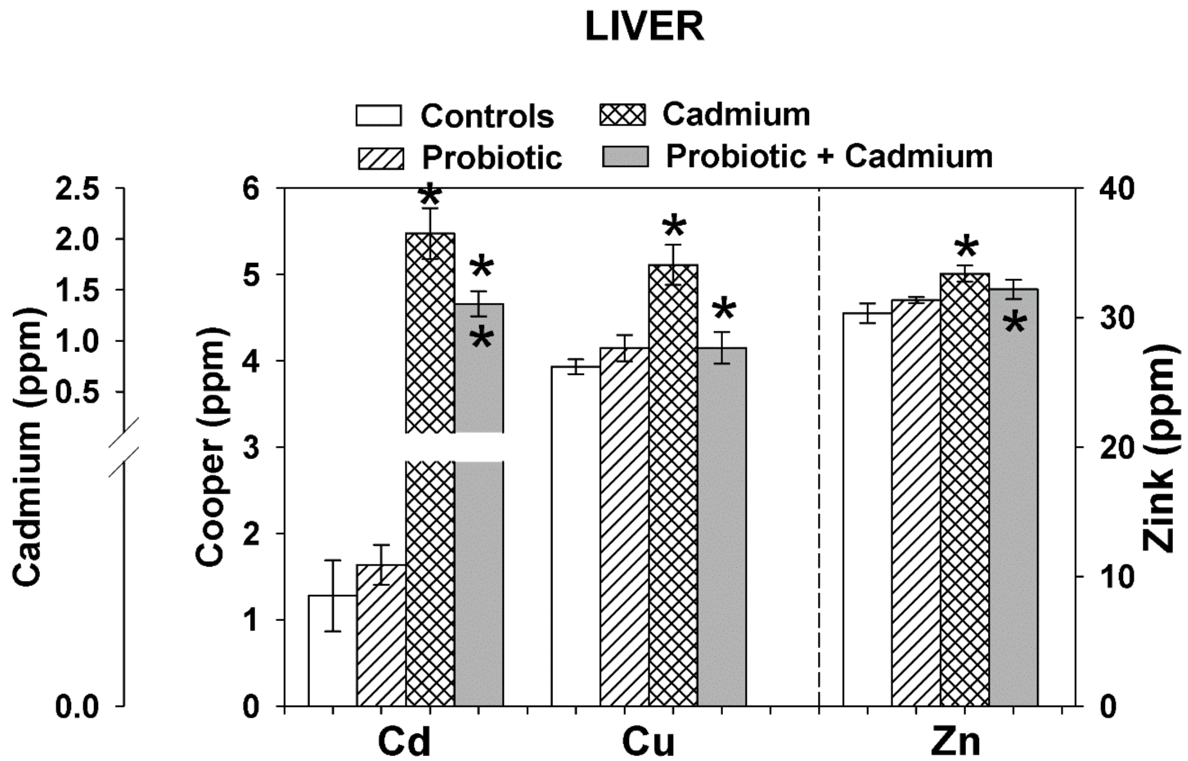


Figure 5. Cadmium, copper and zinc concentration (ppm) in liver of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

The alterations in liver cadmium accumulation are showed at the Fig. 5 which reveals that exposure of rats to cadmium led to the significant increase of Cd level in liver parenchyma if compared to the controls. On the other hand, probiotics administration did not influence the concentration of this metal measured in the liver parenchyma, compared to the control group of animals. When animals were treated simultaneously probiotics and cadmium, the liver Cd content rose significantly comparing to the controls. Although this increase was significant comparing to the control group of animals, it was still less prominent in comparison to the CdCl2 only treated rats. In addition, exposure of rats to cadmium revealed significant increase of copper and zinc levels in liver parenchyma in comparison to control group. Furthermore, adding of probiotic to cadmium chloride significantly decreased levels of both copper and zinc compared with cadmium treated animals. On the other hand, there were no significant changes in the concentration of copper and zinc between probiotic treated and control animals.

6. Kidney changes in cadmium, copper and zinc levels

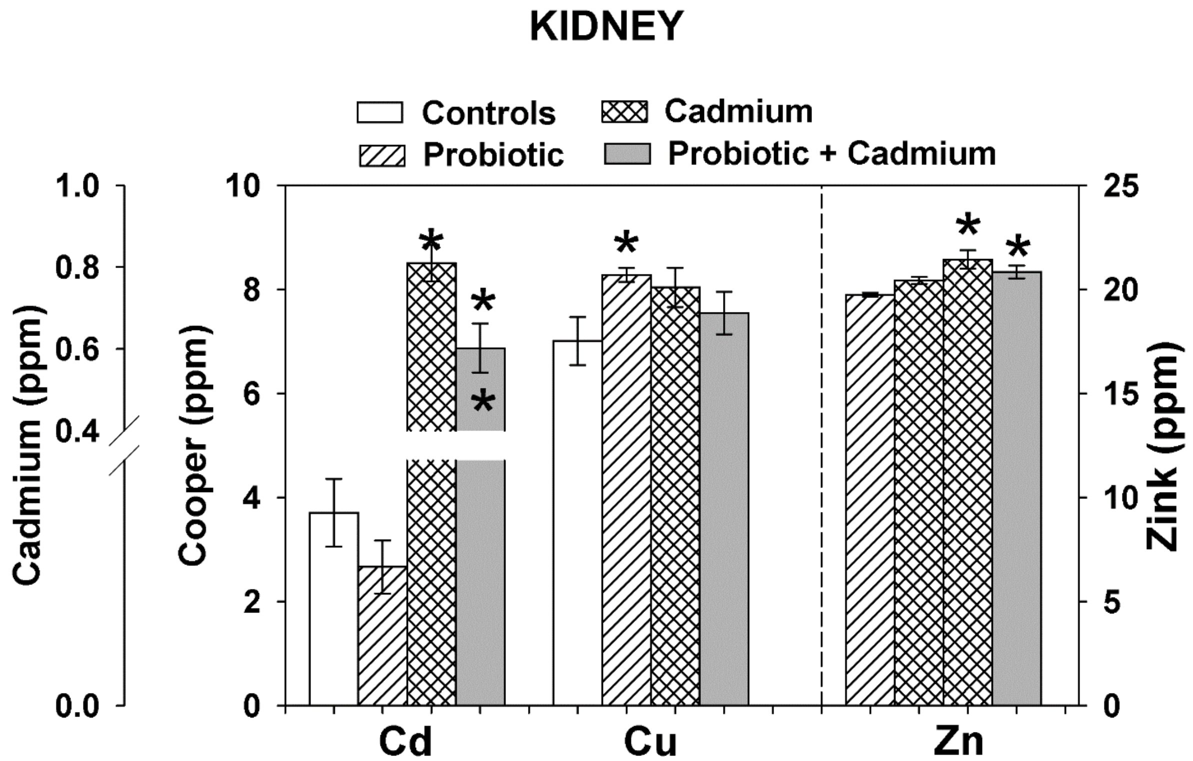


Figure 6. Cadmium, copper and zinc concentration (ppm) in kidney of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

