Name of candidate: Shaimaa Ismail Mohamed **Degree:** M.Sc.
Title of thesis: Biological Effect of Some Capsulated Bacterial Strains in Food On Experimental Rats.
Supervisors: Prof. Dr. Abd-El moneim Afify
Dr. Ramy Romeilah
Prof. Dr. Mona Mohamed Hussein
Department: Biochemistry **Branch:**
Approval: 30 / 7/2013

ABSTRACT

This study evaluated the antioxidant activity of four probiotics strains like *Lactobacillus rhamnosus GG, Lactobacillus L.reuteri*, *Bifidobacterium as well as Probionebacterium,* where investigated through the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging ability of the cell free extract of bacteria of the probiotic strains comparing with the standard antioxidant ascorbic acid and BHT. The results of the $DPPH^{\cdot}$ scavenging potential of cell free extract showed that the maximum antioxidant activity with *Probionebacterium freudenreichii* (97.75 %) and it was significantly increased compared with vit.C and BHT followed by *Lactobacillus L.reuteri* activity (96.74 %). All cell free extracts showed highly scavenging potential against ABTS radical. Free probiotic bacteria under acidic conditions (pH 2 and pH 3/ 3h) showed that *L.GG* intolerance to pH 2, but showed more acid tolerance at pH3. In contrast, the other strains showed more acid tolerance at pH 2 values where incubation of strains for 3h at pH 2 resulted in a decrease of about one log cycle, while at pH 3 the decrease ranged from 0.2 to 0.7 log cycles. In the case of 1% and 2% bile salt for *L.reuteri and B.breve,* the decrease ranged from 0.4 to 0.6 log cycles, while in case of *L.GG* and *P. Jensenii* the decrease in cell viability was about 1.5 log cycle. In the present study two diets have been formulated in biscuits shap first type coated by chocolate syrup supplemented with encapsulated probiotics strains, with $0.3g/10^7$ CFU. while another biscuits coated by chocolate syrup without probiotics strains each biscuits weight 8g. The biological evaluation was studied on albino rats for six weeks, showed that non significant change in total cholesterol and HDL-C but there were significant change in serum triglycerides and LDL-C the values amounted (83.23 and 18.75 mg/dL) respectively compared with normal control (91.57 and 25.07 mg/dL)respectively . Activity of (AST) showed non-significant changes between two treatments (biscuit with probiotic bacteria and without probiotic bacteria diets, (33.84 ± 3.5 and 33.05 ± 3.5 IU/L) respectively and normal control group (30.31 ± 2.5IU/L). Also the same for serum (ALT) showed non-significant changes between two treatments (36.26 ± 2.58 and 37.20 ± 1.84IU/L), respectively and normal control group (33.62 ± 2.69IU/L). (ALP) showed non-significant change between two treatment (141.54 ± 2.72 and 144.43 ± 2.54IU/L) respectively and normal control group (146.76 ±2.33IU/L). Also non significant change in kidney function were recorded. Urea records non-significant different between the two biscuit treatment (29.32±1.77 and 30.50±1.91mg/dl) respectively and control (28.76±1.72mg/dl), on the other side the creatinine showed non-significant change between the two biscuit treatment as (0.66 ± 0.06 and 0.69 ± 0.04mg/dl) respectively, and control group (0.70±0.7mg/dl).

Key words: Probiotic strains, Antioxidant, Encapsulation, Biological experiment, Biochemical evaluation.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Probiotic is a term that means “For Life" and defined as live microorganisms that beneficially affect the host's healthy by improving microbial balance (Burgain, *et al.,*2011) . Nowadays it is well established that there is a strong relation between diet and health therefore probiotic microorganisms has been used for more than 100 years to prevent and treat a variety of human diseases. Recently, there has been an increase in research of probiotics which has led to significant advances in our understanding of those microorganisms. The importance of probiotics is meaningful because have both, an application on industrial product development and a beneficial effect on human health. In this regard, probiotic microorganisms have been widely added to yogurts and other fermented milks, which are leader products of functional foods comprising approximately 65% of the world functional food, market (Figueroa-González *et al.*, 2011).

Probiotics are believed to stimulate growth and healthy as well as to modify the ecology of the intestine in a beneficial manner for the host (Veizaj-Delia *et al.*, 2010). An increasing number of scientific reports have appeared on the effects of probiotic combinations on the health of the host than using single probiotic strain (Collado *et al.,* 2007). Probiotic bacteria are widely used in human and animal nutrition and beneficially influence the balance of the intestinal flora of the host. Probiotic

bacteria provide an array of health benefits which include competition, antagonistic effects, enhancement of digestion, strengthening of the immune system and stimulation of vitamin production (de Baets et *al.,* 2009). Probiotic 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 scavenger glutathione (GSH). Moreover, they promote the production of certain antioxidant biomolecules, such as the exopolysaccharides (EPSs). Finally probiotics exhibit metal chelating activity. All these data suggest that probiotics may have a potential therapeutic role in reactive oxygen species (ROS), characterized gastrointestinal disorders. (Spyropoulos *et al.,* 2011).

