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Immunomodulatory Effects of Probiotics on *Salmonella enterica* Serovar *Typhimurium* Isolated from Diarrheal Children in Albino Male Mice

A dissertation

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Dedication

***Mom** I dedicate this to you; thanks for being there all the time and even now I am sure you are there; death will never pull us apart.*

*To the one and only, my soul mate **Sarmad**; you will always be my knight with shining armor.*

*To the joy of my life, my dearest brothers **Hakam** and **Homam**; I am so proud to have you .*

*To you my Jewels, my precious legacy; **Lanoo** and **Beeso**.*

Safa

Summary

This study aimed to determine the frequency of *Salmonella Typhimurium* and its multi-drug resistance (MDR) status as a diarrheal causative pathogen in children younger than five years old, as well as to investigate the role of four probiotic microorganisms*(L.acidophillus,L.casei,S.cerevisiae,S.boulardi)* in controlling such infection.

For this purpose, 128 stool samples of patients (76 males and 52 females) was included in this study. They were children suffering from diarrhea and fever who admitted to the "Central Pediatric Hospital" in Baghdad during the period from April to September, 2012. Their ages ranged from six days up to five years.

The immunological effects of probiotics and MDR *S. Typhimurium* isolate in the experimentally infected and probiotic-treated mice were investigated via assessing the level of ten cytokines (IFN-γ, IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, GM-CSF, RANTES and IP-10) in the lavage of small intestine.

Accordingly, five groups of mice were used in the *in vivo* part of this study;

Mice in group I received probiotic for 7 successive days, challenged with *S. Typhimurium* on day 8, and dissected on days 14 and 21. Group II was similarly treated, but the probiotic was continued for 14 days. Group III was given the probiotic only, and group IV was challenged with *S. Typhimurium,* while group V was the control.

Results obtained in this study could be summarized as follows:

1. Out of 128 stool samples, *S. Typhimurium* was isolated and identified in 9 samples only (7.03%). All isolates were totally resistant to nalidixic acid, with the exception of one isolate which showed intermediate sensitivity. Furthermore, only one of these isolates (symbolized B) was found to be resistant to three antibiotics (ampicillin, amoxicillin and nalidixic acid).

Therefore, this isolate was considered as an MDR isolate and selected for further experiments in the study.

2. When the susceptibility of MDR *S. Typhimurium* isolate B was further assessed *in vitro* by using four probiotics (*Saccharomyces cerevisiae*, *S. boulardi*, *Lactobacillus acidophilus* and *L. casei*), results showed that *S. cerevisiae* and *L. acidophilus* were the most efficient by recording the highest inhibition zones (12.6 and 16.3 mm, respectively), therefore, they were further investigated for their anti-*S. Typhimurium* effects *in vitro* and *in vivo*.
3. By using the unconcentrated and (one-fold, two-fold, three-fold) concentrated filtrates of these two probiotics, it was found that the three-fold filtrates were most efficient in their antibacterial activity by recording the highest inhibition zones (25.0 mm for *S. cerevisiae* and 31.0 mm for *L. acidophilus*.
4. Significant increases in the values of liver index were observed in mice of group I treated with *L. acidophilus* at 21 days (10.73%) compared to the corresponding group treated with *S. cerevisiae* (7.41%) or other four groups. For spleen, index value in *L. acidophilus* groups was higher than the corresponding groups in *S. cerevisiae*. Mice treated with a probiotic alone or in a combination with the pathogen showed significant increases in the spleen index values of all groups compared to the untreated mice (group V).
5. Both probiotics were effective in reducing *S.* Typhimurium colony forming units per plate (cfu/plate) in the liver and spleen. In liver, mice in group IV showed a count of 224.4 cfu/plate, which was significantly higher than any count in groups of *L. acidophilus* and *S. cerevisiae*. Group II mice recorded better results than group I mice, while the lowest counts (21.6 and 27.8 cfu/plate for *L. acidophilus* and *S. cerevisiae*, respectively), were observed at day 21.
6. The ten investigated cytokines showed different levels in the small intestine wash; such differences were subjected to the group of mice under

investigation and type of probiotics used. In addition, variations in IFN-γ/IL-4, IFN-γ/IL-10, IL-4/IL-10 and IL-17A/IL-10 were also observed.

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List of Abbreviations

AAD	Antibiotic associated diarrhea
ADH	Arginine dihydrolase test
AMC	Amoxicillin
AMP	Ampicillin
ANOVA	Analysis of variance
Api 20E	Analytical profile index 20 Enterobacter
ARA	Arabinose fermentation test
ASM	American Society of Microbiology
AZ	Azithromycin
CD	Cluster designation
CDC	Centers for Disease Control
CFU	Colony Forming Unit
CIP	Ciprofloxacin
CIT	Citrate utilization test
DCs	Dendritic cells
DT	Definitive phage type
ELISA	Enzyme Linked Immunosorbent Assay
ESBL	Extended Spectrum Beta- Lactamase
FADD	Fas-Associated Protein With Death Domain
FAO	Food and Agriculture Organization
GEL	Gelatin liquefaction test
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLU	Glucose fermentation test
GM-CSF	Granulocyte monocyte colony stimulating factor
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INO	Inositol fermentation test

iNTS	invasive non typhoidal salmonella serovar
IWF	Intestinal wash fluid
LAB	Lactic acid bacteria
LCD	Lysine decarboxylase test
LPS	Lipopolysaccharides
LSD	Least significant difference
MDR	Multi-drug resistant
MEL	Melibiose fermentation test
MLNs	Mesenteric lymph nodes
MRS	Manns Regosa Sharpe
MR-VP	Methyl Red -Vogas Proskauer
NAL	Nalidixic acid
NCCLS	National Committee for Clinical Laboratory Standards
NK cells	Natural killer cells
NTS	Non typhoidal salmonella serovars
ODC	Ornithine decarboxylase test
OXI	Oxidase test
PBL	Peripheral Blood Lymphocyte
PBS	Phosphate buffer saline
PLS	Hydrogen sulphide test
R	Resistant
RHA	Rhamnose fermentation test
rpm	Revolution per minute
S	Sensitive
S.D.	Standard deviation
SCVs	*Salmonella* containing vacuoles
SGI-1	*Salmonella* genomic island type-1
SIFs	*Salmonella* induced filaments
sIgA	Secretery IgA
SIV	Simian immunodeficiency virus
SOR	Sorbitol fermentation test

SPIs	*Salmonella* pathogenicity islands
SPSS	Statistical Package for Social Sciences
SS agar	*Salmonella - Shigella* agar
StST	*Salmonella typhimurium* sequence type
SXT	Trimethoprim
TDA	Tryptophane deaminase test
T-fh	T-follicular helper cells
TGF	Transforming growth factor
TGF-β	Transforming growth factor -Beta
Th cells	T helper cells
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor – alpha
Treg	T regulatory cell
TTSS	Type three secretion system
URE	Urease test
VAP	Vacuole associated actin polymerization
WHO	World Health Organization

Chapter One
Introduction and Literature Review

Chapter One
Introduction and Literature Review

1.1 Introduction

Salmonella is one of the principal causative agents of poisoning and food-borne disease in the world, and enteric infections caused by this genus are one of the major causes of morbidity and mortality in infants in developing countries. It can cause a wide variety of diseases; from mild gastroenteritis to typhoid fever, nature and severity of the infection are dependent on many factors; including the serovar involved, the virulence of the strain, the infective dose, the species, the age and immune status of the host (Gordon, 2011; Kaiser and Hardt, 2011). Accordingly, it has been stated that salmonellosis is an important global public health problem caused by the *Salmonella spp.*, which are the main cause of typhoid fever in humans, and responsible for a serious health problem in developing countries. Twenty two million new cases of typhoid fever are registered annually worldwide despite various vaccination programs (Gunn *et al*., 2014), and in children, *Salmonella* infection is an important health risk, and an estimated 17 million cases of typhoid fever (enteric fever) with approximately 600,000 deaths worldwide each year has been reported, in which *S. enterica* serovar Typhimurium (*S.* Typhimurium) is a major cause of food-borne illness, causing intestinal inflammation and diarrhea (Waddington *et al*., 2014). A major concern of *S.* Typhimurium for physicians in developing countries is their antibiotic resistance, and multidrug-resistant (MDR) strains continue to be a worldwide health challenge, and probiotics may have the promise (Tadesse, 2014).

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit in the host, and currently numerous organisms have met the criteria established by the World Health Organization (WHO) for a probiotic (Adam *et al*., 2012). They are suggested to impact metabolism,

endocrine system, proper gut development, and regulation of the immune system, and by modifying the microbial community within the gut, it is possible to prevent or treat gut-associated infections (Cani and Delzenne 2009). Focusing on effects on the immune system, probiotics act on a wide variety of cells in the intestine to modulate immune cells towards pro- or anti-inflammatory actions (O'Flaherty *et al*., 2010). Therefore, new strategies devised the use of probiotics as an alternative therapy for treatment and prevention of bacterial gastrointestinal infections, and there is a growing interest in probiotics as a safe therapeutic agent through their ability to alleviate food allergies, enhance non-specific and specific immune responses, suppress intestinal infections, and have anti-carcinogenic activity (Akin and Tözün, 2014; Walker, 2014).

It has also been evident that probiotics can be effective in the prevention and/or treatment of diarrheal diseases caused by *S*. Typhimurium. The suggested mechanism by which probiotics might exert their protective or therapeutic effect against enteric pathogens include non-immune mechanisms, such as stabilization of the gut mucosal barrier, increasing the secretion of mucus, improving gut motility, and therefore interfering with their ability to colonize and infect the mucosa; competing for nutrients; secreting specific low molecular weight anti-microbial substances, and influencing the composition and activity of the gut microbiota (regulation of intestinal microbial homeostasis) (De Moreno De Leblanc *et al*., 2010). Probiotics also exert their effect as immune adjuvants modulating the mucosal and systemic immune responses. They can modulate inflammatory response, stimulate certain cytokine production and phagocytic activity of macrophages and neutrophils, regulate NK cell activity, and enhance specific humoral responses (De Moreno De Leblanc *et al*., 2011).

1.2 Aims of Study

Due to the importance of *S*. Typhimurium infection especially in children and to assess and understand the role of four probiotics (*Lactobacillus*

acidophilus, *L. casei*, *Saccharomyces cerevisiae* and *S. boulardii*) in controlling such infection in diarrheal cases among children younger than five years old, the study was planned with the following aims:

1. Determining the frequency of *S*. Typhimurium as a causative pathogen in hospitalized diarrheal children under the age five years.
2. Assessing the susceptibility of the isolated *S.* Typhimurium to different antibiotics and MDR isolate which was further assessed for its susceptibility to the investigated four probiotics *in vitro*.
3. The most effective probiotics will be further selected to treat albino male mice experimentally infected with one of the MDR *S*. Typhimurium isolate, and the bacterial load in liver and spleen will be inspected.
4. Assessing the immunological effects of probiotics and MDR *S*. Typhimurium isolate, the small intestine wash of experimentally infected and probotic-treated mice was assessed for cytokines produced by T helper (Th) cells; Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17A) and T regulatory (Treg) cells (IL-10) and chemokines (RANTES and IP-10); in addition to IL-1β, IL-12, IL-21 and granulocyte monocyte-colony stimulating factor (GM-CSF).

1.2 Literature Review

1.2.1 *Salmonella*

Salmonella is enteric bacteria, whic are a major cause of infectious diseases throughout the world. These bacteria infect both humans and other animals and are a common cause of zoonotic disease. The genus *Salmonella* incorporates Gram-negative, facultative anaerobic rod-shaped bacilli that are classified as members of the family Enterobacteriaceae (Mastroeni and Sheppard, 2004).

Salmonella is named after an American bacteriologist, D. E. Salmon, who first isolated *S. choleraesuis* from porcine intestine in 1884. *Salmonella* are a group of bacterial organisms with a high genetic similarity and are differentiated by their serotyping results. The antigenic classification system of various *Salmonella* serotypes is a result accumulated from many years of studies on antibody interactions with surface antigens of *Salmonella* organisms established by Kauffman and White almost 100 years ago. All antigenic formulae of recognized *Salmonella* serotypes are listed in a document called the Kauffmann-White scheme (Popoff and Le Minor, 2001). The World Health Organization Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (WHO Collaborating Centre) is responsible for the updating of the scheme, and every year newly recognized serotypes are reported in the Research in Microbiology, and in a report published in 2004, there were a total of 2,541 serotypes in the genus *Salmonella*. The terms “serotype” and “serovar” are both frequently used, but according to the Rules of the *Bacteriological Code* (1990 Revision) established by the Judicial Commission of the International Committee on the Systematics of Prokaryotes, the term serovar is preferred to the term serotype. Thus “serovar” is used in the Kauffmann-White scheme (Popoff *et al*., 2004).

Salmonella nomenclature is complex and the nomenclature system used at the Centers for Disease Control and Prevention (CDC) for the genus *Salmonella*

is based on recommendations from the WHO Collaborating Centre. According to the CDC system, the genus *Salmonella* contains two species, *S. enterica*; the type species, and *S. bongori*. *S. enterica* consists of six subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*. In subspecies I, serotypes (or serovars) are designated by a name usually indicative of the associated diseases, their geographic origins, or their usual habitats. In the remaining subspecies, as well as those of *S. bongore*, antigenic formulae determined according to the Kauffmann-White scheme are used for those unnamed serotypes (Shelobolina *et al*., 2004). To avoid confusion between serotypes and species, the serotype name is not italicized and starts with a capital letter. When cited at the first time in a report, the genus name is given followed by the word "serotype" (or the abbreviation "ser.") and then the serotype name, e.g., *Salmonella* serotype or ser. Choleraesuis, and *Salmonella* serotype or ser. Typhi. Afterward the name is shortened with the genus name followed directly by the serotype name; e.g., *Salmonella* Choleraesuis or *S.* Choleraesuis, and *Salmonella* Typhi or *S.* Typhi (Popoff *et al*., 2004). Because the type species name, *enterica*, was not approved before 2005, serotype names are used directly after the genus name without the mention of the species. Following the officially approval of "*enterica*" as the type species name from January 2005, the ASM (American Society for Microbiology) Instruction to Authors indicated that, for the species, "*Salmonella enterica*" is used at the first time, and "*S. enterica*" thereafter; for the subspecies, "*Salmonella enterica* subsp. *arizonae*" is used at first, and "*S. enterica* subsp. *arizonae*" thereafter. Serotype names are in Roman type with the first letter capitalized, e.g., *Salmonella enterica* serotype Typhimurium. After the first use, the serotype is used without a species name, e.g., *Salmonella* serotype Typhimurium (Judicial Commission of the International Committee on Systematics of Prokaryotes, 2005).

1.2.1.1 Epidemiology

While many non-typhoidal *Salmonella* (NTS) serovars such as Typhimurium and Enteritidis are generalist pathogens with broad host specificity, a few *S. enterica* serovars including Typhi, Sendai, and Paratyphi A, B, or C are highly adapted to the human host that is used as their exclusive reservoir. These specialist pathogens, collectively referred to as typhoidal *Salmonella* serovars, are the causative agents of enteric fever (also known as typhoid or paratyphoid fever if caused by serovar Typhi or Paratyphi, respectively). Enteric fever is an invasive, life-threatening, systemic disease with an estimated global annual burden of over 27 million cases, resulting in more than 200,000 deaths (Crump *et al*., 2004; Buckle *et al*., 2012). Enteric fever is endemic in the developing world in regions that lack clean water and adequate sanitation, facilitating the spread of these pathogens via the fecal-oral route. In recent years, the incidence of infections with serovar Paratyphi A is on the rise and in some regions of the globe, particularly in South–East Asia, this serovar is accountable for up to 50% of all enteric fever cases (Ochiai *et al*., 2005; Meltzer and Schwartz, 2010).

In contrast to typhoid fever which is common in the developing world, NTS salmonelloses occur worldwide. There are an estimated 93.8 million cases of gastroenteritis due to NTS infection each year, resulting in approximately 155,000 deaths (Majowicz *et al*., 2010). Despite global morbidity, mortality due to NTS infection is primarily restricted to the developing world. In addition to contaminated animal-derived food products such as poultry, eggs, and dairy products, NTS transmission can result from person to person contact or from contact with pets such as cats, dogs, rodents, reptiles, or amphibians (Haeusler and Curtis, 2013). Another important source of infection is consumption of contaminated produce especially sprouts, tomatoes, fruits, peanuts, and spinach which have all been associated with recent outbreaks (Jackson *et al*., 2013; Bayer *et al*., 2014).

While normally NTS infections in humans induces gastroenteritis, in up to 5% of NTS cases, bacteria cause an invasive, extra-intestinal disease leading to bacteremia and focal systemic infections, henceforth referred to as invasive NTS (iNTS). Interestingly, various NTS serovars (e.g., Typhimurium) tend to have more potential to cause extraintestinal infections than others. This implies there is a genetic basis for the emergence iNTS disease; however, these differences are still not understood (Marzel *et al*., 2014). In Sub-Saharan Africa, iNTS is a major cause of bacteremia in adults and children, with an estimated annual incidence of 175–388 cases per 100,000 children and 2000–7500 cases per 100,000 HIV-infected adults. Especially S. Typhimurium sequence type (ST) 313 is associated with invasive disease. Startlingly, in 20–25% of cases, invasive infection results in the death of the patients. Other major risk factors for invasive disease in addition to HIV are co-infection with malaria and malnutrition (Feasey *et al*., 2012; MacLennan, 2014).

1.2.1.2 Clinical Manifestations

Enteric fever caused by typhoidal serovars differs dramatically from the gastroenteritis normally associated with NTS. Infections caused by different typhoidal serovars (e.g., Typhi and Paratyphi A) cannot be distinguished by clinical presentation (Meltzer *et al*., 2005). The average incubation period for typhoidal serovars is 14 days with symptoms persisting for up to three weeks. Patients most typically present with a gradual onset of sustained fever (39 - 40°C). Other frequent symptoms include chills, abdominal pain, hepatosplenomegaly, rash (rose spots), nausea, anorexia, diarrhea or constipation, headache, and a dry cough (Wangdi *et al*., 2012). In contrast to enteric fever, individuals infected with NTS have self-limiting, acute gastroenteritis and watery diarrhea. Nausea, vomiting, abdominal pain, and fever are also common symptoms. With NTS infection, symptoms appear 6–12 hours after the ingestion of the pathogen and clinical symptoms last less than 10 days. In the case of iNTS infections, which are often associated with patients with

immunodeficiency, disease more closely resembles enteric fever in that patients often suffer from high fever, hepatosplenomegaly, and have respiratory complications with intestinal symptoms often being absent (Patel *et al*., 2010).