As for the Cd concentration measured in the kidneys, it can be noticed at the Fig. 4 that $CdCl_2$ administration resulted in the same pattern of tissue accumulation as we obtained for liver, with considerable lower Cd concentration in comparison to this tissue. Namely, we detected statistically significant elevation of Cd concentration in kidney of animals treated with $CdCl_2$ in relation to the control group. The treatment with probiotics only did not change the Cd concentration in the kidneys comparing to the controls. While $CdCl_2$ administration resulted, as already mentioned, in a statistically significant elevation of kidney tissue Cd concentration, the simultaneous administration of probiotics along with $CdCl_2$ exerted an ameliorative effect, decreasing the kidney Cd concentration back toward control levels. Also, our findings revealed that administration of cadmium chloride, alone or in combination with probiotic, resulted in a statistically significant elevation of kidney zinc concentration comparing to control group.

7. Cadmium concentration in feces and blood of treated animals

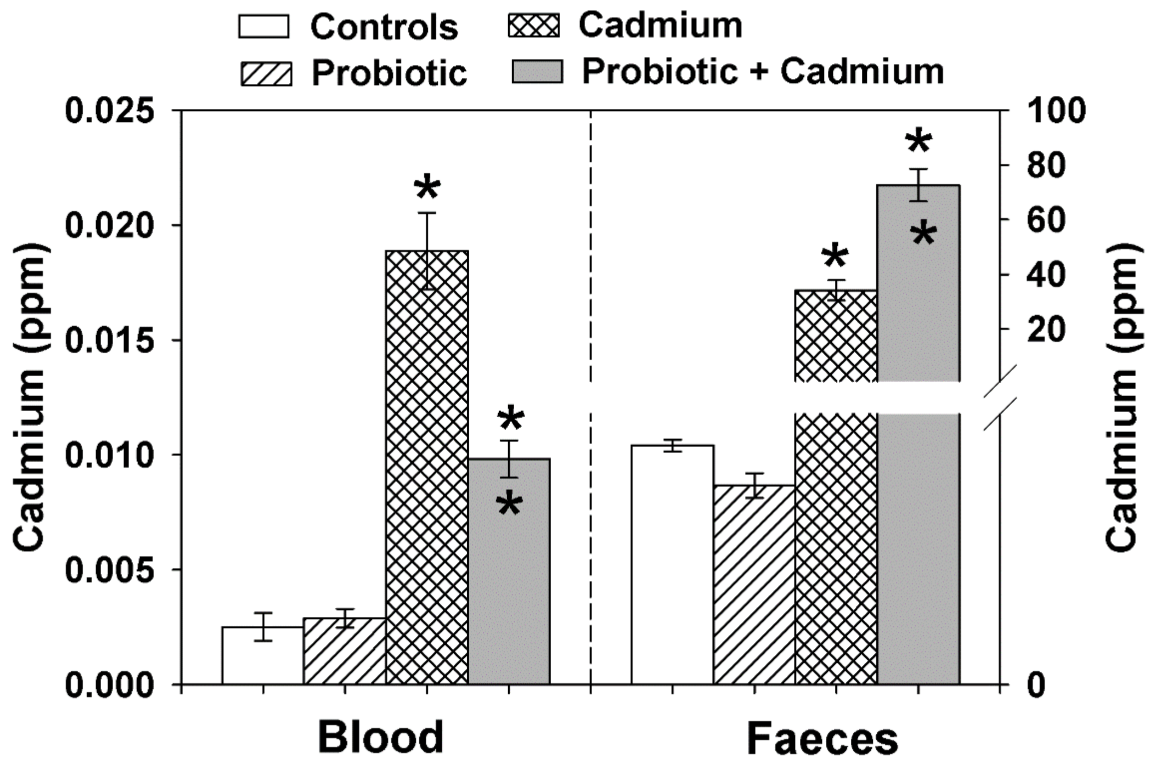


Figure 7. Cadmium concentration (ppm) in feces and blood of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

Regarding the blood and feces Cd concentrations, it can be noticed at Fig. 7 that exposure of animals to $CdCl_2$ increased its concentration in both the blood and feces, compared to the control group of animals. While treatment with probiotics alone did not influence neither the feces nor blood Cd concentration, in the rats treated simultaneously with $CdCl_2$ and probiotics the feces Cd concentration was higher compared to both controls and $CdCl_2$ treated group of animals. Furthermore, although the blood Cd concentration stayed above control levels in the group receiving the probiotics along with $CdCl_2$, this concentration was significantly lower comparing to the group of animals treated with $CdCl_2$ only.

