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Thesis

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Antioxidant efficiency of probiotic bacteria and their role in reducing drug toxicity

Under the Supervision of: **Pr. SIFOUR Mohamed**

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ALP	Alkaline phosphatase
ALT	Alanineaminotransferase
AST	Aspartate aminotransferase
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAT	Catalase
CFU	Colony forming unit
COX	Cyclooxygenase
CPP	Cumulative probiotic potential
DNA	Deoxyribo Nucleic Acid
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
DTNB	5,5-dithiobis-2-nitrobenzoic acid or
EPS	Exopolysaccharides
GI	Gstrointestinal
GPx	Glutathione peroxydase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-transferases
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radicals
LAB	Lactic acid bacteria
LPO	Lipid perxidation
MDA	Malondialdehyde

MRS	de-Man-Rogosa Sharp
NAPQI	Acetyl-*p*-benzoquinone imine
NSAID	Non-steroidal anti-inflammatory drugs
$O^{\bullet}_2$	Superoxide anion radicals
PBS	phosphate buffer solution
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SOD	Superoxide dismutase
TBA	Thiobarbituric Acid
TCA	Trichloroacetic acid solution
UHT	Ultra high temperature
WHO	World Health Organization

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Ouled-Haddar H; Abbas N; **Riane K**; Sifour M; Idoui T (**2017**) Effective chromium removal capacity of probiotic lactic acid bacteria. VII International Conference on Environmental, Industrial and Applied Microbiology **(Bio Micro World 2017)**, 18-20 October 2017, Madrid (Spain)

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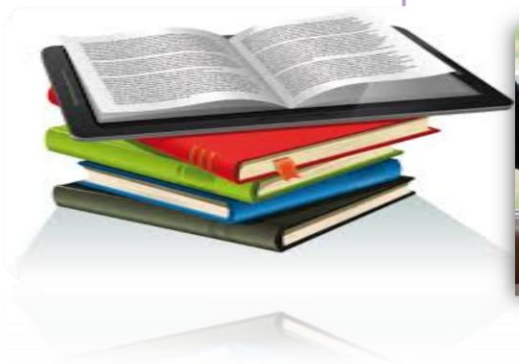
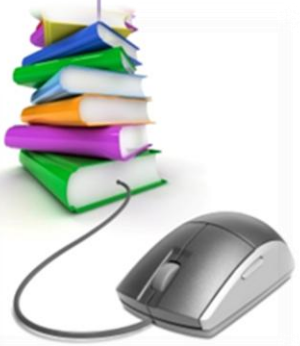
Introduction





Oxygen is an indispensable element for our life; under certain cases it has detrimental effects on human health. Most of the potential harmful effects of oxygen are due to the formation of free radicals and reactive oxygen species (ROS), which have a tendency to donate oxygen to other substances, thereby damaging biologically relevant molecules such as DNA, proteins, carbohydrates and lipids **(Young and Woodside, 2001)**. The negative effects of reactive oxygen species can be balanced by antioxidant defense mechanisms that form the biological antioxidant barrier **(Wang et al., 2009)**. However, in certain circumstances such as age, repeated exposure to pollution, radiation, and other stressors, the defense system fails to protect the body against oxidative stress **(Amaretti et al., 2013)**. In addition, there is evidence that all drugs still have side effects despite the efforts made to produce drugs without side effect. Furthermore, literature report implication of oxidative stress in drug-induced toxicity **(Deavall et al., 2012)**. So it is a reasonable assumption that the antioxidant might alleviate the toxicity induced by these drugs.

Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases **(Zhang et al., 2011)**. Natural antioxidants, such as tocopherols, vitamin C, flavonoids and synthetic antioxidants, such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA), and ethoxyquin have been shown to have some beneficial effects to neutralize the oxidant molecules. However, synthetic antioxidants are not always beneficial for human health **(Lobo et al., 2010)**. There are many controversies around the use of these synthetic antioxidants in foods, since both BHT and BHA seem to have tumor-promoting activities and anti-carcinogenic properties **(Baszczyk et al., 2013)**. Hence, the search for effective, nontoxic natural compounds with anti-oxidative activity has been intensified in recent years **(Nikhade et al., 2019)**.

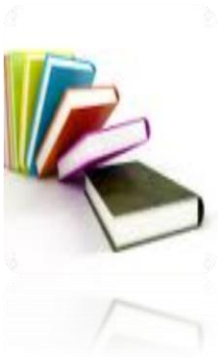
Several studies have supported the potential health benefits of probiotics, such as the improvement of gastrointestinal microbiota ecosystems, stimulation of the immunological system, anticarcinogenic activities, and reduction of oxidative stress **(Martarelli et al., 2011; Moro-Garcia et al., 2013)**. It is reported that consumption of probiotics alone or foods supplemented with probiotics may reduce oxidative damage, free radical scavenging rate, and modification in activity of crucial antioxidative enzymes in human cells. Thus, probiotic supplementation may result in improving antioxidant status **(Mishra et al., 2015; Zamani et al., 2019)**.

Hence, the development of probiotics exerting antioxidant activity and counteracting the oxidative stress in the host may represent a novel pioneering approach. In this direction, the present study aimed to identify probiotic bacteria with antioxidant capacity. In this view, we hypothesized that probiotic bacteria with antioxidant potential are able to reduce oxidative stress induced by administration of the most common drugs with side effects (non-steroidal anti-inflammatory drugs).

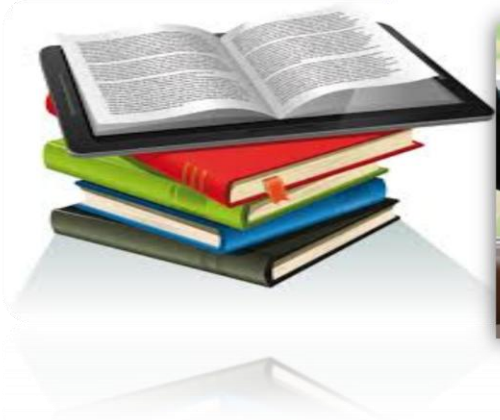
Thus, the main research objective consists in identifying novel probiotic bacteria with antioxidant capacity and assessing its ability to reduce oxidative stress generated by drug administration in rats.

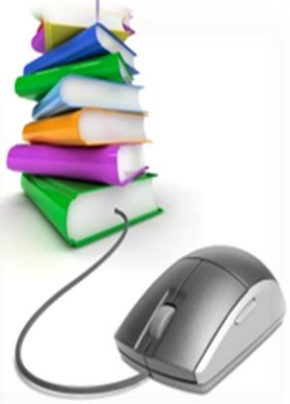
The objectives of the study were to:

- ✓ Find bacteria with antioxidant abilities.
- ✓ Assess antibacterial, bile salt and pH tolerance, and antibiotic sensitivity of the selected strains will be performed to investigate its probiotic potentialities.
- ✓ Evaluate the antioxidant efficiency in *vitro*, through assessment of its ability to scavenge hydroxyl radical, chelate iron ions, and inhibit lipid peroxidation.
- ✓ Evaluate the effect of selected probiotic bacteria on oxidative stress induced by drugs overdose.



Literature review





II.1. Oxidative stress and antioxidants

Oxidation plays a fundamental role in many living organisms within the production of energy necessary for all biological processes **(Esteban et al., 2014)**. One of the paradoxes of life on this planet is that the molecule that sustains aerobic life, oxygen, is not only fundamentally essential for energy metabolism and respiration, but it has been implicated in many diseases **(Palmieri and Sblendorio, 2007; Achuthan et al., 2012; Baba et al., 2014)** such as cancer, emphysema, atherosclerosis, arthritis, diabetes and accelerated aging by causing oxidative stress and oxidative damage **(Halliwell and Whiteman, 2004; Zhang et al., 2011)**. However, this fact is the origin of the disruption of the delicate balance between generation of reactive oxygen species (ROS) and antioxidant scavenging systems, known as oxidative stress which induces serious biological molecules damages **(Lin and Yen, 1999; Heo et al., 2005)**.

The term reactive oxygen species is used by investigators to include both oxygen-centered radicals and non-radical derivatives of oxygen that are continuously produced *in vivo* **(Andersen, 2004).** A large variety of reactive oxygen species can be formed in the human body and in food systems **(Lin et and Yen, 1999)**, including free radicals, namely superoxide anion radicals, hydroxyl radicals, and non-free radical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen **(Wang et al., 2017a).** Oxygen free radicals can develop during several steps of normal metabolic events. Cells under aerobic conditions are always threatened with the insult of ROS which are efficiently taken care of by the highly powerfull antioxidant systems of the cell. Once the balance betweeen ROS production and antioxidant defenses is lost, oxidative stress occurs which through a set of events perturbates the cellular functions leading to various pathological conditions **(Hemnani and Parihar, 1998; Bandyopadhyay et al., 1999)**.

Thus, in a normal cell there is a balance between formation and removal of free radicals **(Shinde et al., 2012)**. However, this balance can be shifted and cause a disruption between the production of FR or ROS, and the antioxidant defense system **(Baynes, 1991)**. Many factors can force organisms to experience oxidative stress such as cigarettes, herbicides, nitrogen oxides, ozone, radiation, some metal and also drugs **(Mikelsaar and Zilmer, 2009; Adly, 2010; Birben et al., 2012)**. Due to their highly reactive nature, ROS can modify other oxygen species, DNA, proteins, or lipids (Figure 01). It is believed that excessive amounts of ROS can cause genomic instability **(Ames et al., 1993)**, leading to a variety of chronic

diseases, including atherosclerosis, arthritis, diabetes, alzheimer's disease, neurodegenerative and cardiovascular diseases **(Eftekharzadeh et al., 2010; Ceriello et al., 2004)**.

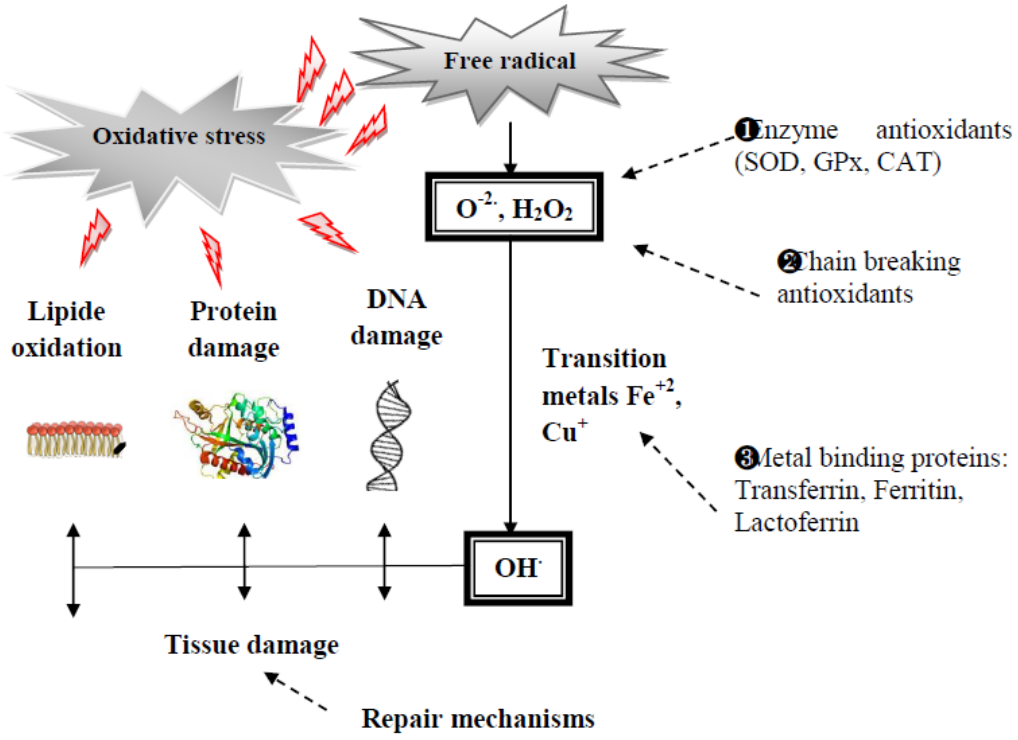


Fig. 01. *Oxidative stress and antioxidant defense* **(Young and Woodside, 2001)**.

Although the harmful effect of ROS, they play a key role for cell signaling **(Morrell, 2008)**. In the past decades, evidences suggest that ROS could serve as second messengers to regulate biological processes **(Finkel, 2011)**. Therefore, oxygen species are important players in maintaining normal physiology. A lack of Oxygen species could have significant physiologic effects, and excessive oxygen species can also contribute to cell injury **(Morrell, 2008)**.
Cells can tolerate moderate oxidative loads by increasing gene expression to up-regulate their reductive defense systems and restore the oxidant/antioxidant balance. But when this increased synthesis cannot be achieved due to enzymes damage, or substrate limitations, or when the oxidative load is overwhelming, an imbalance persists and the result is oxidative stress **(Halliwel, 2005)**.

To counteract the harmful effects of ROS produced in the course of normal physiological conditions, humans have evolved several complex antioxidant strategies **(Rahal et al., 2014)**. The human antioxidant system can be divided into two major groups, enzymatic antioxidants and non-enzymatic antioxidants (Table 01). These protective mechanisms scavenge or

detoxify ROS, block their production, or sequester transition metals that are the source of free radicals. Both enzymatic and non enzymatic antioxidant systems are necessary for sustaining life by maintaining a delicate intracellular redox balance and minimizing undesirable cellular damage caused by ROS **(Rahal et al., 2014)**. Antioxidant can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins **(Young and Woodside, 2001)**.

Table 01. *Classification of antioxidant defenses* **(Carocho and Ferreira, 2013)**.

Enzymatic antioxidants		**Non-enzymatic antioxidants**
The primary defense	- Glutathione peroxidase - Catalase - Superoxide dismutase	- Uric acid - Vitamins (A, C, E,K) - Flavonoids - Glutathione
The secondary defense	- Glutathione reductase - Glucose-6-phosphate dehydrogenase	

Antioxidant enzymes act to scavenge free radicals by converting them to less harmful molecules **(Sies, 1997)**. Among the most known enzymatic antioxidants, we notice superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxydase (GPx) and catalase (CAT). The antioxidant enzymes CAT and SOD constitute the first line of enzymatic antioxidant defense against free radicals. SOD catalyses dismutation of super oxide anion into hydrogen peroxide and molecular oxygen; which is a less harmful product, and then the hydrogen peroxide was removed by CAT an iron-containing hemoprotein and glutathione, byconverting hydrogen peroxide to water and oxygen **(Hsu et al., 2015)**. GPx is an enzyme containing a selenium ion as a cofactor, and to catalyzethe reaction it requires reduced glutathione (GSH), which is provided by glutathione reductase. GPx is one of the most effective antioxidants in erythrocytes. A reduction in GPx activity results in increased H_2O_2 levels and hence severe cellular damageis observed **(Cheeseman and Slater, 1993)**. Chain breaking antioxidants are ‘‘small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable byproducts’’ (**Young and Woodside, 2001)**.

Non-enzymatic antioxidants, such as glutathione, tocopherols, retinols, and ascorbate, play an important role in scavenging ROS. Such antioxidants are divided into lipid phase antioxidants

that scavenge radicals in membranes and lipoprotein particles such as vitamin E and aqueous phase antioxidants as vitamin C which will directly scavenge radicals present in the aqueous compartment **(Young and Woodside, 2001; Powers and Jackson, 2008)**.

Transition metal binding proteins, the major essential transition metals in human diseases are iron and copper that play a key role in the production of hydroxyl radicals through Fenton reaction as demonstrated below **(Stohs and Bagchi, 1995)**:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$$

Transition metal binding proteins such as ferritin, transferrin, lactoferrin, and caeruloplasmin act as a pivotal component of the antioxidant defense system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. Caeruloplasmin may function as an antioxidant via the rapid oxidation of the Fe^{2+} form that drives the Fenton reaction to the less reactive Fe^{3+} form as shown in the reaction below **(Young and Woodside, 2001)**:

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

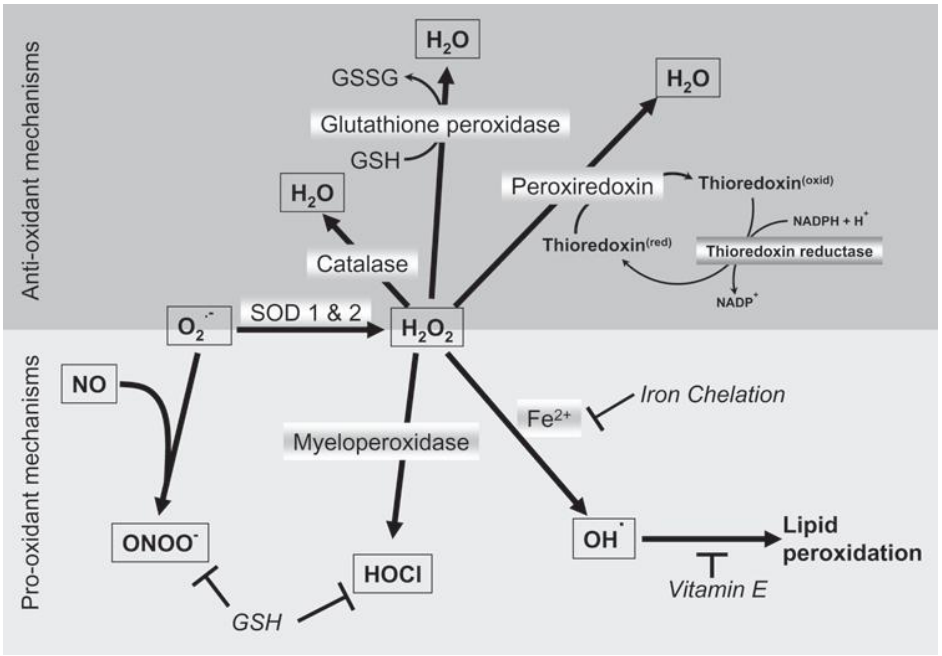


Fig. 02. *Cellular mechanisms of oxidant generation and scavenging* **(Jaeschke et al., 2012)**.

Thus, in order to cope with an excess of free radicals produced upon oxidative stress, the body has several mechanisms to maintain redox homeostasis **(Masella et al., 2005)**. Either

naturally generated *in situ* (endogenous), or externally supplied through foods(exogenous). The role of antioxidants is to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention **(Pham-Huy and Pham-Huy, 2008)**. However, the balance can be lost because of the overproduction of free radicals, by exposure to sources that overwhelm the oxidant defenses, or by inadequate intake of nutrients that contribute to the defense system **(Bandyopadhyay et al., 1999)**.

An antioxidant can be defined as: "any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents the oxidation of that substrate" **(Halliwell and Gutteridge, 1995**). An ideal antioxidant should be readily absorbed, quench free radicals, and chelate redox metals. It should also work in both aqueous and/or membrane domains (**Valko et al., 2006; Al-Dalaen and Al-Qtaitat, 2014)**. Antioxidants from our diet play an important role in helping endogenous antioxidants to protect cells from ROS effect and enhance the antioxidant status **(Brambilla et al., 2008)**. Nowadays, antioxidants are used on a large scale to try to obtain and preserve optimal health. Both synthetic and natural antioxidants are used. However, there are doubts about the safety and long-term effects on health of synthetic antioxidants such as butylatedhydroxytoluene (BHT), which are suspected to have some toxic effects **(Heo et al., 2005; Li et al., 2014)**. Therefore, it is desirable to develop natural nontoxic antioxidants with possible antioxidant and/or radical scavenger properties as alternatives to synthetic ones in pharmaceutical and food industries **(Heo et al., 2005)**.

II.2. Probiotics

The concept of probiotics evolved at the beginning of the 20th century from a hypothesis proposed first by the Russian scientist **Elie Metchnikoff** who got a Nobel Prize. The later suggested that Bulgarian peasants that consume a diet with fermented milk with the bacterium«Bulgarian bacillus » (later was classified as *Lactobacillus bulgaricus*) promote their longevity **(Vasiljevic and Shah, 2008; Soccol et al., 2010; Anandharaj et al., 2014 Franz et al., 2014)**. He believed that these bacteria affect the gut microflora positively and decrease the microbial toxic activity in human intestine **(Kumar et al., 2012; Franz et al., 2014)**. The word probiotic comes from two Greek words « pro bio » meaning « for life » unlike antibiotics which means «against life» **(Vasiljevic and Shah, 2008; Reddy et al., 2011; Chong, 2014; Xiao et al., 2014)**. Several definition for probiotics have been suggested, and one of the most recently adopted cited that probiotics are defined as organisms that confer

health benefit to consumers when administered in the adequate amount **(Fakruddin et al., 2017)**. Microbes used as probiotics are derived from different genera and species and have been studied for a variety of health and disease endpoints. Both yeast (*Saccharomyces cerevisiae*) and bacteria are used as probiotics, including lactic acid bacteria (LAB); (such as species of *Lactobacillus*, *Streptococcus* and *Enterococcus*), *Bifidobacterium*, *Propionibacterium*, *Bacillus* and *Escherichia coli* **(Sanders et al., 2010)**.

An effective probiotic strain must require several basic properties. Survival and adhesion within the gastro intestinal tract, production of antimicrobial substances, antagonism against pathogenic bacteria and safety in human use are considered as the most important selection criteria of a good probiotic strain **(Vasiljevic and Shah, 2008; Yang et al., 2015)**. Probiotics were originally used to improve the health of both animals and humans through the modulation of the intestinal microbiota **(Grajek, 2005)**. In addition, they play an important role in the treatment of diarrhea, lactose intolerance, inflammatory bowel disease, cancer prevention, reduction of serum cholesterol, enhancement of the immune response **(Shah, 2010; Afify et al., 2012; Homayouni et al., 2012; Kechagia et al., 2012)**. Moreover, probiotics, which are capable of colonizing the intestinal tract, are reported to improve metabolic diseases such as obesity and diabetes through modulating intestinal microorganisms **(Gomes et al., 2014; Wang et al., 2015)**. In addition to the beneficial effects mentioned above, in recent decades, probiotics have attracted a great attention as new antioxidant **(Mandal et al., 2013)**.

Mechanisms of probiotic action described to date (Figure 03) include adhesion to the intestinal-lumen interface; competition with pathogens for receptor binding, nutrients and colonization; enhancement of mucosal barrier function; promotion of innate and adaptive immune responses; elaboration of bacteriocins; and modulation of cell kinetics, with further mechanisms of action likely to be identified **(Howarth and Wang, 2013)**.

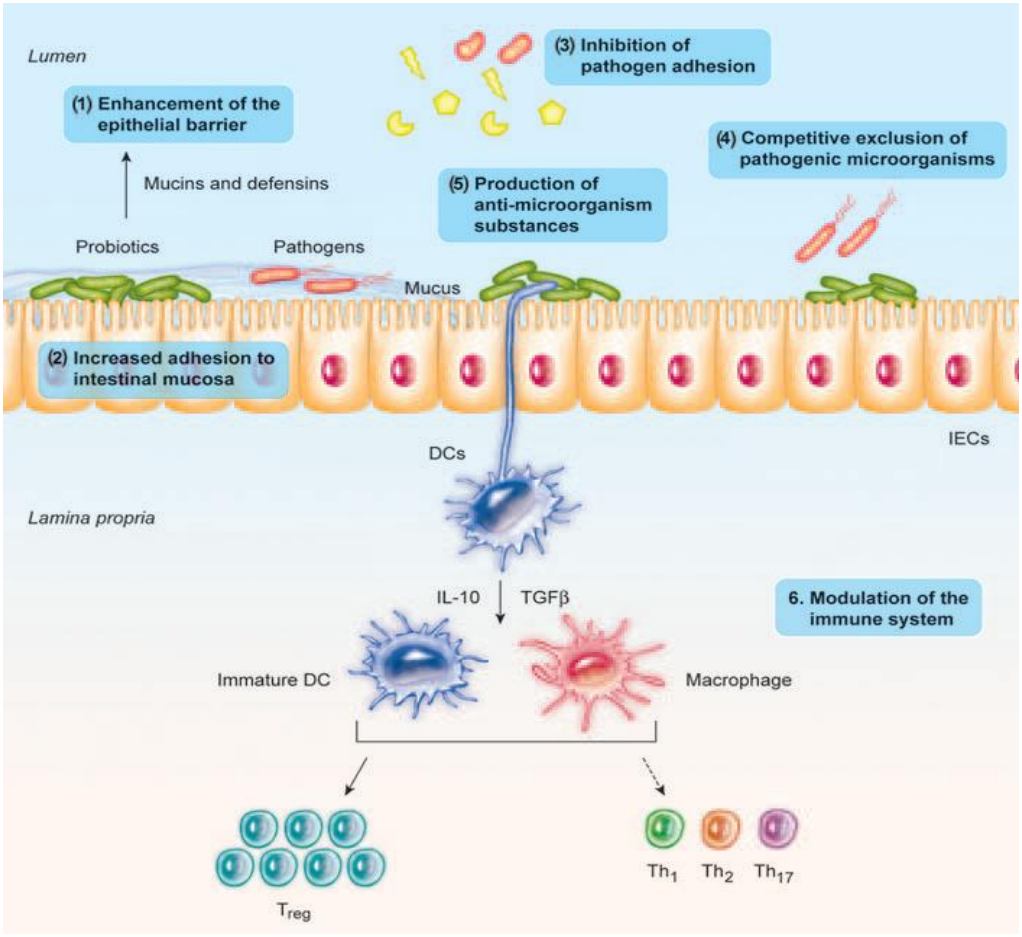


Fig. 03. *Probiotic mode of action* **(Bermudez-Brito et al., 2012)**.

II.3. Antioxidative efficiency of probiotics

As well as their several potential health benefits including reduction and prevention of diarrheas of different origin, improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergy, enhancement of immune potency, and antitumorigenic activities, a number of probiotic strains were demonstrated to possess antioxidative action in *vivo* and in *vitro* **(Songisepp et al., 2004; Kim et al., 2006a; Cinque et al., 2011)**. Three strains of LAB,*Lactobacillus casei, Lactobacillus acidophilus* and *Lactococcus lactis* were studied in fermented milkfor antioxidant activity *in vitro* and *in vivo* by feedinginto the gut of mice with diet supplemented by fermented milk. All three selected strains exhibited potent 2,2-diphenyl-1-picrylhydrazyl, malonaldialdehyde and hydrogen peroxide radical scavenging abilities as well as inhibition of linoleic acid peroxidation activity. These activities were highest in *Lb. casei* as followed by *Lb.acidophilus* and *Lb. lactis.* These results indicate that, selected LAB strains have good antioxidant activity **(Jain et al., 2009)**.

It is reported that *Lactobacillus* and *Bifidobacterium* strains present antioxidative activity, were able to reduce the risk of accumulation of ROS in a host organism and could potentially be used to reduce oxidative stress during ingestion of food **(Kim et al., 2006a; Jonkers and Stockbrugger, 2007; Cinque et al., 2011; Mahrous, 2011; Zhang et al., 2011)**. **Truusalu et al., (2004)** tested the ability of selected probiotic *Lactobacillus* spp. (with high antimicrobial and antioxidative potential in *in vitro* tests) to compete with invasive *Salmonella enterica* serovar *typhimurium* infection and protect the gut mucosa against excessive oxidative stress during inflammatory tissue damage in amouse model. The study concluded that the administration of probiotic *Lactobacilli* of human origin did not increases the colonization resistance against *S. typhimurium* infection in mouse gut, but demonstrated the ability of specific probiotic *Lactobacilli* to reduce pro-oxidant levels and oxidative stress indices, thus improving gut mucosal antioxidative activity.

In addition, Thirty-four strains of lactic acid bacteria (7 *Bifidobacterium*, 11 *Lactobacillus*, 6 *Lactococcus* and 10 *Streptococcus thermophilus*) were assayed *in vitro* for antioxidant activity. Antioxidant assay was carried against ascorbic acid and linolenic acid oxidation (TAA (AA)), trolox-equivalent antioxidant capacity (TEAC), intracellular glutathione (TGSH) and superoxide dismutase (SOD). Wide dispersion of each of TAA (AA), TEAC, TGSH, and SOD occurred within bacterial groups, indicating that antioxidative properties are strain specific. The strains *Bifidobacterium animalis* subsp. *lactis* DSMZ 23032, *Lactobacillus acidophilus* DSMZ 23033, and *Lactobacillus brevis* DSMZ 23034 exhibited the highest TAA (AA), TEAC, and TGSH values within the *Lactobacilli* and *Bifidobacteria*. Furthermore, the administration of probiotics exhibiting high values of antioxidative properties protected rats against doxorubicine induced oxidative damage **(Amaretti et al., 2013)**. In humans *Lb. rhamnosus* a powerful antioxidant strain could enhance redox status and neutralize the effects of reactive oxygen species in Athletes exposed to oxidative stress (**Martarelli et al., 2011)**. However, it should be noted that several studies reported the ability of probiotic bacteria to improve the antioxidant defense system of the host **(Mandal et al., 2013)** and these ability is strain specific. **Table 02** summarized some probiotic strains with their antioxidant effect.

Table 02. *Antioxidative effects of some probiotics.*

Strains	Antioxidative effect	References
***Lactobacillus casei* Zhang**	A decrease of MDA and increase of SOD and GSH-Px in serum and liver	**(Zhang et al., 2010)**
***Lactobacillus fermentum*ME-3**	Enhanced total antioxidative status	**(Songisepp et al., 2005)**
Engineered *Lactobacillus gasseri*	Antioxidant enzymes (SOD) reduce the inflammation in the case of colitis	**(Carroll et al., 2007)**
Lactococcus lactis	Antioxidative superoxide dismutase (SOD) activity	**(Zommara et al., 1996)**
***Lactobacillus casei* KCTC 3260**	Antioxidative capacity caused by chelating metal ions	**(Lee et al., 2005)**
***Lactobacillus acidophilus* KCTC 3111**	Has antioxidative effect	**(Kim et al., 2006a)**
***Enterococcus faecium* (BDU7)**	Has antioxidant property by producing exopolysaccharides	**(Abdhul et al., 2014)**
***Lactobacillus gasseri* NLRI 312**	Reduce DNA damage induced by H_2O_2	**(Kim et al., 2006b)**
***Streptococcus thermophilus* YIT 2001**	A significant decrease of lipid peroxide in the colonic mucosa	**(Ito et al., 2003)**
Bacillus polyfermenticus	Lower plasma lipid peroxidation levels and higher plasma total antioxidant levels	**(Paik et al., 2005)**
Probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei*	Lower values of TBARS and higher values of glutathione in liver and pancreatic tissues	**(Yadav et al., 2007)**
Probiotic yoghurt containing *Lactobacillus acidophilus* LA-5 and *Bifidobacterium* BB-12	An increase of SOD and catalase activity	**(Chamari et al., 2008)**

II.4. Mechanisms of action of probiotics

Many studies indicate that LAB are classified as a good candidates for development of new strains with antioxidant activity, however, little is known about their antioxidant potential mechanisms **(Kullisaar et al., 2012; Wu et al., 2014**). Probiotics ability to act as antioxidant can be attributed to the presence of antioxidant enzymes such as SOD, CAT, GPx, GR, and GST, to the release of antioxidant compounds acting mainly as free-radical scavengers such as glutathione, to the production of extracellular polysaccharide (EPS) biomolecules showing an *in vitro* antioxidant and free radical scavenging activities **(Cinque et al., 2011; Spyropoulos et al., 2011; Afify et al., 2012)**, and to the exhibition of metal chelatingactivity **(Amaretti et al., 2013)**. Some probiotics like *Lb. fermentum* ME-3 possessed substantial and a high antioxidative activity expressed manganese superoxide dismutase, can effectively eliminated hydroxyl radicals and contained the complete glutathione system (GR, GPx) necessary for glutathione recycling, transporting and synthesis (figure 04) **(Kullisaar et al., 2002)**.