Both typhoidal and NTS serovars initially adhere to and invade the intestinal epithelium of the small intestine. Unlike NTS infection, infection by typhoidal serovars does not induce a high inflammatory response during the initial invasion of the intestinal mucosa (Nguyen *et al*., 2004). Minimal intestinal inflammation during enteric fever is correlated with negligible neutrophil transmigration across the intestinal epithelium in contrast to massive neutrophil recruitment during intestinal inflammation caused by NTS serovars. In immunocompetent patients, NTS gastroenteritis is self-limiting, with infection being confined to the terminal ileum and colon. In the case of typhoidal salmonellae, after passing the intestinal mucosa, bacteria gain access to underlying lymphoid tissues and multiply intracellularly within mononuclear phagocytes. Infection quickly becomes systemic with spreading of the pathogen from the intestine to the mesenteric lymph nodes, liver, spleen, bone marrow, and gallbladder. Secondary infection of typhoidal organisms to the small bowel can occur via secretion in the bile through the enterohepatic cycle. The absence of robust intestinal inflammation and the lack of neutrophil transmigration are thought to facilitate the invasion of typhoidal serovars into the deeper tissues of the gut and its dissemination to systemic sites (Gordon, 2008).

It has been reported that up to 10% of convalescing, untreated patients continue to shed *S*. Typhi in their stool for up to three months after infection, and 1 - 4% of individuals infected with *S*. Typhi become asymptomatic, chronic carriers that continue to excrete 10^6 - 10^{10} *S*. Typhi bacteria per gram of feces for more than 12 months. The role of such chronic carriers in disease transmission was notoriously demonstrated by the case of Mary Mallon (Typhoid Mary). During her work at different households as a cook in the New York City area in the early 20th century, Mary Mallon infected between 26 and 54 people (Parry *et*

al., 2002). The suspected site of persistence of S. Typhi in carriers is the gallbladder and gallstones are thought to be an important risk factor for developing chronic carriage as they are conducive for biofilm formation which protects bacteria from anti-microbial compounds and the host immune system (Khatri *et al*., 2009). Long-term carriage of S. Paratyphi has received much less attention and is currently less characterized than S. Typhi, but a recent study in Nepal suggests a similar rate of persistence for serovars Typhi and Paratyphi A in endemic regions (Dongol *et al*., 2012).

Long-term carriage of NTS has not been described. However, even though symptoms usually last only for a few days, adults excrete *Salmonella* on average for a month after infection and children under the age of five years shed bacteria in their feces for an average of seven weeks (Hohmann, 2001). In addition, several studies have shown that treatment with antibiotics can prolong shedding of NTS bacteria, although these findings are controversial. In comparison to NTS serovars, the long-term persistence of typhoidal serovars in humans suggests an enhanced ability of these pathogens to evade the human immune system (Raffatellu *et al*., 2008).

1.2.1.3 Pathogenesis

Salmonella Typhimurium has been considered by some investigators as a pathogenesis model of infection by this enteropathogen (Fàbrega and Vila, 2013). The infection begins with the ingestion of organisms in contaminated food or water. The first obstacle to overcome within the host is the acidic pH of the stomach. To protect itself against severe acid shock, *S*. Typhimurium activates the acid tolerance response, which provides an inducible pH-homeostatic function to maintain the intracellular pH at values higher than those of the extracellular environment (Foster and Hall, 1991). After entering the small bowel, salmonellae must reach and traverse the intestinal mucus layer before encountering and adhering to intestinal epithelial cells. In mice, salmonellae appear to preferentially adhere to and enter the M cells of the

Peyer's patches in the intestinal epithelium, although invasion of normally nonphagocytic enterocytes can also occur (Jones *et al*., 1994). Shortly after adhesion, the invasion process appears as a consequence of engaged host cell signaling pathways leading to profound cytoskeletal rearrangements. These internal modifications disrupt the normal epithelial brush border and induce the subsequent formation of membrane ruffles that engulf adherent bacteria in large vesicles called Salmonella-containing vacuoles (SCVs); the only intracellular compartment in which Salmonella cells survive and replicate (Santos *et al*., 2003). Simultaneously, induction of a secretory response in the intestinal epithelium initiates recruitment and transmigration of phagocytes from the submucosal space into the intestinal lumen. This process is associated with the production of several pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8), and lastly, the apical epithelial brush border reconstitutes (Griffin and McSorley, 2011).

Salmonella-containing vacuoles are initially integrated within the early endocytic pathway. However, they need to be later uncoupled to bypass delivery of lysosomal enzymes. This action depends on Salmonella-directed changes in host endocytic trafficking and function to eventually avoid fusion with secondary lysosomes (Rathman *et al*., 1997). During SCV maturation, Salmonella induces *de novo* formation of an F-actin meshwork around bacterial vacuoles; a process which is termed vacuole-associated actin polymerization (VAP) and is important for maintenance of the integrity of the vacuole membrane (Meresse *et al*., 2001), and then SCVs migrate to a perinuclear position, in close proximity to the Golgi apparatus, presumably to facilitate interception of endocytic and exocytic transport vesicles to obtain nutrients and/or membrane fragments. This event appears to be essential for bacterial replication. In addition, it has been observed that intracellular *Salmonella* can induce the formation of long filamentous membrane structures called Salmonella-induced filaments (SIFs) (Deiwick *et al*., 2006), which are tubular

aggregates along a scaffold of microtubules and originate from the SCVs and extend throughout the cell. Although the biological role of the induction of SIFs is not completely understood, it has been postulated that this process may lead to an increased availability of nutrients that may otherwise be limited within the SCV (Rajashekar *et al*., 2008).

Thereafter, a fraction of these SCVs transcytose to the basolateral membrane. Once across the intestinal epithelium, salmonellae are engulfed by phagocytes. Three types of phagocytes are reported to interact with these invading bacteria: (i) neutrophils, (ii) inflammatory monocytes which differentiate into macrophages, and (iii) dendritic cells; another type of monocytes which function as antigen-presenting cells. The first two types of cells are both recruited from blood in response to the inflammatory signals (Menendez *et al*., 2009). In general terms, since most of the bacterial cells have breached the epithelium through the M cells, they directly reach the Peyer's patches and then the mesenteric lymph nodes (MLNs) via the intestinal lymph, most likely being transported by dendritic cells. Nonetheless, dendritic cells have also been reported to directly take up bacteria from the intestinal lumen by opening the tight junctions and sending dendrites to the lumen (Msefula *et al*., 2012). *Salmonella* are then phagocytosed and internalized again within SCVs, triggering a response similar to that reported inside epithelial and M cells to ensure bacterial survival and replication. Migration of these infected phagocytes, predominantly macrophages, facilitates systemic dissemination of the bacteria via the bloodstream to several additional tissues, such as the spleen and liver, where this pathogen preferentially replicates (Monack, 2012). Alternatively, direct blood access of Salmonella-infected phagocytes from the basolateral side of the intestine has also been suggested to contribute to systemic dissemination. This hypothesis is supported by the finding of infected phagocytes in the blood within minutes after oral infection and is attributed to a manipulation of the motility of the infected cells (Nuccio and Baumler, 2014).

1.2.1.4 Virulence Factors

Salmonella possesses a number of virulence factors that contribute to its pathogenesis. It invades the epithelial cells of the human intestines using a type three secretion system (TTSS) to inject its outer proteins into the host's intestinal cells. The TTSS is a needle-like structure that helps the bacteria find the host and then insert effector proteins. As soon as the TTSS comes into contact with the host cell, it starts secreting these effector molecules. The host cell then engulfs the bacteria, and consequently allowing the bacteria to reproduce and cause infection. The effector proteins that are inserted into the host cell signal the host to take-up the bacteria (Müller *et al*., 2012). This happens by the effectors taking control of the actin polymerization process. The actin polymerizes to the point where it causes the host cell membrane to begin to raise on either side of the bacteria. Once the bacteria is surrounded on all sides by the cell membrane, it is engulfed into the host and encased in a vesicle called SCVs. *Salmonella* mature within the cell inside of SCVs; thereby evading the host's immune response (Malik-Kale *et al*., 2012). Additionally, *Salmonella* have at least 16 adhesion factors which allow them to better adhering to the intestinal epithelium. *Salmonella* has also acquired at least 5 SPI's (*Salmonella* Pathogenicity Islands) via horizontal gene transfer which contribute to its virulence. Specifically, SPI-1 and SPI-2 are the main contributors to *Salmonella*'s virulence (Figueira and Holden, 2012). In addition to its pathogenicity islands, *Salmonella*, like all Gram-negative bacteria, are surrounded by an outer membrane containing lipopolysaccharide (LPS). *Salmonella* lipopolysaccharide contains the lipid A endotoxin, which upon release, can cause shock in the host (Kong *et al*., 2012). Another virulence factor of *Salmonella* is its ability to synthesize enterobactin. Enterobactin, the strongest siderophore known, secreted by *Salmonella* and allows it to commandeer iron from the host's body, and it can be used for growth within the host (Nagy *et al*., 2013).

1.2.1.5 Host Immune Response

A. Innate immune response

During the early phases of infection, the production of a number of cytokines and soluble factors, as well as recruitment of bone marrow derived macrophages and development of organized granulomas have been shown to be critical for controlling *Salmonella* spread and growth in the reticuloendothelial system. Macrophages and neutrophilic granulocytes have been demonstrated to be decisive for controlling the net growth of bacteria during the early phase, and they exert a bactericidal activity in the first few hours of the infection that is replaced later by bacteriostatic functions (Grant *et al*., 2008).

The formation of multicellular pathological lesions at the foci of infection is critical for the effective control of *Salmonella*, particularly in tissues such as the spleen, lymph nodes and liver (Mastroeni *et al*., 1992). Suppression of bacterial growth in the reticuloendothelial system coincided with the formation of macrophage rich multicellular lesions and the administration of anti-TNF-α antibodies exacerbated the course of a *Salmonella* infection in both susceptible and resistant mouse strains by inhibiting their formation (Everest *et al*., 1998). In mice genetically lacking TNF-α, these granulomas were not formed efficiently, and the *Salmonella* were found widely distributed within the liver tissues surrounded by increasing pathological signatures as the infection rapidly worsens (Everest *et al*., 2001). Fluorescence microscopy studies and novel genetic tagging approaches, where individual clones of replicating *Salmonella* can be identified, have dissected the patterns of the formation of individual infection foci and subsequent bacterial escape. Such studies have clarified how the infection develops at a local level, have highlighted the dispersive nature of the infection process, and have indicated escape from granulomas as a key mechanism in *Salmonella* virulence (Grant *et al*., 2008).

Components of the bacterial cell wall such as LPS, DNA, flagella, and certain lipoproteins activate toll-like receptors (TLRs) on host cells, which in

turn induces a robust inflammatory response within tissues, characterized by the production of Th1-like cytokines such as IFN-γ, TNF-α, and IL-1, IL-6, IL-12, and IL-18, as well as macrophage migration inhibitory factor and iNOS (Everest *et al*., 2004). IFN-γ has been shown to be vital for resistance against infections involving intracellular pathogens, including *S*. typhimurium, in part because of stimulation of the antibacterial activity of macrophages. Mice treated with anti IFN-γ antibodies or gene-targeted mice lacking the IFNγR are impaired in their ability to clear a sub-lethal dose of virulent *S*. Typhimurium, and they eventually succumb to infection (Martinoli *et al*., 2007). IL-12 and IL-18, secreted by activated macrophages, have been shown to act both independently and synergistically on natural killer cells and helper T cells to induce the production of further IFN-γ, which activates the macrophages through a positive feedback loop, and in addition, IL-12 was found to be important for the polarization of T-helper cells toward the Th1 response (Gaspal *et al*., 2008).

B. Adaptive immune response

Studies about the mice have suggested that T cells may be activated soon after oral and parenteral *Salmonella* infection, and CCR6+ dendritic cells in the Peyer's patches may be involved in local T-cell activation after oral challenge and B-cells are also implicated in T-cell activation, and effective control and eventual eradication of bacteria during the late phases of a primary infection and the generation of protective immunity against subsequent infections requires the development of a *Salmonella*-specific T-lymphocyte response and the active recruitment of such cells to the site of infection. In primary infections, CD4+ αβTCR+ T cells with a Th1 phenotype have been found to mediate the clearance of the bacteria from the tissues with little or no obvious contribution of CD8+ T cells (Ugrinovic *et al*., 2003). B cells and antibodies are also largely dispensable in the late stages of primary salmonellosis, and αβ T cells appear to be more important than γδ T cells since mice on a susceptible background and deficient in γδ T cells were able to control systemic infection with an attenuated strain of

S. Typhimurium, while mice containing defects in the αβ T-cell receptor were not. During a primary infection, T cells also mediate the regulation of *Salmonella*-specific B-cell activation and maturation, which result in the production of isotype switched antibodies against bacterial polysaccharide and protein antigens (Srinivasan *et al*., 2004). It has been suggested that the T-cell response confers protection via activation of the mononuclear cells where *Salmonella* resides, while antibody targets the bacteria that escape from infected cells and travel, via the extracellular space, to distant sites in the tissues to establish new foci of infection. In this context, CD4+ cells were found to mediate protection via the production of cytokines, in particular IFN-γ, and through IFN-γ-independent mechanisms including the production of other macrophage-activating cytokines (Martinoli *et al*., 2007). In fact, the depletion of Th1-like cytokines such as IFN-γ, TNF-α, and IL-12, by using neutralizing antibodies after vaccination, greatly exacerbated secondary infection, and studies with defined T-cell populations have reinforced the critical role of Th1 and IFN-γ in controlling salmonellosis in murine models (Gaspal *et al*., 2008). CD8+ T cells differentiate into cytotoxic T lymphocytes, which may also play a role in protection by liberating intracellular *S*. Typhimurium from infected macrophages. Thus, the activation of Th1 cells is required not only for the defense against primary infection with *Salmonella* but also for the vaccine-induced resolution of infection (Kupz *et al*., 2014).

Although many studies have demonstrated the importance of Th1 cells in protective immunity to *Salmonella*, recent studies have also suggested a contribution by other effector CD4+ subsets including regulatory T cells (Treg) and Th17 cells. Treg cells suppress effector T-cell responses and either arise from the thymus or develop after activation of naive T cells in the presence of transforming growth factor-β (TGF-β) (Xu *et al*., 2010). In contrast, Th17 cells arise after stimulation of naive CD4+ T cells in the presence of IL-6 and TGF-β and are important in mediating immunity to extracellular bacterial infections

(Curtis and Way, 2009). A recent study examined the development of Th1 cells and Treg cells after *Salmonella* infection of resistant mice and found that alterations in the potency of Treg cells during infection reduced the effectiveness of Th1 responses and increased bacterial growth (Johanns *et al*., 2010). However, it is not yet clear whether similar alterations in Treg potency or its cytokine IL-10 can affect the function of Th1 responses in susceptible mouse model or in humans (Atif *et al*., 2014).

After oral infection with *Salmonella*, cytokines associated with Th17 cells, IL-17, and IL-22, were rapidly produced within the intestinal mucosa (Raffatellu *et al*., 2007; 2008). In these two studies, IL-17 and IL-22 production was induced by innate responses to bacterial infection rather than Th17 cells; however, they still indicate the potential for Th17 cytokines to participate in intestinal defense against *Salmonella*. In a subsequent study, IL-23-dependent production of IL-22, rather than IL-17, was found to contribute to bacterial clearance *in vivo* (Schulz *et al*., 2008). Taken together, these studies suggest an important additional contribution of Th17 cells to protection against *Salmonella* infection, and A Th17 response might be protective by initiating or enhancing neutrophil infiltration to intestinal tissues. In this regard, it was found that IL-17-deficient mice exhibited a defect in the ability to recruit neutrophils to sites of infection during *Salmonella* infection. Interestingly, highly susceptible SIV (simian immunodeficiency virus)-infected macaques were found to have a deficiency in IL-17 and IL-22, but not in interferon-γ, perhaps indicating that Th17 cells represent an important effector cell population particularly in the intestinal mucosa (Raffatellu *et al*., 2008). In addition to neutrophil recruitment, Th17 cytokines induce production of antimicrobial peptides by epithelial cells that are effective against lumenal bacteria, and during *Salmonella* infection of rhesus macaques, IL-22 was important in the production of lipocalin-2, which prevents iron acquisition by bacteria (Raffatellu *et al*., 2009). Accordingly, an additional role for Th17 cells in defense against *Salmonella* in the intestine and a

role for Tregs in modulating the potency of Salmonella-specific Th1 cells *in vivo* have been recently suggested, and a greater study of each of these T-helper populations or their cytokines in the mice model of Salmonella infection and in human Salmonellosis is warranted (Kupz *et al*., 2014).

1.2.1.6 Antibiotic Resistance

Multidrug-resistant (MDR) *Salmonella* is defined as strains of *Salmonella*, which are resistant to all three first-line recommended drugs for treatment; i.e. chloramphenicol, ampicillin, and co-trimoxazole (Kumar *et al*., 2008). In 1948, when chloramphenicol was discovered, it was the most effective and commonly used drug for typhoid fever. Within two years, and due to its rampant and indiscriminate use, chloramphenicol-resistant *S*. Typhi isolates were reported from England. However, it was not until 1972 that chloramphenicol-resistant *S*. Typhi strains became a major problem, with outbreaks being reported in Mexico (1972), India (1972), Vietnam (1973) and Korea (1977). These strains were also resistant to ampicillin, but Co-trimoxazole remained an effective alternative drug in treating these resistant strains until 1975, when resistance to it was reported in France, and by the late 1980s, strains of *Salmonella* resistant to all three first-line drugs were in existence (Mirza *et al*., 1996).

The epidemic of MDR *Salmonella* in the late 1980s compelled pediatricians throughout the world to use ciprofloxacin, despite a lack of data regarding its safety for use in children, but follow-up studies done in children found it to be safe, effective, and less expensive with a very high sensitivity pattern. Thus, fluoroquinolones became the drug of choice for the treatment of MDR *Salmonella* worldwide. However, this was soon followed by reports of isolates showing resistance to fluoroquinolones, with the first case being reported in 1992 in the United Kingdom. Subsequently, similar cases were reported from several other countries (Yoo *et al*., 2004). With the development of quinolone (nalidixic acid) resistance, third-generation cephalosporins were

used for treatment, but sporadic reports of resistance to them also followed (WHO, 2003).

The first multidrug-resistant strains emerged in Southeast Asia in the late 1980s and have since spread throughout the region. Asian countries where *Salmonella* have been reported include China (1985), Pakistan (1987), India (1988), Malaysia (1991), Singapore (1994), Bangladesh (1994), Vietnam (1995), Japan (1999), Thailand (2001), Korea (2003), Nepal (2005) and Indonesia (2009); other countries include Kuwait (1996) and Jordan (2008) (Yanagi *et al*., 2009). A further multi-centric study conducted across five Asian countries (China, India, Indonesia, Pakistan, and Vietnam) that are endemic for typhoid reported the prevalence of MDR *Salmonella* strains ranging from 7 to 65% (Ochiai *et al*., 2008). African countries that have reported MDR *Salmonella* include South Africa (1992), Kenya (2000), Nigeria (2005) and Egypt (2005) (Akinyemi *et al*., 2005). Even developed countries such as the United Kingdom (1990), America (1997) and Italy (2000) have reported MDR *Salmonella* isolates; most of the cases were found among travellers who had returned from regions where MDR strains had caused outbreaks or had become endemic (Scuderi *et al*., 2000).