8. Liver catalase and total SOD activity

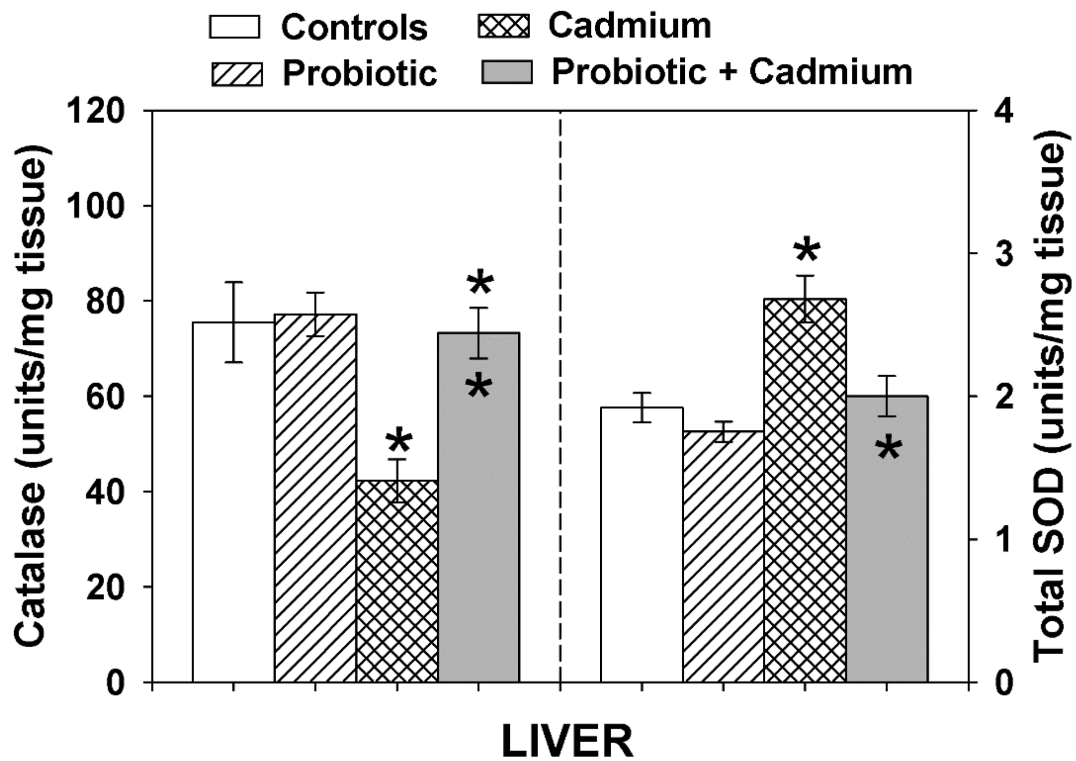


Figure 8. Catalase and SOD activity in liver of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

The activities of catalase (CAT) and total superoxide dismutase (SOD) were measured in the liver of animals treated with probiotics, cadmium or their combination. It can be noticed at Fig.8 that CAT activities significantly decrease as a result of Cd intoxication, while, at same time, total SOD activity increases. On the other hand, treatment with mixture of probiotic and cadmium chloride prevents the loss of CAT and rise of SOD activity.

9. Kidney catalase and total SOD activity

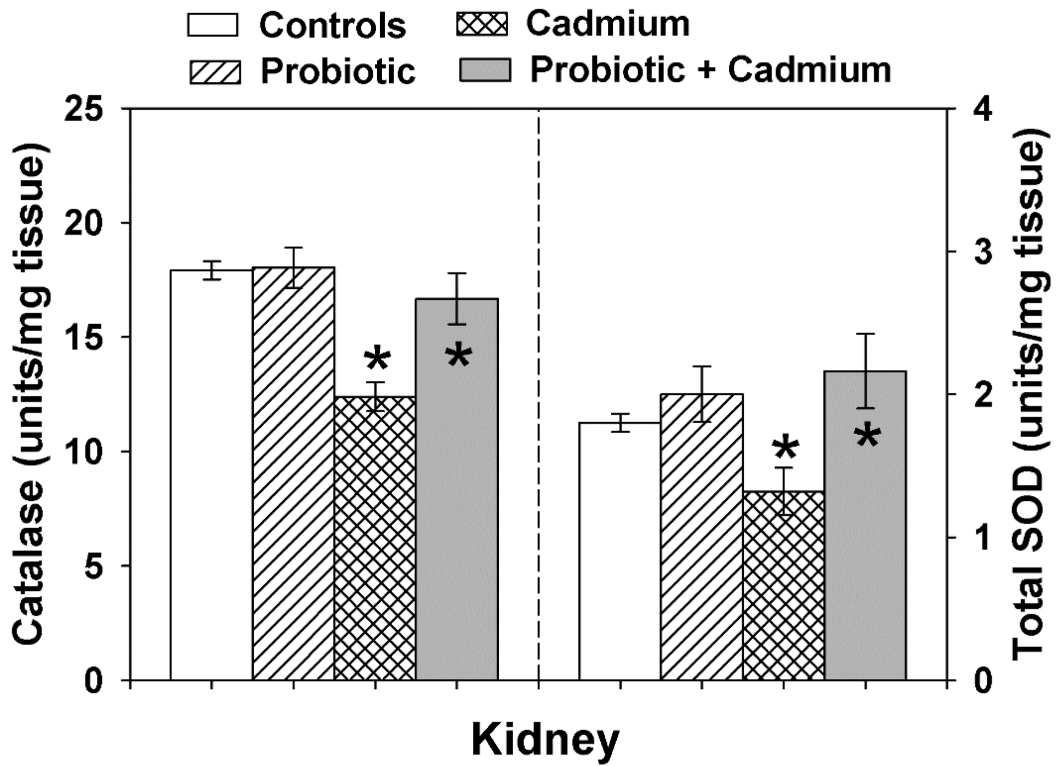


Figure 9. Catalase and SOD activity in kidney of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

The CAT and total SOD activity in the kidney of animals treated with probiotics, cadmium or their combination shows the very same pattern as the one seen in the liver. As a result of the Cd intoxication CAT activity decreases, while total SOD activity increases, with the combined diet effect to abolish above mentioned changes (Fig. 9).