Metabolic activities of probiotic bacteria may have shown the antioxidative effect through the scavenging of oxidant compounds or the prevention of their generation into the intestine. Production of bioactive peptides has been considered as an effective mode of antioxidative activity in foods containing probiotic bacteria **(Coda et al., 2012)**.

Furthermore, the intestinal microflora provides additional enzymatic activities involved in the transformation of dietary compounds, thus increasing the bioavailability of dietary antioxidants **(Davis and Milner, 2009)**. Some authors hypothesize that probiotics exert their protective effects against oxidative stress by restoring gut microbiota **(Forsyth et al., 2009; Nardone et al. 2010)**. In case of *Lactobacillus* spp., it has been concluded that their antimicrobial effect is also expressed via ROS like hydrogen peroxide, superoxide anions and hydroxyl radicals which may have a selective influence on the intestinal microbiota while, similar mechanism has been described for oral *Streptococci* **(Garcia-Mendoza et al., 1993)**.

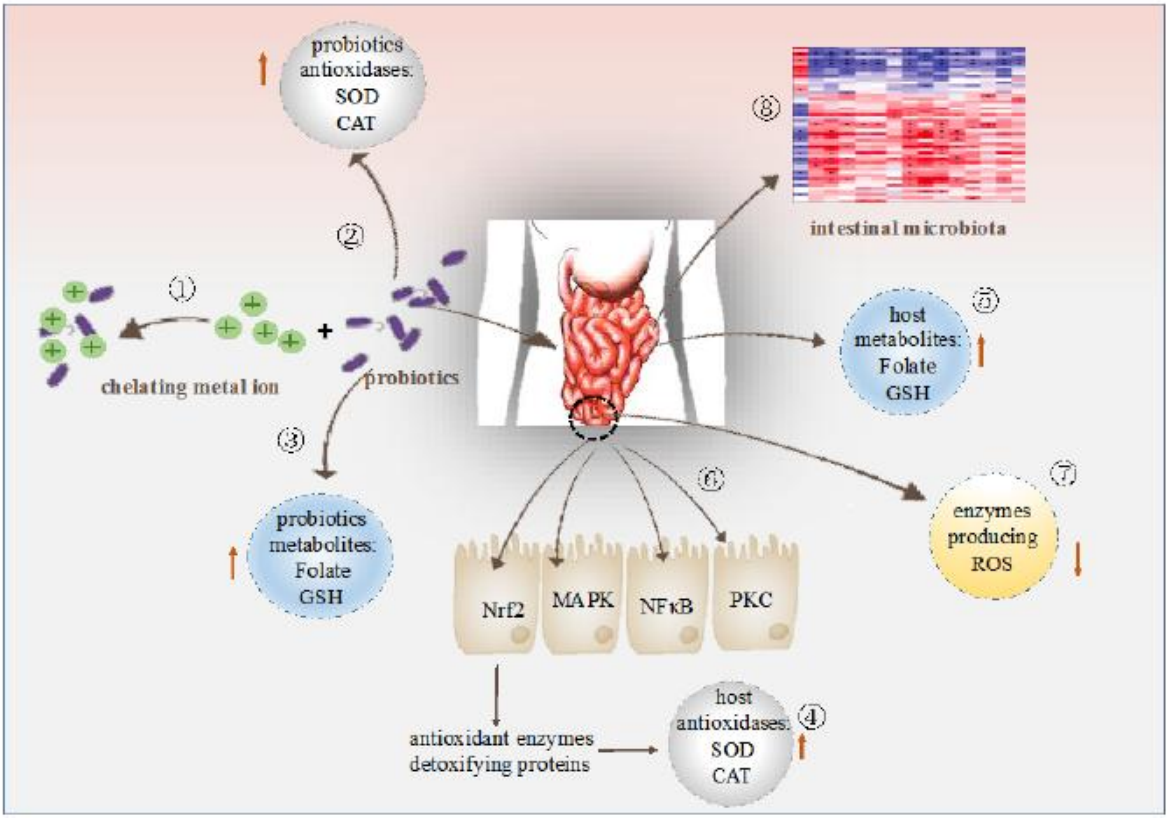


Fig. 04. *Mechanisms of action of probiotics as antioxidant* **(Wang et al., 2017a)**.

(1) *Probiotics chelate metal ion.* ***(2)*** *Probioticspossess their own antioxidases.* ***(3)*** *Probiotics produce antioxidant metabolites.* ***(4)*** *Probioticsup-regulate antioxidase activities of the host.* ***(5)*** *Probiotics increase levels of antioxidant metabolites ofthe host.* ***(6)****Probiotics regulate signaling pathways.* ***(7)*** *Probiotics down-regulate activities of enzymesproducing ROS.****(8)****Probiotics regulates intestinal microbiota*

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II.5. Limitations regarding probiotic as antioxidants

The discussion of **Brambilla et al. (2008)** will be mentioned here “according to the popular idea "if one is good two is better", antioxidants are taken in excess too often and the risk to originate diseases instead of preventing them is quite high. It is noteworthy to underlie that as for all drugs, antioxidants may give important side effects if not correctly used or in combination with other drugs. Vitamin A, E and β-carotene, for instance, have been shown to have prooxidant effects at higher doses or under certain conditions”.

Notwithstanding that antioxidants are effective for many diseases, but on the other hand, they failed to deliver satisfying protection. One of the limitations regarding antioxidants are cell cultures that can be altered with time which were often react with the medium and neutralized

very quickly, thus leading to false results. Another limitation is that several antioxidant assays also pose problems and difficulties when comparing results with different procedures and researchers. As a consequence, serious problems raise that can not help scientific community to move forward **(Carocho and Ferreira, 2013)**.

II.6. Probiotic, gut microbiota and drug: what relationship?

The liver is known to be the major organ responsible for drug metabolism and biotransformation. However, the ability of gut bacteria to metabolize xenobiotics, particularly drugs, is comparable to that of any organs in the body, including the liver. Drug metabolism at the intestinal level brings a significant effect on the drug's efficacy, safety and toxicity **(Stojančević et al., 2014**, **Wilson and Nicholson, 2017; Kim et al., 2018)**.

Drugs which may be exposed to intestinal microflora are those that are orally administered but suffer from low solubility, low permeability or both and reach the lower confines of the gastrointestinal tract, where the host microflora is the densest. Additionally, drugs which are rectally administered in the form of suppositories or enemas, as well as those that undergo enterohepatic cycling, or secretion and diffusion from the systemic circulation into the intestinal lumen may also come in contact with intestinal bacteria **(Stojančević et al., 2014)**.

The gut microbiota have both direct and indirect effects on drug and xenobiotic metabolism **(Wilson and Nicholson, 2017)**. The intestinal microflora is mainly involved in reductive and hydrolytic reactions generating non-polar low molecular weight byproducts **(Sousa et al., 2008)**. In addition, to provide a major source of reductive metabolizing capability, the gut microbiota provides a suite of additional reactions including acetylation/deacylation decarboxylation, dehydroxylation, demethylation, dehalogenation and importantly, in the context of certain types of drug-related toxicity, conjugate hydrolysis reactions. In addition to direct effects, the gut microbiota can affect drug metabolism and toxicity indirectly via e.g., the modulation of host drug metabolism and disposition and competition of bacterial-derived metabolites for xenobiotic metabolism pathways. And, of course, the therapeutic drugs themselves can have effects, both intended and unwanted, which can impact on the health and composition of the gut microbiota with unforeseen consequences (**Wilson and Nicholson, 2017)**.

Since the intestinal microflora plays an important role in physiological, nutritional, metabolic, and immunological processes in human body, there is some interest in the manipulation of its composition and activity by administering probiotics **(Gareau et al., 2010).** Hypothesizing that these probiotics are able to interact with resident microbial community, to affect the respective enzymes or able to provide their own specific enzymatic activities that may consequently change the bioavailability and pharmacological activity of concomitantly taken drugs **(Stojančević et al., 2014)**. **Kim et al. (2018)** reported that the intake of probiotics may make the absorption of orally administered drugs fluctuate through the disturbance of gut microbiota-mediated drug metabolism and that the subsequent impact on microbiota metabolism could result in altered systemic concentrations of the intact drug. Another report, suggest that some probiotics affect drug metabolism involving certain drug-metabolizing enzymes, for example, the metabolism of nifedipine by CYP3A in the intestinal mucosa or the metabolism of sulfasalazine by gut microbiota **(Stojančević et al., 2014)**.

II.7. Drug toxicity

Drugs are prescribed to prevent or treat disease. Those same drugs can be toxic to certain patients **(Taniguchi et al., 2011)**. Drug toxicity can occur as a result of normal therapeutic doses or as a result of an over ingestion of a drug. This can happen if the dose taken exceeds the prescribed dose, either intentionally or accidentally. However, with certain drug, toxicity can also occur in the majority of treated organisms as a result of the nature of the drug (such as cytotoxic agents used for cancer chemotherapy). However, significant toxicity is rare with the majority of commonly prescribed drugs when used at recommended dosage **(Waller and Sampson, 2018)**. In this case, the normally given therapeutic dose of the drug can cause unintentional, harmful and unwanted side effects. These side effects make many patients unwilling to take drugs on a regular basis, and this lack of compliance represents a major practical limitation of pharmacotherapy **(Taniguchi et al., 2011)**. There is considerable interindividual variability in both the nature and severity of adverse reactions, and toxicity can be reduced by taking into account factors that are known to increase susceptibility, such as age, concurrent disease or body weight, when selecting both the drug and the dose **(Waller and Sampson, 2018)**.

It is widely appreciated that drug metabolites, in addition to the parent drugs themselves, can mediate the serious adverse effects because they can interact with a diverse array of receptors to mediate adverse effects *in vivo* **(Baillie and Rettie, 2011; Taniguchi et al., 2011)**.

Sometimes the parent, unmetabolized drug causes toxicity, but often a metabolite of the drug reacts with proteins, DNA, and oxidative defense molecules (such as glutathione) to cause cellular damage and other adverse reactions (Figure 05) **(Taniguchi et al., 2011)**. Thus, drug toxicity depends on the balance between formation of the reactive metabolite from the parent drug, and the rate of elimination of both the reactive metabolite and the parent drug by alternative metabolic routes **(Waller and Sampson, 2018)**.

The most commonly encountered problems are with cardiovascular and hepatic toxicity **(Guengerich, 2011)**. Much progress has been made in understanding mechanisms of toxicities caused by drug metabolites, and the numerous factors that influence individual exposure to products of drug biotransformation (**Baillie and Rettie, 2011)**.

To reduce or overcome problems related to drug toxicity therapeutic interventions are aimed at either increasing elimination of the parent drug or enhancing cytoprotective pathways **(Waller and Sampson, 2018)**.

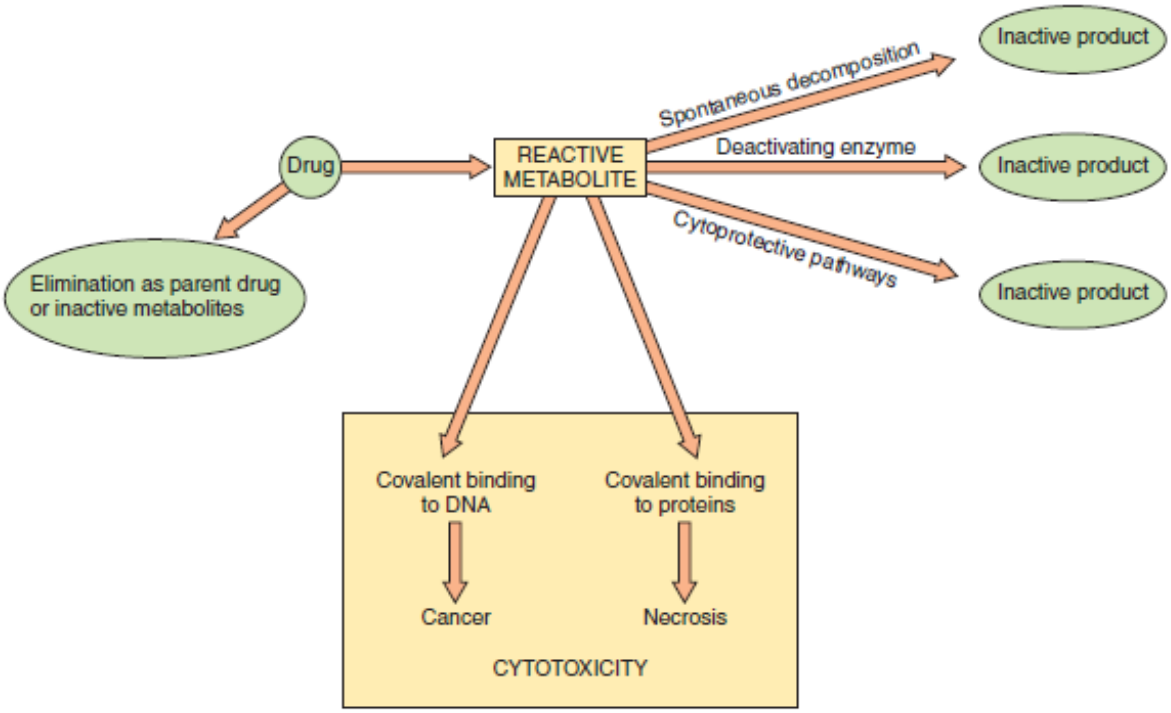


Fig. 05. *Mechanism of drug metabolism induced cellular toxicity* **(Waller and Sampson, 2018)**.

II.8. Oxidative stress as a mechanism of drug toxicity

Drug-induced oxidative stress is implicated as a mechanism of toxicity in numerous tissues and organ systems, including liver, kidney, ear, and cardiovascular and nervous systems**.** Metabolism of a drug may generate a reactive intermediate that can reduce molecular oxygen directly to generate ROS **(Deavall et al., 2012)**. Most of the drugs administered to patients are lipophilic in nature and can enter into the cell easily through plasma membrane. However, to reach the target site for proper adsorption, distribution, and excretion, these drugs must be converted to hydrophilic molecules. The conversion of these nonpolar compounds into polar compounds is termed as drug metabolism. Drug and other xenobiotic metabolism occur through three phases, namely, phase I, phase II and phase III**.** Biotransformation of the drug is a complicated process consisting of several steps that lead to the production of ROS. Many drugs are converted to quinine metabolites during biotransformation. These quinone metabolites are reactive molecular species which forms adducts with macromolecules, anti-oxidant molecules like GSH and deplete the pool of antioxidant molecule like GSH; thereby, generating more ROS. Excessive ROS generation and sequestration of endogeneous antioxidant specieslead to oxidative stress (Figure 06) **(Banerjee et al., 2016)**.

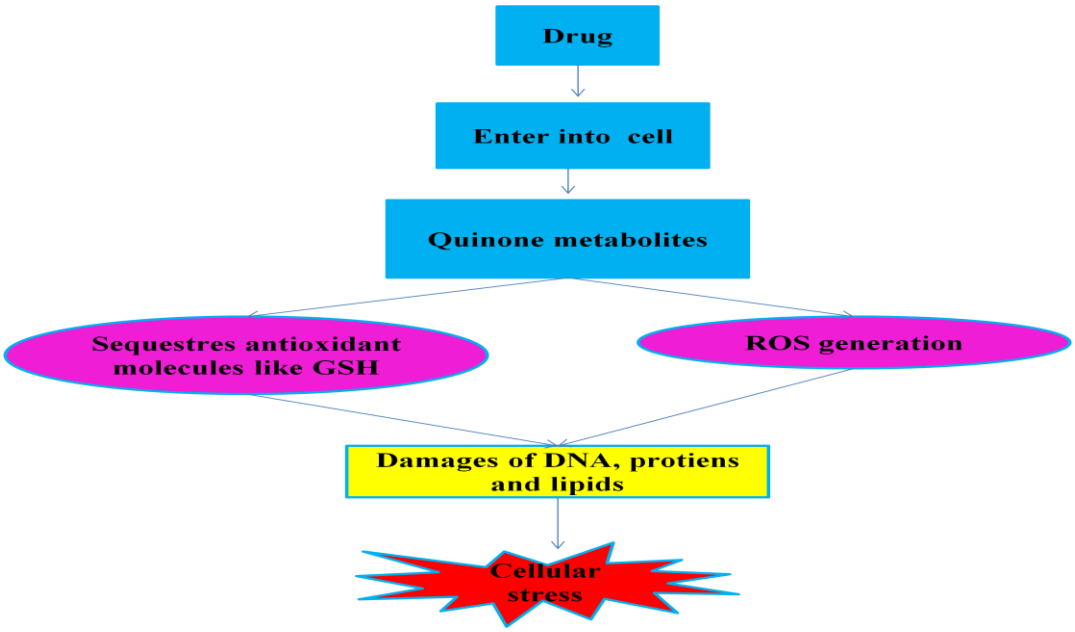


Fig.06. *Flowchart illustrating mechanism of drug metabolism induced cellular stress* **(Banerjee et al., 2016).**

Some of the evidences suggest that a combination of drug metabolite promoting oxidative stress as direct effect and alteration of signal transduction systems result in further loss of

mitochondrial function as indirect effect**.** Mitochondrial stress has since developed in terms of a major aspect of drug toxicity (Figure 07) **(Guengerich, 2011).**

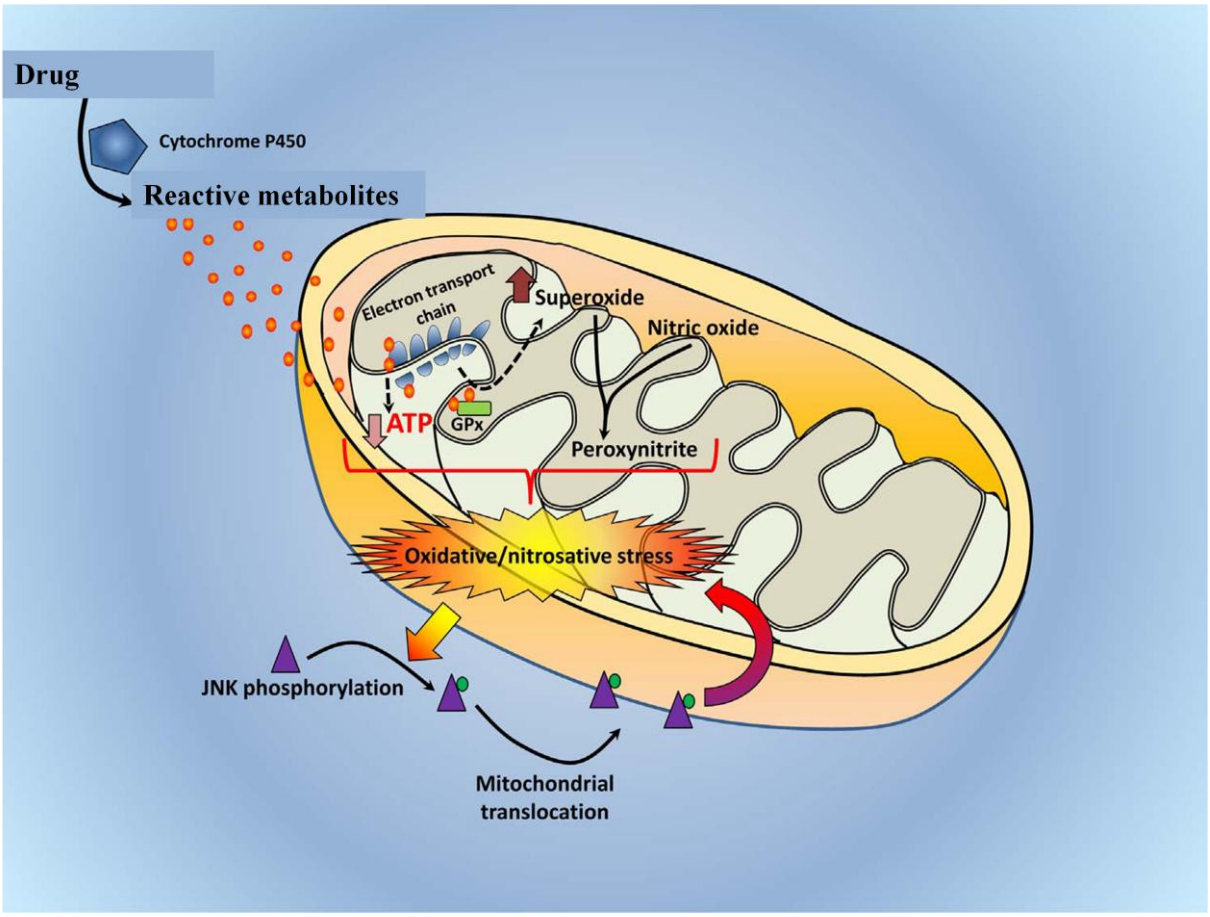


Fig. 07. *Mitochondrial oxidative stress and signaling in drug hepatotoxicity. Metabolism of drug forms the reactive metabolitse, which target proteins, especially mitochondrial proteins. Adduction of ATP synthase and glutathione peroxidase compromises generation of ATP through the electron transport chain and interferes with mitochondrial anti-oxidant capacity. The enhanced generation of superoxide results in its reaction with nitric oxide to produce peroxynitrite, which ultimately produces oxidative/nitrosative stress. This then activates the MAP kinase c-jun-N-terminal kinase (JNK), resulting in its phosphorylation and translocation to the mitochondria, which amplifies the initial oxidative stress* (**Du et al., 2016)**

Cancer therapies, non-steroidal anti-inflammatory drugs (NSAID), antiretroviral agents, antipsychotics, and analgesics are the most interesting example with adverse events to which oxidative stress may contribute (Table 3) **(Deavall et al., 2012)**.

Table 03. *Some toxicities associated with drug-induced oxidative stress* ***(Deavall et al., 2012)***.

Therapeutic class	drug	Example toxicities	Evidence for oxidative stress
Antineoplastic	Doxorubicin	Cardiac toxicity	Reduction of doxorubicin to free radical increase ROS in cardiomyoctes, lipid peroxidation, mitochondrial dysfunction, apoptosis increased ROS and NOS. over expression of SOD and CAT protect against toxicity apoptosis.
Antiretrovial	Azidothymidine	Skeletal myopathy Cardiac toxicity	Oxidative stress generated by a cation radical or redox cycling of intermediates derived from hydroxylation, multifactorial perturbations in mitochondrial dysfunction.
Anti-inflamatory	Diclofenac	Nephrotoxicity hepatotoxicity	Formation of reactive metabolites, depletion of glutathione, activation of proapoptotic proteins, mitochondrial dysfunction, inflammation.
Analgesia	Paracetamol	hepatotoxicity	Increase in superoxide anion, hydrogen peroxide, and hydroxyl radical. Depletion of antioxidants GSH peroxidase and GSH reductase.
Antineoplastic	Cisplatin	Nephrotoxicity ototoxicity	Mitochondrial dysfunction, apoptosis.
Antipsychotic	Chlorpromazine	Dermal toxicity(due to phototoxicity)	Generation of singlet oxygen and superoxide in response to UV irradiation.

II.9. Nonsteroidal anti-inflammatorydrugs toxicity

Non steroidal anti-inflammatory drugs (NSAIDs) are chemicals with anti-inflammatory, analgesic, and antipyretic effects. The most common members of this group of therapeutic agents are acetylsalicylic acid, paracetamol, diclofenac, ibuprofen, and naproxen **(Gómez-Oliván et al., 2012)**. NSAIDs are used widely but have been reported to causevarious disorders due to their use at high doses and in long term use (**Simon and Evan Prince, 2016)**. NSAIDs are the most frequently prescribed drugs worldwide. The clinical utility of NSAIDs is significanlly limited due largely to their ability to cause a diverse array of toxicities, particularly of those in the gastrointestinal tract, renal, and cardiovascular systems. The most common side effects and associated risks of their use due to their toxicities include: Gastrointestinal system.

While short term use of NSAIDs can cause stomach upset, their long term use, especially at high doses, can lead to peptic ulcer and bleeding of the upper gastrointestinal tract in the stomach. NSAIDs such as ibuprofen, diclofenac, loxoprofen and naproxen are known to cause various gastric complications **(Moore et al., 2005)**. Each day about 30 million of the world's population are using NSAIDs. About 1.3% of the patients using NSAIDs have been diagnosed with gastrotoxicity disorders **(Sostres et al., 2013)**. These side effects are caused by NSAIDs inhibiting thecyclooxygenase-1(COX-1) enzyme resulting into a decrease of mucous production in cells lining of the gastrointestinal tract, leaving it vulnerable to gastric acid, bile, enzymes, and alcohol. Gastrointestinal injuries range from heartburn, nausea, erosion and abdominal pain to serious complications such as ulcers and hemorrhage. COX-1 has been found to be responsible for protecting the stomach through mucous membrane and immune cell defense, maintaining blood flow and kidney fonction, and processing sensations **(Schoenfeld et al., 1999; Simon and Prince, 2016)**.

A number of mechanisms have been proposed to explain the mucosal damage induced by NSAIDs, most of which relate to the ability of these drugs to block the synthesis of prostaglandinthrough inhibition of the COX enzymes, COX-1 and COX-2. However, there is irrefutable evidence that COX inhibition is not the sole mechanism of NSAID-induced gastrointestinal injury and that other prostaglandin-independent mechanisms are important in ulcer pathogenesis **(Musumba et al., 2009; Gandhi et al., 2012)**. Direct involvement of oxygen derived free radicals has been implicated in the mechanism of gastrointestinal ulceration. Lipid peroxidation mediated by oxygen free radicals is believed to be an important

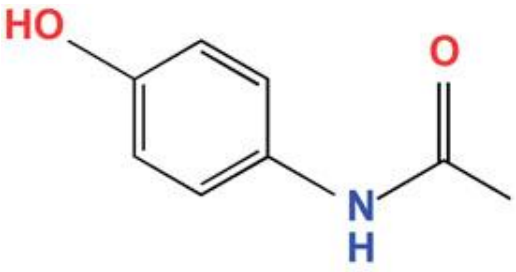
cause of destruction and damage to cell membranes, and attention has been focused on the role of reactive oxygen species in mediating the microvascular disturbances that precede gastric mucosal damage induced by various chemicals, stress, and ischemia–reperfusion. In fact, active oxygen species and lipid peroxidation have been demonstrated to play an important part in the pathogenesis of gastric mucosal injury induced by NSAIDs such as diclofenac **(Sener-Muratoglu et al, 2001)**.

Liver toxicity is also associated with the use of NSAIDs; the long term use of these drugs , especially at high doses, is reported to harm the liver via various complications, which result in hepatic damage. Similar to other hepatotoxic agents, NSAIDs are also known to cause alterations in the morphology of hepatic tissue, hepatic enzyme markers and in antioxidant enzymes **(Boelsterli, 2003)**.

One of the other toxicities associated with the use of NSAIDs is the kidney toxicity. The adverse effect of NSAIDs are mediated via inhibition of prostaglandin synthesis from arachidonic acid by non specific bloking of the enzyme COX leading to vasoconstracted states. This could lead to acute tubular necrosis and acute renal failure when unopposed **(Ejaz et al., 2004)**.

II.9.1. Paracetamol toxicity

Paracetamol is the most widely used non-prescription analgesic in the world. Paracetamol is the drug from analgesics category and derivatives of para amino phenol which is most widely used as aniline antipyretic analgesics to treat headaches with fever and arthralgia, as well as cancer pain and postoperative pain **(Sener et al., 2006; Tatti et al, 2012)**. Paracetamol being a non-steroidal anti-inflammatory drug (NSAID), was first synthesized in 1878 by Morse and first used clinically by von Mering in 1887 (Figure 08) **(Bertolini et al., 2006)**.



CAS: 103-90-2
Molecular weight: 151.16

Fig.08. *Chemical structure of paracetamol.*

The drug is safe and effective at therapeutic doses, its adverse reaction rate is very low under conventional therapeutic dosage conditions **(Rubin et al., 2016)**. Paracetamol overdose is common in life, and some overdoses are unintentional, such as taking more than one drug containing paracetamol at the same time. Another part of overdose intake may be deliberate, such as suicide. Overdose administration of paracetamol can induce severe liver injury and even develop into liver failure **(Liu et al., 2014)**. Paracetamol hepatotoxicity contributes to around 70,000 hospitalizations each year in the US **(Budnitz et al., 2011)**. Overall, paracetamol overdose is responsible for 46% of all cases of acute liver failure in the US and has now grown to be a significant public health problem (**Fontana, 2008)**.

Decades of investigations into the mechanisms of paracetamol-induced liver injury have provided significant insight into the role of paracetamol metabolism and formation of a reactive metabolite in initiating the cascade of events ultimately leading to liver injury. When consumed at therapeutic doses, the majority (80%–90%) of paracetamol is conjugated with glucuronic acid or sulfate and excreted through the kidneys **(McGill and Jaeschke, 2013)**. A minor component is acted upon by cytochrome P450 enzymes such as Cyp2E1 and Cyp1A2 to form a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) (**Nelson, 1990)** (Figure 09). Though highly reactive, NAPQI is rarely harmful after consumption of therapeutic doses because it is rapidly conjugated with abundant glutathione stores in the liver and excreted through the bile. However, this contrasts to the scenarioafter consumption of an overdose of paracetamol where the sulfation pathway is saturated **(McGill and Jaeschke, 2013; Waller and Sampson, 2018)**, and NAPQI generation is significantly elevated in spite of the high capacity of the glucuronidation pathway (**Xie et al., 2015)**. Excessive generation of NAPQI results in its robust reaction with hepatic glutathione stores and the subsequent rapid depletion of glutathione within the liver. This leaves free reactive NAPQI available for reaction with protein sulfhydryl groups to form paracetamol protein adducts **(McGill and Jaeschke, 2013)**, which causes oxidative stress, inflammatory, mitochondrial damage and centrilobular necrosis **(Bandeira et al., 2017; Wu et al., 2015; Zhang et al., 2014)**.