The overall mortality reported during MDR *Salmonella* epidemics is 7 to 16%, and is much higher than the figure of 2% seen in susceptible salmonellosis. The increased incidence of complications and mortality in MDR has been reasoned due to delay in instituting effective antibiotic therapy, higher virulence of bacteria as a consequence of genes present on R-plasmid, much greater bacterial load in tissues due to resistance to conventional agents, higher number of circulating bacteria (Bhutta, 2008). Gastrointestinal complications such as bleeding, intestinal perforation, paralytic ileus, hepatitis, cholecystitis and peritonitis have been described. Respiratory system complications include bronchopneumonia and pleural effusion. Central nervous system complications described are encephalopathy, meningitis, chorea, intracranial hemorrhage,

cerebellar ataxia and seizures. Renal complications include hypernatremia, hypokalemia, acute renal failure and glomerulonephritis. Cardiovascular complications include myocarditis and peripheral circulatory failure. Hematological complications include disseminated intravascular coagulation and bone marrow suppression (Zaki Karande, 2011). Therefore, an alternative therapeutically strategy is certainly required, and employing probiotics in such strategy can be a fruitful choice.

1.2.2 Probiotics

Probiotics are defined as "Live microbial food supplements or compounds of microorganisms which have beneficial effects on human health". Probiotic microorganisms include strains of lactic acid bacteria (e.g. *Lactobacillus* and *Bifidobacterium*), *Clostridium butyricum, Streptococcus salivarius,* nonpathogenic strains of *E. coli* (e.g. *E.coli* Nissle 1917), and *Saccharomyces boulardii* yeast (Harish and Varghese, 2006). However, the most proper definition of probiotic was published by FAO／WHO in October, 2001; in which the definition came as "live microorganisms which when administrated in adequate amounts confer a health benefits on the host" (Khan and Ansari, 2007). These health benefits have been summarized by Lee and Salminen (2009) in the following points:

- Relieve effects and promote recovery from diarrhea.
- Producing lactase, alleviating symptoms of lactose intolerance and malabsorption.
- Relieve constipation.
- Treat colitis.
- Enhance specific and nonspecific immune response.
- Inhibit pathogen growth.
- Stimulates gastrointestinal immunity.
- Reduce chance of infection from common pathogens (*Salmonella, Shigella*, etc.).
- Reduce the risk of certain cancers (colon and bladder).
- Synthesize nutrients (folic acid, niacin, riboflavin and vitamins B1 and B12).

- Increase nutrient bioavailability.
- Increase urogenital health.

Heselmans *et al*. (2005) commented further on the term probiotic; it is a living microorganism that survives passage through the gastrointestinal tract and having the following three beneficial effects on the host: prevent colonization, cellular adhesion and invasion by pathogenic microorganisms, exhibit direct antimicrobial activity and stimulate the host immune response, probiotic bacteria such as lactic acid bacteria (LAB) may have a potential effect in several gastroenterological conditions especially in the disturbance of intestinal normal flora, and lactic acid bacteria may have the ability to inhibit the growth of various Gram positive and Gram negative bacteria due to its ability to produce organic acids such as lactic and acetic acids, hydrogen peroxide, bacteriocins, bactreiocins-like substances and possibly biosurfactants. Numerous studies also proposed the use of probiotics to improve gut health, in the treatment of inflammatory bowel diseases and in the prevention of antibiotic-induced diarrhea (Chaves *et al*., 2011; Song *et al*., 2011). They are also considered as one of the most important antibiotics against several infectious agents, especially against entero-pathogens (Maragkoudakis *et al*., 2010).

The protective effect of certain probiotic strains against specific pathogens is undeniable; however, the scientific basis of the ways through which probiotics confer protection must be well established. A great number of effects have been proposed in such protective effect, Some of these are the stabilization of gut mucosal barrier (Yan *et al*., 2007), the stimulation of goblet cells for mucus secretion (Dogi and Perdigon, 2006), the competition for nutrients, the secretion of antimicrobial substances (bacteriocins), and the modulation of the mucosal and systemic immune responses (Lebeer *et al*., 2008).

1.2.2.1 Mechanism of Action

Different mechanisms have been suggested to explain the probiotic mechanisms of action. They are outlined in the following:

A. Adherence and colonization of the gut

Probiotic microorganisms are able to adhere to the epithelial cells because they have anti–adhesive ability; thereby blocking adherence of pathogens (Lin *et al.,* 2008). Anti-adhesive capability might be due to competitive exclusion for the same receptor by the probiotics and the pathogens, secretion of proteins that destroy the receptor, induction of biosurfactants, establishing a biofilm, and production of receptor analogues (Oelschlaeger, 2010).

B. Competition for limiting resources

Almost all bacteria need iron as an essential element with the exception of *Lactobacillus* which does not need iron in their natural habitat (Weinberg, 1997). It has been demonstrated that *L. acidophilus* and *L. delbrueckii* were able to bind ferric hydroxide at their surface making it unavailable to pathogenic microorganisms. This mechanism is of crucial advantage in competition with other microorganisms which depend on iron (Elli *et al.,* 2000).

C. Anti-invasive property

Not only adhesion but also invasion of epithelial cells is an important property for full pathogenicity of many gut pathogens; the ability to inhibit bacterial invasion of gut epithelial cells by pathogens is rather wide spread among probiotics (Hess *et al.,* 2004). Some researchers confirmed that some probiotics (like *Lactobacillus* and *Bifidobacterium* strain Bb12) had the ability to secrete factors which interfere with the invasion of host epithelial cells by *S.* Typhimurium (Ingrassia *et al*., 2005; Botes *et al*., 2008).

D. Production of antimicrobial substances

Wohlgemuth *et al.* (2009) found that lactic acid bacteria including *L. plantarum* and *L. acidophilus* had the ability to inhibit growth of Gram negative and Gram positive bacteria. This is due to their ability to produce organic acids (lactic acid and acetic acid), hydrogen peroxide, bacteriocens, bacteriocines like

substances, and possibly biosurfactants (Bierbaum and Sahl, 2009; Oelschlaeger, 2010).

1.2.2.2 Anti-*Salmonella* Effects

In the literature, there are several clinical evidences that probiotics could be effective in the prevention and/or treatment of diarrheal diseases due to *Salmonella* infections (Canani *et al*., 2007; Henker *et al*., 2007). The suggested mechanism(s) by which probiotics might exert their protective or therapeutic effect against enteric pathogens include non-immune mechanisms, such as stabilization of the gut mucosal barrier, increasing the secretion of mucus, improving gut motility, and therefore interfering with their ability to colonize and infect the mucosa; competing for nutrients and secreting specific low molecular weight antimicrobial substances (bacteriocins) (Liu *et al*., 2011), and influencing the composition and activity of the gut microbiota (regulation of intestinal microbial homeostasis) (De Moreno de LeBlanc *et al*., 2010). They can also modulate the inflammatory response, stimulate certain cytokine production and phagocytic activity of macrophages and neutrophils, regulate NK cell activity, and enhance specific antibody responses, especially mucosal secretory IgA (De Moreno De Leblanc *et al*., 2011; Wick, 2011)

1.2.2.3 Effects on Immune System

The beneficial effects of the microbiota on the host immune system have allowed the proposal to use some non-pathogenic bacteria, such as probiotics in improving animal health and protection against infectious agents (Galdeano *et al*., 2007). Probiotics have been shown to influence both innate and adaptive immunity through direct contact with epithelial and immune cells, or by their ability to modify the composition and activity of the gut microbiota. They exert their protective effects by multiple immune and non-immune mechanisms. Servin (2004) established that probiotics exert direct anti-microbial activity against pathogens by increasing phagocytosis, modifying cytokine production

by different cell populations or enhancing IgA production (Galdeano and Perdigon, 2006; De Moreno de LeBlanc *et al* ., 2010). In addition, one of the principal mechanisms of protection against gastroenteric infections by probiotics is via modulation of pro-inflammatory (for instance IFN-γ and TNF-α) and anti-inflammatory or immune regulatory (for instance IL-10) cytokines, but the pathways and cells involved in these mechanisms are not clear yet. It is also a fact that not all microorganisms have the same effect on the host, and that probiotic properties are strain and host specific. In this sense, it is not possible to extrapolate the effects found with one probiotic strain to another, or its effect against a specific pathogen to other pathogen (Mieleti *et al*., 2009).

1.2.2.4 Lactic Acid Bacteria

Lactic acid bacteria, especially *Lactobacillus*, are the most commonly used microorganisms as probiotics because of the perception that they are desirable members of the intestinal microflora and because these bacteria have "Generally Recognized As Safe" status (Shokryazdan *et al*., 2014).

A. Usefulness

Many reports showed the usefulness of lactic acid bacteria (LAB) as probiotics for human and animals (Brashears *et al*., 2003; Hamilton-Miller *et al*., 2003). Some LAB strains have been proven to be useful as adjuncts to prevent human from gastrointestinal disorders and from acute enteritis (Fedorak and Madsen, 2004). For these probiotic functions, several factors are usually considered. For example, the adherent capability of LAB became as a barrier to protect the host intestinal epithelium from infection by pathogenic bacteria, such as *Salmonella spp*. or *Escherichia coli* (Coconnier *et al*., 2000). LAB strains also produce acid materials to maintain a competitive advantage to inhibit the other enteric pathogens in gastrointestinal tract (Naidu *et al*., 1999).

B. Inhibitory effects of LAB against gastrointestinal pathogens

Hudalt *et al.* (1997) reported that *L. casei* GG had the ability to promote clinical recovery from acute rotavirus diarrhea in infants, antibiotic associated and traveler's diarrhea in adults, and produced an inhibitory product against *E. coli* and *S.* Typhimurium. It has also been noticed that *L. casei* GG inhibited the Caco-2 cell invasion by *S.* Typhimurium. Moreover, it was observed that the level of cell invasion decreased because of the direct contact of *S.* Typhimurium with the cells free suspension of *L. casei* (Coconnier *et al*., 1997). The latter investigators also found that the cell free suspension of *L. acidophilus* exhibited an inhibitory effect against *E. coli*, *S.* Typhimurium and *Shigella flexneri.*

Rani and Khetarpaul (1998) mentioned that *L. acidophilus* cell culture with 10^5 cfu/ml inhibited growth of *S. dysenteriae*, *S.* Typhimurium, *S.* Typhi, and *E. coli.* Bezkorovainy (2001) also ensured the inhibitory role of *L. acidophilus* against *S.* Typhimurium, and *Campylobacter jejuni*. Felley *et al.* (2001) also reported that *L. johnsonii* La1 when administrated in milk had a long term suppressive effect on the gut colonization by *H. pylori*. In another study by Hudault *et al*. (2001), *L. johnsonii* and *L. casei* when administrated in germ free mice, they were able to produce inhibitory compounds that act against *S.* Typhimurium, which led to delay the death of mice infected with this pathogen.

Annuk *et al*. (2003) and Gomolka *et al* (2003) found that many active inhibitory compounds produced by LAB were able to limit the colonization of Gram negative enteric pathogens *in vitro* and *in vivo*, especially the species belong to the genus *Salmonella*. In a study by Oyetayo *et al.* (2003), *L. acidophilus* and *L. casei* were found to have a liver improvement function. Histopathological data confirmed partial protection of the gastroinstinal tract in rats dosed with *Lactobacillus* isolates and simultaneously infected with *E. coli*. However, enterobacteria were reduced in rats dosed with *L. casei* after three days of feeding trials. Gaon *et al*., (2003) declared that *L. acidophilus, L. casei*

strains and *S. boulardii* were useful in the management of persistent diarrhea in children.

In a recent study, Shokryazdan *et al*., (2014) isolated, identified, and characterized some lactic acid bacterial strains from human milk, infant feces, and fermented grapes and dates, as potential probiotics with anti-microbial activity against some human pathogenic strains. One hundred and forty bacterial strains were isolated and, after initial identification and a preliminary screening for acid and bile tolerance, nine of the best isolates were selected, and then characterized *in vitro* for their probiotic characteristics and their anti-microbial activities against some human pathogens. Their esults showed that all nine isolates were belonged to the genus *Lactobacillus*, and they exhibited good ability to attach to intestinal epithelial cells and were not resistant to the tested antibiotics. They also showed good anti-microbial activities against the tested pathogenic strains of humans.

C. Immunomodulatory effects of LAB

During the 1990s, much interest was focused on the effect of LAB on specific and non-specific immune function. Some strains were found to enhance phagocytosis and secretion of reactive oxygen (Gill, 1998). Immune response could also be modulated by LAB through increasing production of immunoglobulins and phagocytic activity. For instance, *L. casei* GG stimulated local and systemic IgA secretion against rotavirus in children during infection (Kaila *et al*., 1992). In a study by Isolauri *et al*. (1995), levels of IgA and IgM were higher in sera of infants administrated *Lactobacillus* with oral rotavirus vaccine when compared with their levels in sera of infants who took the vaccine only. Oral administration of *L. rhamnosus* or *L. acidophilus* (10^9 cells/day) enhanced protective immunity in spleen, and serum antibody response to orally and systematically administrated antigens (Gill *et al*., 2000). Verse and Schrezenmeir (2002) stated that the orally ingested probiotic microorganisms did not exert health effects exclusively in the intestine, but some strains could

alleviate or prevent bacterial, fungal or viral infections in other organs by stimulating the immune system, as well as preserving or improving barrier function of the intestinal mucosa. They may inhibit translocation of potential pathogens and thus prevent infections of the blood stream and other tissues or organs. Lactic acid bacteria were also used as oral vaccine vector for its ability to stimulate the host immune system (Turner *et al*., 2003). Amdekar *et al*. (2014) also demonstrated that rats with induced arthritis and treated with *L. acidophilus* showed a down-regulation of pro-inflammatory level and up-regulation of anti-inflammatory cytokines level in serum samples. Furthermore, Sharma *et al*. (2014) reported that dietary supplementation of milk fermented with the probiotic *L. fermentum* enhanced systemic immune response (neutrophil functions, interleukin profile, inflammation and antibody responses in the intestine) and antioxidant capacity in aging mice.

1.2.2.5 Yeast

Yeasts are group of unicellular microorganisms most of which belong to the fungi division of Ascomycota. They have been known for thousands years and considered as safe in food industry for various types of fermentations (Olver *et al*., 2002; Muňoz *et al*., 2005); like the production of alcohols, whey, bread, food additives, enzymes, vitamins, flavoring agents, production of microbiologically media and extracts, as alternative source of high nutritional value proteins, and single cell protein (Bekatorou *et al*., 2006).

A. Using yeasts as probiotics

Several *Saccharomyces cerevisiae* strains are commercially available now and used for over a decade in animal production industry due to improving growth and reproduction, and reduction of morbidity and mortality of young animals (Zhang *et al*., 2000). The recorded poor survival of probiotic bacteria in yogurt, led to the incorporation of a probiotic yeast *S. boulardii* due to its ability

to survive in bio-yogurt, and despite inability of yeast to utilize lactose, it is able to utilize available organic acids, as well as galactose and glucose derived from bacterial metabolism of milk sugar (lactose) in dairy products (Lourens-Hattingh and Viljoen, 2001). *S. boulardii* resists the gastric acidity, proteases, and antibiotics (Kühle *et al*., 2005). Czerucka and Rampal (2002) reported some probiotic properties of *Saccharomyces*; for instance, its ability to survive through the gastrointestinal tract and the antagonist interaction with pathogens such as *E. coli*, *Shigella*, and *Salmonella*.

Some strains of *S. cerevisiae* are used in humans as probiotics for many years, because they affect the intestinal microflora with clinical efficiency for prevention and treatment of antimicrobial-associated diarrhea (Elmer, 2001). Pérez-Sotelo *et al*. (2005) stated that some of the mechanisms that help to understand how yeasts are able to protect the hosts against pathogens include stimulation of the immune system, degradation of bacterial toxin by the yeasts proteolytic enzymes, inhibition of bacterial adherence to gastrointestinal epithelial cells by releasing a protease able to digests the bacterial receptors for certain pathogens (such as *Clostridium difficile*), and formation of yeast-bacterial conglomerates by bacterial adhesion to yeast cell wall.

Bekatorou *et al*. (2006) mentioned that *S. boulardii* was first isolated from litchi fruit Indonesia. It is a thermophylic, non-pathogenic yeast, used for more than 50 years as a feed probiotic supplement and a therapeutic agent to treat many gut disorders like diarrhea. Moreover, it is safe, resists antibiotics, achieves high cell number in the intestine in a short time, does not permanently colonize the intestine, and quickly cleared after the cease of administration. Edwards-Ingram *et al*. (2007) declared that *S. boulardii* is considered as a conspecific with *S. cerevisiae*, based on comparative genomic hybridization, genetic finger printing, and gene sequencing. The two yeast species as described by Zanello *et al.* (2009), are differ genetically, metabolically, and physiologically. Also the alternation in the copy number of *S. boulardii* genes

may contribute to increase growth rate, and better survival in acidic environment. Moreover, growing *S. boulardii* is faster than *S. cerevisiae* at 37°C.

B. Immunomodulatory effects of yeasts

Qamar *et al*., (2001) found in their study that feeding mice by *C. difficile* toxoid A in combination with *S. boulardii* led to 1.8 fold increase in total small intestine IgA level, and 4.4 fold increase in specific intestinal IgA. This finding suggested that *S. boulardii* might act as a mucosal adjuvant. Martins *et al.* (2007) stated that higher levels of intestinal contents of sIgA, serum IgA and IgM were observed in mice treated with yeast as compared to germ free mice, and this was probably due to modulation of both local and systematic immunity in mice treated with *S. cerevisiae* 905. Studies on humans and animals revealed that *S. boulardii* was able to enhance the activities of brush-border membrane enzymes and increase secretion of sIgA in intestinal fluid. In humans, *S. boulardii* could also stimulate the intestinal mucosa immune response and cause cellular and humoral changes in the peripheral blood; these effects can be considered reasonable to explain the effects of *S. boulardii* in diarrhea treatment (Fidan *et al*., 2008). Pothoulakis (2009) also discussed that *S. boulardii* and *S. boulardii* secreted protein(s) can inhibit production of pro-inflammatory cytokines by interfering with the global mediator of inflammation nuclear factor κB, and modulating the activity of the mitogen-activated protein kinases. Inhibition of tissue inflammation and bacterial translocation has also been suggested by Martins *et al*. (2013) as one of the protective mechanisms of *S. boulardii* against Salmonella infection in mice. Furthermore, Rajput *et al*. (2014) stimulated chicken bone marrow dendrite cells (chi-BMDCs) were stimulated with *S. boulardii in vitro*, and found that IL-1β, IL-17, IL-4, TGF-β, and IL-10 production levels were higher, while lower concentration of INF-γ and IL-8 were observed.