10. Changes in plasma adrenaline and noradrenaline concentrations of treated rats

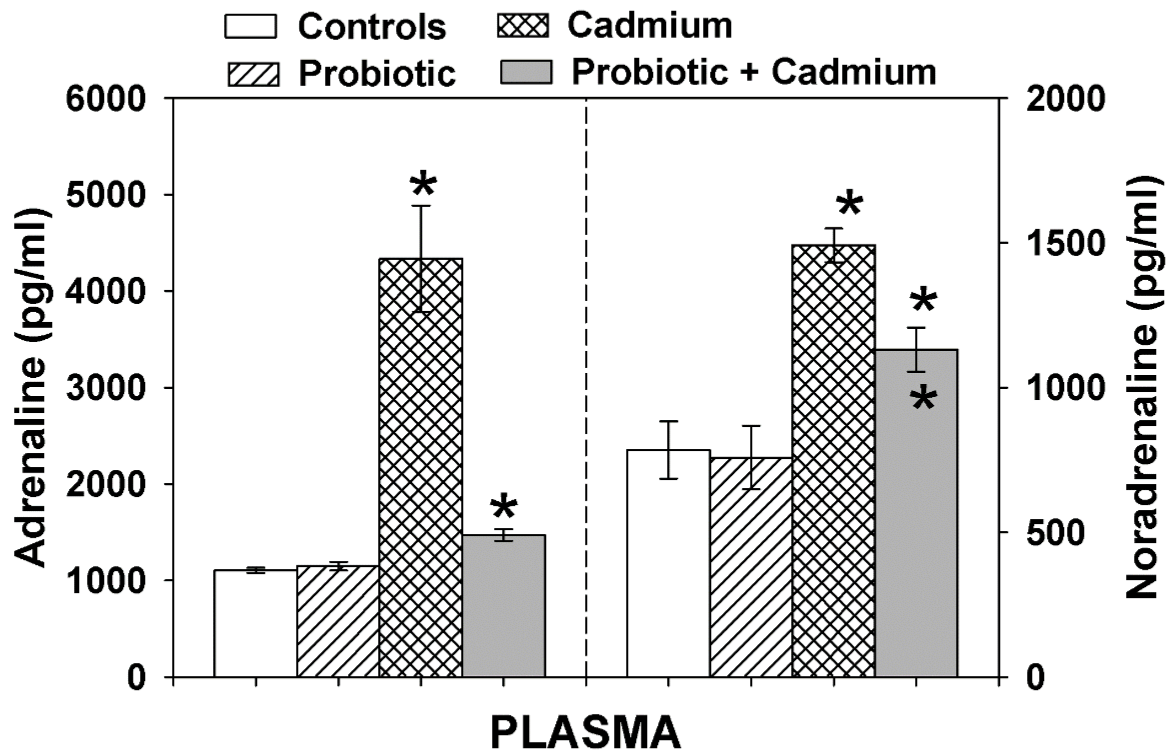


Figure 10. Blood adrenaline and noradrenaline concentrations (pg/mL) in controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

Data from Fig. 10 revealed that blood levels of adrenaline and noradrenaline were significantly increased in animals treated with $CdCl_2$ compared to controls, while probiotic alone did not affect the amount of these catecholamines. As for the adrenaline, probiotic adding to the $CdCl_2$ prevented its elevation in regard to controls. Contrary, administration of cadmium solution in combination with probiotic caused a significant decrease in blood noradrenaline levels comparing to the controls, although significantly lower in compare to one seen in adrenaline.

11. The effect of probiotic on cadmium induced genotoxicity in rat hepatocytes

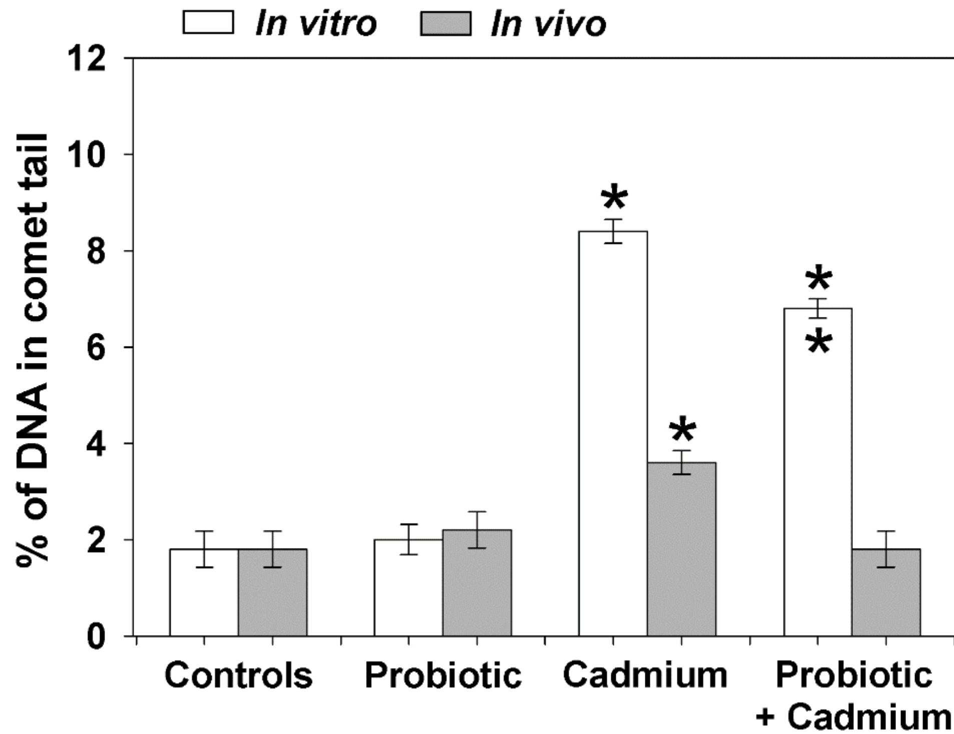


Figure 11. The effect of cadmium and probiotic, applied alone or in combination, on DNA damage in rat hepatocytes *in vivo* and *in vitro*. The level of DNA strand breaks is expressed as the percentage of DNA in the comet tails. Fifty cells were analyzed per experimental point. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

Two experimental procedures were used to study and compare the effect of probiotic on Cd induced genotoxicity in rat hepatocytes: in vivo and in vitro treatment. As shown in Figure 11, the percentage of DNA damage in control and probiotic treated cells was similar in both experimental conditions. The percentage of DNA in the comet tail was 1.71% in controls and 1.53% in vivo and 1.64% in vitro after the treatment with probiotic. The treatment with Cd resulted in significant increase of DNA damage; the percentage of DNA in the comet tail increased from 1.71% to 8.46% in vivo and from 1.71% to 3.43% in vitro. When the animals or cells were simultaneously treated with Cd and probiotic, the significant decrease of Cd induced genotoxicity was observed. The percentage of DNA in the comet tail declined from 8.45% to 6.78% in vivo (20% decrease) and from 3.43% to 1.82% in vitro (48% decrease).