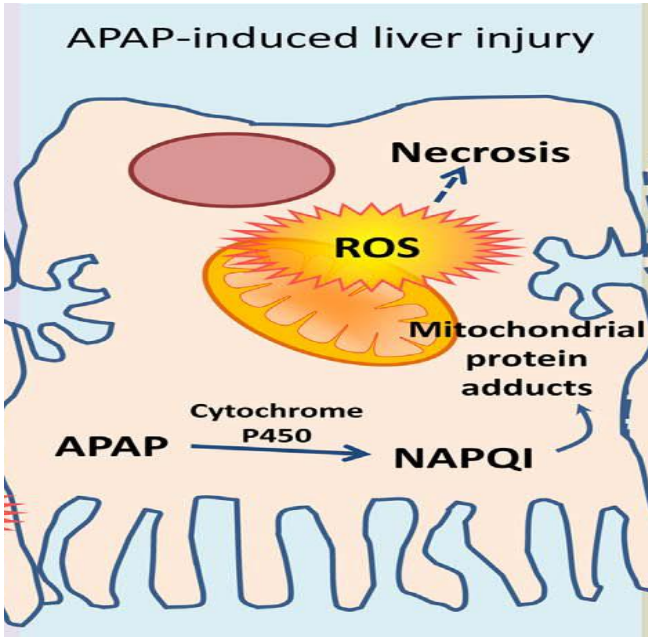


Fig. 09. *Sources of ROS in Paracetamol hepatotoxicity. Paracetamol-induced liver injury, the reactive intermediate NAPQI generated during Paracetamol metabolism forms mitochondrial protein adducts, which cause oxidative stress within the organelle and subsequently initiate signaling cascades resulting in programmed necrosis* **(Du et al., 2016)**.

Overdoses of paracetamol can cause ROS generation and result in alterations in antioxidant status. ROS caused by $O^{\bullet}_2$, $HO^{\bullet}$ and hydrogen peroxide (H_2O_2) usually led to the alteration of the enzymatic antioxidant defense systems in *in vitro* and *in vivo* models. Catalase (CAT), superoxide dismutase (SOD), GSH, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferases (GST) are the primary antioxidant enzymes, and they serve as good redox biomarkers as they are the first-line indicators of the antioxidant state through oxidation/reduction processes. As the most abundant intracellular antioxidant, GSH is involved in the protection of cells against oxidative damage and in various detoxification mechanisms. GSH also acts as a substrate and co-substrate in many essential enzymatic reactions involving GPx, GR, and GST, and a decrease in the GSH level usually impairs cells' response to oxidants **(Wang et al., 2017b)**.

So, Oxidative stress plays a crucial role in the development of paracetamol induced liver damage due to the fact that overdoses of paracetamol can cause ROS generation and resultin severe centrilobular hepatotoxicity and acute liver failure associated with liver congestion, necrosis, and apoptosis **(Karakus et al. 2013; El Morsy and Kamel 2015)**.

II.9.2. Diclofenac toxicity

Diclofenac (2-[2-(2,6-dichlorophenyl amino) phenyl]acetic acid) is a non-steroidal anti-inflammatory drug (NSAID) (Figure 10), developed in the late 1970s for the treatment of pain, fever and inflammatory conditions such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylosis in humans **(Scully et al., 1993, Burke et al., 2006)**.

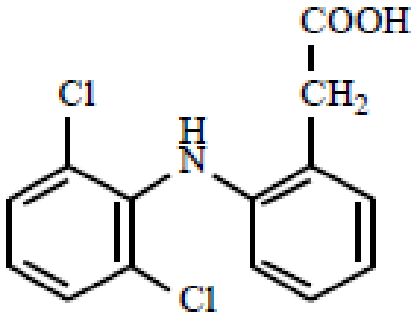


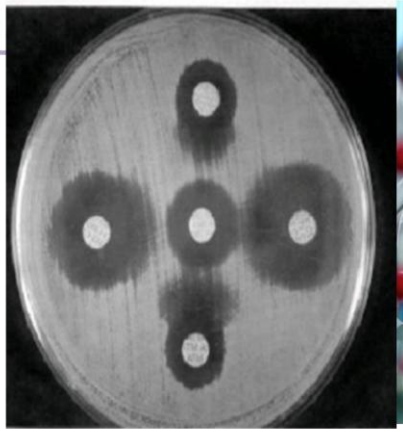
Fig. 10. *Chemical structure of diclofenac.*

Treatment with oral diclofenac (DF) and other NSAIDs has been associated with important side effects including cardiovascular, gastrointestinal, and hepatic complications. Similar to other NSAIDS, DF also causes mild to severe hepatotoxicity. DF-induced hepatotoxicity has been partially attributed to; mitochondrial injury, because, DF causes the opening of mitochondrial permeability pore through the uncoupling of oxidative phosphorylation thereby causing cellular damage **(Bernardi et al, 2006)**. In addition, hepatotoxicity of DF has been attriduted to generation of oxidative stress, alteration of the integrity of covalent protein by reactive metabolites, and immune-mediated mechanisms. Mechanism of DF mediated liver injury remains to be clarified. Hepatic metabolism of DF involves the formation of DF acyl glucuronide. Once formed, DF acyl glucuronide reacts with proteins by transacylation and glycation reactions **(Lassila et al., 2015)**. Albumin adducts of DF c acyl glucuronide have been detected in plasma of patients who were treated with DF **(Hammond et al., 2014)**. Antibodies against DF acyl glucuronide modified proteins have also been identified in serum of patients suffering from DF induced liver injury **(Aithal et al., 2004)**. This mechanism may explain both the allergic and intrinsic hepatotoxicity of DF.

On the other hand, DF is related with extreme gastrointestinal poisonous quality. Gastrointestinal damage occurs *via* systemic inhibition of gastrointestinal mucosal COX

activity and thereby modulating arachidonic acid metabolism **(Kanbayashi and Konishi, 2015)**. DF inhibits lipoxygenases, decreases the production of leukotrienesand suppresses prostaglandin synthesis and thromboxane-prostanoid receptor signaling **(Scheiman and Hindley, 2010)**. DF is metabolized to 4-hydroxydiclofenac and other hydroxylated forms in the liver, after conjugation and sulfation, the metabolites are excreted mainly in the urine and bile **(Kumar et al., 2002)**. Excretion and accumulation of conjugates have been correlated to renal function and end-stage renal disease **(Ahmed et al., 2017)**.

Materials and methods



This work was realized in the

Laboratory of Molecular Toxicology, Faculty of Nature and Life Sciences, University Mohamed Seddik Benyahia of Jijel, Algeria

Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, Campus Regional de Excelencia Internacional "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain

Laboratory of Microbiology, Department of Applied Microbiology and Food Sciences, University of Jijel.

III.1. Materials

III.1.1. Bacterial strains

The present study was conducted with twelve bacterial strains isolated from different origins. These lactic acid bacteria were kindly provided by Prof. Tayeb Idoui from the laboratory of Biotechnology, Environment and Health, University of Jijel, Algeria (Table 04).

Table 04. *bacterial strains used in the study and their origins.*

Strains	**Code**	**Origin**
Lacrobacillus plantarum	J2	Chicken crop
Lactobacillus curvatus	Bj432	Butter Jijel
Lactococcus lactis spp *cremoris*	CHT24	Chamel milk
Streptococcus salivarius spp *thermophilus*	St.sa	Milk
Lactobacillus plantarum	15	Milk
Lactobacillus sp.	45	-
Lactobacillus confusus	Lb. Con	Human feces(API50+Log)
Lactobacillus sp.	05	-
Lactobacillus plantarum	G1	Gizzard
Lb. delbrueckii spp *lactis*	S3	infant stool
Lb. helveticus	J14	Chicken crop
Lb. viridescens	J13	Chicken crop
Methicillin resistant *Staphylococcus aureus*		-
Listeria monocytogenes		-
Salmonella sp.		-

Escherichia coli ATCC 25922		-
Staphylococcus aureus		-
Bacillus subtilis		-

III.1. 2. Media and buffers

Several culture media and buffers were used in this experimental work the main ones were:

- de-Man-Rogosa Sharp (MRS broth and MRS agar) Annex.
- Hyper-sucrose agar Annex
- Phosphate Buffered Saline (PBS, pH 7.4, PBS, pH=2.0, PBS 1% bile salt).

III.1. 3. Chemicals and reagents

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), 1,10-phenanthroline, Trichloroacetic acid solution (TCA), hydrogen peroxide (H_2O_2), ethanol, methanol, $FeSO_4$, phenol, sulfuric acid, ascorbate, Butylated hydroxytoluene (BHT), Thiobarbituric Acid (TBA), HCl, NaOH.

III.2. Methods

III. 2.1. Revivification : Preparation of microorganisms

Twelve LAB strains chosen for the present study were maintained as frozen stocks in MRS medium with glycerol at -20°C. All bacterial strains were revitalized in MRS and incubated at 37^{o}C for 24 h before use.

III.2. 2. Standardisation of inoculum

In This step we estimate the number of cells present in a given OD. The number of cells was determined by counting the total number of colony-forming units (CFU) grown on MRS agar plate from serial dilutions, expressed as CFU per mL of the given OD.

III.2. 3. Screening of antioxidative bacteria

Strains were cultured in MRS broth, incubated at 37°C for 24h and centrifuged at 6000 rpm for 10min at 4°C to obtain the culture supernatant and bacterial pellets. The bacterial pellets were washed twice with sterilized normal saline and resuspended to obtain a concentration of

10^9CFU/ml. Screening of antioxidative bacteria was performed by DPPH method as described by **Mandal et al**. **(2013)**. This method was based on the monitoring of DPPH free radicals scavenging activity by intact cells and cell free supernatant. One ml of freshly prepared DPPH in methanol (0.2mM) were mixed and allowed to react with 0.8ml of intact cells suspension or supernatant for 30min at room temperature. The controls included only sterilized saline water or MRS and DPPH solution, the blanks contained methanol and bacterial suspension. The absorbance was measured at 517nm. The test was performed in triplicate. The DPPH scavenging activity was calculated as fallow:

$$\text{scavenging activity}(\%) = \frac{\mathbf{A}\ \text{control} - \mathbf{A}\ \text{sample}}{\mathbf{A}\ \text{control}}$$

III.2.4. Characterization of selected strains for their probiotic aptitude

III.2.4.1. Acid tolerance

To determine the strain tolerance to low pH, the ability of *S. salivarius* ssp *thermophilus* St.sa and *Lactobacillus plantarum* 15 to grow in acidic condition was studied according to the method described by **Pieniz et al. (2014).** An overnight culture of *S. salivarius* ssp *thermophilus* St.sa and *Lb plantarum* were inoculated separately into 20 ml of 0.1M phosphate buffered saline (PBS) (pH 2.0) adjusted with HCl. At 0, 2, 4, and 6h of incubation at 37°C, cell counts in MRS agar were performed. The results were expressed as growth reduction rates.

III.2.4.2.Bile salts resistance

The strains *S. salivarius* ssp *thermophilus* St.sa and *Lb plantarum* 15 were tested for their resistance to bile salts according to the method reported by **Vijendra and Prasad (2005).** 20ml of 0.1M phosphate buffered saline supplemented with 1% bile salts was inoculated with an overnight culture. Suspension obtained after centrifugation, washed and resuspended in PBS to reach a concentration of 10^9CFU/ml. The culture was then incubated at 37°C. Viability was determined by cell counts in MRS agar media after 2, 4, 6 and 8h.

III.2.4.3. Antimicrobial activity

Agar well diffusion method was used to determine the antipathoginic effect of supernatant according to **Aslim and Kilic (2006),** cultures of *S. salivarius* ssp *thermophilus* St.sa and *Lb plantarum* 15 grown in 100ml MRS broth for 24h at 37°C were centrifuged at 6000/30min, the supernatant was sterilized by passage through 0.22µm Millipore filter. The indicator strains were subcultured on nutrient broth. Plates of Muller-Hinton agar were inoculated with 50µl of indicator bacteria. Wells (6mm diameter) were then cut into each plate. After that, each well was filled with 100µl of filtered supernatant. Then, keeping at 4°C for diffusion. The plates were incubated for 24h at 37°C.

III.2. 4.4.Auto-aggregation

Auto-aggregation was performed according to the methods described by **Balakrishna (2013)** with slight modifications. St.sa and 15 cultures grown overnight were harvested by centrifugation, washed twice, and resuspended in phosphate buffered saline (PBS, pH 7.2) followed by turbidity measurement at 660 nm (ODi). The bacterial suspensions were then kept undisturbed at room temperature for 5h followed by measuring OD at 660nm of upper suspension fluid (ODt). Percentage of auto-aggregation (%AAg) was calculated according to the following formula.

$$\mathbf{AAg\ (\%) = 1-(ODt / ODi) \times 100}$$

OD: optical density

III.2.4.5. Safety profiling *in vitro*

III. 2.4.5.1. Hemolytic activity

The selected strains were tested for hemolytic activity using blood agar (5% horse blood), then incubated for 48h at 37°C. Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) were considered non hemolytic. Strains displaying blood lyses zones around the colonies were classified as hemolytic (β-hemolysis) **(Li et al., 2014)**.The clear zones was observed and measured the diameter of inhibition zones was measured.

III.2.4.5.2. Antibiotic susceptibility

Bacterial sensitivity to antibiotics was determined by the disc diffusion method on agar Mueller Hinton. Four antibiotics were used (Sulphonamide (300µg), Penicillin G (10 unités), Colistin sulphate (50µg), Amoxycillin (25µg)). Briefly, freshly prepared strain cultures (A600nm= 0.6) were seeded on Müller-Hinton agar plates. Then, discs containing each antimicrobial agent were placed on the surface of the plates. After inoculation of culture on Müller-Hinton agar and placement of disks, plates were incubated at 37°C for 18 hours. The diameters of inhibition zones were measured around the discs (mm) **(Liasi et al., 2009).**

III.2. 4.6. Calculation of cumulative probiotic potential (CPP)

Cumulative probiotic potential is the sum of score of acid, bile tolerance, antibacterial potential, acid and alkali and heat tolerance of bacteriocin, antibiotic sensitivity, antibacterial potential and hemolytic activity **(Tambekar and Bhutada, 2010; Halder et al., 2017)**. The probiotic potential of the selected strains was assessed using 5 point scores (acid tolerance, bile tolerance, antibiotic sensitivity, antibacterial potential and hemolytic activity). Probiotic potential was calculated as observed score divided by maximum score into hundred as mention in the equation below

$$\text{probiotic potential} = \frac{\text{observed score} \times 100}{\text{maximum score}}$$

III.2. 5. *In vitro* assays of the antioxidant capacity of the tow strains

Bacterial strain was cultured in MRS broth, incubated at 37°C for 24h and centrifuged at 6000rpm for 10min at 4°C to obtain the cell-free supernatant and the bacterial pellet. The bacterial pellet was washed twice with sterile normal saline and resuspended to obtain a concentration of 10^9CFU/ml.

III.2. 5.1. Resistance to hydrogen peroxide

The resistance of the selected strains to the oxidative stress generated by hydrogen peroxide was determined *in vitro* according to **Zhang et al. (2011)**. Samples of 20mL of PBS with 1mM H_2O_2 were inoculated with cell suspension containing 10^9CFU/ml. The cell suspension was prepared after centrifugation of an overnight culture (3000g, for 15min). The pellets were

washed twice and resuspended into PBS. The inoculated PBS (1mM H_2O_2) was incubated at 37°C. The viability of *S. salivarius* ssp *thermophilus* St.sa and *Lactobacillus plantarum* 15 in stress condition was monitored as follow: Aliquots of the culture appropriately diluted were taken over 2, 4, 6h, and plated onto MRS agar plates for counting the number of cells forming colonies, incubation of plates was performed at 37°C for 48h.

III.2.5.2. Inhibition of lipid peroxidation

To determine the inhibitory capacity of the probiotic strain toward lipid peroxidation, plasma lipids were chosen as a substrate as described by **Ou et al. (2006)**. Briefly, 400µl of plasma, 100µl of $FeSO_4$ solution (50mM) and 0.2ml of the bacterial suspension were mixed and incubated at 37°C in a water bath for 15h. Then, 375µl of trichloroacetic acid (TCA) (4%) and 75µL of butylated hydroxytoluene (BHT) were added and allowed to react for 5 min in ice bath and centrifuged at 3000 rpm for 10 min. Aliquots of 200µl of thiobarbituric acid (TBA) (0.67% in NaOH 50mM) were added to the supernatant, followed by an incubation at 100°C for 30min, the mixture was left to cool, then, the inhibition of lipid peroxidation was determined spectrophotometrically at 532nm. The inhibition rate was calculated using the following equation:

$$\text{inhibition (\%)} = \frac{1 - \mathbf{A}\text{test}}{\mathbf{A}\text{control}} \times 100$$

where (A): absorbance.
The control was prepared as described below, except for the bacterial suspension which was replaced by distilled water.

III.2.5.3. Hydroxyl radical scavenging effect

The method of **Wang et al. (2012)** was used to determine hydroxyl radical scavenging ability of the strain *S. salivarius* ssp *thermophilus* St.sa and *Lb plantarum* 15. In this method o-phenantroline and $FeSO_4$ were used to generate $OH^{\cdot}$ radicals. The hydroxyl radical was generated in the mixture of 1ml of 0.75mM 1,10-phenanthroline, 1ml of 2.5mM $FeSO_4$, 1ml of H_2O_2 (20mM) and 1ml sodium phosphate buffer (0.2M, pH 7.4). Then, to determine the probiotic OH scavenging capacity, 1.0ml probiotic solution was added, the mixture was incubated at 37°C for 30min. The absorbance of the mixture was measured at 536nm. The scavenging activity of hydroxyl radical was determined by the following equation:

$$\text{The scavenging activity on hydroxyl radical (\%)} = \left(\frac{\text{As} - Ap}{Ab - Ap} \right) \times 100$$

Where (As) was the absorbance of the mixture in presence of probiotic, (Ap) in its absence and (Ab) was the absorbance of distilled water instead of H_2O_2 and probiotic sample.

III.2.5.4. Iron ions chelating ability

The chelating ability of iron ions was measured according to the method described by **Zhang et al. (2011).** The reaction mixture contained 0.5ml of cell pellet (10^9CFU/ml), 0.1ml of ascorbic acid (1%, v/v), 0.1ml of $FeSO_4$ (0.4g/l) and 1ml of 0.2M NaOH. 0.2ml of TCA (10%) was added to the mixture. Incubation was carried in a water bath at 37°C for 20min. The mixture was then centrifuged at 6000rpm for 20min and the obtained supernatant was collected and mixed with 0.5ml of phenanthroline (1g/l). After a reaction of 10 minutes, the absorbance was measured at 510nm against a blank. The assay was carried out in triplicate. The chelating ability of iron ions was calculated as follows:

$$\%\ \text{chelating ability on iron ions} = \left(\frac{\text{Abs}_{\text{Blank}} - Abs_{Sample}}{Abs_{Blank}} \right) \times 100$$

III.2.5.5. Producing exopolysaccharides as a mechanism of antioxidant efficiency

The production of exo-polysaccharides was investigated on the hyper-sucrose agar medium at 37°C for 24h **(N'tcha et al., 2016)**. EPS positive were defined as displaying slimy colony morphology, these colonies were tested for compactness or ropiness by touching them with a sterile inoculation loop **(Mostefaoui et al., 2014)**.

III.2. 5.5.1. EPS production

The isolate St.sa, was grown in Erlenmeyer flask containing 50ml of modified MRS broth (20g/l of glucose was replaced by lactose), incubated in shaking incubator at 37°C for 24h **(Savadogo et al., 2004).** Total EPS was estimated in each sample by the phenol–sulfuric acid method, using glucose as standard **(Xu et al., 2000)**.

III.2.5.5.2. EPS extraction

EPS was extracted from the production medium as described by **Li et al., (2014)** with slight modifications. After 24h of incubation, the cells was separated by centrifugation at 12,000xg for 15min at 4°C. Then, trichloroacetic acid (TCA) solution was added to the supernatant to give final concentration of 4% (w/v). The precipitated proteins were removed by centrifugation (12,000 × g for 30min at 4°C), and three volumes ice cold ethanol were added to the supernatant, stirred vigorously and kept at 4°C for overnight. Crude EPS was collected by centrifugation at 15,000×g for 15min, then the EPS pellet was dissolved in distilled water.

III.2.5.5.3. EPS quantification

Exopolysaccharides were estimated as total carbohydrates by phenol-sulfuric acid method. To 800µl of sample (EPS solution), 40µl of phenol reagent (80% w/v) was added followed by 2ml of concentrated sulfuric acid, vortex was applied after addition of each reagent. The absorbance was measured at 490nm against the reagent blank that contains 800µl of distilled water instead of sample. The EPS content of each sample was then calculated using the glucose standard curve. For the preparation of standard curve, the same procedure was investigated using glucose solution (1mg/ml) as standard reagent instead of EPS solution. The results were expressed as milligrams (mg) of EPS per liter **(Xu et al., 2000)**.

III.2.5.5.4. Determination of antioxidant activity of EPS

EPS already extracted were subjected to different antioxidant assays namely DPPH free radical scavenging assay, hydroxyl radical scavenging assay and ferrous ion chelating assay so as to evaluate their antioxidant potential as describe previously (section II.5.) (**Lin and Chang, 2000**).

The probiotic *S. salivarius* ssp *thermophilus* St.sa was selected for the treatment.

III. 2.6. Role of probiotic St.sa in reducing NSAID (diclofenac and paracetamol) toxicity

III.2. 6.1. Rats and experimental design

Female Wistar rats weighing 180-250g provided from Pasteur institute of Algiers (Algeria) were used in this study. The rats were housed, five per cage. Animals were maintained with free access to food and water. Rats were adapted for one week before the indicated treatments.

Paracetamol hepatotoxicity: Sixteen animals were divided into 4 groups (4 animals in each group): 1) Healthy control group rats receiving only physiologic water in all days of treatment; 2) Probiotic control group receiving a daily dose of 10^9CFU of probiotic bacteria for 7 days; 3) Paracetamol induced liver injury group: rats in this group treated by a single dose of paracetamol (200mg/Kg/body weight) (**Lahouel et al., 2004)**, was administered in day 7; 4) Probiotic protective group receiving a daily dose of 10^9CFU of probiotic bacteria for 7 days and a single dose of paracetamol (200mg/Kg/body weight) was administered in the last day. After 24h of paracetamol administration all rats were sacrificed.

Diclofenac toxicity: Twenty animals were divided at random into 4 groups, five animals in each group. The first group comprises control animals that received normal saline in all days of treatment; rats in the second group received 10^9CFU/day of probiotic suspension during 7 days; rats in the third group received a single overdose of diclofenac (100mg/kg bw) in the seventh day and rats in the fourth group received the probiotic suspension during 7 days and a single overdose of diclofenac (100mg/kg bw) in the last day **(Hsu et al., 2008)**. The drug was administered orally by gavage through an intragastric tube. After 24h of diclofenac administration all rats were sacrificed.

After dissection, we focused to study hepatotoxicity of diclofenac and paracetamol in one hand, for this some livers were removed, washed in a 0.9% saline solution and stored at -20°C for biochemical analyses. Blood samples were collected and the serum was separated by centrifugation at 4000/15min. The separated plasma was used for evaluating the liver markers. For histological study, the livers of different groups were immediately fixed in 10% formalin. In the other hand we analyzed gastric toxicity, to conduct this analysis the rats stomachs were removed and opened along the greater curvature, and then were washed with a 0.9% sodium chloride solution. After rinsing with normal saline, some of the stomachs were immediately fixed in 10% formalin for histological study. Also, some of mucosas were stored at -20°C for biochemical analyses.

III.2.6.2. Diclofenac and paracetamol hepatotoxicity

III.2.6.2.1. Hepatic makers as index of liver damage

The liver function markers, including alanineaminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were selected as an index of liver

damage. The assays were performed with an autoanalyzer (DIAMS 2300+) at the Biochemistry Laboratory of Mohamed Seddik Benyahia Hospital (Jijel-Algeria).

III.2.6.2.2. Histology

For histological analyses, pieces of liver and tissue specimens were fixed in 10% formalin buffered with phosphate for one week at 4°C. Tissue fragments were washed in phosphate buffer, dehydrated in graded concentrations of ethanol solution (100% - 5min, 96% - 5 min, 70% - 5min), and then embedded in paraffin. The organs were immersed in paraffin twice, for 2 hours each time. Five micron thick sections were prepared using a microtome; the sections were then stained with hematoxylin and eosin. (Dip slides in alcohol for 5minutes, rinse with water, stain slides in hematoxylin for 4 minutes, after rinsing stain with eosin for 10 minutes, rinse with water, dip slides in alcohol for 1 minute, after rinsing and drying).

The sections were examined under a light microscope (Leica DM6000B) **(Drury and Wallington, 1980)**.

III.2.6.2.3. Non-enzymatic parameters in liver

The amount of GSH in liver tissue was measured according to the method of **Ellmen (1959)** based on the use of Ellman reactif (5,5-dithiobis-2-nitrobenzoic acid or DTNB). Briefly, 1g of liver tissue was homogenized with 3 volumes of KCl (1.15M), and then, fifty µL of the homogenate was diluted with 10ml of phosphate buffer (0.1M, pH 8.0). Twenty µL of DTNB (0.01M) was added to 3ml of the dilution mixture and incubated for 15min. Glutathione content was determined spectrophotometrically at 412nm and the hepatic GSH level was expressed as nmoles/g tissue.

MDA, as an endpoint of lipid peroxidation, was calculated by detecting absorbance of thiobarbituric acid reactive substances at 532nm according to the method described by **Ohkawa et al. (1979)**. 0.5ml of the homogenate was mixed with 1ml of thiobarbituric acid (0.67%) and 0.5ml TCA (20%) and heated for 15min at 100°C. The reaction was stopped by cooling. Two ml of n-butanol was then added to the mixture and centrifuged. The OD of the supernatant was measured at 530nm, MDA levels were expressed as MDA nmol/mg protein.

III.2.6.2.4. Enzymatic activities in liver

Samples of 1g of liver was homogenised with 10 volumes phosphate buffer (50mM /pH 7.0) using a polytron homogenizer and centrifuged at 3000×g for 20min to remove the cell debris, unbroken cells, nuclei and erythrocytes. The supernatant was collected and used for the estimation of the antioxidant enzymes activities.

The superoxide dismutase (SOD) activity was assayed spectrophotometrically based on the formation of formazan salt by the reaction between reduced nicotinamide adenine dinucleotide and phenazinemethosulfate–nitroblue tetrazolium using the method of **Kakkar et al. (1984)**. The superoxide radicals produced *in situ* can reduce nitroblue tetrazolium (NBT) to form formazan crystals (blue), which are detected at 560nm. One unit of enzyme was defined as the activity needed to reach a 50% inhibition of formazan formation in 1 min.

The catalase (CAT) activity was measured according to the method of **Aebi (1984)**. The samples were mixed with 50mM phosphate buffer and 30mM H_2O_2. The amount of hydrolyzed H_2O_2 was calculated by the molar extinction coefficient of H_2O_2 at 240nm ($0.071 mM^{-1}\ cm^{-1}$). The result was expressed as the μM of H_2O_2 decomposed per min per milligram of protein.

III.2.6.2.5. Determination of protein concentration

Protein levels of the cell samples were measured by the Bradford method. Bovine serum albumin was used as the protein standard. 100μL of a protein was mixed with 2.50ml of filtered assay solution (25mg BBC, 12.5ml ethanol, 25ml phosphoric acid, complete the volume to 250ml with distilled water) in tube assay leading to a differential colour change occurs in response to the protein concentration. Consequently, OD at 595nm was established using spectrophotometer, to determine the absorbance value. Therefore, a standard curve was created using bovine serum albumin (BSA) diluted in distilled water by plotting the values of absorbance against concentrations of corresponding protein. The absorbance of unknown protein sample was plotted on the standard curve to evaluate the concentration of the proteins in each sample. This step was performed in triplicate.

III.2.6.2.6. Gene expression analysis

The liver cells were used for the RNA extraction for the antioxidant enzymes genes expression by RT-PCR (reverse transcription-polymerase chain reaction) which is a sensitive method for the detection of mRNA expression levels. Traditionally RT-PCR involves two steps: the RT reaction and PCR amplification. RNA is first reverse transcribed into complementary DNA (cDNA) which is then used as templates for subsequent PCR amplification using specific primers for the SOD and CAT genes **(Orrù et al., 2006)**.

Total RNA was extracted from liver samples by using TRIzol Reagent a follow: 500µl of Trizol was added to each sample incubated 5min at room temperature. 100µl of chloroform was added to each eppendorf (agitated for 15 sec with vortex) and incubated for 15sec. Then, the solution was centrifuged for 15min (4°C, 12000g). Next, the supernatant was recovered (the transparent phase) and 250µl of isopropanol was added with (shake and put for 10 min in ice). Then the tubes were centrifuged for 10min (4°C, 12000g). After removing the supernatant, 500µl of ethanol was added and then centrifuged for 5min (7500g). Next, the ethanol was removed (wait ≈ 5min to evaporate all the ethanol) and 20µl of water (RNAse and DNAse free) was added. The RNA was then quantified and its purity assessed by spectrophotometer (NanoDrop 2000c); the 260nm:280nm ratios were 1.8-2.0. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination.

The extracted RNA was then used for the reverse transcription (RT reaction) of the cDNA. cDNA was synthesized from 1µg of total RNA using the Super Script III reverse transcriptase (Invitrogen) with an oligo-dT18 primer. Then, the mixtures was transferred into a thermocycler (Mastercycler) for 25 cycles using three different temperature 65°C for 5 min, then 50°C for 60 min and terminate the reaction at 70°C for 15 min. The next step is to cool it down at 4°C in the thermocycler for about 1h.

The second step was to evaluate gene expression. The expression of the selected genes was analyzed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10µL of 2×SYBR Green supermix, 5µL of primers (0.6µM each) and 5µL of cDNA template) were incubated for 10min at 95°C, followed by 40 cycles of 15s at 95°C, 1min at 60°C, and finally 15 s at 95°C, 1min at 60°C and 15 s at 95°C. The used primers are listed in table 5.