C. Treatment of gastrointestinal pathogens by yeasts

Gedek (1999) described using viable yeast cells to improve intestinal resistance to bacterial infections. The use of *S. boulardii* as a probiotic instead of antibiotics can be recommended to prevent adhesion of pathogens (*E. coli* and *S.* Typhimurium) to the mucous membrane of gut as an initial step of infection; in addition to the elimination of pathogens from the gastrointestinal tract of infected patients. It is also worth mentioning that *S. boulardii* produces a protease which degrades both toxins A and B of *C. difficile*; the main virulence factors in antibiotic-associated diarrhea (Castagliuolo *et al*., 1999). Another study by Zbinden *et al.* (1999) reported that *S. boulardii* inhibited not only growth of *S.* Typhimurium and *Yersinia enterocolitica*, but also cell invasion which was correlated with reports on clinical efficiency of the yeast.

Dalmasso *et al.* (2006) found that *S. boulardii* was effective against many diseases including inflammatory bowel disease. This yeast was also used worldwide as an alternative therapy to treat both antibiotic-associated diarrhea and *C. difficile* infections (Guslandi, 2006), and in treatment of infections by enteric pathogens such as *C. difficile, Vibrio cholerae, E. coli and S.* Typhimurium (Mumy *et al.,* 2008). In addition, Fidan *et al.* (2008) reported that *S. boulardii* was used in treatment of many intestinal disorders such as acute diarrhea in infants and adults, antibiotic-associated diarrhea, *C. difficile* disease, and traveler's diarrhea. Also, Htwe *et al*. (2008) conducted in 100 hospitalized diarrhea children and showed that *S. boulardii* treatment for 5 days significantly reduces the mean duration of acute diarrhea and frequency of stools, and normalizes stool consistency. These results came to confirm the efficacy of *S. boulardii* for the prevention of acute diarrhea involved 100 children with acute watery diarrhea and reported a significant difference in the incidence of diarrheal episodes in the group receiving *S. boulardii* compared with the control group during two months follow up (Billoo *et al*., 2006). Furthermore, out the 10 controlled trials in adults using *S. boulardii* for the prevention of antibiotic-

associated diarrhea (AAD), 80% showed significant efficacy for the prevention of AAD, and the protective effect of *S. boulardii* and the significant relative reduction in AAD compared with controls ranged between 7.4 and 25% (McFarland, 2010). These results have been further strengthen by Kelesidis and Pothoulakis (2012) who stated the efficacy and safety of the probiotic *S. boulardii* for the prevention and therapy of gastrointestinal disorders.

Chapter Two
Patients, Materials and Methods

Chapter Two
Materials and Methods

2.1 Patients

One hundred and twenty eight patients (76 males and 52 females) were enrolled in the study. They were hospitalized children due to diarrhea and fever, and admitted to the Central Pediatric Hospital in Baghdad during the period April - September, 2012. Their ages had a range of six days and up to five years.

2.2 Materials

General equipment, chemical and biological materials, kits, culture media and antibiotic disks that were employed to achieve the study are given in appendices I, II, III, IV and V, respectively.

2.3 Specimen Collection

A stool sample was taken from each pediatric patient upon admission to the hospital and before taking any medication using the loop then aloopfull was taken from the stool which was transferred to a test tube containing 10 ml sterilized peptone water. The tube was transferred to the laboratory (2-3 hours), and then the sample was incubated at 37°C with a total time of approximately 24 hours.

2.4 Preparation of Media

The media used for isolation and identification of bacteria were prepared according to the instructions of manufacturer, unless otherwise are cited. They were sterilized by autoclaving at 121°C for 15 minutes.

2.4.1 Isolation and Identification Media

A. MacConkey agar: It is a selective and differential medium used for identifying Gram-negative bacteria and detecting their ability to ferment lactose.

B. Motility test medium: The medium was prepared by adding of agar-agar to nutrient broth at a final concentration of 0.2-0.5%, boiled for 1 minute, sterilized, distributed into sterile tubes then inoculated by stabbing. It was used for the detection of bacterial motility (Collee *et al.*, 1996).

C. Pepton water: The medium was prepared by dissolving 10 grams of pepton and 5 grams of NaCl in 800 ml of distilled water and the volume was made up to 1000 ml. The pH was adjusted to 7.4, sterilized and then it was distributed into sterile tubes. It was used for detecting bacterial ability to produce indole from tryptophan (Atlas and Snyder, 2006).

D. MR-VP medium: The medium was used for detecting bacterial ability to ferment glucose, and differentiation of bacteria was based on acid production (methyl red test) and acetoine production (vogas-proskauer reaction) (Tang and Stratton, 2006).

E. Simmon citrate medium: The medium was used for detecting bacterial ability to utilized citrate as a sole source for carbon and energy (Atlas *et al.,* 1995).

F. Kligler iron medium: The medium was used for detecting bacterial ability to ferment sugars and produce H_2S and CO_2 (Atlas *et al.,* 1995).

G. S-S agar medium (*Salmonella-Shigella* agar): The medium was not autoclaved, but it was boiled to 100°C.

H. Glucose fermentation medium: The medium was prepared by dissolving 1 gram of glucose in 100 ml of brain heart infusion broth, and then few drops of phenol red indicator was added, pH was adjusted to 7.2 and the medium was sterilized by autoclaving (Atlas *et al.,* 1995).

I. **Urea agar medium**: It was prepared by adjusting the pH of 95 ml urea agar base (Christensen's media) to 7.2 and then autoclaved. After cooling to 50°C, 5 ml of 40% urea solution (sterilized by filtration using Millipore filter; 0.22 µm) was added, mixed and distributed into sterilized test tubes in a slant position (Atlas *et al.,* 1995).

J. **Luria-Bertani (LB) Broth**: Tryptone (10 grams), yeast extract (5 grams) and NaCl (5 grams) were supplemented with 10mM glucose. After dissolving the components in 1000 ml distilled water, the pH was adjusted to 7.2 and sterilized by autoclaving. For LB agar preparation, agar was added at a final concentration of 1.5% (Maniatis *et al*., 1982).

2.4.2 Cultivation, Maintaining and Preservation Media

A. **Brain heart infusion broth**: It was used for cultivation of bacteria.

B. **Nutrient broth**: It was used for cultivation and maintenance of bacteria.

C. **Nutrient agar**: It was used for cultivation and maintenance of bacteria.

D. **Nutrient broth + glycerol**: The medium was prepared by adding glycerol to nutrient broth at a final concentration of 20%, and then it was dispensed into sterile and well-capped screwed test tube, autoclaved, and then used for preserving bacterial isolates for several months at -20°C (Benson, 2001).

E. **Brain heart infusion agar**: The medium was used for preserving bacterial strains for several days or weeks at 4°C.

2.5 Preparation of Solutions and Reagents

The following reagents were prepared according to methods presented by Colle *et al*. (1996), with the exception of API 20 E reagents:

A. **Catalase Reagent**: It was hydrogen peroxide (H_2O_2) at a concentration of 3%.

B. Methyl Red Reagent: It was prepared by dissolving 0.1 gram of methyl red pigment in 300 ml of ethanol (99%), and then 200 ml of distilled water were added.

C. Voges-Proskaur Reagent: Two solutions were first prepared. The first (A) was prepared by dissolving 40 gram of KOH in 100 ml of distilled water (40% KOH), while the second (B) was prepared by dissolving 5 gram of α-naphthol in 100 ml of absolute ethanol. To prepare working solution, solution A was mixed with solution B in proportion of 3:1.

D. Urea solution: The solution was prepared by dissolving 20 grams of urea in 100 ml of distilled water, sterilized by filtration (Millipore filter; 0.2 μm) and kept in sterilized bottle at 4ºC. It was used for the detection of urease production.

E. API 20 E Kit: The kit consisted of galleries (the gallery is plastic strip with 20 microtubes containing dehydrate reactive ingredients) and API 20 E reagents (Oxidase, Voges-Proskaure reagents [VPI: 40% potassium hydroxide and VPI: 6% alpha-naphthol], 10% Ferric chloride and Kovac's reagents).

2.6 Laboratory Methods

2.6.1 Bacterial isolation and identification from stool specimens

After incubation (section 2.3), 5 ml of cultured stool sample were transferred to 250ml flask containing 50 ml tetra-thionate broth (an enrichment medium for the selection of *Salmonella*), and incubated at 37°C for 24 hours. After incubation a loopful from each flask was streaked on SS agar (selective medium) plates, and incubated at 37°C for 24 hours. The suspected colonies were subjected to further identifications.

2.6.1.1 Tests of Suspected Colonies (Bacterial Isolates)

A. Microscopical test Include

Gram Stain: A smear of the bacterial isolates was made on slide and subjected to the conventional Gram staining. Stained slides were examined for Gram stain pattern under light microscope using oil immersion lens (100X).

B. Identification tests include

A. Catalase test: The test colony was transferred with a sterile loop onto a clean glass slide, and then few drops of 3% hydrogen peroxide (H_2O_2) were added. The release of oxygen bubbles indicated the presence of catalase (Collee *et al.*, 1996).

B. Indole test: Pepton water (3-4 ml) was inoculated with overnight tested bacterial culture and incubated at 37°C for 24-48 hour, and after that, 0.5 ml of Kovac's reagent was added directly to the culture vial. The immediate formation of a red ring at the top of the broth indicated a positive result (Collee *et al.*, 1996).

C. Methyl red test: MR-VP medium (3-4 ml) was inoculated with the tested bacterial culture and incubated at 37°C for 24-48 hour, and then few drops of methyl red solution were added to the broth culture. An immediate red-colored reaction indicated positive result, while yellow-colored reaction was a negative result (Collee *et al.*, 1996).

D. Vogas-Proskauer test: MR-VP broth medium (3-4 ml) was inoculated with the tested bacterial culture and incubated at 37°C for 24-48 hour, and then 3 ml of Vogas-Proskauer solution A and 1 ml of Vogas-Proskauer solution B were added. The appearance of red color after 5 minutes indicated a positive result (Collee *et al.*, 1996).

E. Citrate utilization test: Simmon's citrate agar slant was inoculated with tested bacterial culture by streaking with sterile loop and incubated at 37°C

for 24-48 hour. A positive result was indicated by changing the color of medium from green to blue (Collee *et al.*, 1996).

F. Kligler iron test: Kligler iron slant was inoculated with tested bacterial culture by streaking on the surface and stabbing in button of the medium, and incubated at 37°C for 24-48 hour. The positive result was noticed by changing the color of medium to black and associated with production of H_2S (Collee *et al.*, 1996).

G. Glucose Fermentation Test: The glucose fermentation medium was inoculated with a fresh culture of each suspected isolate and incubated at 37°C for 24 hours. Changing the color from red to yellow indicated a positive result (Atlas *et al*., 1995).

2.6.1.2 Identification of *Salmonella* Isolates

The *Salmonella* was identified by APi 20E system, while *S*. Typhimurium was identified with serotyping.

2.6.1.2.1 API 20E System

Identification of *Salmonella* isolates was carried out by sub-culturing representative colonies from MacConkey Agar plates on APi 20E microtubes system. This system is designed for the performance of 20 standard biochemical tests from a single colony on plate medium. Each test in this system is preformed within a sterile plastic microtube that contains the appropriate substrates and affixed to an impermeable plastic strip (gallery). Each gallery contains 21 microtubes. The biochemical tests included in APi 20E system were:

1. β-Galactosidase test (ONPG).
2. Arginine dihydrolase test (ADH).
3. Lysine decarboxylase test (LDC).
4. Ornithine decarboxylase test (ODC).
5. Citrate utilization test (CIT).
6. Hydrogen sulphide test (PLS).

7. Urease test (URE).
8. Tryptophane deaminase test (TDA).
9. Indole test (IND).
10. Voges-Proskauer test (VP).
11. Gelatin liquefaction test (GEL).
12. Glucose fermentation test (GLU).
13. Manitol fermentation test (MAN).
14. Inositol fermentation test (INO).
15. Sorbitol fermentation test (SOR).
16. Rhamnose fermentation test (RHA).
17. Sucrose fermentation test (SAL).
18. Melibiose fermentation test (MEL).
19. Amygdalin fermentation test (AMY).
20. Arabinose fermentation test (ARA).
21. Oxidase test (OXI).

A. Preparation of galleries: Five milliliters of tap water were dispensed into the incubation tray to provide a humid atmosphere during incubation.

B. Preparation of bacterial suspension: Single colony from plating medium was picked and suspended in 5 ml sterile distilled water by rubbing against the side of the tube and mixed thoroughly.

C. Inoculation of galleries: With a sterile Pasteur pipette, the 21 microtubes were inoculated with the bacteria suspension. According to the manufacture instructions, both the tubes and couple section of CIT, VP and GEH microtubes were filled. After inoculation, couple section of the ADH, LDC, ODC, H_2S and URE microtubes were completely filled with mineral oil.

D. Incubation of the Galleries: After inoculation, the plastic lid was placed on the tray and the galleries were incubated for 18-24 hours at 37°C.

E. Reading the Galleries: All reactions that not requiring reagents were recorded first, and then the following reagents were added to the corresponding microtubes:

- One drop of 3.4% ferric chloride was added to the TDA microtube.
- One drop of Kavoc's reagent was added to the IND microtube.
- One drop of Voges-Proskauer reagent was added to VP microtube.
- One drop of oxidase reagent was added to either H_2S or ONPG

microtube.

The biochemical reaction performed by the APi 20E and their interpretations are listed in table 2-1.

Table 2-1: Interpretation of reactions performed by APi 20E system.

Negative	Positive	Microtube
Colorless	Yellow	ONPG
Yellow	Red/Orange	ADH
Yellow	Orange	LDC
Yellow	Red/Orange	ODC
Pale green/Yellow	Blue-Green	CIT
Colorless/Grayish	Black deposit	H2S
Yellow	Red/Orange	URE
Yellow	Dark brown	TDA
Yellow ring	Red ring	IND
Colorless	Pink/Red	VP
No diffusion	Diffusion of black pigment	GEL
Table 2-1: Continued		
Blue/Blue green	Yellow	GLU
Blue/Blue green	Yellow	MAN
Blue/Blue green	Yellow	INO
Blue/Blue green	Yellow	SOR
Blue/Blue green	Yellow	RHA
Blue/Blue green	Yellow	SAC
Blue/Blue green	Yellow	MEL
Blue/Blue green	Yellow	AMY
Blue/Blue green	Yellow	ARA
Colorless/Light purple	Violet/Dark purple	OXI

F. Identification of isolates: Identification of the isolates using the analytical profile index (Numerical Coding) for rapid identification at species and biotype level were done as supplied by the manufacturer. To use the index, the biochemical profile obtained was transformed into a numerical profile and to compare it with those listed in the index by transforming all 21 biochemical results into a seven-figure numerical profile (seven-digit number), by placing them into groups of three consigning a specific value for each of the positive as follows:

Group 4			**Group 3**			**Group 2**			**Group 1**		
GLU	GEL	VP	IND	TDA	URE	H_2S	CIT	ODC	LDC	ADH	ONPG
4	2	1	4	2	1	4	2	1	4	2	1
Group 7			**Group 6**			**Group 5**		
OXI	ARA	AMY	MEL	SAC	RHA	SOR	INO	MAN
4	2	1	4	2	1	4	2	1

Each positive result is given a value equal to 1, 2 or 4 according to the position of the test in its group. The sum of these three values was given the corresponding figure. Thus, the figure can have a value from 0 to 7 (zero for negative reaction), and the seven numerical digit profile is then looked up in the index and the identification is determined.

2.6.1.2.2 Serotyping of *Salmonella*

Serotyping of *Salmonella* was carried out by the staff of Central Health Laboratories in Baghdad, in which antisera kit was used for the identification of *S.* Typhimurium that was manufactured by BioRad Company and contained the following antisera:

- Anti-Omni serum.
- Anti-OMA serum.
- Anti-O serum.
- Anti-HMA serum.
- Anti-H serum.

2.6.1.3 Maintenance of *S.* enterica serovar Typhimurium Isolates

Bacterial isolates were maintained according to Johnson *et al*. (1988), as the following:

A. Short-term storage (few weeks): Bacterial isolates were cultured on nutrient agar, and incubated at 37°C overnight, before storing at 4°C.

B. Medium-term storage (1 - 3 months): Tubes that contained 5 – 8 ml of sterile nutrient agar in slants position were cultured with the bacterial isolate,

and then incubated at 37°C overnight. After that,40% glycerol was added before storing at 4°C.

C. Long-term storage: A single colony of *S.* Typhimurium was used to inoculate a test tube contained 10 ml of sterile brain heart infusion broth and incubated at 37°C for 24 hours, then 8.5 ml of the cell suspension was mixed with 1.5 ml of glycerol (40%), and stored at - 20 °C until use.

2.6.2 Antibiotic Susceptibility Test of Isolates

Susceptibility of *S.* Typhimurium isolate to different antibiotics was studied by the standard disk diffusion test of National Committee for Clinical Laboratory Standards (NCCLS) of 2002. Five milliliters of sterile brain heart infusion broth were inoculated with 0.1 ml of the fresh culture of *S.* Typhimurium isolate and incubated at 37°C for 4 hours in shaking incubator (100 rpm). Then, ten serial dilutions were prepared, and 0.1 ml of the fourth dilution (10^{-4}) was spread on brain heart infusion agar plate. The inoculated plates were placed at room temperature for 30 minutes (to allow absorption of excess moisture), before antibiotic disks were placed on the inoculated plates (5 discs / plate), and incubated at 37°C fo18 hours. After incubation, diameters of the inhibition zones were measured and compared with that of NCCLS.

2.6.3 Probiotic Susceptibility Test of Isolates

Four probiotics were investigated (*Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Lactobacillus acidophilus* and *Lactobacillus casei*). They were supplied by the Biotechnology Department, College of Science, Al-Nahrain University) as stock samples. After thawing the sample, 1 ml of *Saccharomyces* stock was inoculated into 99 ml of Sabouraud dextrose broth, and incubated at 28°C for 48 hours, while for *Lactobacillus*, 1 ml of the stock was inoculated into 99 ml of MRS broth, and incubated anaerobically at 37°C for 48 hours (Piard *et al*., 1990). By using Izgü and Altinbay (1997) method, 5 mm diameter wells were made in a nutrient agar plate that was already spreaded

with 100 µl of *S.* Typhimurium isolate from a previous overnight culture. Then, each well was filled with 50 µl of *Saccharomyces* or *Lactobacillus* culture, and the plate was incubated at 37°C for 24 hours. After incubation, the inhibition zone diameters were measured in millimeter (mm) and then two probiotics (with the highest inhibitory zone based on three replicates of each probiotic) were selected for further experiments.