12. The fecal profile of bacteria

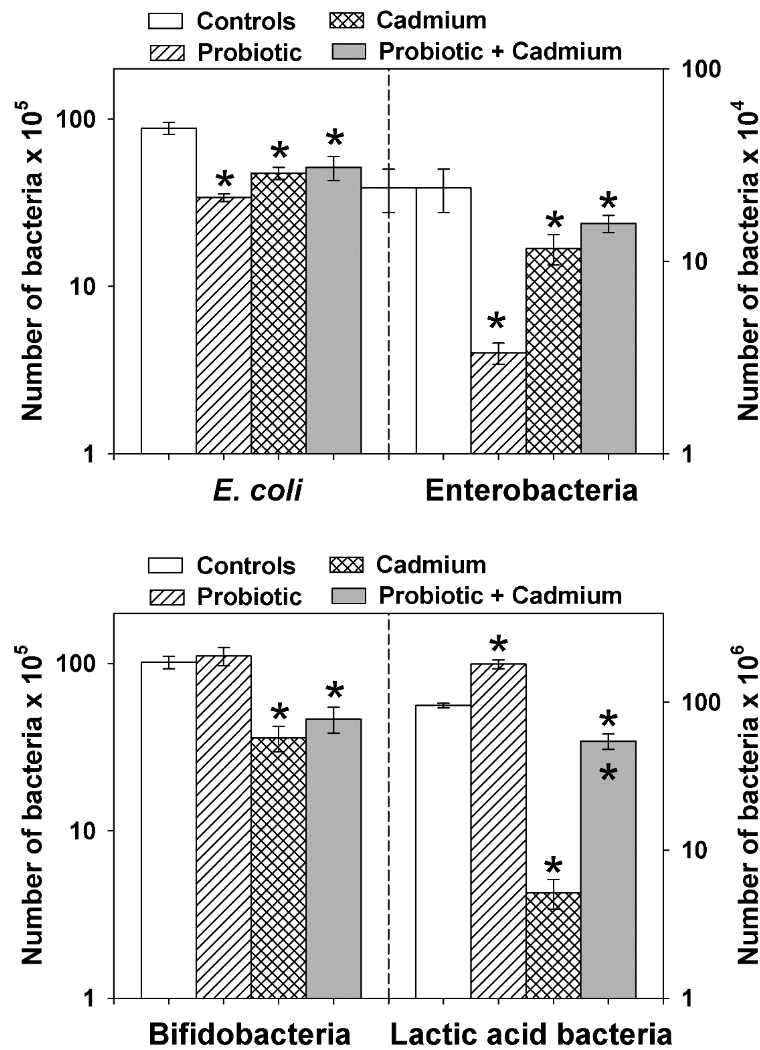


Figure 12. The number of E. coli, enterobacteria, bifidobacteria and lactobacilli in feces of controls and rats treated with probiotics, cadmium or their combination. An asterisk above the columns indicates a significant difference between control and treated group of animals. An asterisk inside the columns indicates a significant difference between cadmium group and combined diet (probiotic+cadmium) group of animals. The values represent Means of six animals ± S.E.M.

The compositions of microflora in feces of differently treated rats is shown in Figure 12. The counts of *E. coli* and enterobacteria in probiotic treated animals were lower in comparison with controls. Exposure to Cd also resulted in reduction of both enterobacteria (by 44%) and E. coli (by 48%) counts compared with controls. However, adding probiotic to Cd caused non-significant changes as compared to that of probiotic or Cd.

While the counts of bifidobacteria in feces of control and probiotic treated group were not significantly different, exposure to Cd significantly decreased abundance of bifidobacteria compared with control group (by 64%). Adding probiotic to Cd resulted in increase of bifidobacteria count, but this difference was not statistically significant.

The counts of lactobacilli in feces of probiotic treated animals were significantly higher compared with controls. While exposure to Cd significantly reduced the number of lactobacilli compared with control group (by about 95%), in animals co-treated with Cd and probiotic the number of lactobacilli was significantly increased comparing to Cd treated animals.

Discussion

Cadmium is a highly toxic and dangerous heavy metal with wide distribution in atmosphere. The major sources of exposure to cadmium are intake of polluted food, water, inhalation tobacco smoke or contaminated air (Nawrot *et al.*, 2006). For most mammals including humans, food is the primary source of cadmium in accidental intoxication, and gastrointestinal tract (GIT) thus represents the first target organ in cadmium poisoning (Klassen *et al.*, 2009). This also means that the GIT mucosa faces the severest cadmium exposure and is a crucial for protection of the host when oral intoxication is involved (Hulinska *et al.*, 1988). In the GIT only small percent of ingested inorganic cadmium is absorbed (according to different authors from 0.5 to not more than 5%), while remaining part is excreted in feces.

After uptake from the GIT, cadmium is transported in blood plasma bound to albumin and is preferentially taken up by the liver (Kowalczyk *et al.*, 2003). There, cadmium induces synthesis of metallothionein (MT) and forms cadmium-MT complexes. Few days later, these complexes appear in the blood plasma and because of their low molecular weight they are filtered through glomeruli. Nephrocytes of proximal tubules reabsorb cadmium-MT complexes by pynocytotic vesicles and send them to lysosomal compartment for degradation. Released cadmium induced synthesis of MT in nephrocytes, forms complexes with it and remains there for long period.

Although simplified, the description of cadmium route from absorption to accumulation points to liver and kidney as another two important target organs for cadmium intoxication. It is well known from numbers of earlier works that cadmium provokes significant histopathological lesions in all these organs which may be considered as biomarkers for the health evaluation of organisms exposed to toxicant, or, in other words, may serve as indicators of the severity of intoxication.

In our study, administration of cadmium at dosage 70 ppm in drinking water during five weeks showed that exposure to cadmium provoked clear symptoms of toxicity with reference to reduced body weight gain. Conversely, adding probiotic with cadmium resulted in improving of the body weight gain, which is in agreement with previous reports (Salim *et al.*, 2011). At the same time, there were no significant changes in food and water intake in cadmium treated and co-treated animals as compared to the control and probiotic treated animals, which is also in consistency with previous studies (Asagb and Eriyamremu, 2007). This phenomenon has already been reported in various species (Erdogan et al., 2005; Han et al., 2006; Rencuzogullari and Erdogan, 2007; Sant'Ana et al., 2005) and it was proposed that the action of the metallothioneins could be associated with diminished body weight gain. Namely, long-term exposure to Cd causes depletion of liver and muscular glycogen, due to its action on enzymes involved with glycogenesis (Toury et al., 1985), resulting in changes in the energetic metabolism.

In this study, we observed that the presence of cadmium in the Cd-exposed rats in liver, kidney and whole blood was significantly increased relative to control animals. It has been reported that accumulation of cadmium in both liver and kidney is greater than in any other organ (Andersen *et al.*, 1988), because these organs contain most of the metallothionein, which has a high affinity for cadmium. Taking probiotic with Cd resulted in a statistically significant decrease in cadmium level in liver, kidney and blood compared to cadmium treated animals, findings that are supported by studies carried out by Halttunen *et al.* (2007).

The level of cadmium in feces of cadmium treated rats was significantly increased compared to control animals, in concordance with reports that most of cadmium is excreted by feces (Klaassen and Kotsonis, 1977, Shim *et al.*, 2009). At the same time, cadmium accumulation in feces was significantly increased in co-treated rats comparing to control animals or cadmium treated group, and followed with concomitant decrease in cadmium

concentration in blood, liver and kidney. This results provide evidence that probiotics contributed to removal of cadmium from the body.