Evaluation and screening of natural substances that have antioxidant activity is the new research trend of biology, medicine and food science (Afify *et al.,* 2011a; Afify *et al.,* 2011b; Afify *et al.,* 2012). As a probiotic, lactic acid bacteria have been widely used in food and medicine. Lactic acid bacteria can reduce cholesterol in plasma, regulate the balance of intestinal flora, inhibit and reduce the risk of cancer in addition to anti-aging, antioxidant and other important physiological functions (Gao, 2012).

Microencapsulation is often mentioned as ways to protect bacteria against sever environmental factors. The goal of encapsulation is to create a micro–environment in which the bacterial will survive during processing and storage and released at appropriate sites (e.g. small intestine) in the digestive tract. The benefit of encapsulation is to protect probiotic against low gastric pH have been shown in numerous reports (Weinbreck *et al.,* 2010).

The objective of the present study was to determine antioxidant activity using of DPPH radical and ABTS radical scavenging assay of cell free extract and pH and bile resistance of four Probiotic strains [*Lactobacillus rhamnosus GG, Lactobacillus reuteri (ATCC 20016), Bifidobacterium breve* (*ATCC 15700) and Probionebacterium freudenreichii ssp. Shermanii (ATCC 1907*). Also study their biochemical evaluation through lipid profile parameter (serum triglyceride, HDL, LDL and cholesterol) and safety through liver and kidney functions (AST, ALT, ALP, Creatinine and Urea) when incorporated into biscuit coated with chocolate syrup.

REVIEW OF LITERATURE

1. Probiotics bacteria

a. Definition of probiotics

Foods are no longer considered by consumers only in terms of taste and immediate nutritional needs, but also in terms of their ability to provide specific health benefits beyond their basic nutritional value. Currently, the largest segment of the functional food market is provided by the foods targeted towards improving the balance and activity of the intestinal microflora (Saarela *et al.,* 2002). Consumption of foods containing live bacteria is the oldest and still most widely used way to increase the numbers of advantageous bacteria in the intestinal tract. Such bacteria are called 'Probiotics'.

More recently, probiotics have been referred to as "live microorganisms when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001).

Havenaar and Veld (1992) have defined probiotics as "mono- or mixed cultures of live microorganisms when applied to animal or human, beneficially affect the host by improving the properties of the indigenous microflora". When these probiotic bacteria are present in yogurt and other fermented foods, they may beneficially alter the normal gut flora (Metchnikoff, 1907).

Probiotics have also been defined by the European Union (EU) Expert Group on Functional Foods in Europe (FUFOSE) to

be “viable preparations in foods or dietary supplements to improve the health of humans and animals” (FUFOSE working group, 1999).

b. Probiotic bacteria

Strains of LAB, such as *Lactobacillus, Bifidobacterium, Eubacterium* and *Streptococcus*, have traditionally been used in the manufacture of fermented dairy products and are generally regarded as safe (GRAS) (O’Sullivan *et al.,* 1992).

Berg (1998) stated that these bacteria are desirable members of the intestinal microflora. Lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens are some of the general criteria that have been used for the selection of probiotics (Ouwehand *et al.,* 2002).

Mitsuoka (1990) reported that the prevalence of *lactobacillus* and *bifidobacterial spp.* in the intestinal tract of humans is not known accurately. *Lactobacillus crispatus*, *L. gasseri*, *L. salivarius*, and *L. reuteri* have been reported as the major species of the *Lactobacillus* microflora. Whereas, *Lactobacillus johnsonii*, *Lactobacillus ruminis*, *Lactobacillus casei*, and *Lactobacillus brevis* have been detected occasionally. *Bifidobacterium longum* has been found predomonently in adult human GIT, while *Bifidobacterium bifidum* was detected occasionally. In contrast, *Bifidobacterium infantis* and

Bifidobacterium breve were detected predominantly in infant feces, while *B.longum* and *B. bifidum* detected occasionally (Biavati *et al.,* 1984).

c. Health benefits associated with the ingestion of probiotic bacteria

Health benefits associated with the ingestion of probiotic bacteria includes: reduction in colon irritation, constipation, traveler's diarrhea, inhibition of the adhesion of pathogenic genera including *Escherichia, Clostridium, Salmonella* and *Campylobacter* to the intestinal lumen, synthesis of B vitamins, lowering of blood ammonia levels, cholesterol absorption and inhibition of tumor formation (Ziemer and Gibson, 1998).

d. Properties of Probiotic Bacteria

The GIT of the human body is a complex ecosystem with a diverse and concentrated microbial population that mediates numerous interactions with the chemical environment, such as digestion, adhesion and colonization in the GIT. The mucosal surface area increases by: circular folding which contributes to about a 3-fold increase, through the production of villi, for a 7- to 10-fold increase, and by the formation of intestinal microvilli, which results in a 15- to 40- fold increase (Holzapfel *et al.,*1998).