For each mRNA, gene expression was corrected by the RNA β-actin subunit content in each sample. The results are expressed with respect to the control group, which was normalized to 1. Data of gene expression are represented as fold decrease or increase obtained by dividing each sample value by the mean control value. Values higher than 1 express an increase while values lower than 1 express a decrease in the indicated gene.

Table 05. *PCR primer sequences*.

Gene	Sense	antisense
SOD	CACTCTAAGAAACATGGCG	CTGAGAGTGAGATCACACG
CAT	ATGGCTTTTGACCCAAGCAA	CGGCCCTGAAGCTTTTTGT
ß-Actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC

III.2.6.3. Diclofenac gastric toxicity

III.2.6.3.1. Histology

For histological analyses, pieces of Stomach tissue specimens were fixed in 10% formalin buffered with phosphate for one week at 4°C. The histological study was conducted as described in section II.6.2.2. The estimation of gastric mucosal damage was estimated as the length of epithelium layer and surface rates (The surface rate is the relationship between the white spaces and the surface of the epithelium of the stomach). The estimation of epithelium layer length and surface rates was done by Cristobal Espinosa at the University of Murcia.

III.2.6.3.2. Biochemical analyses

MDA, GSH, and the antioxidant enzymes activities were realised as described in sections II.6.2.3. and II.6.2.4. respectively.

III.2.7. Incorporation of St.sa in milk and evaluation of their properties

Several studies reported that some probiotic strains present antioxidative activity, were able to reduce the risk of accumulation of ROS in a host organism and could potentially be used to reduce oxidative stress during ingestion of food **(Songisepp et al., 2004; Kim et al., 2006a; Jonkers and Stockbrugger, 2007; Cinque et al., 2011; Mahrous, 2011; Zhang et al.,**

2011). Therefore, this part aimed to evaluate the properties and antioxidant activity of fermented milk by St.sa.

III.2.7.1. Milk fermentation and storage

Milk was prepared as follows: Ultra High Temperature (UHT) milk was mixed vigorously with 12% of skimmed milk powder then treated at 100°C for 30 min followed by cooling to 40°C before inoculation with 2% of probiotic cultures (*S. salivarius* ssp *thermophilus* St.sa). Milk was divided and tow equal portions while one portion was used as a control. The preparation was incubated for fermentation in a shaker incubator at 37°C and terminated when pH reached 4.5 (approximately after 24h). After the incubation period, samples were stored in a refrigerator at 4 °C for 15 days and aliquots were collected each 2, 5, 9 and 15 day and submitted to different analyses **(Sah et al., 2014)**.

III.2.7.2. Viability of probiotic bacteria

The enumeration of probiotic bacteria in milk was determined by spread plate method using MRS agar medium. Suitable dilutions were realized in PBS for each sample, and then plates were incubated at 37°C for 48h. The enumeration was carried out either before fermentation and after 2, 5, 9 and 15 days of storage **(Baba et al., 2014; Li et al., 2014)**.

III.2.7.3. Determination of pH and titratable acidity (TA) during storage

The pH and titratable acidity of milk were monitored during milk storage using a pH meter and a standard solution of sodium hydroxide (NaOH, 0.1N) and phenolphthalein as an indicator, respectively. Titratable acidity was expressed as percentage of lactic acid **(Madhu et al., 2012)**.

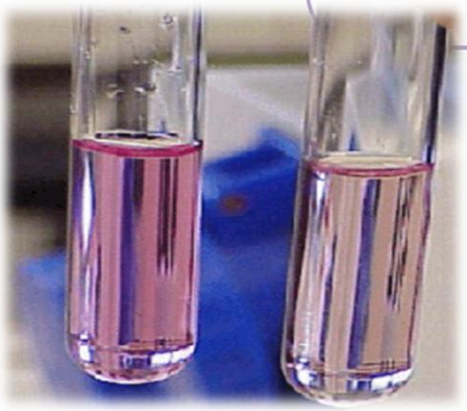
III.2.7.4. DPPH scavenging activity

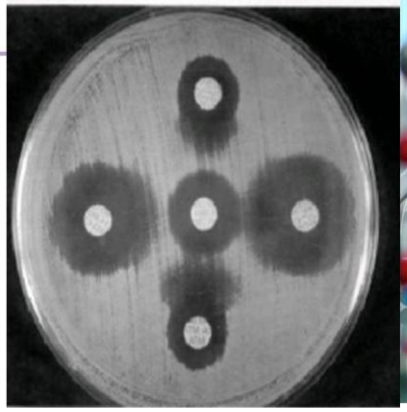
The antioxidant capacity of each milk samples during storage was determined with a stable radical substrate DPPH. Briefly, 800 µl of methanolic DPPH solution (0.1 mM of DPPH in methanol) was mixed with 200 µl of milk sample, vortexed well and incubated for 30 min. The samples were centrifuged for 10 min at 13,000 rpm, and the absorbance of samples was measured spectrophotometrically at 517 nm **(Madhu et al., 2012)**. The scavenging activity was determined as described above.

III.3. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Post hoc comparisons have been performed using the *t-test* when ANOVA was significant. The values were expressed as means ± S.E.M. *P-Values* < 0.05 were considered significant. All statistical analyses were carried out using Excel SPC software package. Results were considered to be significantly different when $p < 0.05$ and highly significantly different when $p<0.001$. For statistical analysis of stomach histology the data were analysed by the computer application SPSS for Windows® (version 15.0; SPSS Inc., Chicago, IL, USA).

Results and discussion





Research claims that the use of probiotics may represent an effective strategy to prevent deficiencies of antioxidant, thus new probiotic strain with antioxidative activity are needed. It is also important to note that interventions with ability to prevent or scavenge ROS are the most promising therapeutic targets against drug toxicity **(Waller and Sampson, 2018)**. Our study was focused first to select a probiotic bacterium with an antioxidant efficiency, then, to characterize the antioxidant potential of this probiotic bacterium *in vitro* for the first time and to reveal its protective effect against drug toxicity induced by two common used drugs (paracetamol and diclofenac); although the exact mechanism of such effect is not clear. For this, the expression level of some antioxidant enzyme genes will be considered, in an attempt to clarify a possible mechanism by which probiotic protects against paracetamol and diclofenac induced oxidative stress.

IV.1. Screening of antioxidative bacteria

Several reports are available on bacteria producing active antioxidants. Most of them reported species from *Lactobacilli* group of bacteria. Several commercial yogurt starter cultures like *Lb. bulgaricus, Lb. casei, Lb. acidophilus* and *Lb. rhamnosus* have shown different levels of antioxidant activity **(Kim et al., 2005)**. In the present work twelve lactic acid bacteria were tested for their antioxidant activities by DPPH method.

DPPH is commonly used for screening of potent antioxidant probiotics **(Afify et al., 2012; Coda et al., 2012)**. The interaction between antioxidants and DPPH neutralizes the DPPH free radical character, and changes the color of the reaction mixture from purple to yellow, due to their capability to accept an electron **(Mukherjee et al., 2014)**. DPPH assay is a useful method to screen potent antioxidant probiotics **(Afify et al., 2012; Coda et al., 2012)**. All tested bacterial strains were capable of scavenging DPPH radical, indicating an antioxidant potentiel of our strains. Results summarized in figure 11indicated that the level of free radical scavenging activity varied with the different strains from 23.34 % to 89.43% and 25.21% to 84.92% for intact cells and supernatant, respectively.

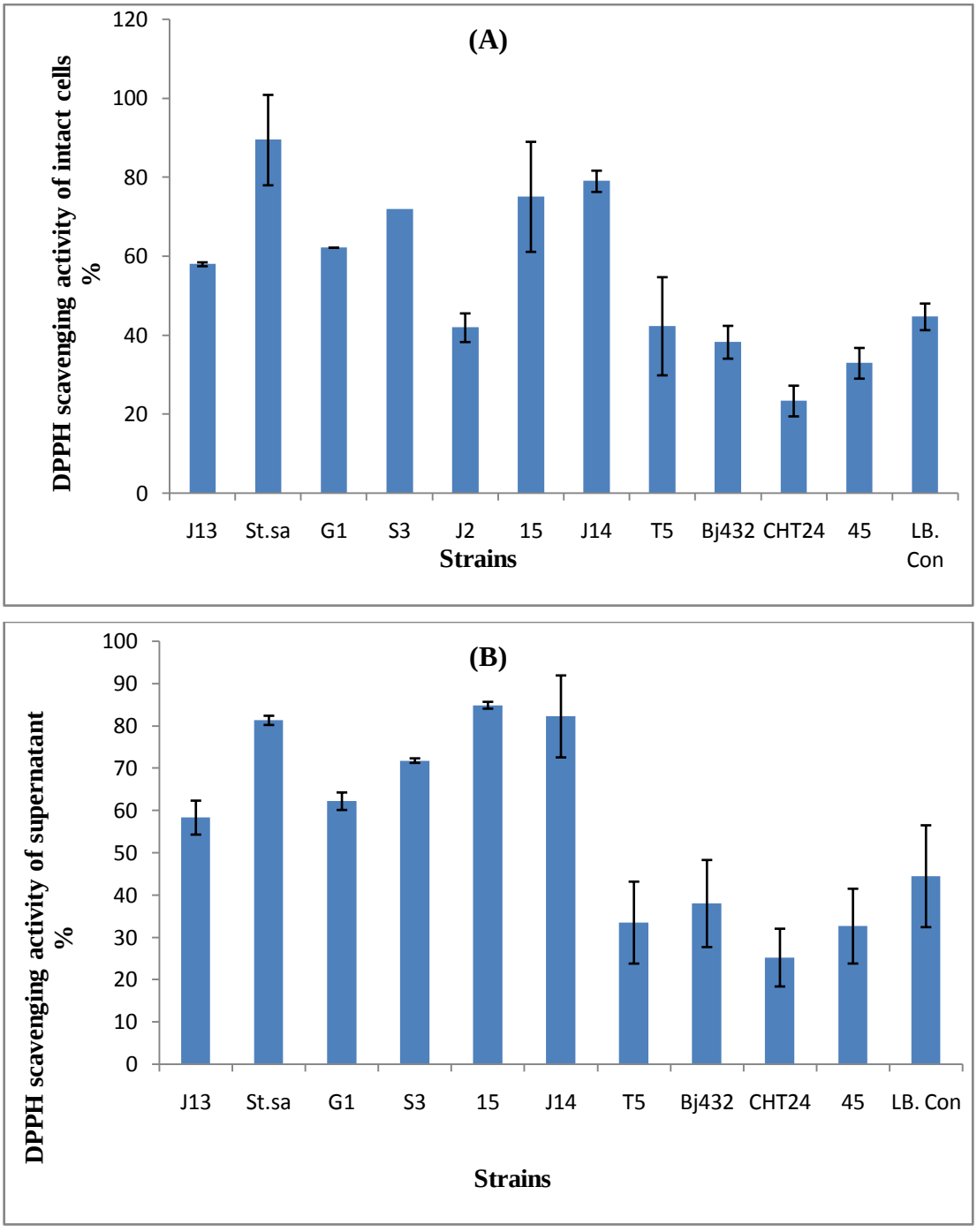


Fig. 11. *DPPH scavenger effect of probiotic bacteria.* ***(A)*** *for intact cells* ***(B)*** *for supernatant*J13: *Lb. viridescens,* St.sa: *Streptococcus salivarius* ssp *thermophilus,*G1: *Lb. plantarum,*S3: *Lb. delbrueckii* ssp *lactis,*J2: *Lb. plantarum,*15: *Lb. plantarum,* J14: *Lb. helveticus,* T5: *Lactobacillus* sp.,BJ432: *Lb. curvatus,*CHT24: *Lc. lactis* ssp. *cremoris* , 45: *Lactobacillus* sp and LB Con: *Lb. confusus*

Our results are correlated with those obtained by **Afify et al. (2012)** who demonstrated that *Propionibacterium freudenreichii* CFE and *Lb. retueria* had high antioxidant activity with

percentages of 97.75 % and 96.74 % respectively, scavenging of DPPH radical. Also, **Lin and Chang (2000)**, reported that *Lb. acidophilus* showed the ability to scavenge DPPH free radical by 43.2%–52.1%.

The results obtained demonstrated that scavenging of DPPH radical is strain specific features. This is in accordance with suggestions of **Amaretti et al. (2013)** who suggested that antioxidant characteristics of probiotic bacteria are strain-specific properties. Also, both intact cells and cell free supernatants exhibited antioxidant potential.

Taking in consideration the scavenging DPPH rate of intact cells we show that the strain *S. salivarius* ssp *thermophilus* St.sa has the highest level with a percentage of 89.43% followed by the strain *Lb. plantarum* 15. But the supernatant of *Lb plantarum* 15 had a higher scavenging DPPH rats then *S. salivarius* ssp *thermophilus* St.sa. Based on this result we selected *S. salivarius* ssp *thermophilus* and the strain *Lb. plantarum* 15 to evaluate their probiotic and their antioxidant properties. These strains have showed the highest level of DPPH scavenging activity in both intact cells and cell free supernatants.

DPPH, as a free radical, make possible to elucidate the structure-antioxidant activity relationships of the chemical compounds containing SH-, NH- and OH- groups **(Salah et al. 1995)**. Thus, the effect observed with our strains may not be attributed to the antioxidant enzymes released from the bacteria but rather to the presence of other molecule released from the bacterial strain **(Spyropoulos et al., 2011; Afify et al., 2012)**.

IV.2. Probiotic characterization of the selected strains

Probiotics are a group of organisms that confer health benefit to consumers. To be used as probiotic, an organism should possess several properties such as adhesive ability, bile and acid tolerance and significant antibacterial activity and the most important criterion is that it should be non-pathogenic **(Fakruddin et al., 2017)**.

IV.2.1. Bile salts and acid tolerance

Bile and acidic tolerance is a prerequisite for colonization and metabolic activity of probiotic bacteria in intestinal tract. Thus, it is important for predicting the survival of a strain *in vivo* when consumed in non-protected way because gastric juice contains bile salt and HCl **(Kim et al., 2006b; Mandel et al., 2013)**. Therefore, toqualify a strain as probiotics, it must have fulfilled certain physiologicalcharacteristics, including survival in the gastrointestinal tract,

tolerance to low pH and tolerance to bile salts **(Ji et al., 2015)**. The pH value of 3.0 has been considered standard for such investigation on probiotic strains **(Halder et el., 2017)**.
The results of the survival rate of the tested strains *S. salivarius* ssp *thermophilus* St.sa and *Lb. plantarum* 15 under low pH (2.0) and bile salts (1%) conditions are shown in figure12. As seen in this figure, the strains were found to be resistant after 6h of incubation at low pH value and bile salts condition, the viability of *Lb. plantarum* 15 under low pH is high (86.40%) but, for bile salts conditions no remarkable differences were shown.

The ability of LAB to maintain viability at low pH and in the presence of bile salts was reported in other studies, like the report of **N'tcha et al., (2016)** and **Song et al., (2015)**, which have demonstrated that *Lactobacillus* strains remained viable when exposed to acid pH values of 2.5-4.0, as well as bile salts concentration. In one hand, acid-tolerant strains have an advantage in surviving in the low pH conditions of the stomach (as low as pH 2.0), where hydrochloric and gastric acids are secreted, on the other hand, the physiological concentration of human bile ranges from 0.3% to 0.5%. Therefore, resistance to bile acid is an important characteristic that enables probiotic strains to survive, grow, and remain active in the small intestine **(Song et al., 2015)**. In the same line, according to **Kim et al. (2006a)** the ability of probiotic bacteria to survive the passage through the stomach was reported variable and strain dependent; these differences might be due to differences in the cell wall structure, because the survival of bacteria was reduced generally by destroying their cell membranes. The survival rates of *S. salivarius* ssp *thermophilus* St.sa and *Lb. plantarum* 15 are relatively high (Figure 12), suggesting the potentiality of our strains to survive in stomach and intestinal conditions.

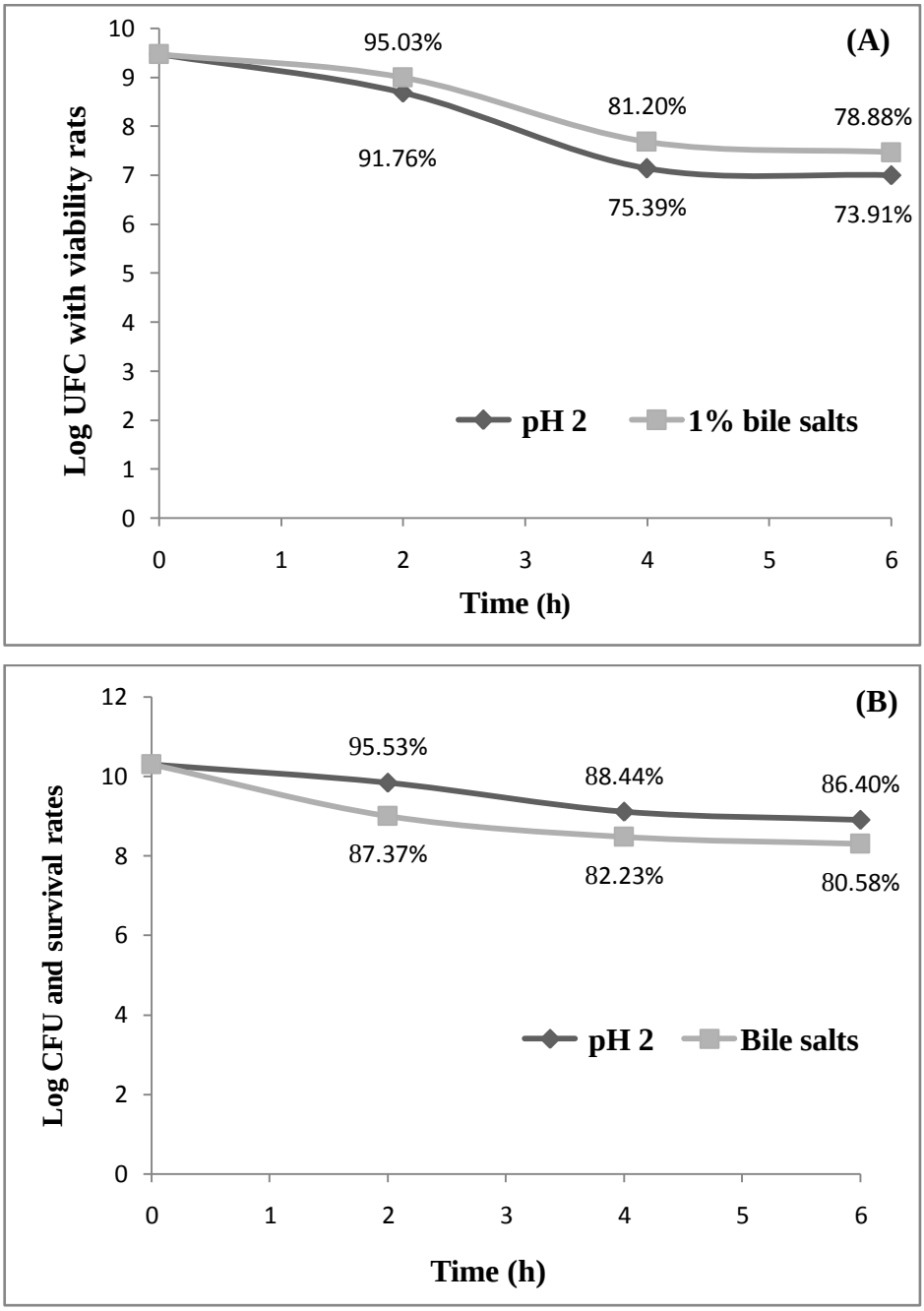


Fig. 12. *Viability of S. salivarius* ssp *thermophilus St.sa (A) and Lactobacillus plantarum 15 (B) in pH 2.0 and in 1% bile salts at 37°C in PBS.*

IV.2.2. Auto-aggregation

Auto-aggregation is among the most important criteria for probiotic strains selection. It can be considered as a marker for adhesion and colonization to mucus of the digestive tract. Auto-aggregation does not depend on the pH of the medium but on the presence of auto-aggregation factors in the supernatant **(Behira, 2012)**. Several authors have proposed that the aggregation test should be the first step to be performed since it has been demonstrated that

strains with a high aggregation have also a high hydrophobocity, this means a strong adhesion to the intestinal mucus **(Taheri et al., 2009)**.

Auto-aggregation is assessed by the rate of sedimentation. The Auto-aggregation of *S. salivarius* ssp *thermophilus* St.sa and *Lb. plantarum* 15 was evaluated and the results obtained are summarized in figure 14. The results demonstrated that auto-aggregation is increasing over time, and St.sa strongly autoaggregating with an autoaggregation percentage equal to 89% after 5h of incubation. While at the same conditions, the strain 15 showing only moderate auto-aggregation (50%). In a similar study conducted by **Gomaa (2013)**, it was shown that several probiotic strains: *Lb. plantarum, Lb. paracasei* and *Lb. acidophilus* have a self-aggregating power ranging between 51.12% and 78.17%.

The high autoaggregation capacity allows the cells to form a biofilm on the cells of the intestinal mucosa and to form a barrier to prevent pathogenic bacteria from attaching to the cells of the host mucosa **(Schachtsiek et al., 2004)**.

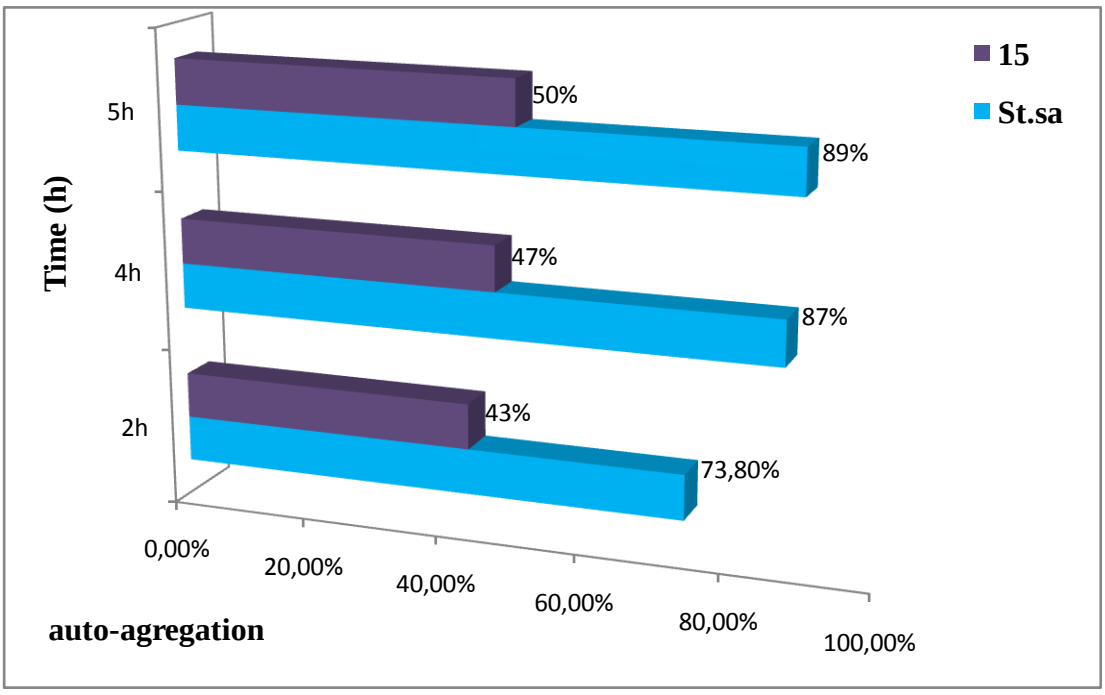


Fig. 13. *Auto-agrégation rate of S. salivarius* ssp *thermophilus St.sa and Lactobacillus plantarum* 15.

IV.2.3. Antimicrobial activity

To select any strain as a potential probiotic, it is important to assess its antimicrobial activity **(Fakruddin et al., 2017)**. This test allows highlighting the characteristics possessed by lactic acid bacteria to inhibit the growth of some pathogenic strains. It is clear from the results presented in Table 06 and figure 14 that the studied strains present a pronounced antagonism against pathogenic bacteria.

The diameters of the inhibition zones ranged from 11to 15.5mm, with a good inhibitory effect against *Bacillus subtilis* (15.5±0,1mm) for both strains. These results indicate that the bacterial isolates are able to synthesize inhibitory substances having antibacterial activity. Lactic acid bacteria are known to produce a variety of antimicrobial compounds such as organic acids, bacteriocins, diacetyl and hydrogen peroxide **(Titiek et al., 1996; Aslam et Qazi, 2010)**. The *in vitro* inhibitory capacity of lactic acid bacteria against pathogens seems like a good probiotic property, as it can play a role in maintaining the hygienic quality of food **(Ammor et al., 2006)**.

Table 06. *Inhibition zones diameter (mm) of the supernatant of S. salivarius* ssp *thermophilus St.sa and Lb. plantarum 15 against some pathogenic bacteria.*

Tested strain	*Listeria monocytogenes*	*Escherichia coli* ATCC 25922	*Bacillus subtilis*	*Salmonella* sp.	*Staphylococcus aureus*	*MRSA*
St.sa	14,6±0,05	12,2±0,09	15,5±0,1	12,7±0,05	12±0	11±0
15	12.5±0.15	12±0.1	14.2±0.05	12.3±0.05	12±0.14	12±0

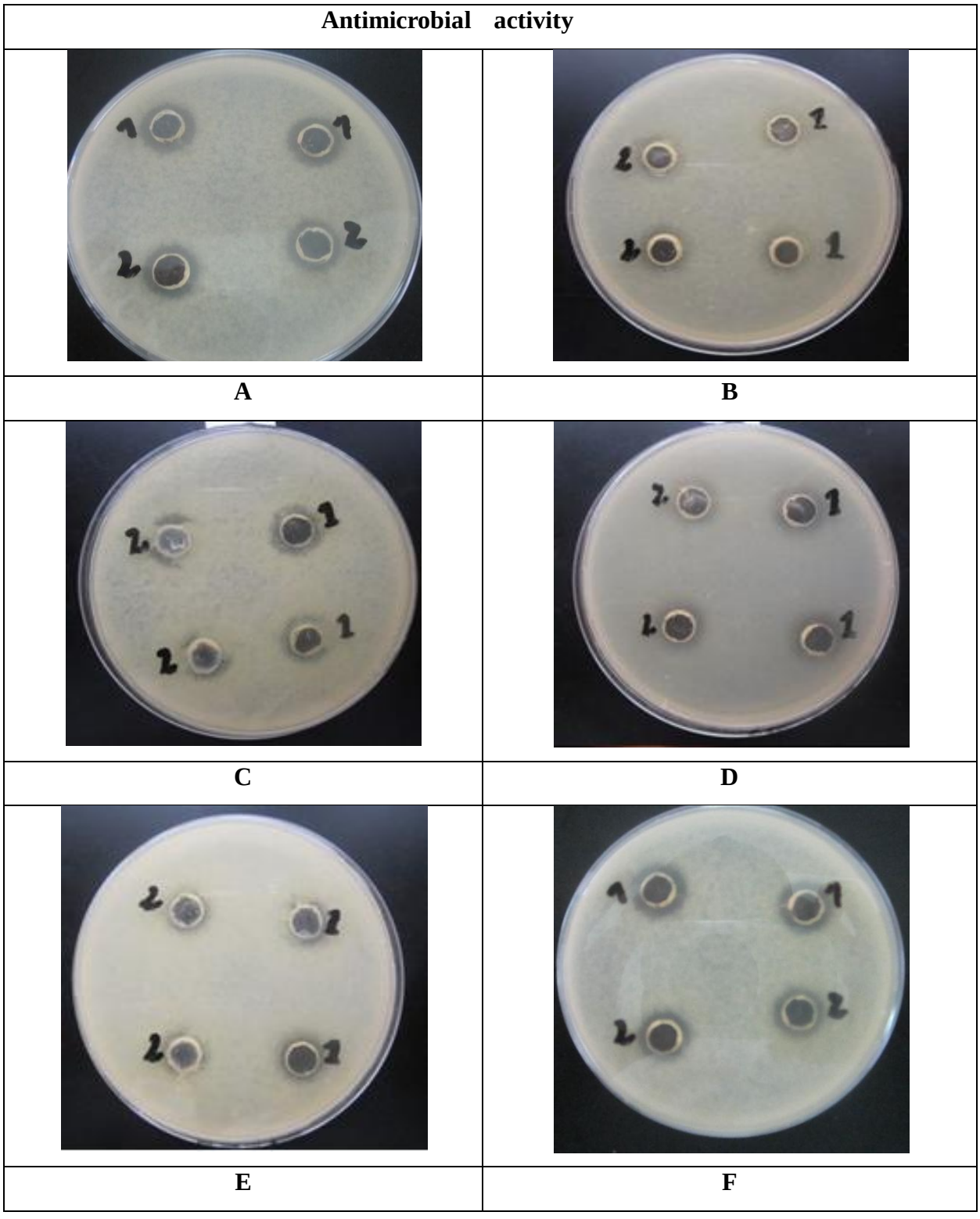


Fig. 14. *Antimicrobial activity of S. salivarius* ssp *thermophilus St.sa* (1) and *Lactobacillus plantarum* 15 (2) *supernatant on Mueller Hinton agar.* ***A*** *(Listeria monocytogenes),* ***B*** *(Escherichia coliATCC 25922),****C*** *(Bacillus subtilis),* ***D*** *(Salmonella),* ***E*** *(Staphylococcus aureus) et* ***F****(Methicillin resistant Staphylococcus aureus).*

IV.2.4. Safety profiling *in vitro*

The safety profile of the strains was determined by testing their hemolytic activity and antibiotic susceptibility.

IV.2.4.1. Antibiotic susceptibility

The results of resistance and susceptibility of strains to antibiotics are summarized in Table 08. Strains with an inhibition zone diameter less than 15mm are considered resistant **(Dalache et al., 2003)**. The obtained results indicated that the strain *St. Salivarius* ssp *thermophilus* St.sa has resistance to two antibiotics; it is sensitive to Penicillin G and Amoxycillin. In contrast, the strain 15 resists to all tested antibiotics. It should be noted that this resistance and sensitivity, can be related to the concentration of each antibiotic, hence to confirm the results it is important to test several concentrations. Several studies have shown the natural resistance of a wide range of lactic acid bacteria to antibiotics **(Botes et al., 2008)**.

Resistance to antibiotics indicates that the strain if introduced in patients treated with antibiotic therapy may be helpful in controlling intestinal disorders. On the other hand, sensitivity indicates that patients taking these antibiotics will not be appropriate for probiotic treatment. Moreover, the resistance of probiotics to antibiotics may be a problem if it can be transmitted to pathogens where the therapeutic resistance could have adverse consequences. The use of different lactic acid strains as probiotics must be the subject of extremely attentive work **(Hummel et al., 2007)**. Similarly, the European Food Safety Authority suggests that probiotics should not have acquired resistance to antibiotics **(Zago et al., 2011)**.