Well diffusion method of Wilkins (1949) was used to determine the anti-microbial activity. *Saccharomyces* that was grown in Sabouraud dextrose broth (section 2.6.3) was centrifuged at 5000 rpm for 15 minutes to obtain cell-free culture solution, and then the suspension was filtrated using Millipore filter (0.22 µm). (Filtrate). The filtrate was concentrated by evaporating 100 ml of it in a vacuum oven at 40-45°C to obtain the one-fold filtrate (50 ml), which was tested for its anti-microbial activity. To carry out such assessment, *S.* Typhimurium culture was prepared by spreading 100 µl of *S.* Typhimurium broth on surface of a nutrient agar plate, and then wells with depth of 5 mm were made by a 5 mm cork borer. Each well was filled with 50 µl of the yeast filtrate to be tested for the anti-microbial activity, which was based on three replicates, and addition a blank well was filled with Sabouraud dextrose broth, as a negative control. The one-fold filtrate was further evaporated to 25 ml (two-folds), and to 12.5 ml (three-folds), and the filtrates were tested for their anti-microbial activity. A similar procedure was followed to test the antimicrobial activity of *Lactobacillus in vitro*.

2.7 Anti-Microbial and Immune-Modulation Effects of Probiotics *in vivo*

In this part of the study, two probiotics (*L. acidophilus* and *S. cerevesiae*) which were showed the best results of inhibition among four types of probiotics, were tested *in vivo* for their anti-microbial effects against one isolate of *S. Typhimurium* that showed multi-drug resistance (MDR). At the same time, liver

and spleen indices and cytokine profile of small intestine wash were determined in treated mice.

2.7.1 Laboratory Animals

Seventy two BALB/c male mice were the laboratory animals in the study. They were supplied by the Biotechnology Researches Center at Al-Nahrain University. Their ages at the start of experiments were 5-6 weeks, and the weight of each was 20-22 grams. They were hosted at 20-25°C,water and food were supplied daily , lighting was supplied as 12 hrs dark and 12 hrs light.

2.7.2 Experimental Design

The experimental design was adopted from De Moreno De Leblanc *et al*. (2010). For each probiotic (*L. acidophilus* or *S. cerevesiae*), the mice were distributed into 5 groups, and each group was kept in a separate plastic cage for the entire period of experiment.

A. Group I:Mice in this group (12 mice) received the probiotic for 7 successive days, and on day 8 was challenged with *S.* Typhimurium. On days 14 and 21 were dissected (6 mice for each period) for laboratory evaluation. In this design, the preventive effect of probiotics was assessed.

B. Group II: Animals in this group (12 mice) received the probiotic for 14 successive days, and on day 8 was challenged with *S.* Typhimurium. On days 14 and 21 were also dissected (6 mice for each period) for laboratory evaluation. In this design, the reversal effect of probiotics was assessed.

C. Group III: Animals in this group (6 mice) received the probiotic (*Lactobacillus acidophilus ,Sacharomyces cereviseae*) for 7 successive days, and dissected on day 8 (a probiotic group).

D. Group IV: Animals in this group (6 mice) were challenged with *S.* Typhimurium on day 1without taking the probiotic milk or anything , and dissected on day 8 (only pathogen group).

E. Group V: Animals in this group (6 mice) were left untreated (a control group).

2.7.3 Preparation of Microbial Cultures and Doses

The two probiotics (*S. cerevesiae* and *L. acidophilus*) was grown as previously decribed in the *Probiotic Susceptibility Test of Isolates* (section 2.6.3) The cells were harvested by centrifugation at 5000 rpm for 15 minutes, washed three times with fresh sterilized PBS and then re-suspended in non-fat milk diluted with sterile distilled water to reach the concentration 10% (v/v), as suggested by De Moreno De Leblanc *et al*., 2010).

S. cerevesiae or *L. acidophilus* was administered to the mice as a drinking solution (10% non-fat milk) to reach a concentration of 1×10^8 cfu/ml, and mice had a free access to it (*ad libitum*), but it was changed every 24 hours with a fresh diluted milk supplemented with the probiotic. For the pathogen, 200 µl from overnight culture of *S.* Typhimurium isolate was placed in a test tube containing 5 ml of sterile brain heart infusion broth before incubation for 24 hours. Concentration of *Salmonella* culture was adjusted to 1×10^8 cfu/ml in PBS. Each mouse was challenged with 100 µl of 1×10^8 cfu/ml of *S.* Typhimurium given by gavage. Such dose was suggested by De Moreno De Leblanc *et al*. (2010).

2.7.4 Organ Index and Bacterial Load in Liver and Spleen

At the end of each experiment, the mice were sacrificed by cervical dislocation and dissected by using sterile instruments after cleaning of abdominal area with alcohol. Then, a longitudinal incision was made and the two organs (liver and spleen**)** were removed. First the index of liver and spleen was obtained by dividing the weight of organ by the weight of mouse, and the outcome was multiplied by 100. After cutting the organs into two pieces, a loopfull from each of them was diluted in 10 ml of sterile saline. An aliquot of the later solution (100 µl) were spread onto the surface of MacConkey agar

plate, which by then was incubated at 37°C for 24 hours. After incubation, the plate was inspected for the formation of bacterial colonies, which were scored as number of colonies per plate (CFU/plate).

2.7.5 Determination of Cytokine Levels in Small IntestineLavage

From the dissected mice in section 2.7.4, the small intestine was obtained, and its contents were collected with 2 ml of sterile cold physiological saline (0.85% NaCl) in a test tube (intestinal wash fluid; IWF). The fluid was immediately centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was distributed into aliquots (0.25 ml) in Eppedorf tubes, and by then they were kept at -20°C until assessment of cytokines. The IWF was assessed for the level of ten cytokines (IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF and RANTES) by means of an ELISA method that was based on similar principles.

The murine IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES kit (PeproTech; United Kingdom) is a sandwich enzyme-linked immunosorbent assay designed for quantitative measurement of natural or recombinant IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES in mouse serum, plasma and other biological fluids, in which an anti-mouse IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES coating antibody (**Capture Antibody**) is adsorbed onto wells of 96-well plate. Mouse cytokine present in the sample or standard binds to antibodies that were adsorbed to the wells. A biotinylated anti-mouse cytokine antibody is added and binds to mouse cytokine captured by the first antibody (**Detection Antibody**). Following incubation, unbound biotinylated anti-mouse cytokine antibody is removed during a wash step, and avidin horseradish peroxidase (HRP) conjugate is then added and binds to the biotinylated anti-mouse cytokine antibody. Following incubation, unbound avidin-HRP conjugate is removed during a wash step, and a substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of

mouse cytokine present in the sample or standard. The color development is monitored with ELISA plate reader and absorbance is measured at a wavelength of 405 nm. A standard curve is prepared from standard dilutions and mouse cytokine sample level is determined from a curve fitting equation.

A. Kit Contents

- ELISA plate: Blank 96-well plate
- Capture antibody: Goat anti-mouse IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES antibody.
- Detection antibody: Biotinylated anti-mouse IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES antibody.
- Standards: Recombinant mouse IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES.
- Avidin-HRP conjugate.
- ABTS liquid substrate solution.
- Washing buffer: 0.05% Tween-20 in phosphate buffer saline (PBS).
- Block buffer: 1% bovine serum albumin (BSA) in PBS.
- Diluent: 0.05% Tween-20 and 1% BSA in PBS.

B. Assay Procedure

Before carrying out the assay procedure of IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES determination, the kit was left at room temperature (18-25°C) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

- The wells of plate were coated with capture antibody by dispensing 100 µl of anti-mouse IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES antibody in each well, and the plate was sealed and incubated overnight at room temperature (18-25°C).

- The day after, the contents of wells were discarded and each well was washed four times with washing buffer (300 µl/well/wash), and then the plate was inverted to remove residual buffer and blotted on a towel paper.
- In each well, 100 µl of block buffer was dispensed and the plate was incubated at room temperature for 60 minutes, and then the washing step was repeated.
- An aliquot (100 µl) of each standard of cytokine (7.8125, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 pg/ml) and IWF samples was dispensed into separate wells. The plate was incubated at room temperature for two hours, and then the washing step was repeated.
- An aliquot (100 µl) of detection antibody (biotinylated anti-mouse IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF or RANTES antibody) was dispensed in each well. The plate was incubated at room temperature for two hours, and then the washing step was repeated.
- An aliquot (100 µl) of avidin-HRP conjugate was dispensed in each well. The plate was incubated at room temperature for 30 minutes, and then the washing step was repeated.
- Finally, 100 µl of substrate solution was added, and color development was monitored with ELISA plate reader and absorbance was measured at a wavelength of 405 nm. Three reading were taken (3, 6, and 9 minutes) and the mean absorbance was considered for calculations of sample results.

C. Calculation of Sample Results

The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the samples by using standard curve fitting equations for IL-1β, IL-4, IL-10, IL-12, IL-17A, IL-21, IFN-γ, IP-10, GM-CSF and RANTES (Figures 2-1, 2-2, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9 and 2-10 respectively).

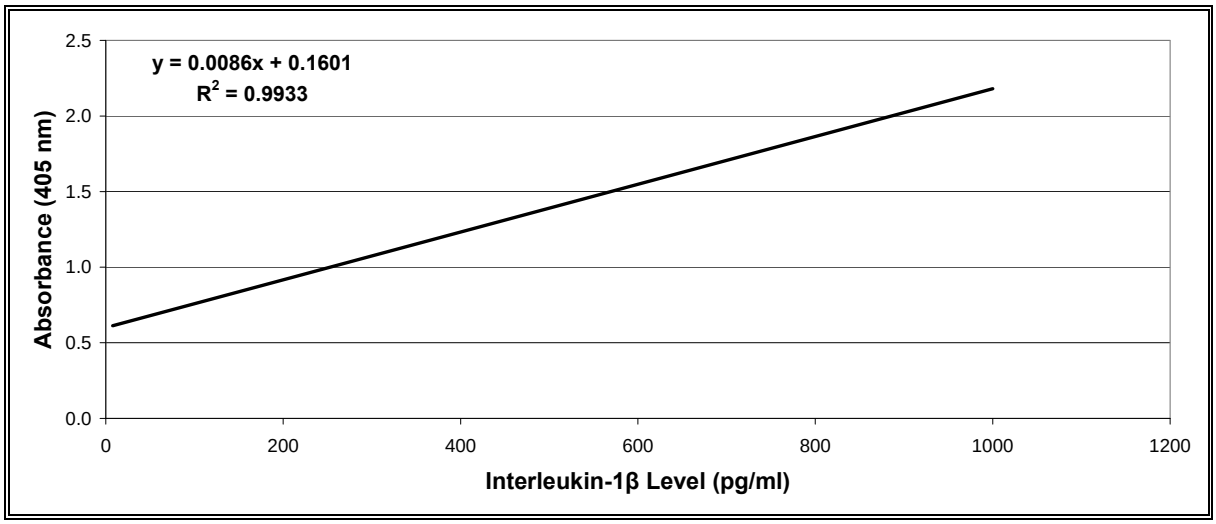

Figure 2-1: Standard curve of IL-1β.

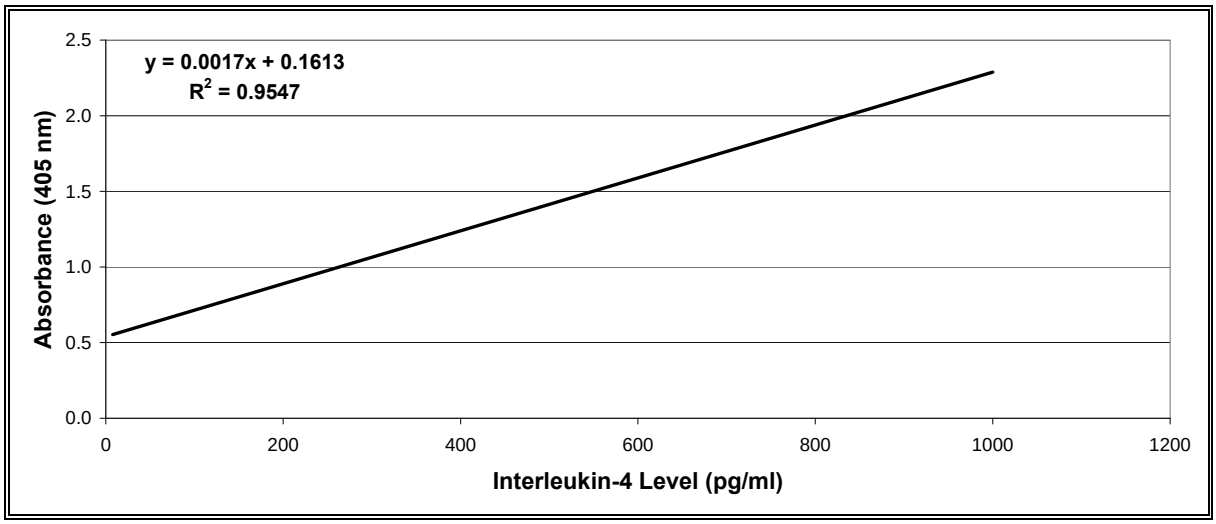

Figure 2-2: Standard curve of IL-4.

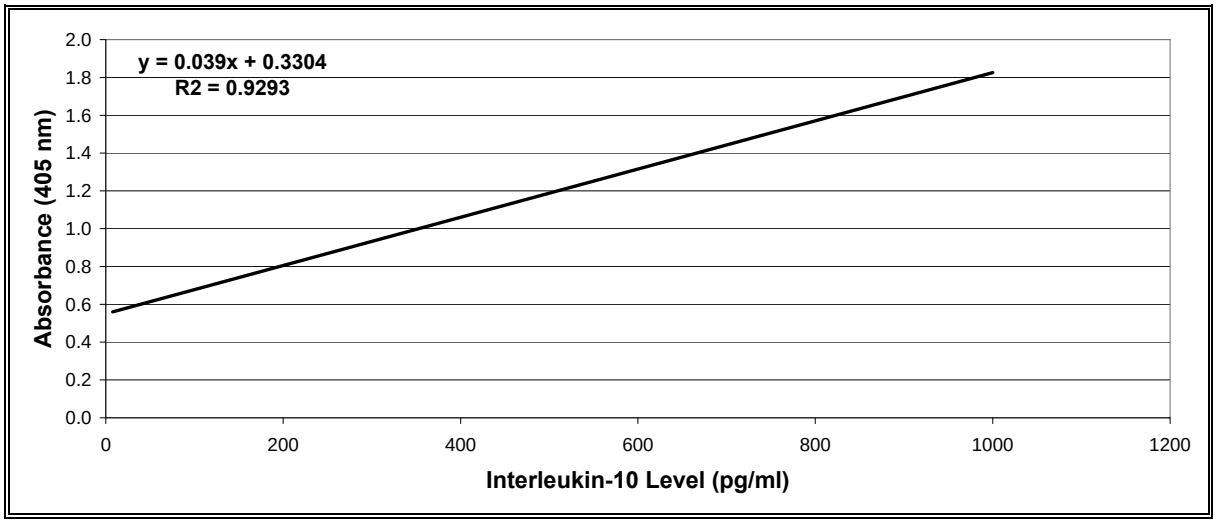

Figure 2-3: Standard curve of IL-10.

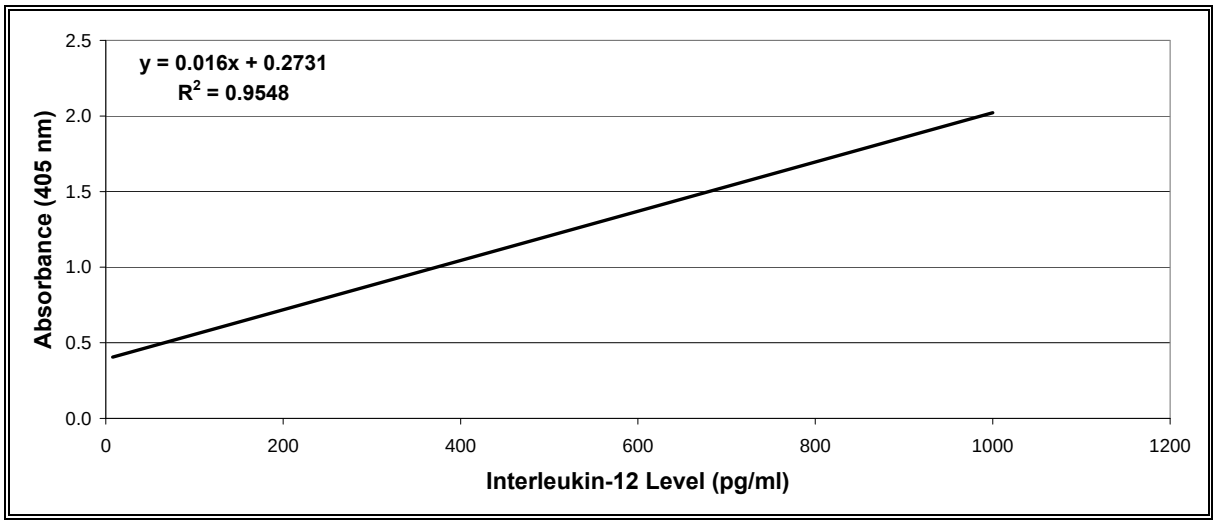

Figure 2-4: Standard curve of IL-12.

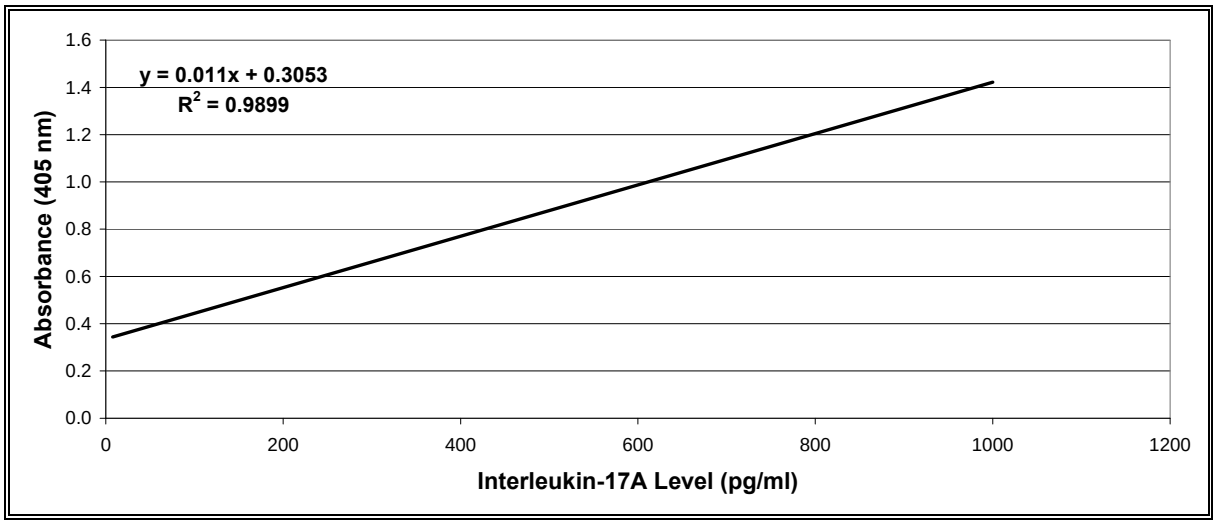

Figure 2-5: Standard curve of IL-17A.