It has been reported that cadmium intoxication induces misbalance of bioelements such as Zn, Cu, and Mg in biological fluids, blood and urine, and other organs (Bulat, 2012). However, several studies provided evidence that supplementation with certain essential elements, especially zinc (Zn), selenium (Se) and natural herbs, for instance Nigerian-like diet, or naringenin and taurine (Bulat, 2012, Asagba and Eriyamremu, 2007, Renugadevi and Prabu, 2010, Sinha *et al.*, 2008) can have protective role against Cd toxicity. Furthermore, copper might have potential to reduce the accumulation and toxicity of cadmium (Kaji *et al.*, 1992a). Our results demonstrate significant increase in Cu level of the liver in Cd-exposed animals, with no change in concentration of this metal in the kidney. These results are partly in accordance with previous reports, showing an increase in the level of Cu in the liver of Cd treated rats (Stonard and Webb, 1976)

In this study, administration of cadmium chloride produced a significant increase in Zn concentration in both kidney and liver, and this increase could be related to the intervening effect of cadmium in absorption and distribution of Zn (Bunn and Matrone, 1966), or by metallothionein which synthesis was induced by cadmium (Nordberg, 1978). An increase in zinc levels in liver and kidney was reported in rats treated with cadmium (Eybl *et al.*, 2004), with explanation that zinc has protective effects against cadmium induced cytotoxicity (Kaji *et al.*, 1992b). On the other hand, administration of probiotic mixture showed no significant decrease in the level of zinc in liver comparing to control animals. In general, probiotic did not cause systematic alterations in zinc levels.

In line with the elevated cadmium levels in liver and kidney are results of histopathological analysis, showing that Cd alone provoked serious histopathological lesions in both tissues. Effects of cadmium intoxication on liver parenchyma were manifested by

serious histological destruction and inflammatory cell infiltration. Patterns of alterations observed are typical for cadmium intoxication and are previously reported and explained by others. In general, liver injury is mainly based on initial damage of hepatic endothelium, leading to ischemia, activation of Kupffer's cells and development of inflammatory response (Rikans and Yamano, 2000, Kuester *et al.,* 2002). Cadmium hepatotoxicity is explained by its direct effects on hepatocytes, especially on their mitochondria, as well as indirect effects mediated by damage of hepatic endothelium and activation of Kupffer cells, leading to ischemia and inflammation, respectively, and followed by all further pathological changes of structure and function (Rikans and Yamano, 2000, Kuester *et al.*, 2002). In addition, Kupffer cells also contribute to the hepatotoxicity of cadmium, by release of a variety of cytotoxic mediators, including reactive oxygen species, nitric oxide, and cytotoxic proteins that can directly damage hepatocytes. They also can release chemokine that promote the infiltration of other inflammatory cells to the liver. These in turn release cytotoxic agents that promote the progression of liver injury.

In kidney, tubular damage precedes progressive and irreversible glomerular changes and may explain impaired renal filtration and reabsorption (Järup *et al.*, 2000). The observed renal histological lesions are mainly localized in the re-absorptive (proximal tubules) and in the filtering part (glomeruli) of the nephron. The changes in the proximal convoluted tubules include vacuolization, loss of brush border, and in few places necrosis. The decrease in the intensity of PAS reaction that was evident in the brush border of the main tubules of rats treated with cadmium, and in less extent in those simultaneously treated with cadmium and probiotic, could indicate damage to the re-absorptive surface of epithelial cells. Our results are in line with similar changes observed and reported by other authors (Renugadevi and Prabu, 2009). Also, early pathological changes have been reported in rat’s kidneys after 6 weeks exposure to cadmium (Aughey, 1984), so it clear that significant nephrotoxicity comes after chronic

environmental or occupational exposure to cadmium (Kajikawa *et al.*, 1981, Itokawa *et al.*, 1978, Goyer, 1991). Although the exact mechanisms of cadmium-induced nephrotoxicity are still not completely understood, there are some evidences that it mediates oxidative stress, mitochondrial dysfunction and stress of endoplasmic reticulum.

When exposure to cadmium causes severe damaging on the liver, it can be manifested with increase in serum concentration of some of the liver specific marker enzymes (Kowalczyk *et al.*, 2003). Our study demonstrated a statistically significant elevation in serum of both aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as compared with control group. These findings are in consonance with other published reports which quoted that the concentrations of these parameters are increased during cadmium intoxication (Al-Hashem *et al.*, 2009, Kowalczyk *et al.*, 2003). The reason of this is the fact that apoptosis is a major model of elimination of critically damaged cells in acute cadmium hepatotoxicity and it precedes necrosis (Habeebu *et al.*, 1998, Kondoh *et al.*, 2002), and increased levels of both ALT and AST can be used as marker of hepatic cell injury and dead (Kowalczyk *et al.*, 2003). When the hepatocytes are damaged, as we proved by microscopically observation, these enzymes are released into the extracellular fluid which results in rise of their concentrations in circulation (Johnson, 1989).

However, our results also showed that probiotic supplementation together with cadmium diminished the cadmium induced rise of the AST and ALT activities, in line with those of Adawi and coworkers (Adawi *et al.*, 2001), which reported that administration of *Lactobacillus* and *Bifidobacterium* reduced the levels of hepatic enzymes in acute liver injury. This results are also in agreement with our histopathological analysis, showing considerably reduction of histopathological lesions, including necrosis, in case of co-administration of probiotics together with cadmium, as well as with the results showing significant reduction in

Cd blood and tissue content, followed with its simultaneously concentration increase in feces (Figs. 4 and 5).

Every kind of intoxication followed by tissue injury represents a stressful event for an organism. Since sympatho-adrenomedular system is one of the central systems of stress reactions, we decided to determine the blood concentrations of adrenaline and noradrenaline, as measure of its activity. Adrenaline and noradrenaline are hormones/neurotransmitters with several vital physiological effects in the cardiovascular, neuronal, metabolic and endocrine systems of mammals (Hoffman and Taylor, 2001). They are secreted during stress reaction from adrenal medulla and nerve endings of sympathetic nerve system. As can be seen from our results, blood adrenaline and noradrenaline levels were highly elevated in the animals exposed to the $CdCl_2$, preparing the animals for so called "fight or flight" reaction. These findings are in line with some previously published papers (Rastogi and Singhal, 1975), which showed that i.p. injection of $CdCl_2$ increased adrenal weights and augmented the levels of adrenal noradrenaline and adrenaline as well as the activity of adrenal tyrosine hydroxylase. Anyway, adding of a probiotic mixture to cadmium chloride restored the levels of adrenaline all the way back to the control values, while noradrenaline concentration remained elevated, although at lower level compared to that observed in $CdCl_2$ only exposed animals. This finding represents the direct proof of the findings regarding probiotics protective role in Cd intoxication, given that stress reaction is clearly diminished.