Table 07. *Results of antibiotic susceptibility*

	Sulphonamide (300µg)	Penicillin G (10 unités)	Colistin sulphate (50µg)	Amoxycillin (25µg)
S. Salivarius ssp *thermophilus*St.sa	- No zone (R)	21mm (S)	- No zone (R)	28mm (S)
Lb. plantarum 15	- No zone (R)	- No zone (R)	- No zone (R)	08mm (R)

(R): resistant, (S): sensible

IV.2.4.2. Hemolytic activity

Hemolytic activity is the ability of the isolate to cause lysis of red blood cells in the host. The results obtained after 48h of incubation in blood agar plates showed that *S. salivarius* ssp *thermophilus* St.sa and *Lb. plantarum* 15 were γ-hemolytic indicating that the strains St.sa and 15 did not exhibited any effect (non-hemolysis) and no red blood cell lysis activity observed on the blood agar. In contrast for hemolytic bacteria, an inhibition zone appeared (β-hemolysis), in this case, bacteria are able to break down the epithelial layer of the host cells and would cause invasive diseases in the host **(Nurhidayu et al., 2012**). Therefore, the determination of hemolytic activity is considered as a safety aspect for the selection of probiotic strains, which indicate that our strains are non-virulent in nature **(Pieniz et al., 2014)**, and according to **Kumar and Murugalatha (2012)** the strain may be safe for human use.

IV. 2.5. Calculation of probiotic potential

Cumulative probiotic potential (CPP) of the strains has been considered as an improved criterion for a successful probiotic validation (Table 09). The probiotic potential based on the cumulative probiotic score was 86.6% and 63.2% for St.sa and strain 15, respectively. The probiotic potential of our selected seems to be similar to that of commercial probiotic preparations available in the market, the last presented a CPP ranging from 75% to 85% **(Halder et al., 2017)**. Similarly, **Tambekar and Bhutada (2010)** compared the excellent probiotics isolated from milk for their probiotic potential with commercial probiotic preparations (Sporlac powder, LactoBacil plus, P-Biotics kid, Gastroline, Pre-Pro kid and standard probiotic bacterial strains *Lb. plantarum* (MTCC 2621) and *Lb. rhamnosus* (MTCC 1048)). Isolated LAB exhibited excellent probiotic characteristics than commercial probiotic preparations and standard probiotic bacterial strains **(Tambekar and Bhutada, 2010)**.

We can consequently consider that our strains meet the criteria of FAO/WHO, indetermining the status of a safe probiotic according to biomedicine **(Halder et al., 2017)**. Furthermore, **Tambekar and Bhutada (2010)** suggested that probiotic bacteria with a good CPP can be help to prevent or control the intestinal infections and contributes health benefits to consumers.

Table 08. *Cumulative probiotic potential (CPP) score*

Probiotic characters	Indication	Score for strain15	Score for strain St.sa
Acidic pH tolerance	Resistant = 1 Sensitive = 0	1	1
Bile salt tolerance	Resistant = 1 Sensitive = 0	1	1
antibiotic sensitivity	Sensitive = 1 Other resistance = 0	0	1
Antagonistic activity(Average)	14-16 mm 1 17-20 mm 2 > 21 mm 3	0.16	0.33
Hemolytic activity	α hemolytic=0 $^{\beta}$hemolytic=0 $^{\gamma}$hemolytic=1	1	1
Total score	5	3.16	4.33
CPP for strains		63.2%	86.6%

IV.3. *In vitro* assays of the antioxidant capacity of the tow strains

Oxidation process involves a cascade of reaction steps. Therefore, the antioxidant activities can be exhibited through multiple reaction mechanisms. In the absence of a universal method which can give unambiguous results, the best way to provide comprehensive information about total antioxidant capacity of the compound tested is to use different methods instead of one. In this context, various methodologies have been adopted to characterize the antioxidant efficiency of probiotic such as procedures involve the use of synthetic antioxidants (total antioxidant content) or free radicals to assay free radical scavenging activity (DPPH and hydroxyl radical), few are specific for lipid peroxidation and require animal or plant cells (inhibition of plasma lipid peroxidation) (**Mishra et al., 2015)**.

Thus, several different assays must be performed to provide comprehensive information about total antioxidant capacity of the compound tested. To characterize the antioxidant efficiency of *S. salivarius* ssp *thermophilus* and *Lb. plantarum* 15, different assay methods were employed such as free radical scavenging activity (DPPH and hydroxyl radical), inhibition of plasma lipid peroxidation and iron chelating activity.

IV.3.1. Resistance to hydrogen peroxide

Hydrogen peroxide is not a free radical but it is highly important because of its ability to penetrate into cells **(Nordberg and Arner, 2001)**. It was reported to be an intermediate during endogenous oxidative metabolism. It therefore mediates radical oxygen formation (**Hussein, 2011)**. Based on the results of the study of the impact of H_2O_2 on the viability of *S.salivarius* ssp *thermophilus* St.sa and *Lb. Plantarum* 15, the strains can be classified as tolerant to 1mM H_2O_2, after being exposed to 1mM H_2O_2 for 6h, the cells exhibited a survival rate of 77.08% and 76.12% for *S.salivarius* ssp *thermophilus* St.sa and *Lb. plantarum* 15, respectively which is acceptable (Figure 15). According to **Wang et al. (2009**) this tolerance is not unexpected, because hydrogen peroxide is one of the antimicrobial compounds produced by some lactic acid bacteria. These finding were similar to the results obtained by **Lee et al. (2005)** which found that *Lb. casei* and *Lb. rhamnosus* remained viable for 8h in presence of 1mM H_2O_2. However, *Lb. cas*ei had lost viability after less than 1h. It is then suggested that the resistance to hydrogen peroxide may be strain dependent.

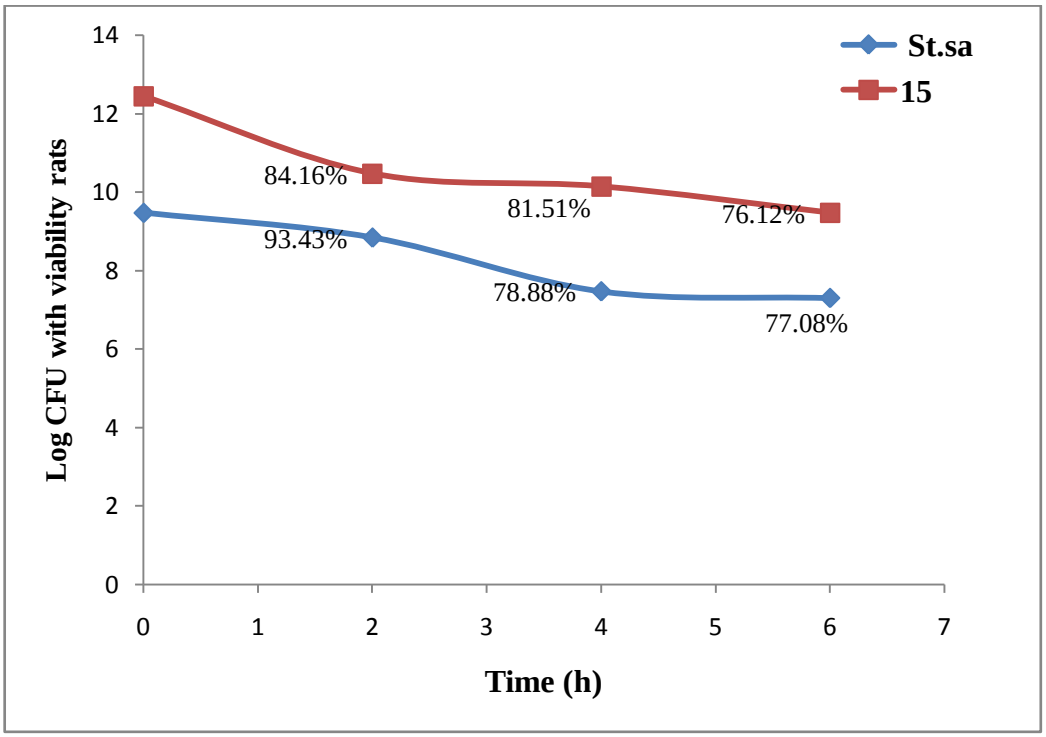


Fig. 15. ***V****iability of probiotic S.salivarius ssp thermophilus St.sa and Lb. plantarum 15 to 1mM hydrogen peroxide, in phosphate buffer at 37°C.*

IV.3.2. Hydroxyl radical scavenging effect

Among reactive oxygen species, hydroxyl radical is considered to be the most reactive oxygen radicals, which can react with all biomacromolecules functioning in living cells and can induce severe damage to cells. Therefore, scavenging of hydroxyl radical plays a critical role in reducing oxidative damage **(Xu et al., 2010; Zhang et al., 2011)**.

In this study, Fenton chemistry reaction was used to test the scavenging ability of *S. salivarius* ssp *thermophilus* St.sa and *Lb. plantarum* 15. The strains were found to be effective in scavenging hydroxyl radical (47% and 46.15%, respectively) (Table 09). Similar results were reported by **Lin and Chang (2000)**, which found that *Lb. delbrueckii* ssp *bulgaricus* possesses a good hydroxyl radical scavenging ability (134µM). Furthermore, **Lee et al. (2005)** found that *Lb. casei* KCTC 3260 is able to survive until 7h and resist to hydroxyl radicals while *Lb. rhamnosus* GG showed no viability in the presence of hydroxyl radicals. It is reported that the scavenger ability of the different types of ROS is considered as one of the main mechanisms of antioxidants presented by LAB. Hydroxyl radicals produced in the biological systems involved in the initiation of lipid peroxidation are highly reactive free radicals. Thus, hydroxyl radical is the main factor to cause oxidative damage *in vivo* due to its strong reactivity and oxidizing capacity on almost every type of molecules in living cells **(Nordberg and Arner, 2001; Kim et al., 2006; Kumar et al., 2014)**.

Table 09. *The different antioxidant activity assays*

Assay	**Activity of *Lb. plantarum* 15**	**Activity of *S. salivarius* ssp *thermophilus***
DPPH scavenging activity of cell pellet	75.06%	89.43%
DPPH scavenging activity of the supernatant	84.92%	81.34%
The scavenger capacity of hydroxyl radicals	46.15%.	47%
Iron ions chelating ability	20.52%	33.2%
Inhibition of plasma lipide peroxidation	nd	57%

nd: not determine.

IV.3.3. Iron ions chelating ability

Since it mediates catalyzing transition metals during lipid peroxidation, metal chelating activity is claimed as one of antioxidant mechanism. Among the transition metals, iron is consideredas the most important lipid oxidation pro-oxidant owing to its high reactivity **(Liu et al., 2011)**. It was reported that chelating agents, which form s-bonds with metal are

effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the iron ion **(Li et al., 2014)**.

The ferrous ion chelating effects of intact cells, was investigated.The strain *S. salivarius* ssp *thermophilus* St.sa and *Lb. plantarum* 15 showed chelating capacity against iron ions with rate of (33.2% and 20.52%, respectively) as shown in table 10. Previous study conducted by **Kim et al. (2006a)**, focused to evaluate the iron chelating ability of *Lb. casei* 01 illustrated that this strain was able to chelate iron ions with range of 72.06%. In addition, the intact cells ability to chelate metal ions have been also reported by **Zhang et al. (2011)**, in which *Lactobacillus* strains isolated from yogurt have showed the highest chelating ability by 50.55%, besides **Lee et al. (2005)** have reported that *Lb. casei* KCTC 3260 exhibited high metal ion chelating ability.

According to **Kęska and Stadnik (2017)**, chelating agents could fight toward oxidative stress due to their ability to mask the effect of metal ions. Iron ions are highly active metal involved in oxidative processes and participate in hydroxyl radical formation through Fenton reaction **(Kim et al., 2006a; Kęska and Stadnik, 2017)**.

IV.3.4. Inhibition of lipid peroxidation

Lipid peroxidation arises from a series of reactions between free radicals and lipids. The product of lipid peroxidation includes malondialdehyde (MDA) which is considered as an important feature of cellular injury, due to its ability to damage proteins, nucleic acids, and other biological macromolecules resulting in numerous pathological events, including inflammation, metabolic disorders and cell aging **(Mukherjee et al., 2014)**. Inhibition of lipid peroxidation is commonly determined for the analysis of antioxidant activity. In this study, lipid peroxidation was significantly inhibited in presence of intact cells of St.sa with a rate of 57% (Table 09). **Ou et al. (2006)** obtained similar results when they studied the antioxidant activity of the yoghurt bacteria *S. salivarius* ssp *thermophilus* ATCC19258 and *Lb. delbrueckii* ssp *bulgaricus*. In addition, **Wang et al. (2016)** reported that free radicals were produced through redox cycle by metal ions, and promoted lipid oxidation. The inhibitory effect of probiotic *S. salivarius* ssp *thermophilus* against lipid peroxidation could be related to its ability to scavenge free radicals and /or to chelate metal ions. Thus, if *S. salivarius* ssp *thermophilus* could scavenge free radicals or chelate metal ions it may decrease or inhibit lipid peroxidation. Moreover, **Namiki (1990)** reported that scavenging of different

types of ROS was thought to be one of the main antioxidant mechanisms of antioxidant action exhibited by lactic acid bacteria.

IV.4. Producing exopolysaccharides as a mechanism of antioxidant efficiency

LAB are useful microorganisms in dairy technology and they also contribute to the benefic effects on the health and their utility is reinforced by their demonstrated probiotic properties **(Liu et al., 2011)**. In addition, they exhibit antioxidant activity in all major way; they may reinforce the inherent cellular antioxidant defense by secreting enzymes like superoxide dismutase (SOD). They also release and promote the production of the major non-enzymatic antioxidant and free radical scavengers. Moreover, they promote the production of certain antioxidant biomolecules, such as the exopolysaccharides (EPS) (**Afify et al., 2012)**. Our result showed that the strains St.sa and 15 were capable to grow on hyper-sucrose agar. In addition, St.sa and15 colonies have a mucoid appearance indicating their ability to produce EPS (Figure16). St.sa showed better EPS production; consequently we select EPS produced by St.sa for antioxidant potential evaluation.

Fig. 16. *Exopolysaccharides produced by S. salivarius* ssp *thermophilus St.sa on hyper sucrose agar.*

In the same line, during the last few decades exopolysaccharides produced by LAB are the subject of an increasing number of studies. Due to their generally recognized as safe, EPS from LAB are potentially useful as safe additives to improve texture and viscosity of natural fermented products, moreover it has been suggested that some EPS produced by LAB may confer health benefit to the consumer **(Li et al., 2014)**.

IV.4.1. EPS quantification

Exopolysaccharides were estimated as total carbohydrates by phenol-sulphuric acid method using glucose as standard (appendix 3). This method is simple, rapid, and sensitive, and gives reproducible results **(Dubois et al., 1956)**. The method detects virtually all classes of carbohydrates, including mono-, di-, oligo-, and polysaccharides (**Nielsen, 2010**). The basic principle of this method is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid, produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectible color (**Albalasmeh et al., 2013**). In the present study, the tested strain has shown EPS production with a concentration of 34.4 mg/l. Other similar studies have obtained high concentrations of EPS compared with our results. **Savadogo et al. (2004)** has obtained a range of 100-600 mg/l of EPS produced by *Lb.lactis* subsp. *lactis.* In the same topic, **N'tcha et al. (2016)** have found greater EPS, *Lb. casei* (1130 mg/l), *St. thermophilus* (750 mg/l), *Lb. fermentum*(700 mg/l) and *Enterococcus faecium* (330 mg/l). The amount of EPS production differs between genera, also, these differences may probably due to the production coditions such as temprature, pH and medium composition **(Lai, 2014)**. Therefor, we suggest further studies to optimise EPS production.

IV.4.2. Determination of antioxidant activity of EPS

After the evaluation of EPS antioxidant activity, it has been shown that EPS produced by the strain St.sa showed high free radical scavenging activity and metal ion chelating ability as illustrated in table 10, these results revealed that EPS is one of the mechanisms responsible for the antioxidant activity of this strain. The results obtained by **Li et al. (2014)** demonstrated that EPS of *Lb.helveticus* MB2-1 had strong scavenging acticity on superoxide anion (71.82%), the EPS minimized the concentration of ferrous ion in the Fenton reaction and, the sccavenging effect of the EPS might be due the active hydrogen donating ability of the hydroxyl substitutions of EPS. Therefore, it was reported that chelating agents, which form s-bonds with metal are effective as secondary antioxidants because they reduce the redox potential there by stabilizing the oxidized form of the iron ion **(Li et al., 2014)**.

Table 10. *Antioxidant ability of EPS produced by the strain St.sa*

Assays	Scavenging DPPH	Scavenging hydroxyl radical	Chelating ferrous ions
Percentage of EPS activity	44%	42%	70%

The strain St.sa was selected for the *in vivo* study because of its probiotic potential and its antioxidant ability.

IV.5. Role of probiotic *S. salivarius* ssp *thermophilus St.sa* in reducing NSAID (diclofenac and paracetamol) toxicity

IV.5.1. Diclofenac and paracetamol hepatotoxicity

IV.5.1.1. Hepatic markers and histology study

Earlier studies have shown that Non-steroidal anti-inflammatory drugs (NSAIDs) are the most common drugs responsible for adverse drug reactions (**Bolat and Selenk, 2013)**. NSAIDs are commonly used for treatment of arthritis and other musculoskeletal disorders **(Maity et al., 2008)**. They are the most common toxicity-inducing drugs which can be fatal in some cases **(Dhikav et al, 2003)**. Liver as a major organ involved in drug metabolism is susceptible to the injury when exposed to drugs, which is generally indicated by the elevated levels of serum enzymes in the liver (**Amacher, 1997; Dass and Sattigeri, 2018)**. The level of aminotransferase activity is considered as serum biochemical marker of hepatic injury **(Amacher, 1997)**.

In order to evaluate liver damage, AST, ALT and ALP enzymes were analyzed. During treatment, no particular mortality was recorded in either group. The results illustrated in figure 18 showed that AST, ALT and ALP content in blood were similar in probiotic fed rats and control rats indicating that probiotic administration didn't affect the levels of hepatic markers in rats of probiotic group. This confirms the safety criteria of our probiotic strain. Significant ($p<0.01$) increase in AST, ALT, ALP levels in rats received overdose of paracetamol or diclofenac. It is reported that diclofenac may cause an asymptomatic increase of plasma transaminase in approximately 15% of patients **(Yano et al., 2012)**, suggesting its ability to induce hepatotoxicity **(Bort et al., 1998)**. The elevated serum enzyme levels are indicative of

cellular leakage and functional integrity of cell membrane in liver **(Sathesh Kumar et al., 2009).** The liver marker enzymes (AST, ALT and ALP) are cytoplasmic in nature; upon liver injury these enzymes enter into the circulatory system due to altered permeability of membrane **(Zimmerman and Seeff, 1970)**. AST level provides a general estimation about any cellular injury occurred as its level increases in case of disease and cellular injury. On the other hand, ALT more specifically indicates liver cell damage and higher serum cholesterol. Increased ALP has been linked with increased osteoblastic activity and lack of bile flow and higher serum cholesterol **(Fakruddin et al., 2017)**. The obtained results demonstrated the severity of paracetamol and diclofenac toxicity leading to tissue damage. Our findings are in agreement with the results reported in some other studies. According to **Ramm and Mally (2013**) the liver toxicity of diclofenac could be related to the metabolites excreted during diclofenac metabolism. In addition, this toxic effect may be induced through various mechanisms such as mitochondrial permeability transition, activation of cytochrom P450 and generation of ROS.

The obtained increases were significantly reduced by the probiotic pretreatment as shown in group fed with probiotic cells for 7 days before the administration of a diclofenac and paracetamol overdose (Figure 17). Indicated that the probiotic strain can significantly protect the integrity of cell membrane ($p<0.05$) against paracetamol and diclofenac induced alterations of these parameters, which revealed the possible protective role of *S. salivarius* ssp *thermophilus St.sa* against paracetamol and diclofenac hepatotoxicity associated with a non-toxic effect of this strain.

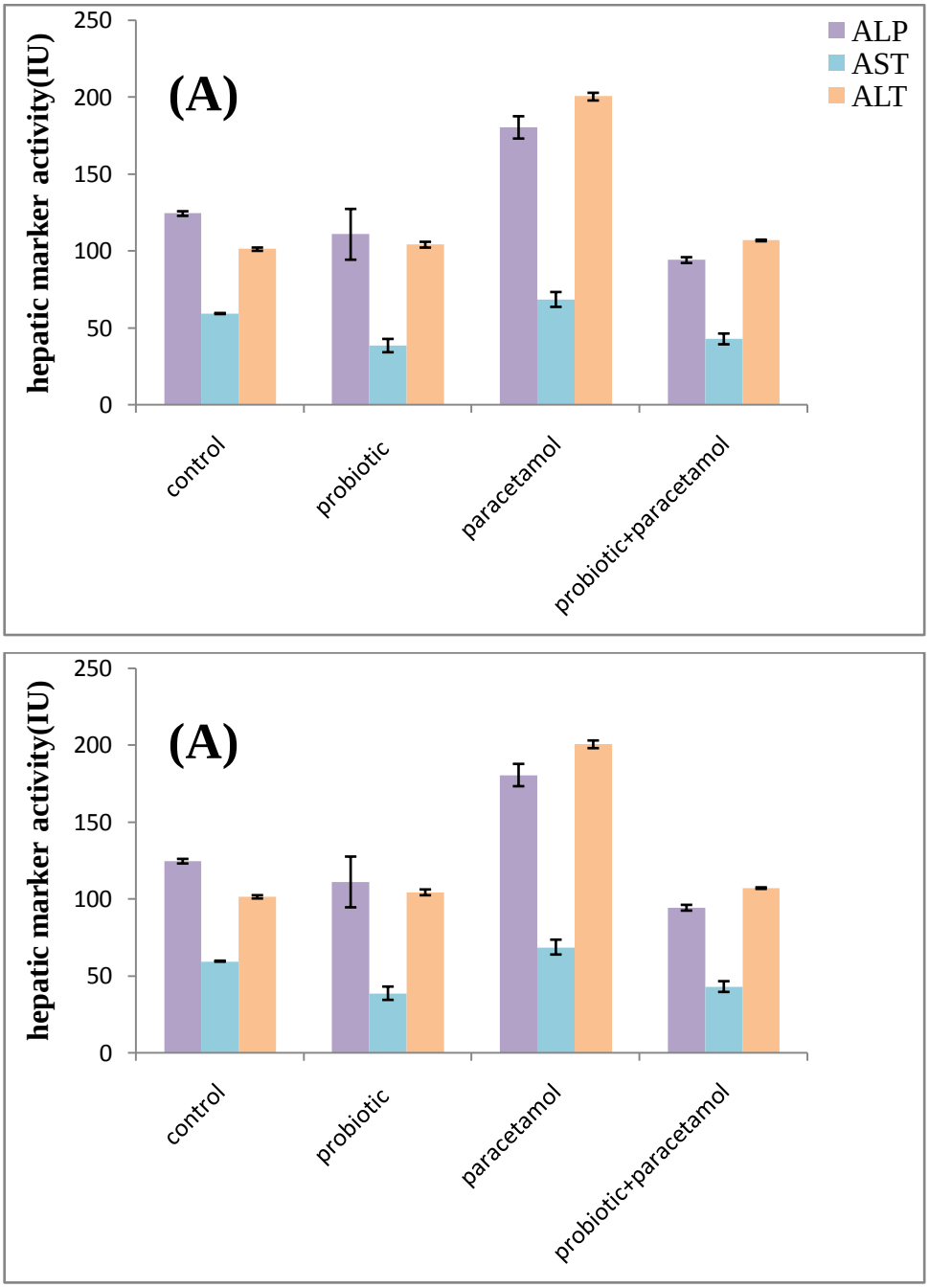


Fig. 17. *Variation in ALP, AST, and ALT (UI) determined in serum from rats specimens unexposed or exposed to drug overdose (paracetamol (A) and diclofenac (B)) with or without probiotic pre-treatment. Results are expressed as mean ± SEM. Asterisks (*) denote significant differences between control rats and paracetaml or diclofenac group ($P < 0.05$). (#) denote significant differences between paracetaml or diclofenac treated group with probiotic pretreatment relative to rats without probiotic pretreatment.*

Additionally, the micrographs of liver sections presented in figure 18 revealed normal liver architecture in control (Fig. 18.A) and probiotic treated group (Fig 18.B). These observations demonstrate that the probiotics are safe and well tolerated. In contrast, liver tissue of diclofenac treated group showed a damaged liver architecture with severe alterations such as infiltration of inflammatory cells. Changes in diclofenac treated rats are commonly found in other researches that reported that diclofenac at high doses induced hepatocellular necrosis as well as a severe hepatotoxicity **(El-Kordy and Makhlouf, 2014; Huang et al**., **2016)**. Impressively, the pretreatment with probiotics reduced the typical hepatotoxicity characteristics of liver. The results of hepatic markers and histological analysis together indicated that a single overdose of diclofenac administration to rats caused hepatotoxicity. In addition, the pretreatment with probiotics for 7 days before diclofenac administration can minimize histological injury. It could be concluded that a correlation between hepatic markers levels in serum and histological analyses exists; suggesting that cellular damage to liver tissue including necrosis areas observed by histological study increased the levels of ALP, AST, and ALT in serum.

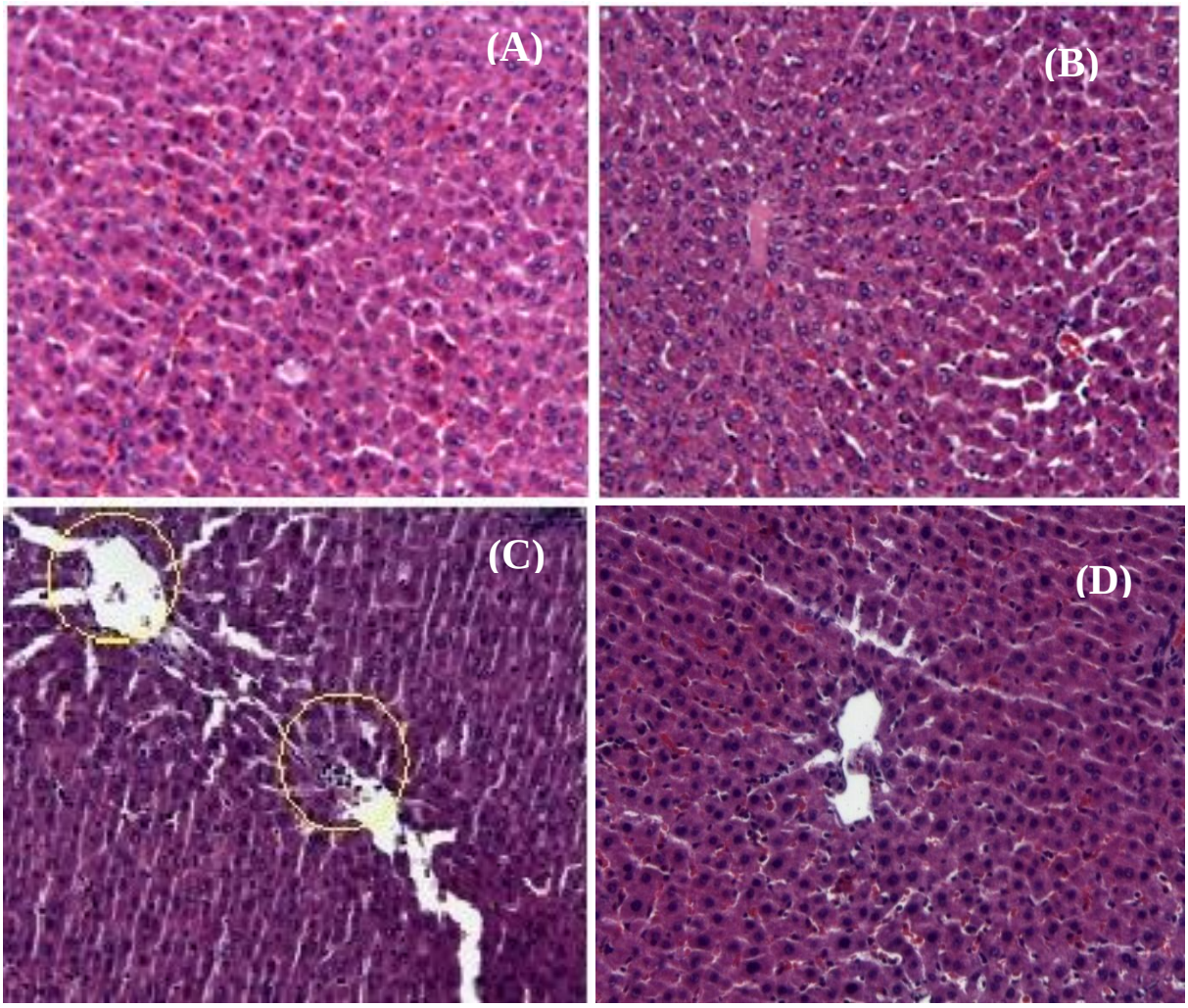


Fig. 18. *Representative micrographs of liver sections of: controlrat (A), probiotic pretreated rat (B), rat exposed to over dose of diclofenac (C), rat treated with over dose of diclofenac after probiotic pretreatment (D) (X400).*

IV.5.1.2. Non-enzymatic antioxidants

Cellular biomembranes are one of the major targets of ROS, where they induce lipid peroxidation resulting in the production of malondialdehyde (MDA) **(Wang et al., 2012)**. MDA is accepted as a marker of oxidative stress because it is one of the final products of lipid peroxidation formed during oxidative degeneration, and an increase in ROS causes over production of MDA **(Bolat and Selenk, 2013; Mandal et al., 2013)**. Evaluation of MDA production in liver of rats in our study demonstrated that its level in probiotic group was similar to that of control rats. In contrast, the level of MDA in paracetamol and diclofenac treated group, showed a significant elevation ($p<0.05$) compared with the control which

indicated that paracetamol and diclofenac had induced oxidative stress. It is reported that NSAIDs affect the mitochondrion and therefore oxidative phosphorylation, increased ROS production may occur, particularly of $O\cdot_2$, leading to an increase in MDA, and these processes may be explained the increase found in liver of rats treated with an overdose of diclofenac and paracetamol. However, a highly significant ($p<0.05$) reduction of toxicity in probiotic pretreated rats was noted,(Figure 19A).This may be due to the powerful antioxidant activity and free radical scavenger effect of the probiotic strain. The results are in agreement with the finding of **Chen et al. (2013)** who indicated that probiotic supplementation recovered antioxidant capacity and decreased lipid peroxidation. These findings suggest that the strain *S. salivarius* had the ability to attenuate oxidative stress by decreasing the lipid peroxidation level in paracetamol and diclofenac treated rat liver.