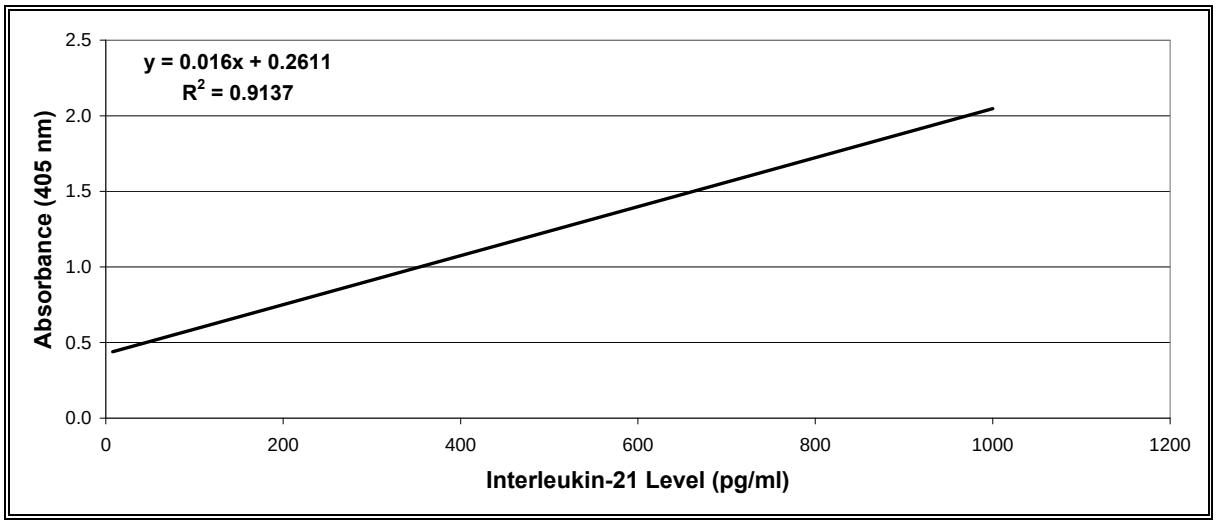

Figure 2-6: Standard curve of IL-21.

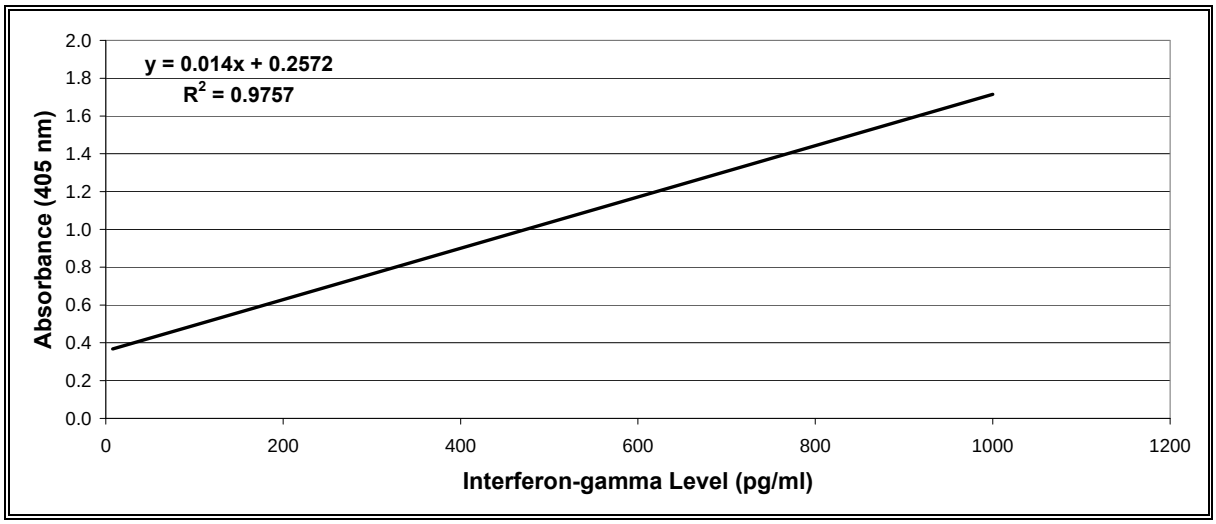

Figure 2-7: Standard curve of IFN-γ.

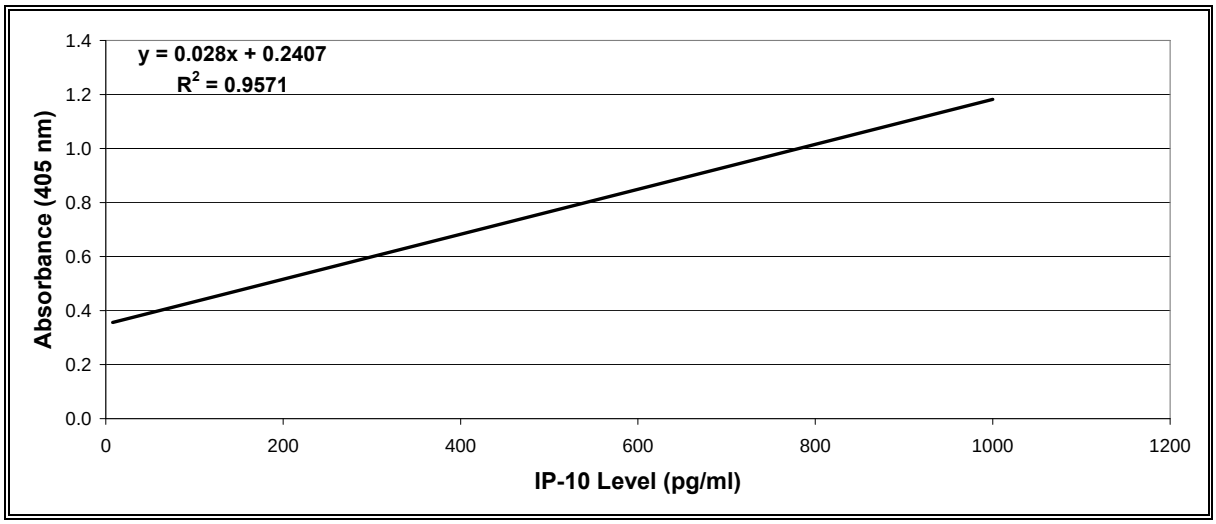

Figure 2-8: Standard curve of IP-10.

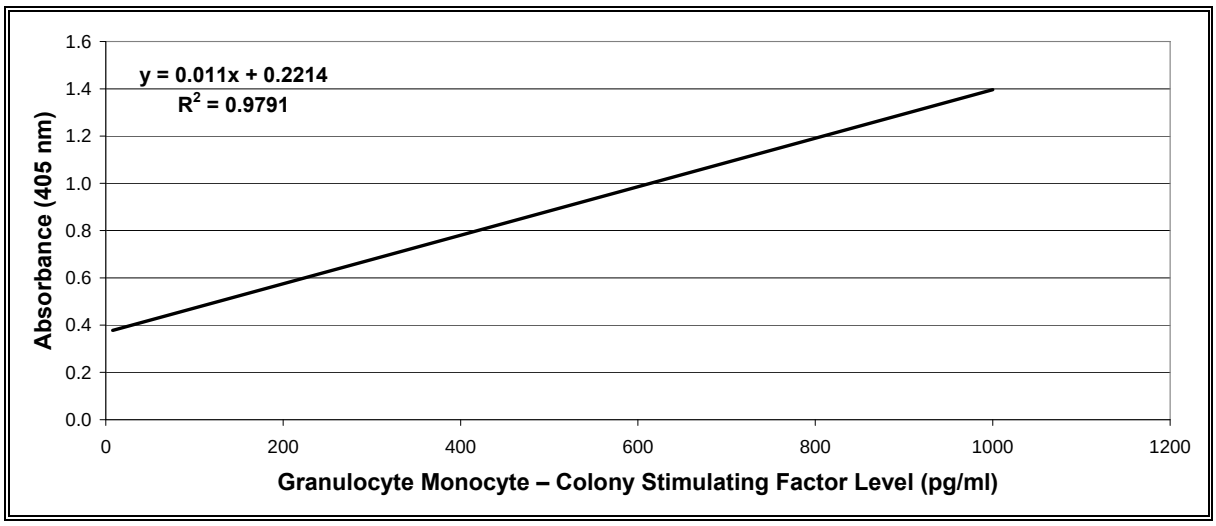

Figure 2-9: Standard curve of GM-CSF.

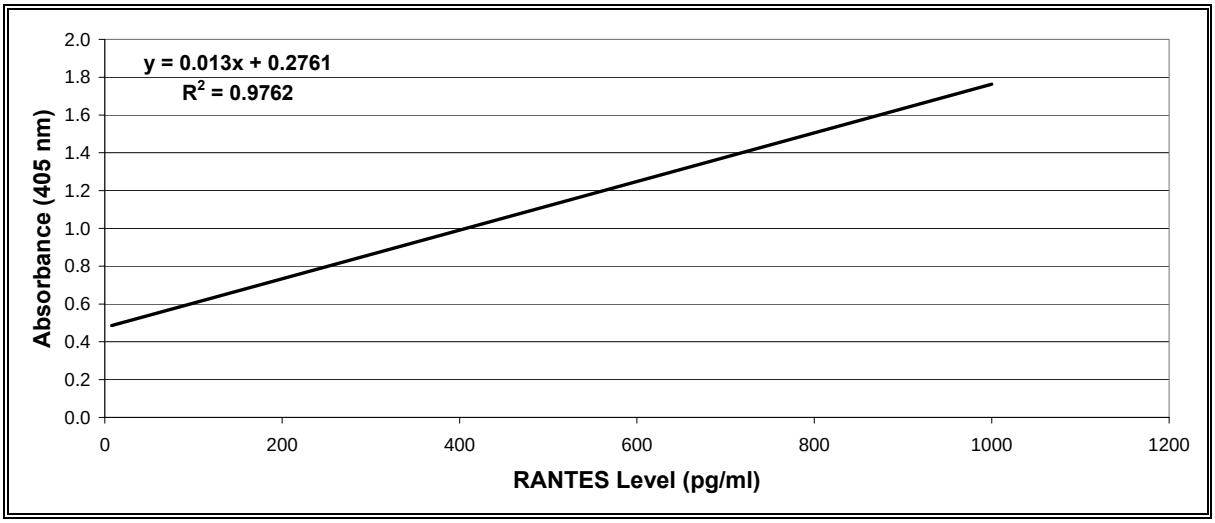

Figure 2-10: Standard curve of RANTES.

2.8 Statistical Analysis

Data were presented as mean ± standard deviation (S.D.) and differences between means were assessed by ANOVA (analysis of variance) followed by either LSD (least significant difference) or Duncan test. Three levels were considered; $P \leq 0.05$, 0.01 and 0.001. The analyses were carried out using the software SPSS version 13.0 (statistical package for social sciences).

Chapter Three
Results and Discussion

Chapter Three
Results and Discussion

3.1 Isolation and identification of *Salmonella* enterica serovar Typhimurium

Out of 128 diarrhea specimens, *Salmonella* Typhimurium was isolated and identified from nine samples only (7.03%), which were belong to nine children; their ages ranged from six days to five years. All children had severe diarrhea that was associated with fever. It has been well-documented that enteric infection with *Salmonella spp*. is an important cause of children diarrheal disease worldwide, but the frequency of children with the infection shows variations between studies. A study from Bolivia of 133 consecutive children less than 5 years old presenting with bloody diarrhea revealed bacterial etiology in 41%, and out of this percentage, *Salmonella* spp. accounted for 4% (Townes *et al*., 1997). However, in a further study from Vietnam, no *Salmonella* infection was reported in 587 children with diarrhea under the age 24 months, although other potential pathogens were identified in 67.3% of children with diarrhea, including Gram negative bacteria (Vu Nguyen *et al*., 2006). In a more recent study, *Salmonella spp*. Infection was reported in 6.2% of 260 diarrheal children from Ethiopa (Beyene and Tasew, 2014). These findings together with present study findings confirm that globally, intestinal *Salmonella spp*. remain major contributors to acute enteric infections.

The isolation of *S*. Typhimurium was performed by cultivating the stool samples in flasks containing 50 ml tetra-thionate broth medium, which was used as a selective enrichment for the cultivation of *Salmonella spp*, that may be present in small numbers and compete with intestinal flora. Selectivity is accomplished by the combination of sodium thiosulfate and tetra-thionate, which suppresses common intestinal organisms (Eckner *et al*., 1994) For pure cultures, 100µl aliquots from tetra-thionate broth cultures were transferred and

spread onto the surface of *Salmonella-Shigella* agar plates (S.S. agar), which is highly selective and differential medium formulated to inhibit the growth of most coliform organisms and permit the growth of *Salmonella* and *Shigella* species from environmental and clinical specimens (Murray *et al*., 2007). The high bile salt concentration and sodium citrate inhibit all Gram positive bacteria and many Gram-negative organisms including coliforms. Lactose is the sole carbohydrate and neutral red is the indicator for acid detection. Sodium thiosulfate is a source of sulfur and the production of H_2S is indicated by black precipitate formed with ferric citrate (Atlas *et al*., 1995). The growth of *Salmonella spp*. in S.S. agar showed colorless colonies with black centers owing to H_2S production, while *Shigella spp*. does not blacken (Wellsand Butterfield, 1997). As a result, nine isolates, which showed black colonies in SS agar, were suspected as *Salmonella spp*. and subjected for further identifications.

3.2 Morphological and Cultural Characterization

The suspected isolates were first identified depending on their Gram stain and microscopical characteristics. *Salmonella* isolates were found to be Gram negative, rod or bacilli and non-spore forming. Morphological characteristics of the suspected isolates were done depending on the colonial shape and form when re-cultured on the surface of MacConkey agar and SS agar. The colonies were pale yellow or nearly colorless, 1-3 mm in diameter and non lactose fermented. In addition, the colonies in SS agar appeared to be circular, colorless, 1-2 mm in diameter with gas bubbles and black spots due to the production of H_2S. Such characteristics were applied to only nine isolates that were suspected to be candidate for the biochemical tests.

Biochemical tests were achieved on the suspected 9 isolates that showed growth and morphological characteristics, which were closely related to *S*. Typhimurium. For this purpose eight biochemical tests were carried out. Results showed that the nine isolates gave negative reactions for indole, urease and

Voges-Proskaur tests, but they gave positive reactions for citrate utilization, catalase, methyl red and glucose fermentation. In triple sugar iron test, all isolates were H_2S and gas producers. Table 3-1 demonstrates that the suspected nine isolates were identified as *S*. Typhimurium by the criteria of Bergey's Manual of Systematic Bacteriology ((Holt *et al*., 1994).

Table 3-1: Morphological, microscopical, and biochemical characteristics of locally isolated *Salmonella Typhimurium*.

Characteristics and Tests										
Shape	Rod		Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	-		-	-	-	-	-	-	-	-
Motility test	M		M	M	M	M	M	M	M	M
Indole test	-		-	-	-	-	-	-	-	-
Methyl red test	+		+	+	+	+	+	+	+	+
Voges-proskaur test	-		-	-	-	-	-	-	-	-
Citrate utilization test	+		+	+	+	+	+	+	+	+
Urease test	-		-	-	-	-	-	-	-	-
Catalase test	+		+	+	+	+	+	+	+	+
Glucose fermentation test	+		+	+	+	+	+	+	+	+
Triple sugar iron tests K/A	H_2S	+	+	+	+	+	+	+	+	+
	Gas	+	+	+	+	+	+	+	+	+
	Alkaline /acid	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

M: Motile; +: Positive; –: Negative

Enterobacteriaceae members are Gram-negative bacteria and can grow in the intestinal tract of human and animals (Prescott *et al*., 1996). Many convention biochemical tests have been used to distinguish between Enterobacteriaceae family. *Salmonella* is a pathogenic enterobacter, which can be identified from coliforms in many tests; for instance, H_2S production, blackening and non- lactose fermentation (Woo *et al*., 2001). Within the genus

Salmonella, species can be characterized by carrying out more biochemical tests (i.e. fermentation of different sugars), serological and genetic examinations; however species of *Salmonella* also differ from each other in the conventional biochemical tests; for instance, *S*. Typhimurium can utilize citrate but not *S*. Typhi or *S*. Paratyphi A (Tindall *et al*., 2005).

A further identification of the nine *S*. Typhimurium isolates was achieved by using Api system (Api 20E). Results of these isolates were able to give positive results for ornithine decarboxylase, citrate utilization, H_2S production, gelatin liquefaction, and they were glucose, arabinose, sorbitol, rhamnose, melibiose, inositol and manitol fermentors. While, they gave negative reactions for β-galactosidase, arginine dihydrolase, lysine decarboxylase, urease, tryptophane deaminase, indole , Vogas-proskauer , amygdaline fermentation and sucrose fermentation. The results mentioned above were in agreement with those described by Holt *et al*. (1994); indicating that the nine isolates were belong to the genus *Salmonella*., and by applying serotyping, they were confirmed as *S*. Typhimurium.

3.3 Antibiotic Susceptibility of *Salmonella* Typhimurium

Disc diffusion method was used for testing the susceptibility of *S.* Typhimurium to six antibiotics (ampicillin, amoxicillin, nalidixic acid, ciprofloxacin, trimethoprim/sulfamethoxazole and azithromycin.

The nine tested isolates showed different susceptibilities to the tested antibiotics, and most of them were sensitive, others showed intermediate sensitive, and resistant isolates were also observed. All isolates were resistant to nalidixic acid, with the exception of isolate 1.11, which showed intermediate sensitivity. Only isolate B was resistant to three antibiotics, which were ampicillin, amoxicillin and nalidixic acid; therefore this isolate was considered as multi-drug resistance (MDR) isolate and selected for further experiments in the study that included probiotic susceptibility *in vitro* and *in vivo*, in addition to

the pathogen and probiotic effects on cytokine production in small intestine of treated mice.