Environmental pollutions can stimulate production of reactive oxygen species (ROS), which cause significant oxidative damage to the cells of living organisms (Stohs and Bagchi, 1995). The results obtained regarding activities of SOD in the liver and kidney indicate that co-exposure during administration is able to increase the oxidative stress in the liver. SOD and CAT are important antioxidant enzymes that protect from enhanced peroxidation of lipids, which cause the damage in cells and tissues. SOD catalyzes the reaction of superoxide anion

radical (O_2^-) dismutation to hydrogen peroxide (H_2O_2), whereas CAT degrades H_2O_2 into a molecule of oxygen and a molecule of water (Stohs and Bagchi, 1995).

Our results showed that the increased activity of SOD in the liver as the result of cadmium intoxication is followed with decrease in the activity of CAT. Opposite to that, there is SOD activity decrease in kidney under influence of cadmium, while the CAT activity is decreased. However, in co-exposed animals aforementioned changes back to the control levels.

This results are fully supported with result of Dzobo and Naik (2013), showing liver catalase decrease and total SOD increase, as well as the kidney decrease of both total SOD and CAT activities in cadmium induced oxidative stress, or Štajn *et al.* (1997), showing kidney decrease of both total SOD and CAT activities under influence of cadmium

The decrease in catalase activity both in liver and kidneys of rats exposed to Cd might be a result of metal deficiency. It is known that Cd decreases the levels of iron (Fe) in the liver (Jurczuk *et al.,* 2004), and because Fe is a component of the active site of catalase, a decrease in Fe might result in a decrease in catalase activity.

SOD activity is known to be inhibited by Cd as Cd can replace Zn on the SOD molecule therefore resulting in an inactive enzyme (Bauer *et al.,* 1980), which can explain this enzyme changes in kidney. The SOD activity increase in liver could be linked with Cd-induced increase in lipid peroxidation, as SOD would have to remove excess ROS (Dzobo and Naik, 2013, Radosavljević *et al.,* 2012). At the same time, it should be noted that some authors showed SOD activity decrease in liver under influence of cadmium (Ognjanovic *et al.,* 1995).

It is known that heavy metals have inhibitory effects on the growth of a number of bacteria (Forsberg, 1978, Ravikumar *et al.*, 2007). In this study we noticed significant reduction of counts of all studied bacteria in the feces of rats treated with cadmium, compared to the control group. The most sensitive bacteria to the toxic effect of Cd were lactobacilli (which

toxicity increase in order *L. rhamnosus* (40%), bifidobacteria (64%), and *L. acidophilus* (95%), indicating the highest resistance of *L. rhamnosus* to $CdCl_2$), while *E. coli* showed the highest resistance to this heavy metal. Our results are similar to that of Fazeli *et al.* (2011), who reported that cadmium has toxic effect on gastrointestinal bacteria in mice, probably through oxidative deterioration of biomolecules, including DNA, proteins and lipids. Our investigation also demonstrated that treatment with the mixture of probiotic bacteria along with cadmium led to the increase in number of all studied bacteria, although that increase was significant only in the case of lactobacilli.

It is interesting that the treatment with probiotics alone did not affect the number of bifidobacteria and lactobacilli, but significantly reduced the number of *E. coli* and other enterobacteria compared to controls. Adawi *et al.* (2001) reported that oral administration of different probiotics decrease the population of *Entrobacteriaceae* in the gut, while Telez *et al.* (2012) found that probiotics can modulate the intestinal microflora in chickens and turkeys and provide the high resistance to *Salmonella* sp. infection. Itoh and Freter, (1989) reported that numerous probiotic strains may have controlled and inhibited the growth of *E. coli.* We presume that the cause of decreased number of enterobacteria may be the competition for space and nutrients with lactic acid bacteria continuously provided by probiotic. Adawi *et al.* (2001) reported that oral administration of different probiotic decrease the population of *Entrobacteriaceae* in the gut. Furthermore, it has been reported that a numerous of probiotic strains may have controlled and inhibited the growth of *E.coli* (Itoh and Freter, 1989). Feeding with strain of *B.longum* can lead to several effects that benefit to the host, such as production of short-chain fatty acids, reducing levels of ammonia and PH in faecal (Rowland *et al.*, 1998) inhibition of proliferation of pathogens and immunomodulation. The possible mechanisms by which probiotic decrease pathogenic bacteria in gut could be production of antimicrobial substances, competition between probiotic and pathogenic bacteria on nutrients or creating an

anaerobic conditions (Timmerman *et al.*, 2004). These conditions may be contributing factors which reduce a number of microorganisms in gut.

A number of reports convincingly demonstrate genotoxic potential of Cd in animals and mammalian cells (Waisberg *et al.*, 2003). In the present work we investigated protective effect of probiotic bacteria against liver genotoxicity induced by Cd, using Comet assay. The experiments were performed *in vivo* and *in vitro*, and genotoxic effect of Cd was significant in both cases. From our data it is obvious that after chronic *in vivo* exposure to Cd (5 weeks) the percent of DNA damage was higher comparing to short *in vitro* exposure (15 min). It is known that the genotoxicity of Cd involves induction of oxidative stress and inhibition of different DNA repair mechanisms. It is to be expected that both processess have occured during the chronic *in vivo* exposure, leading to substantial DNA damage. In the case of *in vitro* exposure, the time was probably too short for saturation of antioxidative defence of the hepatocytes and the amount of DNA lesions was consequently lower. In addition, low concentration of vitamine C, present in PROBIOTIC® preparation, could have acted as antioxidant and reduced oxidative stress.

Nowdays, there is an increasing interest in use of probiotic bacteria to mitigate the toxic/genotoxic potential of heavy metals. On the basis of our results it is clear that protective effect of probiotic bacteria aginst Cd induced genotoxicity exists. The protection was more pronounced in *in vitro* conditions (48% comparing to 20% *in vivo*), indicating that the most probable mechanism was direct binding of Cd^{2+} to probiotic bacteria.

The surface of lactic acid bacteria is composed of thick layer of peptidoglycan, teichoic acid, proteins and polysaccaharides; moreover some *Bifidobacterium* strains and *L. rhamnosus* GG are known to produce exopolysaccaharides. These structures contain different kinds of negatively charged groups like carboxyl, hydroxyl and phosphate groups. Therefore, probiotic bacteria have a great number of different possible ligands able to bind cations such as Cd^{2+}. It

is possible that, in the mixture of liver cells, probiotic bacteria and Cd *in vitro*, bacterial cells bind Cd^{2+} more efficiently than in the rat intestines, preventing more effectively the induction of DNA damage. The ability of probiotic strains to perform auto-aggregation, as well as co-aggregation with other bacteria in the intestines, might reduce binding of heavy metals (Kos *et al.*, 2003, Salim *et al.*, 2011), which could be one of the reasons for weaker protection observed *in vivo*.