It should be mentioned that glutathione (GSH) has a very important role in protecting against ROS damage **(Costantini and Verhulst, 2009)**. Similarly, treatment of rats with diclofenac and paracetamol alone caused a significant decrease ($P < 0.001$) in liver reduced glutathione level when compared with control group. This significant depletion in liver glutathione content suggests a detoxification function of the glutathione system which is well reported **(Forrest and Clements, 1982; Aouacheri et al., 2009; Ji et al., 2012)**. Glutathione depletion following diclofenac exposure has been shown to occur using an *in vitro* hepatotoxicity model **(Pourahmad et al., 2011)**. Similarly **El-maddaway and El-Ashmawy (2013)** found that diclofenac administration to rats increased lipid peroxidation and decreased GSH content in liver tissue which indicates the generation of an oxidative stress.

The depletion of GSH content found may be explained by the fact that drug metabolism involves GSH conjugation. The administration of paracetamol toxic dose elevated the level of MDA and decreased GSH levels indicating a failure of antioxidant defense machinery due to the formation of a reactive intermediate NAPQI that immediately reacts with glutathione **(van de Straat et al., 1987)** and /or ROS generation. On the other hand, the main products of diclofenac metabolism are hydroxylated metabolites, with predominant of 4′-hydroxy diclofenac (OH-DCF), that can form reactive quinone imines that adduct and deplete glutathione causing a state of oxidative stress **(Tang et al., 1999)**. Hence, higher dosage of diclofenac can lead to the depletion of GSH thereby causing reduced antioxidant status and accumulation of toxic metabolites. Interestingly, the pretreatment for one week with probiotic strain, significantly restored the liver GSH content ($p<0.05$). Similarly, **Mandel et al. (2013)**

showed that oral administration of *Lb. ingluviei* ADK10 at a dose of 10^9CFU to paracetamol induced oxidative stress in rats, significantly increased GSH and MDA level in liver.

This beneficial effect may be due to the intake and/or the induction of synthesis of non-enzymatic antioxidants such as GSH by probiotics **(Amaratti et al., 2013)**. **Spyropolous et al. (2011)** showed that the beneficial effect of probiotics on health is due to the production of GSH by probiotics in the gut. In addition, intake of probiotics could induce the transcription of genes involved in the biosynthesis of GSH in the intestinal mucosa of the host **(Lutgendroff et al., 2009)**.

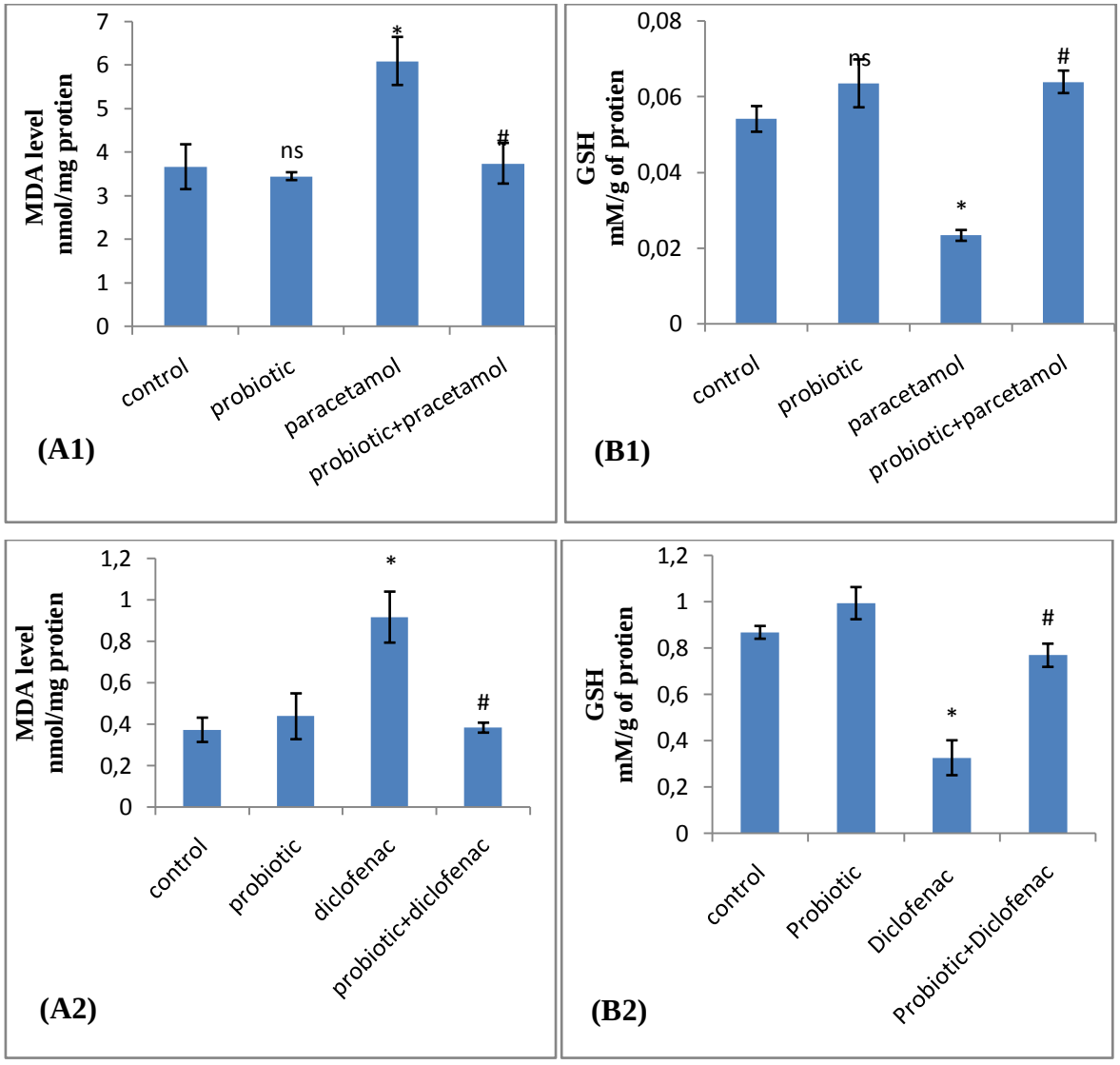


Fig. 19. *Levels of MDA and GSH in rat liver. (A) level of MDA; (B) level of GSH*

IV.5.1.3. Enzymatic antioxidants

The hepatotoxicity is not usually caused by free radicals only; but it is formed also due to exhaustion of SOD, CAT, and GSH. Their depletion is majorly caused by oxidative damage of these proteins **(Soto, 2003)**.

As shown in figure 20 A**,** no significant difference was found in control and probiotic alone groups for studied parameters except for CAT activity, which was found to be lower in probiotic treated group. Inhibition of CAT activity in rats treated with *S.salivarius*is in accordance with the findings reported by **Tabak and Bensoltane (2011)** who reported a decrease in catalase activity in rats fed with a probiotic strain. Decreases in antioxidant enzyme activities in the group pretreated with probiotic could be attributed to the loss of the central metal ion essential for reactivity. Since it contains iron ion in its heme group attached to the peptide chain. In addition, decreased activity of catalase was found in both diclofenac and paracetamol administered rat liver as compared to control animals, indicating the inhibition of the most important antioxidant enzyme. Pretreatment of animals with probiotic was found to increase ($P < 0.01$) the CAT activity highly significantly as compared to the corresponding paracetamol and diclofenac treated rats.

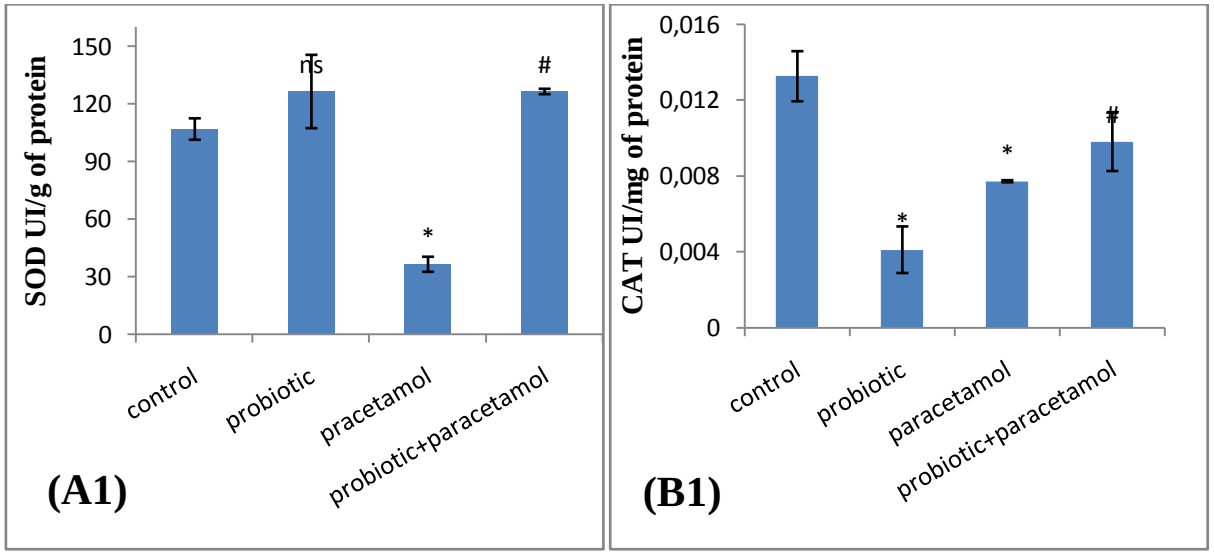
The Activity of superoxide dismutase significantly decreased in diclofenac and paracetamol treated rats, indicating that the endogenous antioxidant system was damaged. While pretreatment with St.sa could restore this activity.

Our investigation demonstrated that diclofenac and paracetamol administration induced a significant depletion of CAT and SOD activities ratifying the toxic effect of pracetamol and diclofenac, as a result of ROS production, confirmed by the high levels of MDA. The pretreatment with probiotic bacteria can normalize these activities (Figure 21) where an increase in the activity of these enzymes compared to the group receiving the toxic dose of diclofenac and paracetamol was noticed. However, this effect is not significant with paracetamol toxicated rats. These findings are similar to previous studies on a mouse model and diclofenac-induced liver injury, where SOD and CAT activities showed a significant decrease in animals treated with diclofenac alone compared with the normal group **(Jianchun et al., 2016)**.

Mandal et al. (2012) reported that paracetamol administered to a toxic dose resulted in decreasing or inhibiting of SOD and CAT activities following the generation of ROS. Indeed, the drug causes increased production of microsomal superoxide and hydrogen peroxide in mice. The depletion of SOD and CAT enzymes could be a response toward increased ROS

generation as well as the observed increase of MDA **(Arafa et al., 2005; Koti et al., 2009)**. The antioxidant enzymes CAT and SOD constitute the first line of enzymatic antioxidant defense against free radicals. SOD catalyses dismutation of super oxide anion into hydrogen peroxide which is a less harmful product, and then the hydrogen peroxide was removed by CAT and glutathione **(Hsu et al., 2015)**. The protective effect of probiotics *S. salivarius*s could be related to the secretion of enzymes such as SOD by bacteria to resist against oxidative stress, as several selected strains have very high levels of SOD activity **(Re et al., 2005; Mandal et al., 2012)**.

Antioxidant enzyme activities had contradictory changes toward oxidative stress **(Ciobica et al., 2011)**. In normal organisms, antioxidant enzymes activities should increase under oxidative stress conditions to protect the organisms against harmful effects. However, if oxidative condition was very severe, the body will exhausted, so the enzyme activity may decreases, although oxidative stress is present. It depends on the state of the cell/ tissue. Thus, after a mild increment in reactive oxygen species generation, cells are able to increase the antioxidant response and overcome stress. However, after high oxidative damage, cells are no longer able to protect themselves and even antioxidant enzymes could be degraded **(Ciobica et al., 2011; Porokhovnik et al., 2015)**. **Oliveira et al., (2015)** reported that paracetamol exposure may induce deleterious effects including enzyme inactivation. Thus, the significant decrease in the antioxidant enzyme activities may be explained as a consequence of direct inactivation of these enzymes caused by reactive metabolites generated after paracetamol or diclofenac administration.



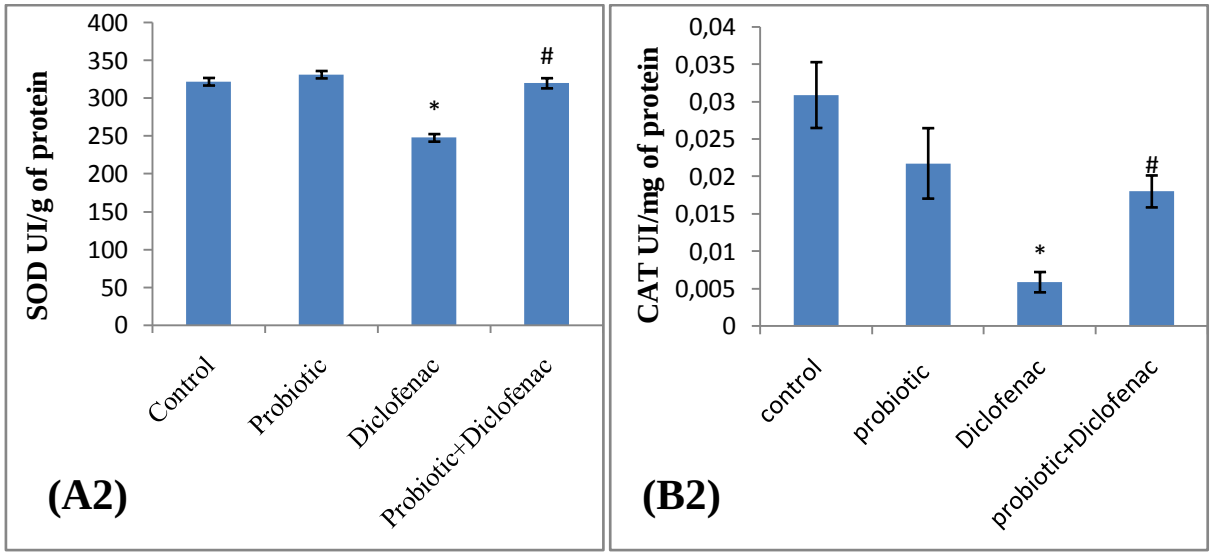


Fig. 20. *Effects of probiotic on the expression of antioxidant enzymes rat liver. (A) SOD activity; (B) CAT activity. All values are expressed as mean±S.E.M*

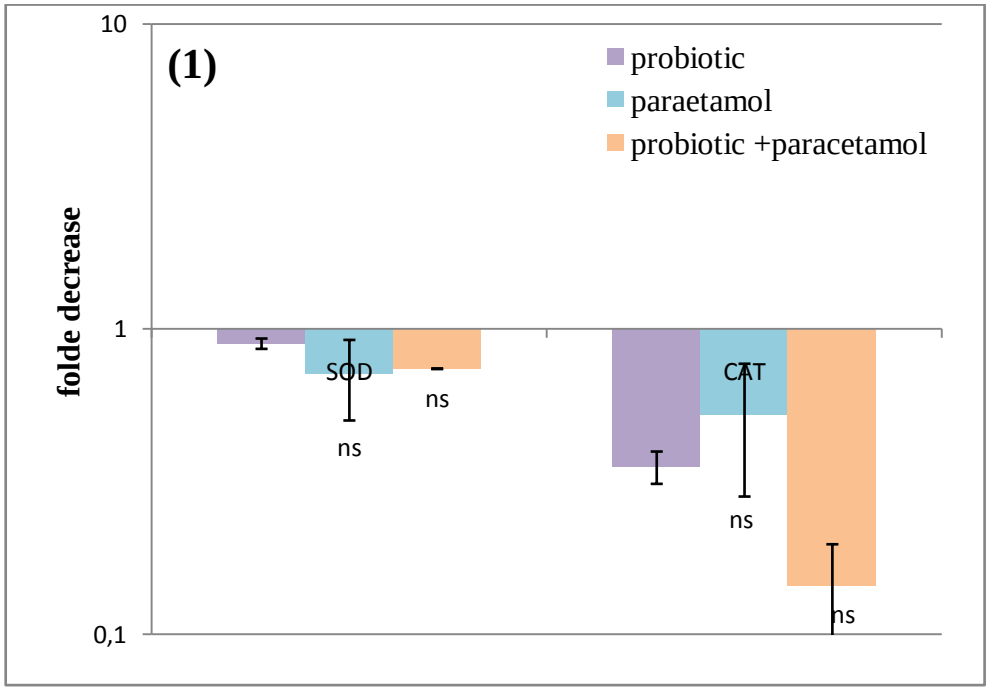
Each value is expressed as mean±S.E.M. (*) denote significant differences between control rats and diclofenac group (P < 0.05). (#) denote significant differences between diclofenac treated group with probiotic pretreatment relative to rats without probiotic pretreatment. SOD: superoxide dismutase CAT: catalase.

IV.5.1.4. Gene expression

Castex et al. (2012) associated the reduction of antioxidant enzymes activity with decreased oxidative stress and free radical activities. The body expressed fewer antioxidant enzymes due to the lower level of oxidative stress and free radical. The levels of expression of body-protecting chemical enzymes as well as the balance among them are critical to harm or influence oxidative injury in cells. These data allowed us to focus our investigations to clarify a possible mechanism by which drugs (diclofenac and paracetamol) and probiotic exert their effects, through analyzing the expression of the antioxidant enzyme genes. In this part, we analyzed whether the level of mRNA expression of antioxidant enzyme SOD and CAT is affected by administration of paracetamol and diclofenac toxic doses and probiotic.

Our results showed no significant differences in the expression of both SOD and CAT in all groups except for rats of the group to which diclofenac was administered without probiotic presented a significant increase in the expression of SOD and CAT (Figure 21). This could be due to the mechanisms by which ROS can regulate the transcription factors of gene expression **(Koziorowska-Gilun et al., 2015)**. First, the results concerning paracetamol toxicity

revealed that the changes in oxidative stress parameters may not always require an over-expression of antioxidant enzymes during paracetamol toxicity **(Cigremis et al., 2009)**, and this may be due to the fact that paracetamol toxicity was a direct action of its known reactive metabolite NAPQI, rather than a consequence of gene regulation **(Ruepp et al., 2002)**. Similar findings were obtained when the effect of paracetamol toxicity on hepatic mRNA expression of SOD, CAT and GSH-Px was investigated. No change was observed in mRNA expression of SOD and CAT in response to paracetamol toxicity in rabbit's liver **(Cigremis et al., 2009)**.



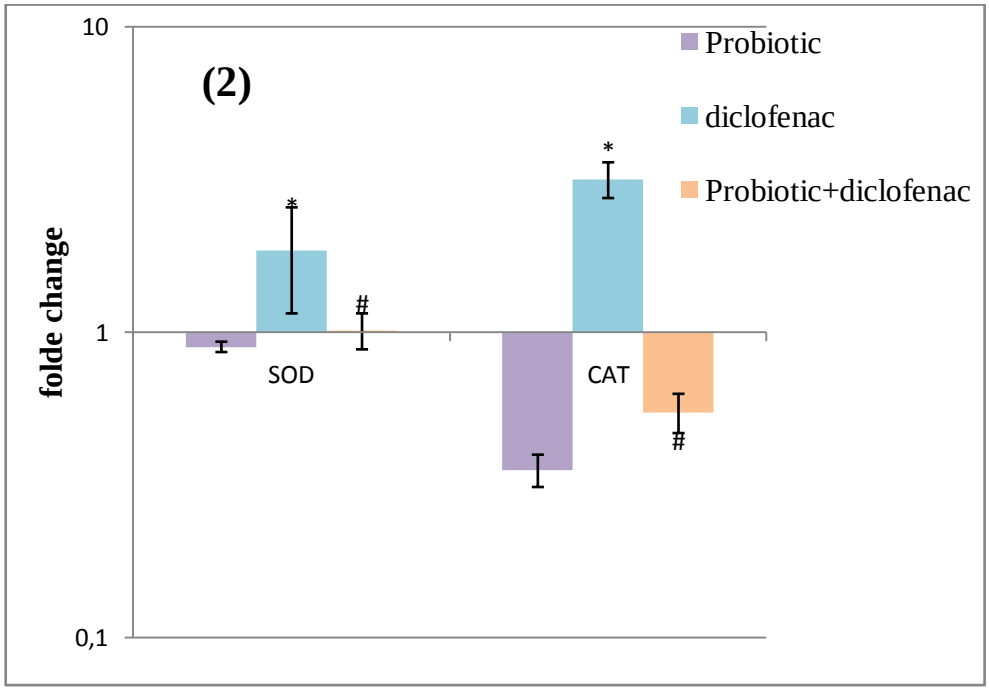


Fig. 21. *Effects of probiotic on the expression of antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) genes determined by real-time PCR in the liver of rats after paracetamol (1) and diclofenac (2) overdose administration. All values are expressed as mean±S.E.M. (ns) denotes no significant differences between control rats and drugs administered groups (p > 0.05). (*) denote significant differences between control rats and diclofenac group. (#) denote significant differences between drug treated group with probiotic pretreatment relative to rats without probiotic pretreatment.*

In the second part we showed that diclofenac overdose induce gene expression of antioxidant enzymes. This induction of gene expression could be related to the over generation of radical induced by diclofenac overdose. Many researchers reported that higher free radical production is associated with over expression of the antioxidant **(Costantini and Verhulst, 2009**), in this case and like many other cases **(Bolat and Selenk, 2013)**, there is an over generation of free radicals after diclofenac administration resulting from diclofenac metabolism and the preventive capacity of probiotics may be then due to their powerful scavenging ability of free radicals. In contrast to our results, **Hassan et al. (2017**) reported that oral administration of diclofenac sodium leads to significant reduction in renal SOD and CAT gene expression versus normal control group. These contradictory results could be explained according to **Franco et al. (1999)** who suggested that the response of antioxidant enzyme genes to oxidant stress differ in a tissue-specific manner**.** On the other hand, our results showed that oral administration of an over dose of paracetamol in rats didn't affect gene expression of the enzymes in liver while diclofenac overdose administration increases the expression of SOD and CAT significantly in the same tissue (liver). Thus, it appear that response of antioxidant enzymes gene not only related to the specificity of tissues but also, related to other factors such as the nature of pro-oxidant and dose **(Ciobica et al., 2011)**.

When we compare SOD and CAT at mRNA level and SOD and CAT activity levels, the results revealed that SOD and CAT mRNA expression was induced after diclofenac administration. However, the level of their enzyme activities decreased significantly. Furthermore, probiotic pretreatment reversed this situation. In the same time paracetamol administration in rats didn't affect the level of mRNA with a significant decrease in their enzyme activity. This unparallel change of antioxidant enzymes activities and its mRNA expression indicate the existence of important differences between changes in transcript levels and changes in enzyme activity levels.

In diclofenac treated group, SOD and CAT activities decreased despite the significant increase in their mARN levels, suggesting that ROS are actually targeting the catalytic reaction or the translational level to reduce an oxidative stress and not the transcriptional step. On the other hand, rats that received probiotics as preventive treatment and diclofenac, showed a reversed situation, normal enzymes activities versus normal transcription levels, indicating that the presence of probiotics restored the redox-status most probably by reducing ROS levels. Furthermore, both activities decreased significantly in response to paracetamol induced oxidative stress. However, no significant change was observed on their mRNA expression in the same experimental group. The conflicting results concerning SOD and CAT enzyme activities and their mRNA expression is an interesting observation consist with other findings that showed no significant differences in mRNA levels of SOD and CAT enzymes face of a significant oxidative stress **(Wu et al., 2019)**.

To explain this situation we suggest that reduction in SOD and CAT activities is related to increased utilization of these enzymes in scavenging and neutralizing the free radicals and lipid peroxides **(Singh et al., 2011)**. The activities of SOD and CAT may be affected by several factors, such as post-transcriptional, translational and post-translational modifications, metal binding, H_2O_2 inhibition and are not only related to gene expression **(Franco et al., 1999; Wu et al., 2019)**. In this context, **Wu et al. (2019)** showed that the transcriptional responses of SOD and CAT genes to a stress induced in *Oxyachinensis* by lead (Pb) were variable. mRNA levels of icCuZnSOD2, ecCuZnSOD1, ecCuZnSOD2, MnSOD and CAT1 were affected by Pb administration, while the transcription of icCuZnSOD1 and CAT2 had no significant changes. The author suggested that the obtained results implied that different regulation mechanisms modulated these genes expression under lead stress. In this case, differences between enzyme activity and gene expression lead to conclude that transcriptional and post-translational modifications might be related to Pb-induced oxidative stress **(Wu et al., 2019)**.

Thus, the regulation of antioxidant enzymes at mRNA level and protein level during oxidative stress is complex and may depend on multiple factors **(Cigremis et al., 2009)**. Also, transcriptional expression was usually instantaneous *in vivo*, and as a kind of transient changes, was acuter and easier to be observed. While the changes of enzymes and proteins may relatively lag compared with transcriptional responses **(Liu et al., 2017)**.

Finally, we suggest that probiotic protective effect could be related to the reduction of ROS accumulation through their free radical scavenging ability rather than affecting gene expression. Furthermore, this protective effect could be related to many activities such as secretion of enzymes like SOD, metal-chelating activities, promotion of the production of antioxidant biomolecules such as exopolysaccharides showing an *in vitro* free radical scavenging activities **(Amaretti et al., 2013; Wang et al., 2017)**. According to **Du et al. (2016)** the enhencement of endogenous antioxidant defense systems including vitamin E, glutathione peroxidase (GPx), thioredoxins and iron chelation are generally sufficient to limit lipid peroxidation after paracetamol overdose. Alternative explanations for the protection can be off-target effects of the natural product, *via* inhibition of drug metabolism **(Jaeschke et al., 2011)**.

The health benefits of probiotics including the antioxidant effect, are being extensively investigated recently, however, mechanism of most of them are very complex and thus not clearly understood. In our case we suggest to further analyze the antioxidant system expression by transcriptomics- proteomics approaches to better understand the mechanism of action of each of the studied element namely diclofenac, paracetamol and probiotic bacteria.

IV.5.2. Gastric toxicity of diclofenac

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) that is prescribed to alleviate symptoms associated with ankylosing spondylitis, osteoarthritis, rheumatoid arthritis, and migraine **(McNeely and Goa, 1999; Scialis et al., 2015)**. Diclofenac is generally well tolerated although long term usage has been implicated with a variety of adverse drug events in a subset of patients. Gastrointestinal damage induced by non-steroidal anti-inflammatory drugs (NSAIDs) is one of the most frequent adverse effects **(Gandhi et al., 2012)**.

The most common side effects of diclofenac are discomfort, ulceration, and bleeding in the gastrointestinal (GI) tract. These adverse drug reactionsare related to diclofenac pharmacodynamics, namely chronic inhibition of cyclooxygenase (COX) enzymes causing a decrease of prostaglandins that protect the GI mucosa **(Menasse et al., 1978; Wallace, 2008)**. Gastrointestinal injury following diclofenac administration has been demonstrated to occur in animal models of acute exposure. Rats given a single dose of diclofenac developed intestinal ulcers within 24 hours of administration **(Atchison et al., 2000)**.

It has been reported that the use of probiotic bacteria may have therapeutic effects in gastrointestinal disorders **(Rembacken et al., 1999; Bibiloni et al., 2005)**. The effect of LAB on acute gastric lesions induced by chemical agents in experimental models was also reported. The administration of probiotic lactobacilli as *Lb. gasseri* OLL2716, *Lb. casei* GG, *Lb. paracasei* subsp. *paracasei* NTU 101 and *Lb. plantarum* NTU 102 to rats inhibits the gastric mucosa injury in HCl/ethanol-induced ulcer and pyloric ligation models **(Lam et al., 2007a; Liu et al., 2009; Uchida and kurakazu, 2004)**. In addition it was shown that pre-treatment of rats with *Lb* GG at 10^9CFU/ml markedly reduced ethanol-induced gastric mucosal lesions **(Lam et al., 2007a)**. Furthermore, probiotic strain *Lb* GG was determined to enhance the healing of acetic acid- induced gastric ulcer in rats, via the attenuation of cell apoptosis to cell proliferation ratio and increase the angiogenesis **(Lam et al., 2007b)**.

In this part of our work, we investigated whether St.sa has prophylactic effects on diclofenac induced gastric damage in rats.

IV.5.2.1. Histopathological study

The histological study was used to evaluate the effect of probiotic on diclofenac induced gastric mucosal injury. The gastric mucosa of control animals given probiotic appeared normal in both morphological and histological examinations. Treatment with diclofenac sodium causes extensive gastric erosions on the glandular mucosa. Moreover, diclofenac provoked lesions that seriously affected the mucosa; specifically, histological structure and glandular epithelium were altered. By contrast, pre-treatment with probiotic caused a reduction in ulceration (Figure 22). It is reported that the oral administration of ibuprofen (100 mg/kg) for 6 consecutive days induced ulceration of the gastric mucosa. Oral co-administration of *Saccharomyces boulardii* (Biocodex, France) significantly reduced the numbers of gastric ulcers and the ulceration surface of the gastric mucosa **(Girard et al. 2010)**. **Rodriguez et al.** demonstrated the potential preventive effect of fermented milks with EPS-producing *Streptococcus thermophilus* CRL 1190 on acetyl-salicylic acid induced gastritis *in vivo* in BALB/c mice. Other EPS-producing strains, *S. thermophilus* CRL 804 and CRL 638, and *Lb. casei* CRL 87 were also evaluated *in vivo* assays but with unsuccessful results (**Rodriguez et al., 2009; Rodriguez et al., 2010)**.