Table 3-2: Susceptibility of *Salmonella* Typhimurium to six antibiotics.

Isolate Symbol	AMP	AMC	NAL	CIP	SXT	AZ
1.1	**S**	**S**	**R**	**S**	**I**	**S**
1.2	**S**	**S**	**R**	**S**	**S**	**S**
1.3	**S**	**S**	**R**	**S**	**S**	**I**
1.5	**S**	**S**	**R**	**S**	**S**	**S**
1.6	**I**	**S**	**R**	**S**	**S**	**S**
1.7	**S**	**S**	**R**	**S**	**S**	**S**
1.11	**S**	**S**	**I**	**S**	**S**	**S**
A	**S**	**S**	**R**	**S**	**S**	**S**
B	**R**	**R**	**R**	**S**	**S**	**S**

AMP: Ampicillin, AMC: Amoxicillin/clavulanic acid, NAL: Nalidixic acid, CIP: Ciprofloxacin, SXT: Trimethoprim/sulfamethoxazole, AZ: Azithromycin, I: Intermediate sensitivity, S: Sensitive, R: Resistant

As shown in table 3-2, *S.* Typhimurium B isolate was sensitive to three antibiotics (CIP, SXT and AZ), but it resisted AMP, AMC and NAL. In agreement with such presentation, *S.* Typhimurium has been documented to be frequently associated with MDR (Soto *et al*., 2003; Biendo *et al*., 2005), in part due to the worldwide emergence of *S.* Typhimurium definitive phage type (DT) 104, which contains the chromosomal *Salmonella* genomic island type I (SGI-1). SGI-1 harbors genes that confer the ACSSuT phenotype (i.e., resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (Mulvey *et al*., 2006). Although *S.* Typhimurium DT104 is the main example of MDR in *S. enterica*, many antimicrobial resistance genes have been reported also in isolates of other serotypes (Michael *et al*., 2006).

Non-typhoidal *Salmonella* infections generally result in mild-to-moderate self-limiting gastroenteritis, and antimicrobial treatment is only required in severe cases occurring in vulnerable patient groups or to combat invasive

infections. However, due to the increasing resistance of this bacterium to the conventional antimicrobial agents used in the treatment of salmonellosis, amoxicillin, third-generation cephalosporins, and fluoroquinolones have become further treatment options. Resistance to β-lactams in *S. enterica* is mainly due to the production of acquired β-lactamases (Michael *et al*., 2006). Among these, TEM-1, PSE-1, and OXA-1 have been described as the enzymes most frequently related to ampicillin and amoxicillin resistance (Güerri *et al*., 2005; Biendo *et al*., 2005). The resistance of *Salmonella* to third-generation cephalosporins is primarily mediated by the production of extended spectrum β-lactamases (ESBL) of the TEM, SHV, and CTX-M types, which are associated with different mobile genetic elements (Michael *et al*., 2006). ESBL have been described not only in clinical *Salmonella* isolates but also in isolates from animals and food (Coque *et al*., 2008). Mobile genetic elements such as plasmids and transposons, possibly containing integrons, are able to disseminate antimicrobial resistance by horizontal transfer in Enterobacteriaceae. Integrons are genetic elements that capture and incorporate gene cassettes by using a site-specific recombination mechanism (Cambray *et al*., 2010).

Resistance to amoxicillin among *S. enterica* isolated from different hospitalized cases has become increasingly widespread, accompanied by the emergence of ESBL-producing isolates, detected in human samples. This resistance may be related to resistance gens possessed by the bacteria (Wain *et al*., 2013).

Levings *et al*. (2005) also found that this pathogenic bacterium was resistant to gentamycin and streptomycin, and during the period 1999 - 2008, Matheson *et al*., (2009) mentioned that, *Salmonella* strains increased their resistance to chloramphenicol; a matter which may reflects that such new strain may carry mobile genetic elements conferring resistance to multiple antibiotics. High sensitivity of *S.* Typhimurium for ciprofloxacin and its resistance to ampicillin were also recorded by Mitra *et al*. (2009). Another study performed

by Kasimoglu Dogru *et al.* (2010) found that *Salmonella spp.* isolated from chicken carcasses possessed MDR toward tetracycline, cephalothin, and streptomycin.

Risk factors for the development of resistance in *Salmonells* spp. include overuse, misuse, and inappropriate antibiotic prescribing practices. Factors such as patient and time pressures and diagnostic uncertainties are some of the main forces behind irrational prescription of antimicrobial combinations (Sharma *et al*., 2005). Easy availability of drugs at the pharmacy without a prescription, use of allopathic drugs by traditional medicine practitioners such as homeopaths, unani and ayurvedic practitioners, and uncontrolled use of antibiotics in agriculture, animal husbandry and fisheries has further aggravated the problem. Moreover, in some countries such as India, local production of many different antimicrobial drugs with questionable quality and potency control, coupled with poor compliance of patients to costly antimicrobials adds to the threat of antimicrobial resistance (Tunger *et al*., 2009). Antibiotics are unnecessarily prescribed for infections such as the common cold, cough and diarrhea, which are usually of viral etiology and can be resolved by the immune system. Emphasis is placed on treatment instead of finding the causative organism and reaching a proper diagnosis. This leads to patients being treated with broad spectrum antibiotics, which results in the emergence of MDR organisms (Zaki and Karande, 2011).

3.4 Susceptibility of *Salmonella* Typhimurium to Probiotics

The susceptibility of MDR *S.* Typhimurium B isolate was further assessed *in vitro* by using four probiotics, which were *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Lactobacillus acidophilus* and *Lactobacillus casei*. The results revealed that *S. cerevisiae* and *L. acidophilus* recorded the highest inhibition zones (12.6 ± 0.6 and 16.3 ± 0.7 mm, respectively), which were significantly different ($P \leq 0.01$) from the recorded zones in *S. boulardii* and *L.*

casei (8.3 ±1.6 and 5.3 ± 0.4 mm, respectively) (Figure 3-1). Therefore, *S. cerevisiae* and *L. acidophilus* were further investigated for their anti- *S.* Typhimurium effects *in vitro* and *in vivo*.

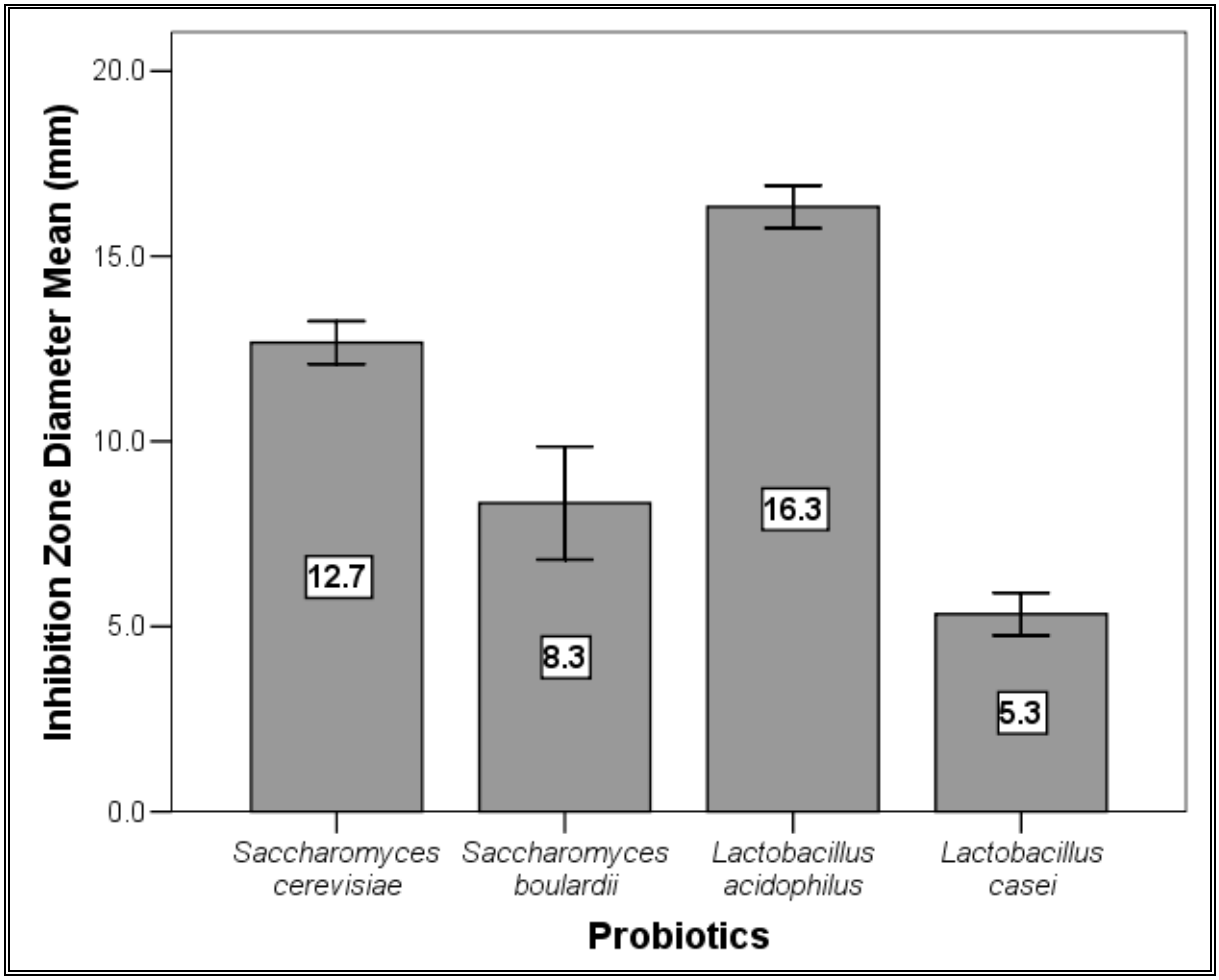

Figure 3-1: Inhibition zone diameter of *S. cerevisiae*, *S. boulardii*, *L. acidophilus* and *L. casei* against growth of *S.* Typhimurium isolate B.

Each of the two probiotics (*S. cerevisiae* and *L. acidophilus*) that showed the inhibition zone by using the whole microorganism, were tested further against MDR *S.* Typhimurium isolate B by using their filtrates at one-fold, two-fold and three-fold concentration. It was found that the inhibition zone was fold- and probiotic-dependent. The three-fold filtrate recorded the highest inhibition zone for both probiotics (*S. cerevisiae* and *L. acidophilus*), which were 25.0 ± 1.0 and 31.0 ± 1.0 mm, respectively, and they were significantly different from the other fold filtrates (one-fold: 7.3 ± 0.6 and 10.0 ±1.0; two-fold: 12.0 ± 1.0 and 16.0 ± 1.0, respectively). In addition, *L. acidophilus* was better than *S.*

cerevisiae in recording a significantly larger inhibition zones in the investigated folds of filtrate (Figure 3-2).

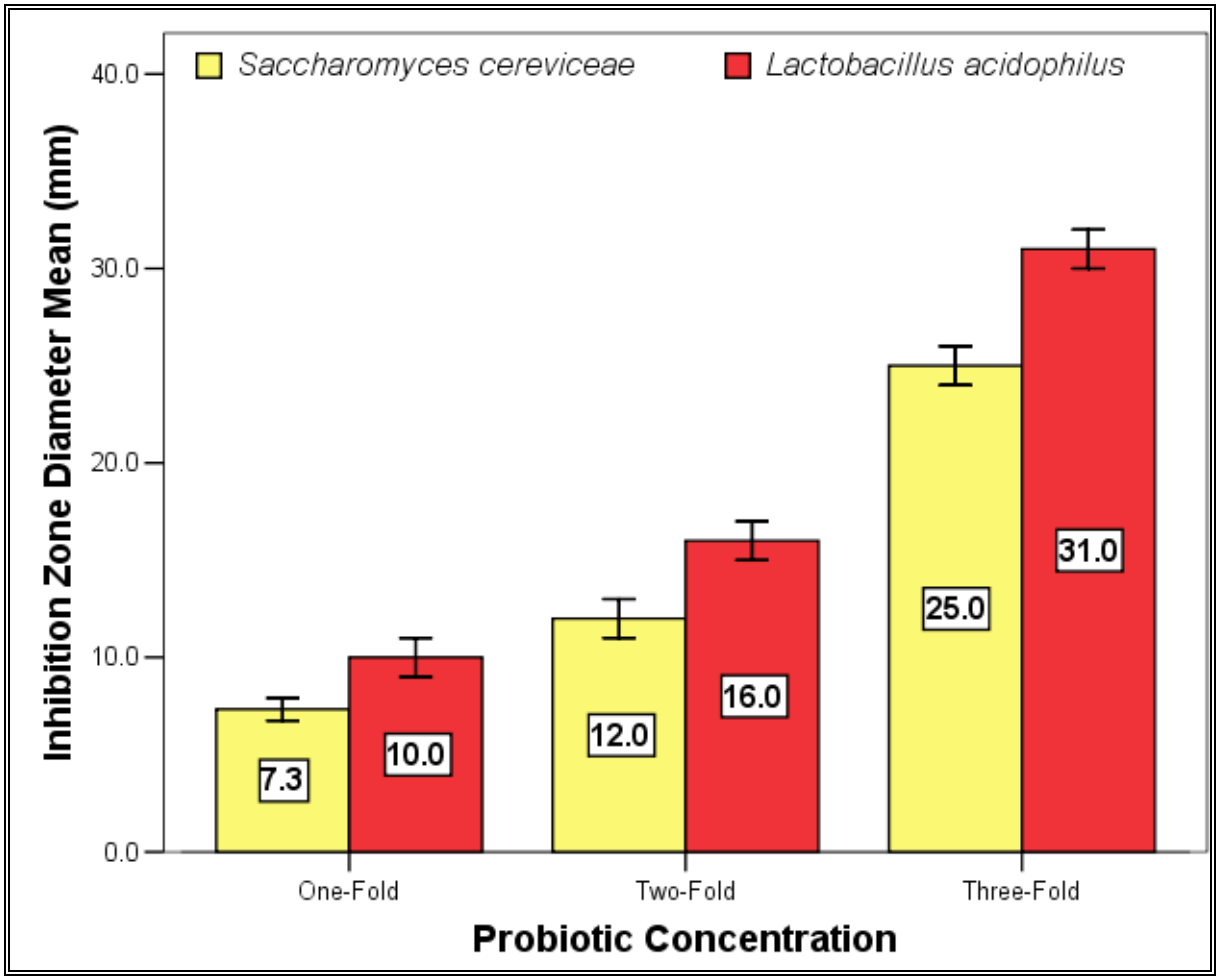

Figure 3-2: Inhibition zone diameter of *S. cerevisiae* and *L. acidophilus* filtrates against growth of *S.* Typhimurium isolate B.

These results suggest that filtrates of both probiotics (*S. cerevisiae* and *L. acidophilus*) were effective in limiting the growth of *S.* Typhimurium isolate B, which was resistant to three antibiotics. With respect to *S. cerevisiae* antagonism of microorganisms by this yeast has been attributed primarily to competition for nutrients, pH changes in the medium as a result of growth-coupled ion exchange or organic acid production, secretion of anti-bacterial compounds and release of anti-microbial compounds such as killer toxins or mycocins, which are extracellular proteins or glycoproteins that disrupt cell membrane function (Golubev, 2006). The well known mechanisms of the killer toxin are the interruption of cell division by blocking the DNA synthesis, inhibition of

synthesis of the cell wall component β-1,3-glucan (Izgu and Altinbay, 2004), and ion leakage caused by the formation of channels on the cytoplasmic membrane (Schmitt and Breinig, 2002).

The present results are also compatible to those obtained by Gedek (1991) who found antagonist activity of *S. cerevisiae* culture against enteric pathogens (*Salmonella spp*. and *Shigella spp.*). Brandão *et al.*, (1998) also reported that *S. cerevisiae* produced inhibitory compounds similar to *S. boulardii* in its anti-bacterial effect against *Vibrio cholerae*, *Clostridium difficle* and enterobacterial infections especially *Salmonella spp.* and *Shigella spp*. Czerucka and Rampal, (2002) reported further that *S. cerevisiae* and *S. boulardii* were able to reduce growth of various members of the enterobacteriaceae family e.g. *Salmonella* Typhi, *Salmonella* Enteritidis and *Shigella dysenteriae*. Anti- *Escherichia coli* effects of *S. cerevisiae* have also been reported by Etienne-Mesmin *et al*. (2012), and they ascribed such growth inhibitory effect to the ability of such proibiotic to produce ethanol.

With respect to *L. acidophilus*, the observed inhibitory effect might be due to the production of inhibitory compounds, especially organic acids and bacteriocins, as well as some lactobacilli play this protective role by producing compounds such as hydrogen peroxide, lactic acid and biosurfactants, which inhibit the growth of potential pathogens (Pascual *et al*., 2008). In addition, Zakaria Gomaa (2013) demonstrated that all tested lactobacilli isolates produced biofilm on polystyrene surface in all media tested to different degrees, but *L. acidophilus* showed the highest biofilm formation in Rogosa medium.

The results of present study agreed with Jacobsen *et al*. (1999) who found that the antagonist activity of lactic acid bacteria against diarrheal causing bacteria might be referred to its ability in produce organic acids that can lower the pH, bacteriocins, and competition on the nutrients with the pathogenic bacteria. Jin *et al.* (1996) found that a strain of *Lactobacillus* isolated from chickens was able to inhibit the growth of *Salmonella spp*. and *E. coli*, and

Jacobsen *e*t *a*l. (1999) who obtained different *Lactobacillus* strains found their inhibitory activity against enteric bacteria may depend on the strain. Rolfe (2000) also pointed out to the ability of lactic acid bacteria to inhibit *in vitro* growth of many enteric pathogens causing broad range of gastrointestinal disorders in both humans and animal. The present results are also in a good agreement with Fernández *et al.* (2002) who proved that *L. acidophilus* and *L. gasseri* (of human origin) were able to inhibit growth of many enteric pathogens including *Salmonella*, *Listeria*, and *Campylobacter*. Also Hütt *et al.* (2006) noticed the antagonist activity of five probiotic lactobacilli and two bifidobacteria strains against *Salmonella enterica, E. coli* and *Shigella sonnei in vitro* in solid and liquid medium.

The results also showed that the inhibition zone diameter was increased by increasing the filtrate concentration and this was perhaps due to the presence of the inhibitory compounds secreted by *L. acidophilus* in the growth medium, which become more lethal by increasing the concentration. This result agreed with Barefoot and Klanhammer, (1983) who found that the death of the tested bacteria was increased by increasing the concentration of *L. acidophilus* filtrate due to increasing the concentration of the inhibitory compounds especially the bacteriocins. A similar finding was obtained by Kingamkono *et al*. (1994) and Olukoya *et al*. (1994) who noticed the disappearance of *S.* Typhimurium and *Shigella Flexneri* from the medium when subjected to *Lactobacillus*. Mishra and Lambert (1996) noticed further the killing action of the bacteriocins as they bind with the cytoplasmic membrane, affects its permeability, and cause death of the sensitive cell. Acetic acid and lactic acid are mainly produced by lactic acid bacteria; they affect the cytoplasmic membrane and diffuse in the cytoplasm quickly and cause bacterial death (Ogawa *et al*., 2001). In addition, Sreekumar and Hosono (2000) found that lactic acid bacterial filtrates showed no inhibitory effects against *E. coli*, but such effect was developed upon increasing the concentration folds of the filtrates.