Conclusion

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host. Knowledge is accumulating that lactic acid bacteria modulate gut physiology, immunological functions, and may produce many beneficial effects (Erickson and Hubbard, 2000). Administration of probiotics enhances the intestine immune system, decreases bacteria translocation and prevents the overgrowth of enteric pathogens; it also has beneficial effects on acute hepatocellular disease (Fernandes *et al.*, 1987, Mao *et al.*, 1996, Adawi *et al.*, 2001).

Our results showed that cadmium has inhibitory effects on the growth of the all of examined gut bacteria, in line with results from other reports. (Ravikumar *et al.*, 2007). In the group of rats treated with probiotic the number of *L. acidophilus* and *L. rhamnosus* in feces significantly increased in comparison to the control, while the number of bifidobacteria remained unchanged. In the very same way, in group treated with Cd plus probiotic, the number of *L. acidophilus* and *L. rhamnosus* significantly increased, comparing to Cd treated group, while the increase of bifidobacteria counts was insignificant.

Obtained results point at different behaviour of lactobacilli and bifidobacteria in relation to binding Cd^{2+}: it seems that only lactobacilli possess some capacity to bind Cd^{2+}, which enables their better survival. Bearing in mind report that feeding rats with *Bifidobacterium longum* decreased the levels of ammonia in the caecum and reduced the incidence of preneoplastic lesions induced by azoxymethane (Rowland *et al.*, 1998), we can conclude that probiotic potential of different bacterial strains, even within the same species, differs: different strains of the same species may have different areas of adherence (site-

specific), specific immunological effects, and the actions on healthy/inflamed mucosa may be distinct from each other.

The mechanisms by which probiotics exert biological effects are still poorly understood. The adsorption of heavy metals to the bacterial surface appears to be the main mechanism for their removal by probiotics. Halttunen *et al.* (2008) found that different lactic acid bacteria were effective in removal of heavy metals, mycrocystine-LR and aflatoxin B1. Moreover, they found clear differences in metals and toxins removal efficiency between the strains. The assessment of Cd^{2+} removal also indicated a strongly pH-dependent process, with the highest binding at neutral pH (Halttunen *et al.*, 2007). Knowing that pH values in different parts of gastrointestinal tract of rats vary from 5.3-7.4, we can propose that the highest binding of Cd^{2+} occurs in the large intestine where pH values are close to neutral.

The current study was designed to determine the impact of feeding mixture of probiotics on the biological effects of cadmium toxicity in the rat model.

1. Exposure of rats to cadmium chloride (70ppm) alone resulted in significant decrease in body weight gain compared with the control group. The body weight in co-treated animals was increased compared with the cadmium treated group. Overall, there were not significant changes between all of the experimental groups in both food and water intake.
2. The present results revealed that probiotics in a mixture with cadmium acted beneficially to an organism, increasing the cadmium concentration in feces, and consequently decreasing its concentration in blood and both liver and kidney. On the other hand, administration of cadmium alone caused a significant increase of zinc concentrations in both organs, kidney and liver.

3. Administration of cadmium showed alterations in histopathological changes in liver. Parenchyma of liver was evidently changed after Cd-treatment. Sinusoids were dilated, especially in the proximity of the central vein, and hepatocytes often contain nuclei with compacted chromatin, while nucleoli were not visible. Kupffer cells showed morphological characteristics of activation. At the same time, in co-treated rats the degenerative changes of liver parenchyma are extenuated but still evident.
4. It was showed in this study that administration of cadmium increased the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum of the cadmium treated animals. On the other hand, adding probiotics simultaneously with cadmium chloride restored the altered values to the normal level.
5. The histopathological changes in kidneys of rats were also evaluated. In the cadmium treated group, proximal tubules epithelium consists of swollen or necrotic cells, necrosis of the whole tubules was reported, there is no visible pas-positive brush border, nor inducing glomerular shrinkage. At the same time, in co-treated rats the degenerative changes of kidney are extenuated. There were no differences observed between the probiotics and the control group.
6. The activities of antioxidant enzymes such as total superoxide dismutase (SOD) and catalase (CAT) were measured in. The activity of CAT decreased in both liver and kidney in the cadmium treated animals, with no changes in the co-treated group compared to the control group. On the other hand, pattern of SOD activity changes differ between liver and kidney: in liver, cadmium causes SOD activity increase, while in kidney this enzyme activity decreases. However, in the co-treated animals SOD activities back to control level.

7. Adrenaline and noradrenaline were significantly increased in animals treated with $CdCl_2$ compared to the controls, while probiotics alone did not affect the amount of these catecholamines. At the same time, levels of both adrenaline and noradrenaline decreased in the co-treated animals.
8. The treatment by cadmium chloride resulted in significant increase of DNA damage in both exposures *in vitro* and *in vivo.* On the contrary, cadmium given simultaneously with probiotics produced a significant decrease in genotoxicity which was induced by cadmium.
9. Administration of cadmium resulted in significantly reduced numbers of all different types of microflora which were accounted for in this study. At the same time, adding probiotic to Cd resulted in increased only of lactobacilli comparing to Cd treated animals.
10. Overall, present results indicate that probiotics actively contribute in cadmium removal from an organism, probably by binding to their bacterial cell wall, as proposed earlier.
11. Further researches are needed to define specific probiotic combination that would be most effective in binding Cd^{2+} and other heavy metals, as well as different toxins and carcinogens.

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Прилог 1.

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Изјављујем

да је докторска дисертација под насловом

Protective Effects of Probiotics in the Model of Cadmium Toxicity in Rats

Заштитни ефекат пробиотика на моделу токсичности кадмијума код пацова

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, **16.07.2013.**

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора **mr Adel Masoud Jama**

Број уписа **2423** (број пријаве докторске дисертације **10/35-9.05.2013.**)

Студијски програм ____

Наслов рада

Protective Effects of Probiotics in the Model of Cadmium Toxicity in Rats

Заштитни ефекат пробиотика на моделу токсичности кадмијума код пацова

Ментор **др Синиша Ђурашевић, ванредни професор Биолошког факултета Универзитета у Београду**

Потписани **mr Adel Masoud Jama**

изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, **16.07.2013.**

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Protective Effects of Probiotics in the Model of Cadmium Toxicity in Rats

Заштитни ефекат пробиотика на моделу токсичности кадмијума код пацова

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство

2. Ауторство - некомерцијално

3. Ауторство – некомерцијално – без прераде

4. Ауторство – некомерцијално – делити под истим условима

5. Ауторство – без прераде

6. Ауторство – делити под истим условима

(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанда

У Београду, **16.07.2013.**