The bacteria detected in feces reflect the bacteria present in the distal colon, thus studies of the human GIT microflora usually involve analysis of fecal samples (Moore *et al.*, 1978).

Rowland (1989) reported that various short chain fatty acids (SCFA), such as acetate, propionate and butyrate, are end products of anaerobic bacterial fermentation. Thus, measurement of these acids in feces can be correlated with specific bacterial metabolism in the intestine. For example, *Lactobacillus casei GG* fed to children with an intestinal infection significantly increased the total Short Chain Fatty Acid SCFA concentration (Siigur *et al.* 1996).

Ling *et al.* (1994) indicated that increases or decreases in specific enzymes for example, β-glucuronidase and β-galactosidase, in feces can also point to the metabolic activities of certain groups of bacteria. Reduction in β-glucuronidase levels was reported in humans during ingestion of *L. casei* GG.

Favier *et al.* (1997) recorded a significant correlation has been observed between the levels of faecal β-galactosidase and numbers of *bifidobacteria.* While many fecal enzymes, such as azoreductase and nitroreductase are mainly produced by the species *Bacteroides, Eubacterium* and *Clostridium.* More studies are needed to accurately correlate specific fecal enzymes with specific groups of bacteria as reported by (Rowland, 1989).

e. Viability of probiotic microorganisms

Fermented dairy products such as milk and yogurt are the most accepted food carriers for live probiotic delivery to the

human GIT. A recommended intake of probiotics is 10^8-10^9 CFU/ml or g product viable live cells daily.

Standards have been made in many countries for the numbers of viable probiotic bacteria that are present in commercial fermented products. For example, in Japan, fermented milks and Lactic Acid Bacteria LAB containing beverages must contain a minimum of 10^7CFU/mL or gram of product (Robinson, 1987).

Despite the importance of viability of these beneficial *bifidobacteria*, surveys have shown poor viability of *bifidobacteria* in yoghurt preparations (Akalin *et al.*, 2004).

Several factors, like acidity of the product, post acidification (acid produced during storage), level of oxygen in the products, sensitivity to antimicrobial substances produced by bacteria, temperature of storage during manufacture and storage of yoghurt, have been found to reduce the viability of probiotics (Lankaputhra and Shah, 1995; Dave and Shah, 1997).

Thus, maintaining viability of *bifidobacteria* until the products are consumed in order to ensure the delivery of live organisms has been of much interest. Viability of probiotic bacteria in a product at the point of consumption is an important consideration for their efficacy, as they have to survive during the processing and shelf life of food and supplements, transit through high acidic conditions of the stomach and enzymes and bile salts

in the small intestine. The consumption of probiotics at a level of 10^8-10^9 CFU/g per day is a commonly quoted figure for adequate probiotic consumption, equating to 100 g of a food product with 10^6-10^7CFU/g (Kebary *et al.,* 1998).

2. Antioxidative Properties of Probiotic

Oxidative stress occurs when the available supply of the body's antioxidant is insufficient to handle and neutralize free radicals (Halliwell and Gutteridge, 1989).

Battino *et al*. (1999) reported that free radicals are highly unstable molecules that interact with other molecules in our bodies to destroy cellular membranes; enzymes and DNA. They accelerate aging and contribute to the development of many diseases, including cancer and heart disease.

Antioxidants are chemical compounds that can prevent, stop, or reduce oxidative damage. Antioxidants can protect the human body from free radicals and retard the progress of many diseases. Therefore, the development and utilization of effective antioxidants are desired (Lai *et al.,* 2001).

During the past decade, 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. The most widely studied probiotics are *Lactobacillus* and *Bifidobacterium*. Most *Lactobacillus* species

are normal and non pathogenic inhabitants of human and animal intestines, and their presence is important for the maintenance of the intestinal microbial ecosystem (Jacobsen *et al.,* 1999).

Annuk *et al* . *(*2003) found that *Lactobacilli* have been shown to possess inhibitory activity towards the multiplication of enteropathogens, and they are highly competitive, largely due to their production of several antimicrobial compounds.

Few studies have explored the antioxidative properties of probiotics and those few investigated a limited number of strains (An *et al.*, 2010).

a. The Role of Probiotics and Prebiotics in Intestinal Antioxidant Defense Mechanisms

Against oxidative stress, aerobic cells like those of intestinal mucosa are equipped with a complex antioxidant defense system which includes enzymatic and non-enzymatic components having synergistic and interdependent effects on each other. The inherent antioxidant enzymatic network includes proteins, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and glutathione-s-transferase (GST), while non-enzymatic antioxidant defense consists of low-molecular-weight antioxidant molecules acting mainly as free-radical scavengers, such as glutathione (GSH), a-tocopherol (vitamin E), ascorbic acid (vitamin C) and melatonin (Mutlu-Tu¨rkog˘lu *et al*., 2000).