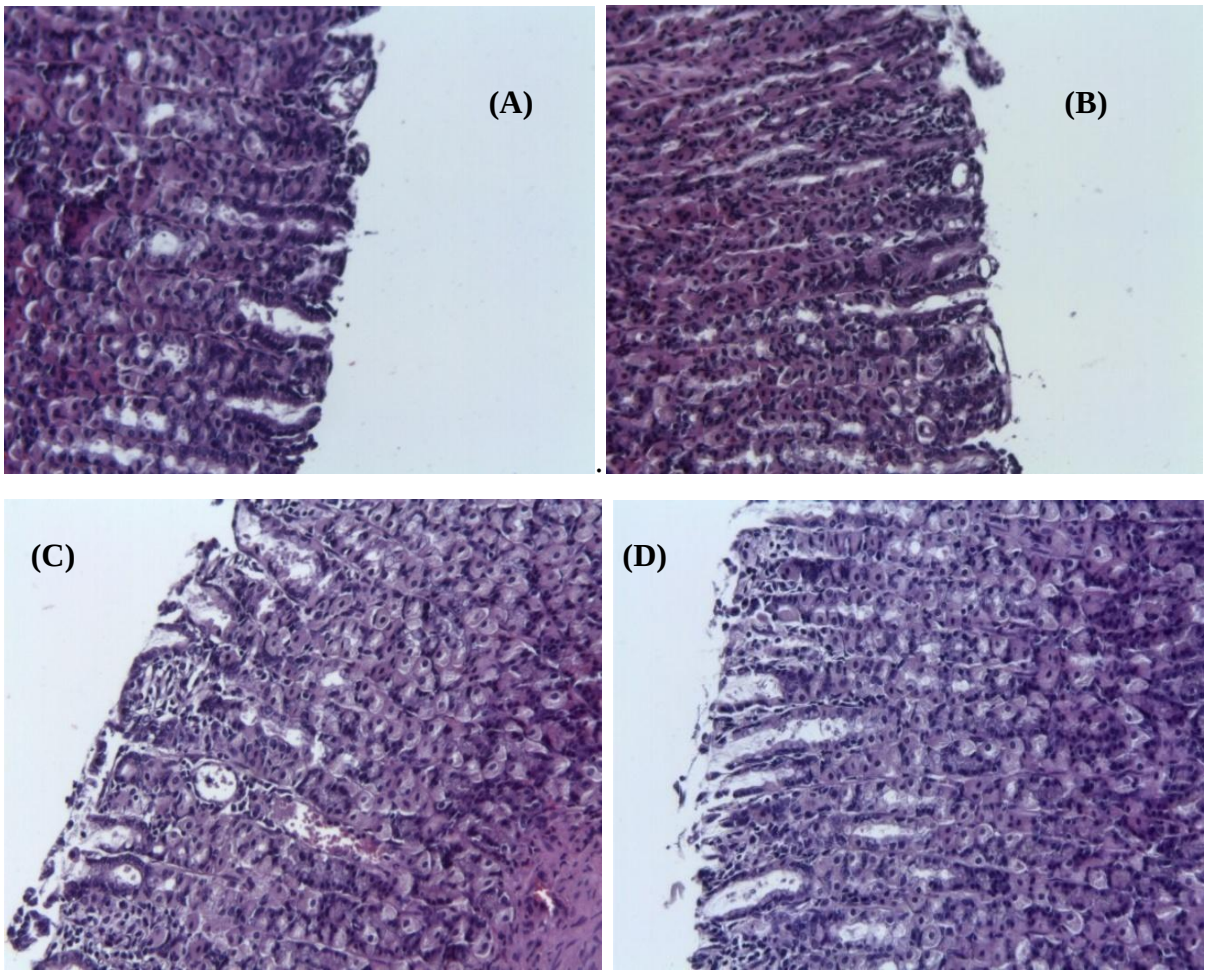


Fig. 22. *Representative micrographs of stomach sections of: control rat (A), probiotic pretreated rat (B), rat exposed to over dose of diclofenac (C), rat treated with over dose of diclofenac after probiotic pretreatment (D) (X400).*

and epithelium layer were found to be significantly differed among control and diclofenac, diclofenac and probiotic pretreated with diclofenac administred rats as shown in figure 23. These results are in agreement with other studies reported by **El-ashmawy et al. (2016)** which have demonstrated that indomethacin induce loss of epithelial layers, decreased mucosal thickness, distortion of the mucosa in the stomach of rats treated with diclofenac while nebivolol pre-treatment, before indomethacin, normalized gastric state.

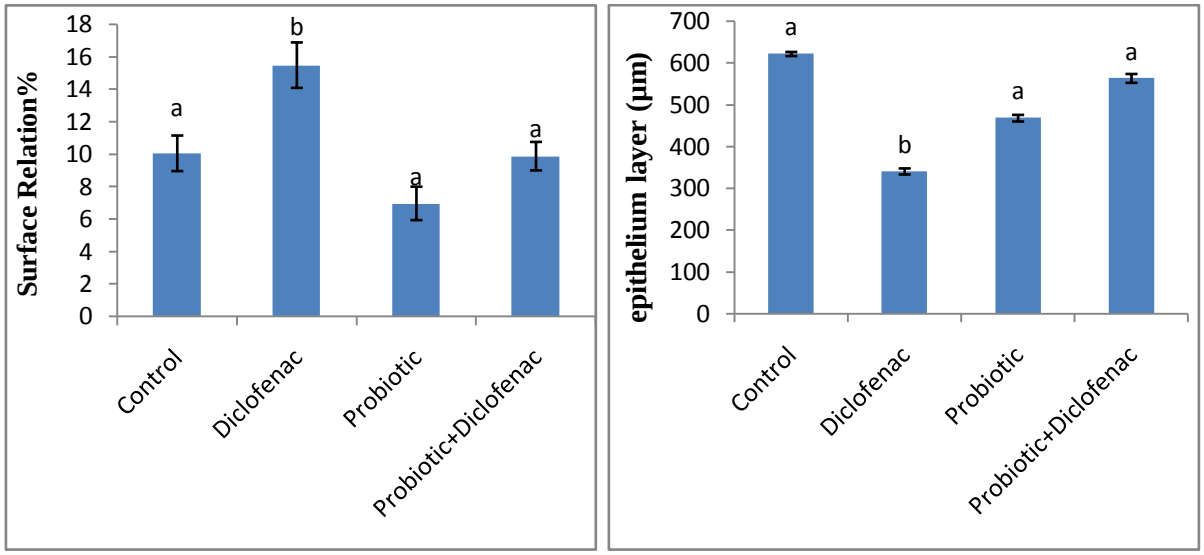


Fig. 23. *(A) Surface relation (%); (B) Epithelium layer (µm) in different experimental groups. (a) denote no significant differences among groups, (b) denote significant differences camper to other groups ($p<0.05$).*

The surface relation rates were made as a relationship between the white spaces and the surface of the epithelium of the stomach. The white spaces are the spaces with white color in the epithelium; these spaces indicated the presence of disorganization (Figure 24). The treated animals with diclofenac showed disorganization and more white spaces into the epithelium.

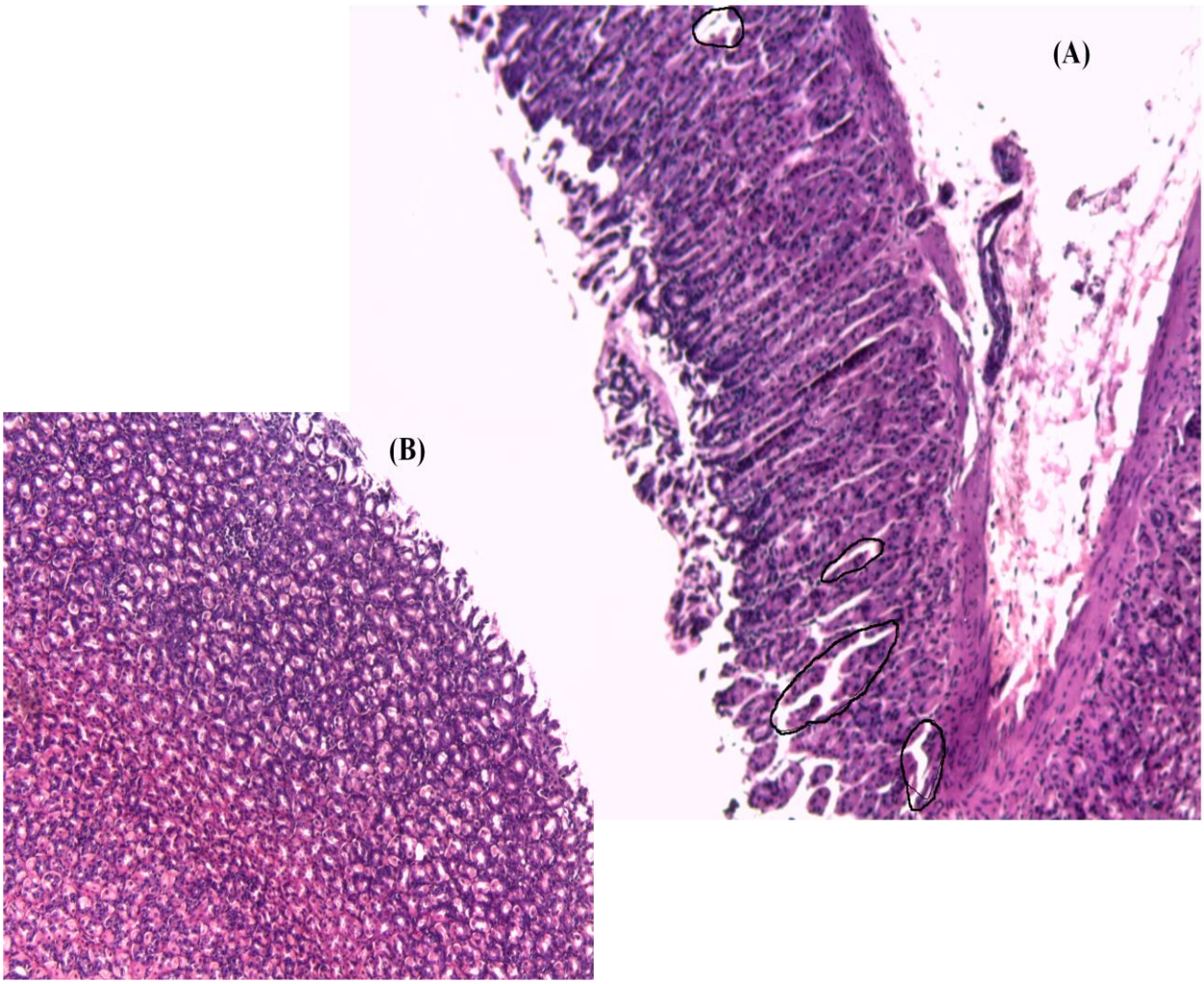


Fig. 24. *Representative micrographs of white spaces in stomach of diclofenac treated groupe (A) and control (B).*

IV.5.2.2. Biochemical study

In order to explore the effects of antioxidant defenses on the ulceration process in all gastric tissues, the antioxidant levels (superoxide dismutase, catalase, and glutathione) and MDA as a marker of tissue damage were evaluated. The levels of lipid peroxidation products and GSH in gastric mucosa of experimental animals are shown in figure 25. The level of lipid peroxidation products was significantly increased ($p < 0.001$) in diclofenac treated rats with a significant decrease ($p < 0.05$) in GSH. In the proboitic diclofenac group, it appears that the presence of probiotic significantly attenuated diclofenac induced lipid peroxidation and GSH depletion compared with those in the diclofenac group (figure 25). MDA levels, which indicate oxidative damage, were significantly increased after diclofenac administration, indicating local damage. However, this increase in lipid peroxidation may be due to the free

radicals generated during diclofenac metabolism. It is well known that NSAIDs induce peptic ulcer not only by denaturing mucous glycol-protein but also by free radical formation **(Maheshwari et al., 2014)**.

Pretreatment with probiotic prevented the increase in MDA level, demonstrating that they prevented the damage induced by diclofenac. Several reports indicate that tissue injury induced by various stimuli is coupled with GSH depletion. Similar finding was obtained in our study. While MDA level increased, GSH levels were decreased in gastric mucosa, revealing that oxidative damage was counteracted by GSH. According to **Reiter et al. (2000)**, GSH plays an important role in the maintenance of cellular proteins and lipids in their functional state, and provides major protection in oxidative injury by participating in the cellular defense systems against oxidative damage.

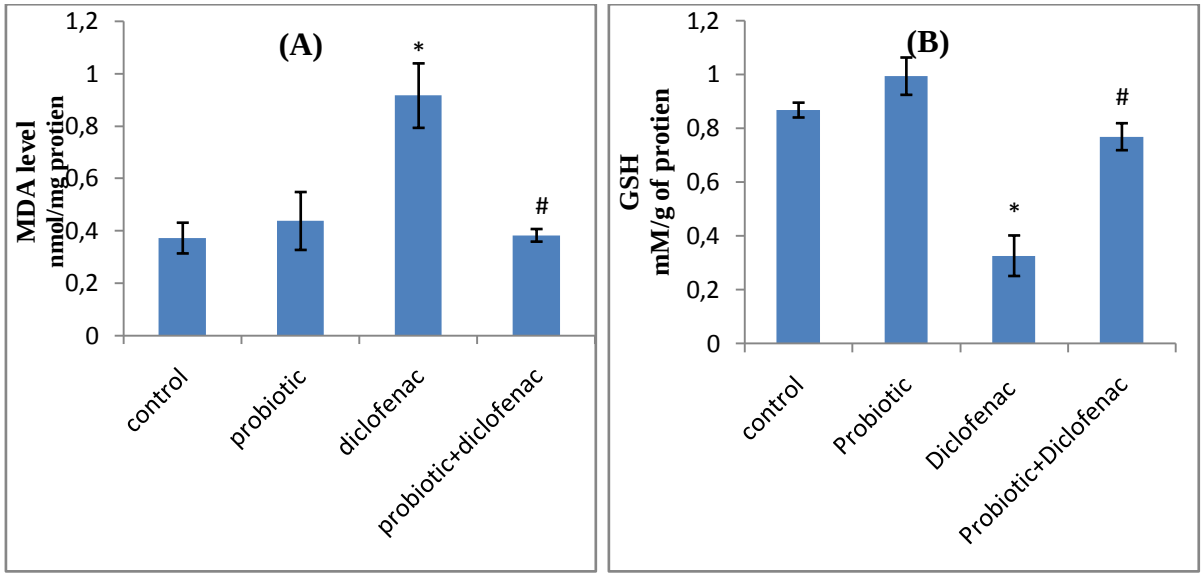


Fig. 25. *Levels of MDA and GSH in rat stomach. (A) level of MDA; (B) level of GSH. Each value is expressed as mean±S.E.M. (n = 5). Asterisks (*) denote significant differences between control rats and diclofenac group (P < 0.05). (#) denote significant differences between diclofenac treated group with probiotic pretreatment relative to rats without probiotic pretreatment.*

On the other hand, CAT and SOD activities were found to be significantly decreased in the stomachs of rats treated with diclofenac in comparison with the control group as a response of reactive oxygen generation (figure 26). These results are in agreement with the hypothesis that ROS play an important role in NSAID induced gastropathy **(Sa´nchez et al., 2002)**.

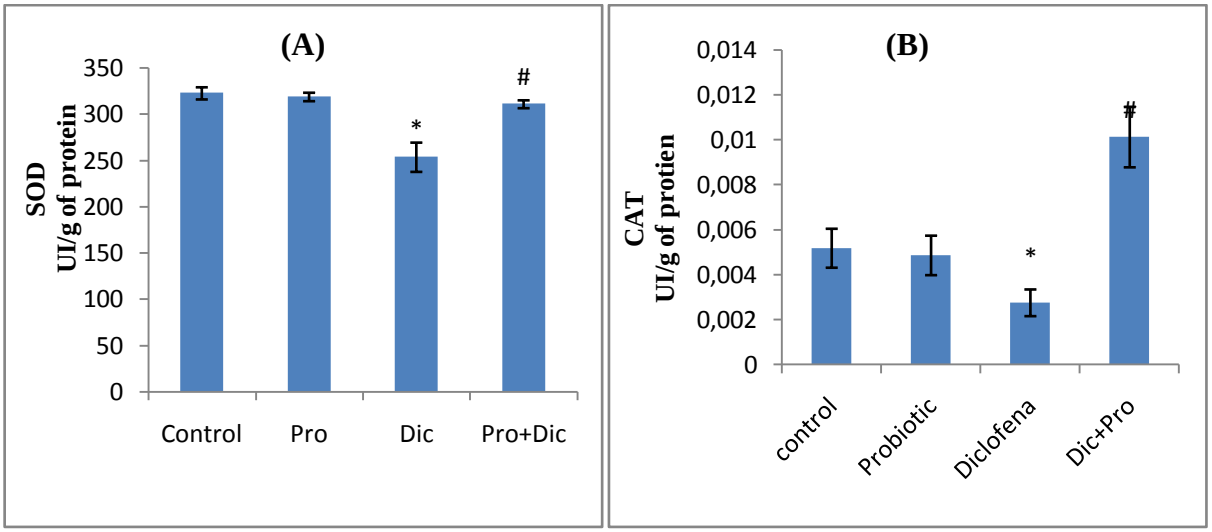


Fig. 26. *Effects of probiotic on the antioxidant enzymesactivities in rat stomach. (A) SOD activity; (B) CAT activity. Pro: probiotic, Dic: diclofenac.*

Pretreatment with probiotic appeared to be able to restore the antioxidant enzyme activities, MDA and GSH levels. In addition, histological observations support the biochemical findings that probiotic can reduce the gastric damage induced by diclofenac treatment. These results suggest that inhibition of oxidative damage is very important in prevention of diclofenac induced gastric damage. Although, the preventive effect of our probiotic against diclofenac damage should be at least through its free radical scavenger and antioxidant properties.

Pretreatment with St.sa succeed to enhance liver and stomach defense to face oxidative stress accompanying high dose of paracetamol and diclofenac in rats. Necessarily the antioxidant properties of St.sa (scavenge free radical, chelate ions, producing EPS…) play an important role in the defense against oxidative stress. According to the literature, probiotics may modulate the host's defenses against oxidative stress through metal ion chelating, regulating signaling pathways, modulating the intestinal microflora, etc. In addition recently, some reports were determined that *Lb. rhamnosus* could play an important role in antioxidant effect by using some peptide to remove oxygen radicals **(Rajoka et al., 2017**). The cell surface compounds such as proteins or polysaccharides of *Lb. plantarum* C88 have an antioxidant activity by decrement of free radicals **(Li et al., 2012)**.

Finally, additional studies are needed to understand and clarify the mechanisms involved in the modulation of antioxidant defense.

IV.6. Incorporation of *S. salivarius* ssp *thermophilus* in milk and evaluation of their properties

Humans have consumed LAB fermented foods for centuries although the role of these microbes was not established at the time. Because of this long history of safe use in food they are considered non-toxic and food-grade microorganisms with a wide acceptance in food, products by the society for varying applications **(Franz et al., 2010)**. Since the renewed interest in probiotics, different types of products were proposed as carrier foods for probiotics by which consumers can ingest large numbers of their cells for the therapeutic effect. More than 90 probiotic products containing one or more groups of probiotic organisms are available worldwide **(Arjmand, 2011)**.

Different Food products containing probiotics are considered as nutraceutical products. Mostof these products are made from milks. Fermented dairy products are the key sector among the probiotic foods that include: yogurt, kefir, various types of cheese, others like ice creams, milk-based desserts, butter, mayonnaise and fermented food of vegetable origin **(Soccol et al., 2010)**. There are also fermented non-dairy products like soy yogurt, yosa, ogi, fruit, vegetable and malt beverages **(Mishra and Mishra, 2012)**.

Fermented milks make up an important contribution to the human diet in many countries because fermentation is an inexpensive technology, which preserves the food, improves its nutritional value and enhances its sensory properties **(Mocanu and Botez, 2012)**. Fermented milk is one of the dairy products obtained by the use of appropriate probiotic bacteria which results in loweringof pH with or without coagulation of milk **(Balakrishnan and Agrawal, 2014)**.

Our fermented milk is typically obtained by fermentation of milk using a single strain *S. salivarius* ssp *thermophilus*. The fermentation generally requires from 18 to 24 hours due to the fastidious nature of Streptococci. The food industries always prefer short fermentation periods in order to reduce microbial contamination **(Macedo et al., 1999)**.

In addition, during the fermentation process, organic acids production, as a result of metabolic activity was occurred, due to the ability of *S. salivarius* ssp *thermophilus* to ferment lactose and produce acids at the end of fermentation, that caused a decrease of pH values in milk to 4.53. As a result we observed a visual appearance indicated complete coagulation as shown in figure 27.

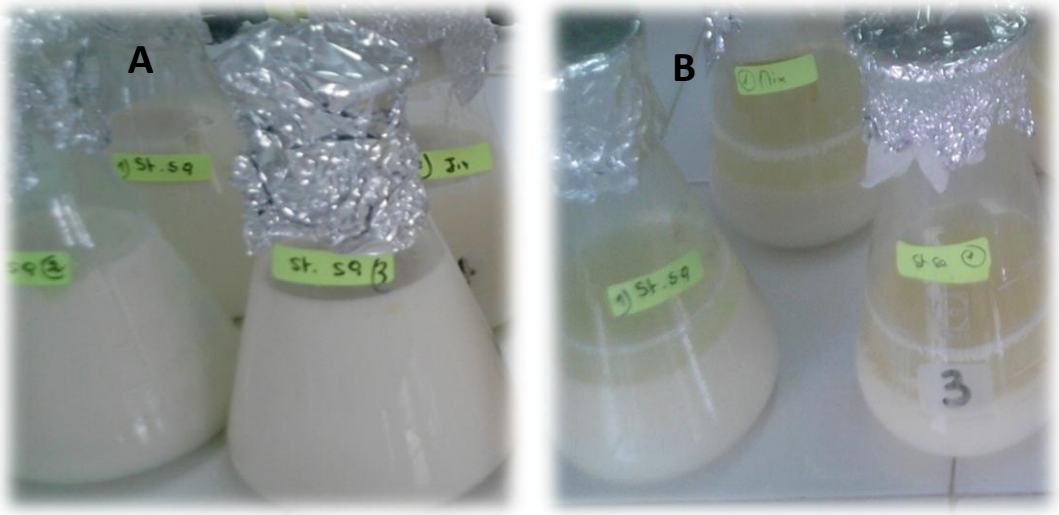


Fig. 27. *Probiotic fermented milk. (A) milk before fermentation. (B) milk after fermentation.*

IV.6.1.Viability of probiotic bacteria

During the preparation of fermented milk using probiotic bacteria as starters, it is essential that these bacteria be alive and abundant in the product at the time of consumption. Ensuring a high viability and metabolic activity of probiotic bacteria during the production as well as over the predicted shelf life is important for any probiotic product to be preferred by the consumers. Their survival in food matrices after exposure to gastrointestinal tract conditions is the most critical parameter as it determines their health efficiency **(Kurmann and Rasic, 1991).**

Although there are no set standards concerning the population of the probiotic organism at the end of the product shelf life, the minimum levels of 10^6 CFU/ml are usually considered as acceptable, in order to provide health benefits **(Georgieva et al., 2009, Korbekandi et al., 2011)**. The viability of *S. salivarius* ssp *thermophilus* in fermented milk and during storage was investigated. As shown in figure 28, the viable cell counts increased rapidly during the first 24h of fermentation and reached its maximum of 12.9 log CFU/ml. During the cold storage period, the viable count of these bacteria declined with minimal differences ranging from 0.06 to 0.9 log CFU/ml. These results were in agreement with a previous study that found a low decrease in the viable counts of *Bifidobacterium longum* and *B. infantis* after cold storage **(Chou and Hou, 2000)**. This indicated that the strains were less metabolically active under the storage condition. From the obtained results, it can be seen that the concentration of *S. salivarius* ssp *thermophilus* remained above the recommended levels during cold storage. **Daneshi et al. (2013)** reported that *Lb. acidophilus* and *Lb. rhamnosus* remained viable above

the critical level of 10^6 CFU/ml in probiotic milk/carrot juice drink for 20 days. The changes of viable cell counts of all the strains during cold storage are insignificant ($p < 0.05$)**.**

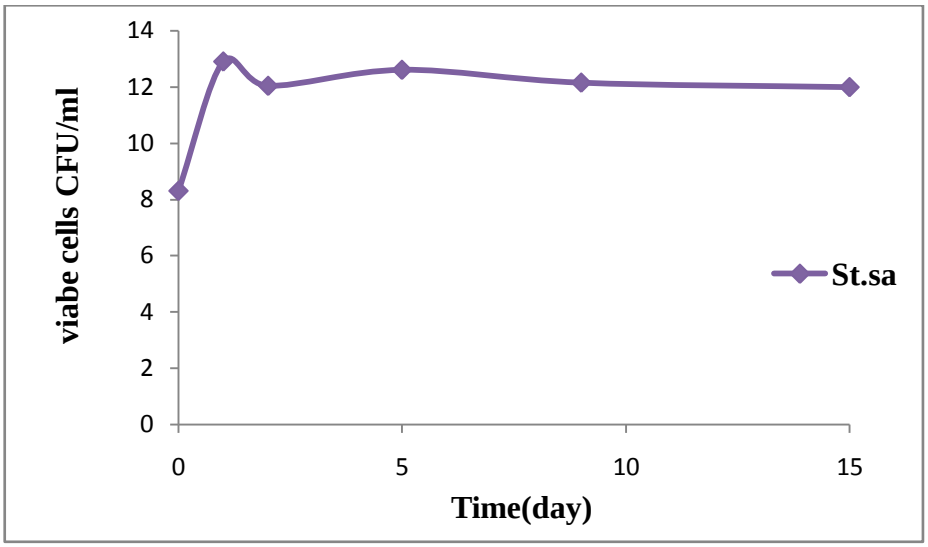


Fig. 28. *Viable cells of probiotic St. salivarius bacteria in fermented milk during cold storage*

IV.6.2. Changes in pH and titratable acidity values

The results of the statistical analysis pointed out significant differences ($p < 0.05$) for pH and titratable acidity values in probiotic St.sa fermented milk after 24h of fermentation. The pH values of milk during fermentation with St.sa decreased from initial 6.47(time 0) to final 4.53 (24h) (Figure 29). The initial titratable (TA) acidity changed from 1.5 (time 0) to a final acidity of 3.3 (24h) due to the metabolic activity of culture during fermentation (Figure 30). In contrast to our results, **Matejčeková et al. (2016)**, showed no significant changes of pH values (0.00-0.24 units) in case of UHT milk (3.5% fat) during the growth and multiplication of *Lb. plantarum*. This can be explained by the low ability of *Lb. plantarum* to utilize lactose and convert pyruvate to lactate in a rate to match the glycolysis **(Jyoti et al., 2004)**.

During storage, remarkable increase in titratable acidity values ($P < 0.05$) was observed in the fermented milk when compared with that of control milk and no observable changes in pH values (Figure 30). **Fan et al. (2009)** reported that changes in titratable acidity do not necessarily have an effect on pH values. Observed changes of acidity values were similar to those reported for 14 commercial fermented milks that varied from 0.79 to 1.16% **(Gueimonde et al., 2004)**. Generally, a high TA value and low pH indicate a higher bacterial activity and an increased acid production. During fermentation, acid production results in

finer coagulation of casein, which may also contribute to the greater protein digestibility **(Madhu et al., 2012)**.

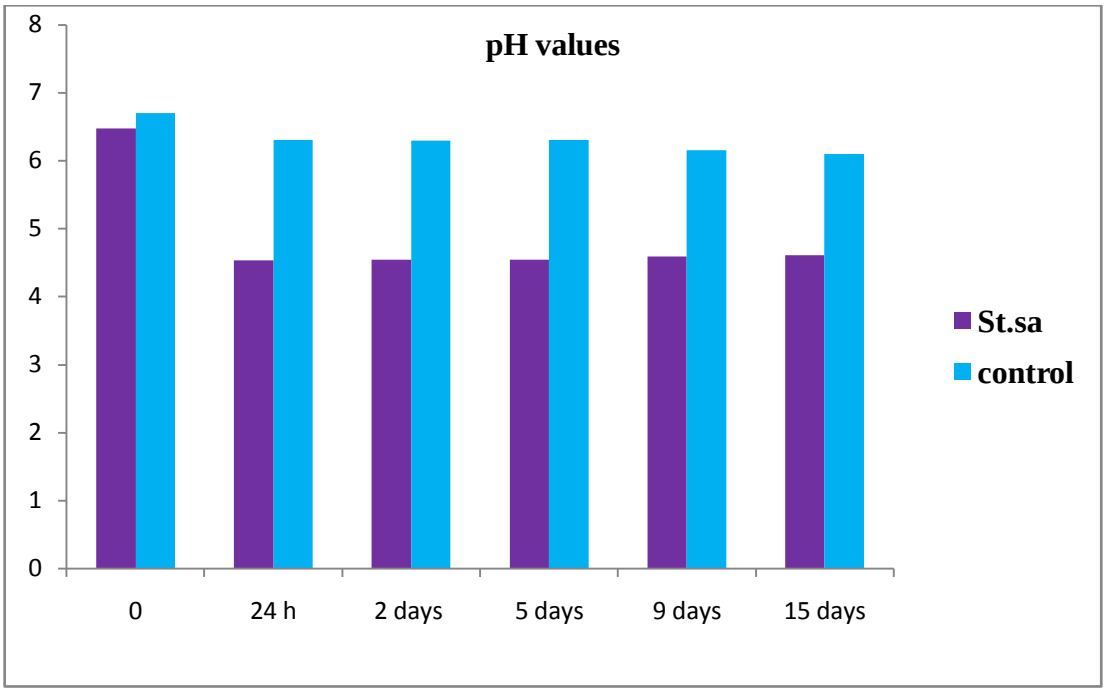


Fig. 29. *Changes in pH of milk samples during fermentation and cold storage.*

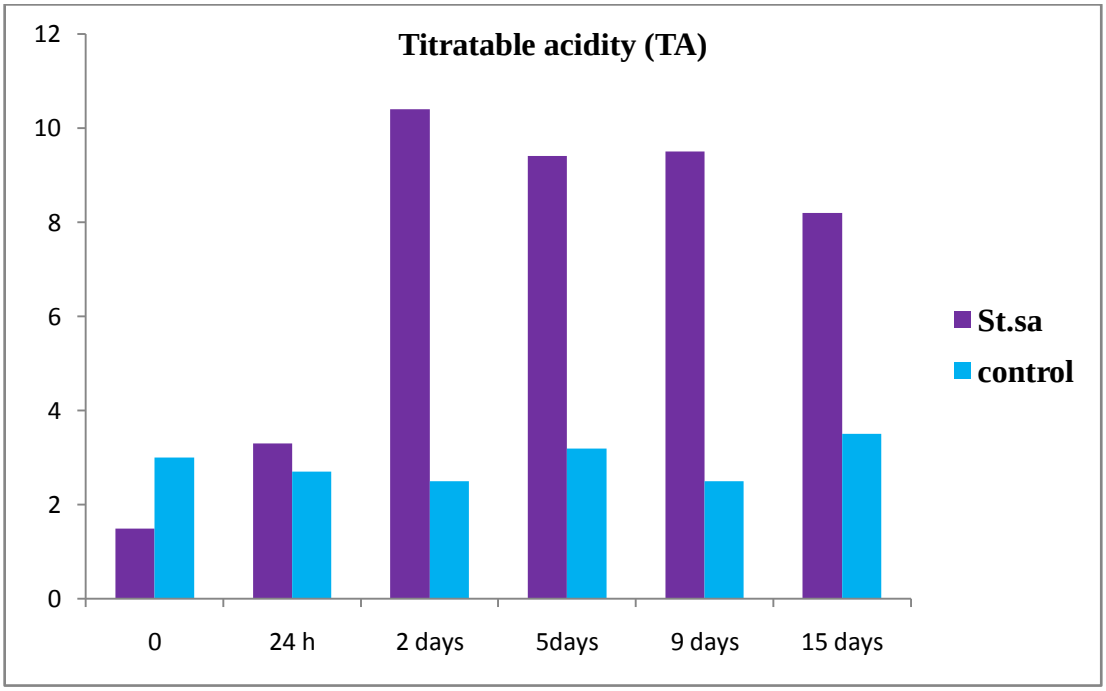


Fig. 30. *Changes in titratable acidity during fermentation and cold storage*.

IV.6.3. Antioxidant capacity using DPPH

The antioxidant activity of our strains using DPPH was measured by their ability to scavenge free radical in fermented milk. **Table 11** shows the radical scavenging activity exhibited by fermented milks before and during fermentation and storage. It shows that the DPPH scavenging rates values ranged from 86.67% to 88.20% for probiotic fermented milk. As can be seen from the results, the antioxidant potential of probiotic fermented milk was significantly ($P < 0.05$) higher when compared with that of control milk (65%) under similar conditions. It's also demonstrated that these antioxidant activity remained stable during 15 days of cold storage.

The increase in radical scavenging activity during fermentation with probiotic bacteria may be due to peptides released during hydrolysis of milk proteins **(Abubakr et al., 2012; Baba et al., 2014; Balakrishnan and Agrawal, 2014)**. It is reported that peptides obtained from hydrolyzed food proteins have been shown to possess antioxidative activities. A pool of selected lactic acid bacteria was used for sourdough fermentation of various cereal flours with the aim to synthesize antioxidant peptides. The radical scavenging activity of water/salt-soluble extracts from sourdoughs was significantly higher than that of chemically acidified doughs. The highest activity was found for whole wheat, spelt, rye, and kamut sourdoughs. Twenty-five peptides having the size from 8 to 57 amino acid residues were identified. Almost all the sequences shared compositional features which are typical of antioxidant peptides. All the purified fractions showed *ex vivo* antioxidant activity on mouse fibroblasts artificially subjected to oxidative stress **(Coda et al., 2012)**.

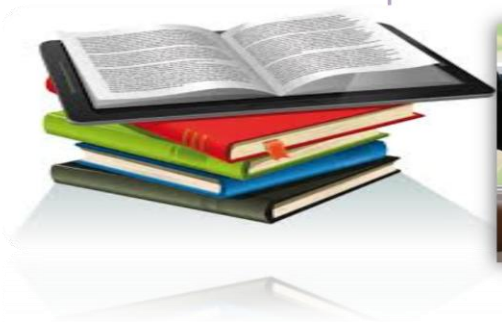
Table 11. *Antioxidative activities of milk fermented with probiotic bacteria*.

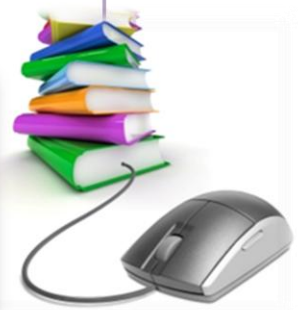
Strain	Inhibition rate %				
	Fermentation period	Storage period			
	day1	Day 2	Day 5	Day 9	Day 15
Fermented milk with St.sa	87.45±0.51	88.20±0.79	88.20±0.08	86.67±0.084	88.20±0.25
Control milk	65.20±1.28	64.27±1.32	65.78±1.27	65.61±1.27	65.61±1.27

Radical scavenging activities of fermented milks suggest that they could be used as natural antioxidant supplement for improving human health **(Abubakr et al., 2012; Balakrishnan and Agrawal, 2014)**. Similarly, **Liu et al. (2005)** reported that *Lb. helveticus* isolated from kefir grains that possessed high proteolytic activities produced fermented milk and soy milk with good antioxidant activity.

This study clearly demonstrated that by incorporating St.sa in milk it is possible to further increase the antioxidant status of fermented milk and thus, improve nutritional value of fermented dairy products. In addition, storage did not adversely affect the antioxidant activities.

Conclusion





Since, the usages of synthetic antioxidants such as BHA, BHT and TBHQ found to have toxic and carcinogenic effects; the development and utilization of harmless antioxidants derived from natural origin are desirable. In this area we conducted this work.

Accumulating evidence suggests that probiotics exert various biological roles through several mechanisms, one of the most debated being the antioxidant activity. In fact, among probiotics beneficial effects, the protection against oxidative stress in humans has been reported by several authors. In this light, the present study aimed to investigate the antioxidant capacity of some lactic acid bacteria and select one of them to assess its ability to reduce oxidative stress induced by drugs.

A preliminary study was carried out in order to select a strain with antioxidant activity. Twelve strains of lactic acid bacteria were studied for scavenging DPPH radical. All tested strains exhibited potent DPPH radical scavenging ability. This activity was highest in *S. salivarius* ssp *thermophillus* St.sa and *Lb. plantarum* 15.

Probiotics are defined as the viable microorganisms that exhibit a beneficial effect on health of the host. To be a successful probiotic, an organism should possess several properties such as adhesive ability, bile and acid tolerance and significant antibacterial activity and the most important criteria is to be non-pathogenic. In order to investigate probiotic properties of *S. salivarius* ssp *thermophillus* St.sa and *Lb. plantarum* 15we tested the ability of these strains to tolerate the effects of bile salt and acid condition. We observed a significant survival rate of both strains in acidic condition and in bile salts existence, suggesting the potentiality of these strains to survive in acidic and intestinal conditions. In addition, after calculating the cumulative probiotic potential, it is clear that *S. salivarius* ssp *thermophillus* is a safe probiotic. Moreover the safety of our strain was further ensured after oral administration to rats. During the trial, no differences were detected in animal growth and in food consumption, all animals appeared in a good health condition and AST, ALT and ALP content in blood were similar in probiotic fed rats and control rats.

In second part of our study the antioxidant capacity of the two strains was evaluated by various antioxidant assays *S. salivarius* ssp *thermophillus* St.sa and *Lb. plantarum* 15showed antioxidant activity such as DPPH radical scavenging, hydroxyl radical scavenging, inhibition of lipid peroxidation, and metal chelating activity, an essential property for antioxidant activity. The differences in antioxidant activity exhibited by the different methods may be due

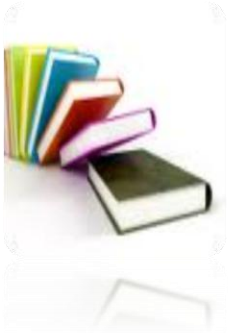
to differences in free radical scavenging mechanisms. The strain *S. salivarius* ssp *thermophillus* St.sa showed also production of exopolysaccharides with antioxidant capacity.

Therefore, we suggested that our probiotic strain could be a potential source of antioxidant compounds so that it can reduce the elevated level of reactive oxygen species (ROS), In this context, we used probiotic *S. salivarius* ssp *thermophillus* St.sa to contract oxidative stress induced by two common drugs (paracetamol and diclofenac) in rats. In this study, we found that probiotics effectively restored liver function markers and the oxidant/antioxidant balance by decreasing level of ROS. In addition, our probiotic strain appeared to be able to reduce the gastric damage induced by diclofenac treatment. Finally, results clearly emphasized the protective role of probiotics against ROS generated during the treatment with the two studied drugs.

The last part of our study aimed at developing probiotic fermented milk while monitoring its characteristics (viable cells, pH, TA, and DPPH radical scavenging activity) during 15 days of storage. *S. salivarius* ssp *thermophillus* St.sa has antioxidant benefit as it has inhibition of lipid peroxidation, scavenging hydroxyl radical, chelating iron ions and producing EPS with antioxidant efficiency. Besides probiotic contributes to health benefit in the prevention of gastro intestinal tract disorders, immunity enhancement and improvement of lactose intolerance disorders. Considering the above benefits then incorporating *S. salivarius* ssp *thermophillus* St.sa to milk is beneficial, as it results in additional value.

In conclusion, the selected strain *S. salivarius* ssp *thermophillus* St.sa has proved to be a potent antioxidant. Determination by other methods is necessary to obtain more evidence on its antioxidant potential. Further experiments are also required to understand the exact mechanisms responsible for the antioxidant activity. In this thesis work we suggest that the protective effect of the probiotic *S. salivarius* ssp *thermophillus* St.sa is at least partly due to the ability to increase glutathione (GSH) levels and to the ability to scavenge free radicals which decreases the prooxidants levels.

Further research is needed to focus on the pathways involved in drug reducing toxicity by probiotic bacteria. Also, a study has to be carried out to identify encoded genes and precise structural characterization of exopolysaccharides. Furthermore, Incorporation of this exopolysaccharides and the strain into food systems and also as therapeutic agents are to be further confirmed by studies on animal models.



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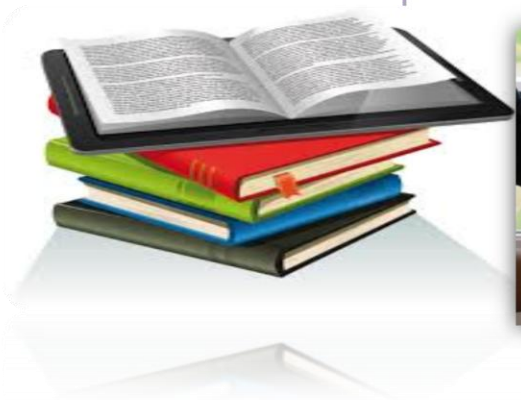

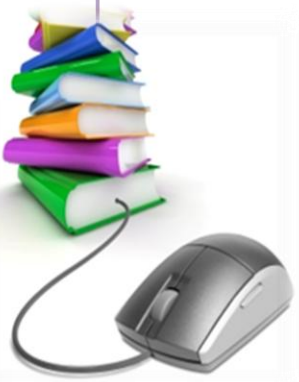

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Annex 1

Component of MRS agar

Peptone…… 10g

Beef extract ……. 8g

Yeast extract ……. 4g

Sodium acetate……. 5g

Phosphate bipotassique……. 2g

Ammonium citrate…….. 2g

Magnesium sulfate……..…….. 2g

Manganes sulfate……..……... 0.05g

Glucose…… 20g

Tween 80……. 1 ml

Agar……. 15g

Cysteine……... 0.1g

Distilled water……. 1000ml

pH: 6,4

Autoclaved for 15 min at 120 °C

For the MRS broth, it contains the same components of MRS agar without agar.

Annex 2

Hyper-sucrose agar

polypeptone ……2.5 g

yeast extract ……...03g

beef extract ……10g

sucrose ……..150g

NaCl ……1g

magnesium sulfate ……0.2 g

agar ……15g

The pH was adjusted to 6.8

Annex 3

Standardisations of inoculum

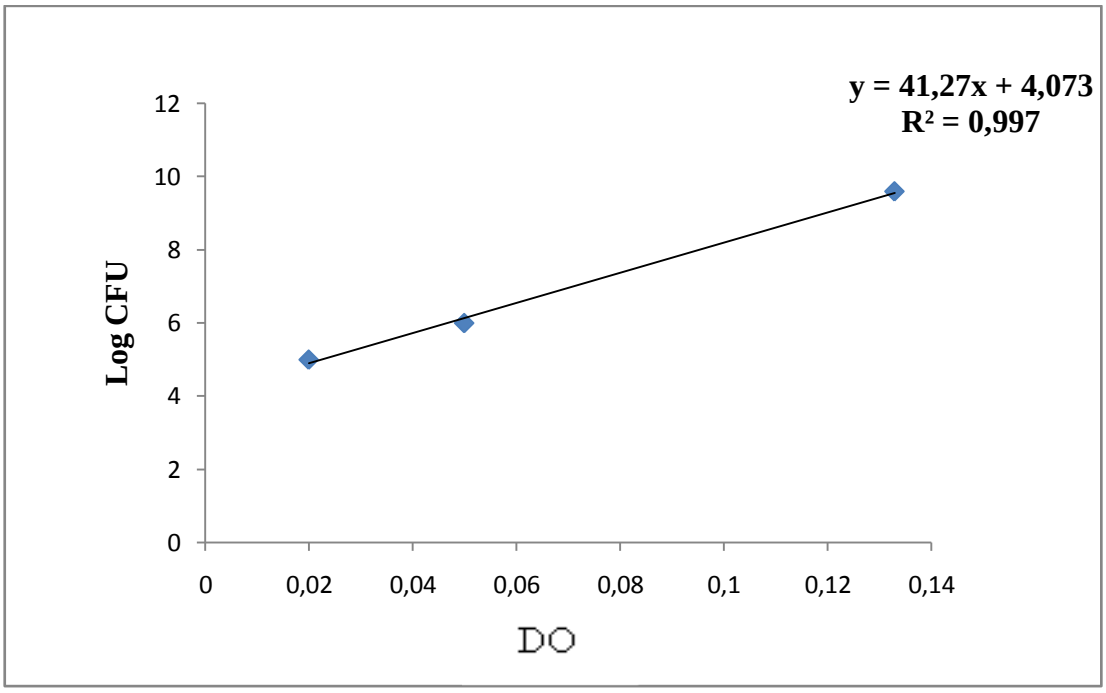

Figure1: counting cells of strain 15 *Lactobacillus plantarum* (CFU/ml)

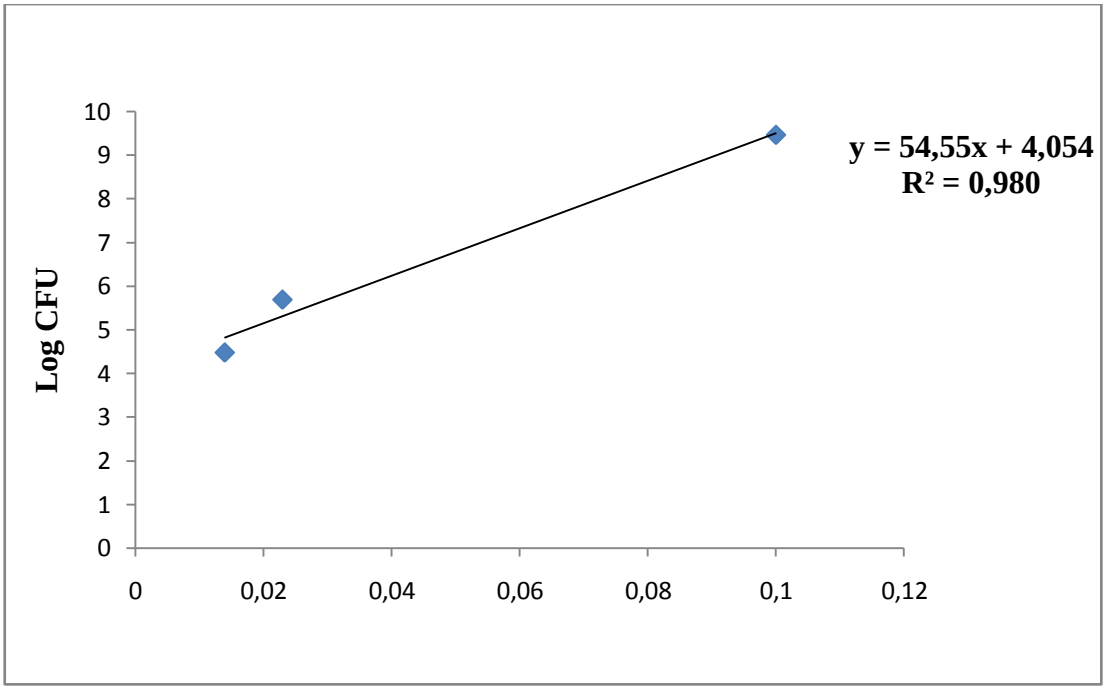

Figure 2: counting cells of st sa *Streptococcus salivarius* ssp *thermophilus* (CFU/ml).

Annex 4

Stander curves

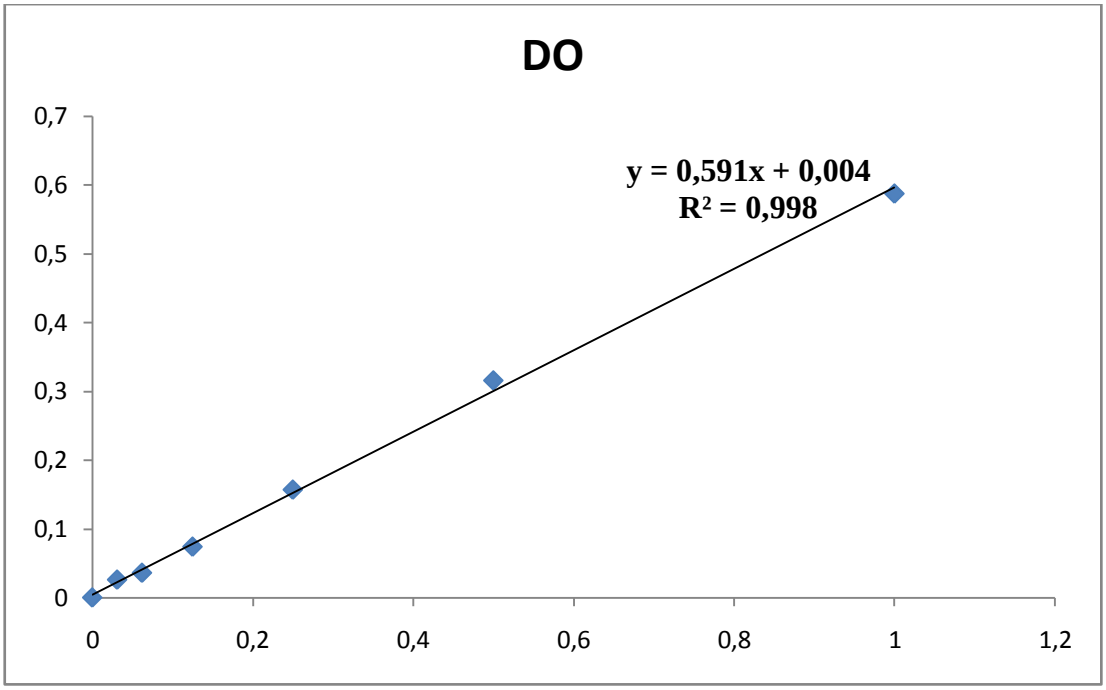

Figure 3: Standard curve for protein level (Bovine serum albumin as the protein standard).

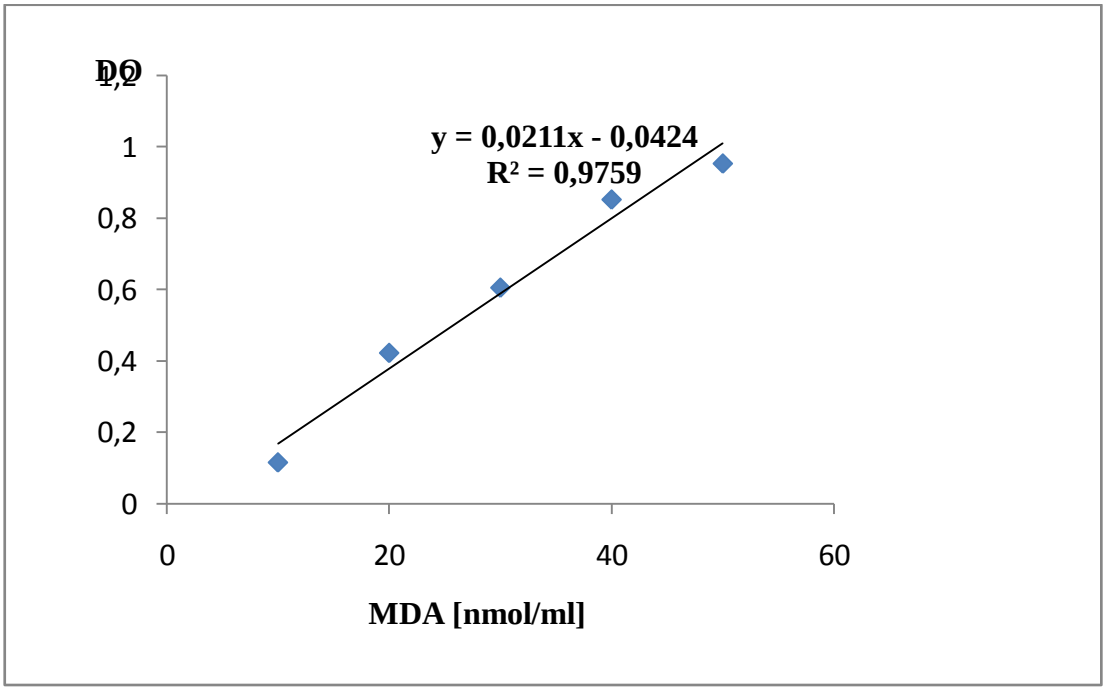

Figure 4: Standard curve for MDA (TMP was used as a stander).

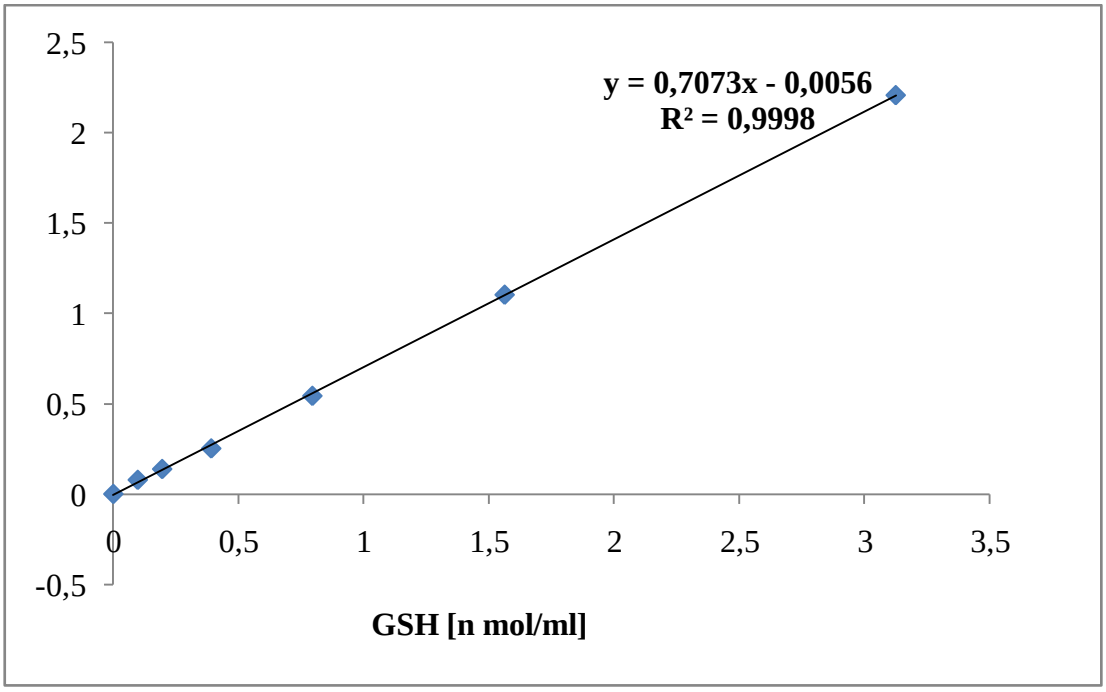

Figure 5: Standard curve for GSH.

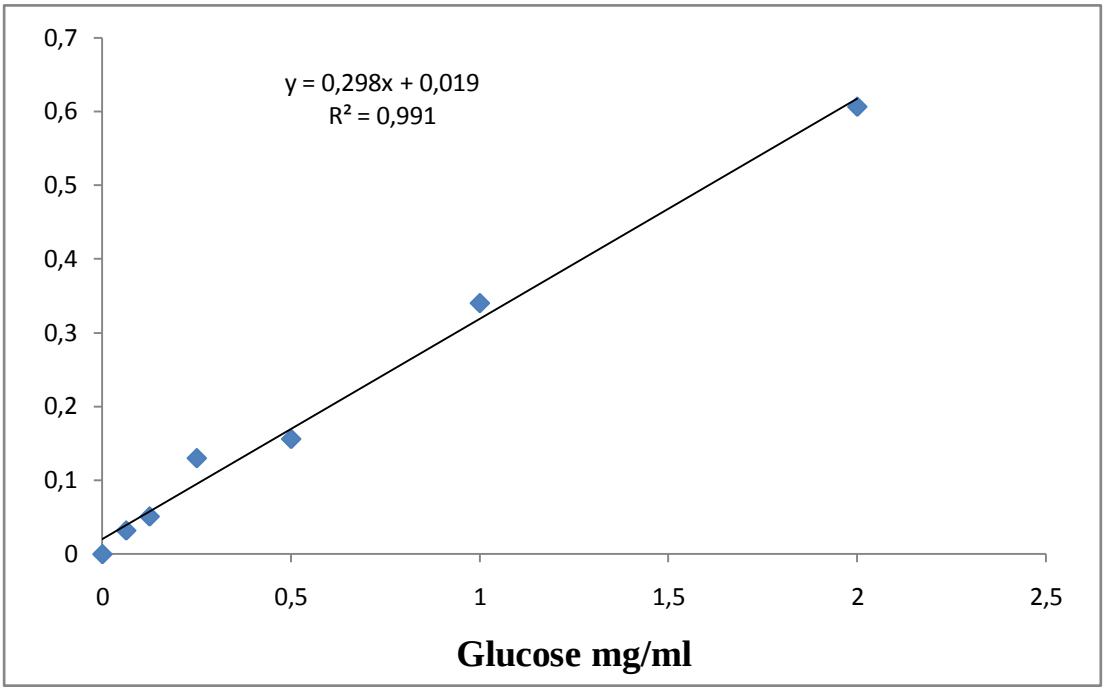

Figure 6: Standar curve for glucose

Realised by:
Riane karima

Supervisor:
Pr. Mohamed Sifour

PhD Thesis

Antioxidant efficiency of probiotic bacteria and their role in reducing drug toxicity.

Résumé

Les probiotiques ont attiré beaucoup d'attention visant à développer des antioxydants naturels non toxiques, en raison de leur rôle dans la diminution du risque d'accumulation des espèces réactives de l'oxygène (ROS). Par conséquent, le but de cette étude était d'évaluer l'activité antioxydante d'un probiotique *Streptococcus salivarius* ssp *thermophillus* St.sa *in vitro* et d'évaluer l'effet protecteur du St.sa contre le stress oxydatif induit par une dose toxique de deux médicaments (paracétamol et diclofénac) chez les rats Wistar. Les résultats ont démontré un bon effet inhibiteur contre la peroxydation des lipides plasmatiques (54,36%). En outre, avait montré une capacité considérable de piéger le radical libre DPPH (89,43%) et il avait également une bonne résistance aux radicaux hydroxyles (47%) et une capacité considérable à chélater les ions de fer (33,21%). D'autre part, la souche sélectionnée avait un effet protecteur contre le stress oxydatif induit par le paracétamol dans le foie et par le diclofénac dans le foie et l'estomac. Les résultats soulignent clairement le rôle protecteur des probiotiques contre les ROS générés pendant le traitement avec les médicaments étudiés.

Mots clés: Probiotiques, *Streptococcus salivarius*, paracétamol, diclofenac, Activité antioxydante

Abstract

Probiotic have attracted a lot of attention aiming to develop natural non-toxic antioxidants, because of their role in decreasing the risk of reactive oxygen species (ROS) accumulation. Therefore, the purpose of this study was to assess the antioxidant activity of a probiotic *Streptococcus salivarius* ssp *thermophillus* St.sa *in vitro* and to evaluate the protective effect of St.sa against the oxidative stress induced by a toxic dose drugs (paracetamol and diclofenac) in Wistar rats. The results demonstrated a good inhibitory effect against plasma lipid peroxidation (54.36%). Also, had shown a considerable ability to scavenge DPPH free radical (89.43%) and it had also a good resistance to hydroxyl radicals (47%) and considerable ability to chelate iron ions (33.21%). On the other hand, the selected strain had protective effect against oxidative stress induced by paracetamol in liver and by diclofenac in both liver and stomach. Results clearly emphasize the protective role of probiotics against ROS generated during the treatment with the tow studied drugs

Key words: Probiotic**,** *Streptococcus salivarius*, paracétamol, diclofenac Antioxidant activity.

الملخص

اجتذبت البكتيريا البروبيوتيكية الكثير من الاهتمام بهدف تطوير مضادات أكسدة طبيعية غير سامة ، وذلك بسبب دورها في تقليل خطر تراكم الأجناس الأكسجينية التفاعلية (ROS). لذلك ، كان ال هدف من هذه الدراسة هو تقييم النشاط المضاد للأكسدة للبكتيريا البروبيوتيكية St.sa *Streptococcus salivarius* مخبريا ، ثم بعد ذلك تقييم تأثيره ا الوقائي ضد الإجهاد التأكسدي الناجم عن جرعات سامة من الأدوية (الباراسيتامول والديكلوفيناك) عند الفئران .أظهرت النتائج المخبرية فعالية معتبرة في تثبيط أكسدة الدهون البلازمية (54.36٪) ،كما أظهرت قدرة كبيرة على كسح الجذور الحرة (89.43٪ ل (DPPH) و (47 ٪) لجذور الهيدروكسيل). إضافة إلى فعاليتها في قنص أيونات الحديد (33.21 ٪). من ناحية أخرى ، كان للسلالة المختارة تأثير وقائي ضد الإجهاد التأكسدي الكبدي الناجم عن الباراسيتامول و الإجهاد التأكسدي الكبدي و المعدي الناتج عن الجرعة السامة لديكلوفيناك. النتائج تظهر بوضوح الدور الوقائي للبكتيريا بروبيوتيك ضد الأجناس الأكسجينية التفاعلية المولدة أثناء العلاج بالأدوية التي خضعت للدراسة.

الكلمات المفتاحية: بروبيوتيك، *Streptococcus salivarius* ،براسيتامول، ديكلوفيناك، النشاط ضد التأكسدي