According to the results above, the two most efficient probiotic strains *S. cerevisiae* and *L. acidophilus* were chosen for further *in vivo* study through evaluating their protective effects in mice against ingested *S.* Typhimurium.

3.5 Organ Index and Bacterial Load of Liver and Spleen

3.5.1 Liver Index

A significant increase in liver index was observed in mice treated with *L. acidophilus* in group I that was a preventive group after 21 days (10.73 ± 3.14%) compared to the corresponding group in *S. cerevisiae* (7.41 ± 2.04%) or all other groups. In the latter group, the index was also significantly increased compared to other groups in *S. cerevisiae*. The lowest index was recorded in group V (2.76 ± 1.22%), which included untreated animals (Table 3-3).

Table 3-3: Liver index in mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	Liver Index Mean Level ± S.D. (%)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	6.67 ± 2.02^{B}	6.59 ± 2.30^{A}	N.S.
	21	10.73 ± 3.14^{A}	7.41 ± 2.04^{A}	**0.05**
II	14	5.16 ± 1.09^{B}	5.48 ± 3.04^{A}	N.S.
	21	4.67 ± 0.82^{BC}	4.89 ± 0.64^{AB}	N.S.
III	8	6.71 ± 1.53^{B}	6.59 ± 1.188^{A}	N.S.
IV	8	4.24 ± 2.11^{BC}	4.24 ± 2.11^{AB}	N.S.
V	8	2.76 ± 1.22^{C}	2.76 ± 1.22^{B}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

The presented results highlighted that the liver index showed variations between groups of mice that were treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and challenged with *S.* Typhimurium, and in all groups the index was increased in the range of two- to five-times compared to untreated control

mice. This probably due to the increase of the bacterial load by the pathogen, because the usual pathological site of *salmonella* infection is the lymphoid tissue of the gastrointestinal tract, but hematogenous dissemination of the organism or its endotoxin results in systemic involvement that can affect almost all major organs, including the liver, central nervous system, gall-bladder, kidney, lung, and heart. It has also been suggested that liver involvement is commonly observed in patients with typhoid fever, although severe hepatic involvement with a clinical feature of acute hepatitis is a rare complication (Albayrak *et al*., 2011). However, Everard *et al*. (2014) suggested that the yeast *S. boulardii* was able to modulate energy homeostasis via a mechanism other than energy intake. Importantly, they found that *S. boulardii* treatment reduced hepatic and systemic inflammation in mice. Because liver lipid accumulation is associated with liver and systemic inflammation, they postulated that the decreased inflammatory tone may be related to the lower liver and whole-body fat accumulation. It has also been, claimed that fermented feed enriched in lactic acid bacteria, including *Lactobacilli* and *Bifidobactria*, influence metabolism in the host tissue, in particular the gastrointestinal mucosa and the liver (Rabot *et al*., 2010). In addition and due to the immunomodulatory effects of lactic acid bacteria, they can decrease glutathione peroxidase levels to reduce hepatic oxidative stress (Chen *et al*., 2013).

3.5.2 Spleen Index

In spleen index, two general observations can be established. In the first, the index in L. *acidophilus* groups was higher than the corresponding groups in *S. cerevisiae*, but none of these differences attended a significant level. The second observation highlights that a treatment with a probiotic alone or in a combination with the pathogen *S.* Typhimurium caused a significant increase in the spleen index of all groups compared to untreated mice in group V, in which the index scored the lowest percentage (0.316 ± 0.205%) (Table 3-4).

Table 3-4: Spleen index in mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	Spleen Index Mean Level ± S.D. (%)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	1.325 ± 0.542^{A}	1.228 ± 0.636^{A}	N.S.
	21	1.349 ± 0.395^{A}	0.924 ± 0.388^{A}	N.S.
II	14	0.952 ± 0.252^{A}	0.936 ± 0.312^{A}	N.S.
	21	0.908 ± 0.145^{A}	0.879 ± 0.200^{A}	N.S.
III	8	1.141 ± 0.166^{A}	0.981 ± 0.122^{A}	N.S.
IV	8	1.009 ± 0.235^{A}	1.009 ± 0.235^{A}	N.S.
V	8	0.316 ± 0.205^{B}	0.316 ± 0.205^{B}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

Spleen index in mice that were treated with the investigated probiotics (*L. acidophilus* or *S. cerevisiae*) and challenged with *S.* Typhimurium in different treatments, revealed some fluctuations but in general it was significantly increased compared to untreated mice in group V, which maintained a lowest index compared to other groups. This might be expected, because during systemic infection, *Salmonella* spp. replicate extensively within phagocyte populations of the GALTs, spleen, liver and bone marrow (Tam *et al*., 2008). The early innate immune response to infection invokes chemokine-dependent recruitment of neutrophils and monocytes to the infected site, where they can be activated to produce inducible NO synthase and kill bacteria (Rydstrom and Wick, 2007). However, recruitment of phagocytes can also be counterproductive, because these newly recruited phagocytes provide a rich source of new cells for additional infection, and prior depletion of phagocytes was found to increase host resistance to *Salmonella* infection (Wijburg *et al*., 2000). In addition to recruitment of phagocytes, *Salmonella* infection also induces marked activation and expansion of CD4+ and CD8+ T cells; the majority of which appear to be *Salmonella*-specific and eventually control

bacterial replication (Srinivasan *et al*., 2007). Thus, a hallmark of *Salmonella* infection is increased cellularity of the spleen, owing in part to recruitment and expansion of phagocyte and lymphocyte populations responding to infection. Although the splenomegaly accompanying *Salmonella* infection is sometimes attributed to this increase in splenic leukocytes, the contribution of other cell populations is also considered, and under conditions of stress and acute infections, erythroid differentiation can become dysregulated, and a large increase in erythrocytes can be initiated in the spleen (Jackson *et al*., 2010). However, using probiotics may encounter such effects, but in group III of mice that were treated with either *L. acidophilus* or *S. cerevisiae*, the spleen index increased to 1.141 and 0.981%, respectively compared to untreated mice (0.316%). In this regard, it has been suggested that probiotic bacteria can cause infective episodes if they translocate from the gastrointestinal tract to extraintestinal sites, such as regional lymph nodes, spleen, liver, bloodstream, heart valves, or other tissues (Liong, 2008). However, cases of probiotic administration leading to bacteraemia or fungaemia have been rare, and in 2003 an expert panel concluded that 'Current evidence suggests that the risk of infection with probiotic *Lactobacilli* or *Bifidobacteria* is similar to that of infection with commensal strains, and that consumption of such products presents a negligible risk to consumers, including immunocompromised hosts' (Hickson, 2011).

3.5.3 Bacterial Load in Liver and Spleen

Both probiotics were effective in reducing *S.* Typhimurium colony forming units per plate (CFU/plate) in the liver and spleen, although some differences were observed. In liver, mice challenged with the pathogen and untreated (group IV) showed a count of 224.4 ± 62.7 CFU/plate, which was significantly higher than any CFU count in groups of *L. acidophilus* and *S. cerevisiae*. Probiotic-treated groups, which were received continues treatment with a probiotic (group II) recorded better results than group I mice, which received the probiotic 7 days

pre- *S.* Typhimurium challenge. The lowest count of CFU was observed in group II at day 21, which was 21.6 ± 7.9 and 27.8 ± 10.0 CFU/plate, respectively for *L. acidophilus* and *S. cerevisiae*, and the reduction was almost 90% compared group IV. Although, no significant difference was observed between the groups of *L. acidophilus* and *S. cerevisiae*, the former probiotic was better than the latter in reducing the CFU count in all investigated groups (Table 3-5).

Table 3-5: Bacterial load in liver of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	Mean ± S.D. (Colony Forming Unit/Plate)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	78.1 ± 14.7 ^{B}	88.6 ±15.5 ^{B}	N.S.
	21	96.8 ±10.6 ^{B}	115.3 ± 36.9 ^{B}	N.S.
II	14	38.3 ±13.6 ^{C}	64.6 ± 18.9 ^{B}	N.S.
	21	21.6 ± 7.9 ^{C}	27.8 ± 10.0 ^{C}	N.S.
III	8	No Growth		
IV	8	224.4 ± 62.7 ^{A}	224.4 ± 62.7 ^{A}	
V	8	No Growth		

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

For spleen, almost similar results were obtained, but group II at 14 days of *L. acidophilus* showed a significantly lower count (P ≤ 0.001) than the corresponding group in *S. cerevisiae* (21.6 ± 6.6 *vs.* 79.6 ± 12.1 CFU/plate). However, in group II at 21 days, the CFU of *Salmonella* was also significantly decreased; moreover it was also approximated in *L. acidophilus* or *S. cerevisiae* treated mice (24.5 ± 7.8 and 23.8 ± 13.9 CFU/plate, respectively), and there was no significant difference between the two means. The reduction in this group was almost 90%. Accordingly, a continuous treatment with the probiotics recorded better results at 21 days of the treatment (Table 3-6).

Table 3-6: Bacterial load in spleen of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	Mean ± S.D. (Colony Forming Unit/Plate)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	64.6 ±13.3 ^{C}	78.1 ± 16.1 ^{B}	N.S.
	21	108.6 ± 24.8 ^{B}	84.6 ± 15.6 ^{B}	N.S.
II	14	21.6 ± 6.6 ^{D}	79.6 ± 12.1 ^{B}	**0.001**
	21	24.5 ± 7.8 ^{D}	23.8 ± 13.9 ^{B}	N.S.
III	8	No Growth		
IV	8	206.6 ± 66.9 ^{A}	206.6 ± 66.9 ^{A}	
V	8	No Growth		

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

The presented results suggest that *L. acidophilus* and *S. cerevisiae* were effective in controlling the growth of *S.* Typhimurium *in vivo*, and such results came to confirm the *in vitro* findings, and *L. acidophilus* was better than *S. cerevisiae* in controlling the infection. Several mechanisms of *Lactobacillus* have been suggested to inhibit *Salmonella* invasion. For instance, some metabolic products such as lactic acid and/or bacteriocins produced by *Lactobacillus* may inhibit the growth of pathogenic bacteria (Forestier *et al*., 2001). Furthermore, *Lactobacillus* strains that maintain adhesive properties and the ability to colonize the human gastrointestinal tract may hinder the association or invasion between the epithelial cells and the pathogenic bacteria (Jankowska *et al*., 2008). In a study by Ho *et al*. (2011), the adhesion of five *Lactobacillus* strains to colonize Caco-2 cells (a cell line model of the intestinal barrier) was investigated and their results suggested the competitive exclusion of adhesion of *S.* Typhimurium. This was explained by the consideration that the entry of *Salmonella* into a given environment can be prevented if the space is already occupied by probiotic organisms that are better suited for establishing and maintaining themselves in the environment or those that excrete substances that inhibit the growth of *Salmonella*.

In the present study, a consumption of *L. acidophilus*, especially after continuous treatment (group II at 21 days) was associated with higher antagonistic effects against the MDR *S.* Typhimurium B strain and a reduction of liver and spleen pathogen was observed, and such findings were favored by Vesterlund *et al*. (2005). Rishi *et al*. (2009) also found that the probiotic *L. acidophilus* decreased the translocation of *S*. Typhimurium and reduced liver damage. Other researchers indicated that lactic acid bacteria such as *Lactobacillus* may possess immune-enhancing properties or bacteriocin that can reduce the occurrence of infection by pathogens (Cheikhyoussef *et al*., 2008; Simova *et al*., 2009). *L. acidophilus* also produces lactic acid as the major metabolic end-product of carbohydrate fermentation, and the resultant pH may be sufficiently low to inhibit the growth of other microorganisms including the most common human and animal pathogens (Salminen *et al*., 2004). Therefore, *Lactobacillus* has been the most commonly studied organisms for their probiotic properties in controlling *Salmonella* infections (Fayol-Messaoudi *et al*., 2007; Jain *et al*., 2008).

With respect to *S. cerevisiae*, the results were also encouraging, and such probiotic was also able to control *S.* Typhimurium efficiently, although it was resistant to three antibiotics. In agreement with such theme, it has been shown that *S. cerevisiae* was able to colonize and survive in the gastrointestinal tract of germ-free and conventional mice and to protect them against experimental infections with *S*. Typhimurium and *Clostridium difficile* (Martins *et al*., 2005). Additional findings revealed that *S. cerevisiae* was able to reduce the translocation of *S. Typhimurium* and to stimulate the immune system in mice, and at the histological level, *S. cerevisiae* conferred protection to intestine and liver tissues, decreased inflammatory foci in liver, and promoted an increase in the number of Kupffer cells after experimental infection with *S. Typhimurium* (Martins *et al*., 2007). Further data demonstrated that this yeast protected against bacterial translocation, preserved gut barrier integrity, and stimulated the

immune system in a murine model of intestinal obstruction (Generoso *et al*., 2010). In addition, Martins *et al*. (2011) demonstrated that Oral treatment with *S. cerevisiae* inhibited weight loss and increased survival rate after *Salmonella* challenge. In a more recent investigation, other *Saccharomyces* species (*S. boulardii*) was investigated in a mouse model of *S.* Enteritidis infection, which included pretreatment with *S. boulardii*, to reveal the protection mechanisms of *S. boulardii* against *S.* Enteritidis infection, including the translocation of *S.* Enteritidis to the liver 10 days after *S.* Enteritidis challenge, and the colonization of *S.* Enteritidis and the formation of hepatic tissue lesions in mice after *S.* Enteritidis challenge on the 10th day. Their results revealed that compared to *S.* Enteritidis infection in mice, *S. boulardii* decreased *S.* Enteritidis translocation to the liver by 96%. *Saccharomyces boulardii* also abated hepatic tissue injury caused by the infiltration of neutrophilic granulocytes, lymphocytes, and plasmocytes by decreasing the translocation of *Salmonella* to the liver (Wu *et al*., 2014).

3.6 Cytokine Levels in Small Intestine Lavage

In this part of the study, the level of 10 cytokines was determined in the intestinal scrubbing fluid of *S.* Typhimurium-infected and probotic-treated mice that were distributed into five groups (as shown in the experimental design presented in chapter two: patients, materials and methods). The cytokines were those produced by Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17A) and T regulatory (Treg) cells (IL-10), as well as chemokines (RANTES and IP-10); in addition to IL-1β, IL-12, IL-21 and granulocyte monocyte-colony stimulating factor (GM-CSF).

3.6.1 Interferon-gamma (IFN-γ); a T helper 1 Cytokine

The highest level of IFN-γ in *L. acidophilus* groups was observed in group II on day 21 (treatment group), which was 21.30 ± 11.37 pg/ml, and the difference was significant ($P \leq 0.05$) compared to the levels in the other groups.

In *S. cerevisiae* groups, a similar pattern was observed (10.48 ± 3.35 pg/ml) but the difference was significant in comparison with group I on day 14 (preventive group). Comparing *L. acidophilus* and *S. cerevisiae* groups revealed that IFN-γ level was significantly increased ($P \leq 0.001$) in group II (21 days) of the first probiotic compared to the same group in the second probiotic (Table 3-7).

Table 3-7: Level of IFN-γ in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IFN-γ Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	5.74 ± 2.51^{BC}	4.89 ± 2.48^{BC}	N.S.
	21	7.19 ± 1.71^{B}	8.34 ± 2.77^{AB}	N.S.
II	14	5.16 ± 1.88^{BC}	8.52 ± 4.06^{AB}	N.S.
	21	21.30 ± 11.37^{A}	10.48 ± 3.35^{A}	**0.001**
III	8	8.45 ± 6.59^{B}	7.52 ± 4.28^{AB}	N.S.
IV	8	8.49 ± 2.41^{B}	8.49 ± 2.41^{AB}	N.S.
V	8	3.14 ± 1.83^{C}	3.14 ± 1.83^{C}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference ($P \leq 0.05$) between means of columns.

In addition the presented results, IFN-γ level was also affected as a consequence of a treatment by any of the two probiotics or by the pathogen, and it was almost leveled at 8 pg/ml, while in untreated control mice (group V), it was 3.14 ± 1.83 pg/ml. however, an administration of the probiotic and the pathogen resulted in a fluctuation from that in controls (groups III, IV and V); either decreased or increased. Interferon-γ is produced by activated T cells and NK cells, and has been shown to play an important role in host defense against intracellular pathogens such as *S.* typhimurium (Benbernou and Nauciel, 1994). Following *Salmonella* challenge, production of IFN-γ by Peyer's patches lymphocytes has been reported in experiments conducted both *in vitro* and *in vivo*, and early production of IFN-γ mRNA has been demonstrated in gut-

associated lymphoid tissues and spleens of mice challenged orally with *S.* typhimurium (Ramarathinam *et al*., 1991). *In vitro* studies have also shown that epithelial cells and fibroblasts are resistant to *S.* typhimurium invasion in the presence of IFN-γ, and that IFN-γ activates mouse peritoneal macrophages, resulting in enhanced *S.* typhimurium killing (Hess *et al*., 1996). *In vivo* experiments have shown that intraperitoneal (i.p.) administration of IFN-γ can protect mice against a lethal *S.* typhimurium infection, but in contrast, administration of anti-IFN-γ antibody is reported to enhance greatly the susceptibility of mice to intravenous (i.v.) bacterial challenge (Bao *et al*., 2000). More recently, It has been shown that mice rendered IFN-γ deficient by targeted gene deletion are susceptible to i.v. challenge with *S.* typhimurium aroA deletion mutants. These mice also demonstrated elevated serum-specific antibody levels compared with normal mice: the patterns of serum antibody were shifted from immunoglobulin G2a (IgG2a) to IgG1, and the production of the Th2 cytokines IL-4 was increased compared with normal mice. Therefore it is believed that activation and/or recruitment of lymphocytes which produce IFN-γ is an important factor in determining the outcome of *Salmonella* clearance following i.v. challenge (Hashizume *et al*., 2008). In addition, IFN-γ is known to negatively regulate the barrier properties and self-renewal of the intestinal epithelium, thus modulating epithelial homeostasis and exacerbating mucosal inflammation (Capaldo and Nusrat, 2009). Probiotics also has their positive effects on IFN-γ production, and a number of investigations suggest that lactic acid bacteria can stimulate several functions of the immune system such as IFN-γ production (Ibnou-Zekri *et al*., 2003; Matsuguchi *et al*., 2003). In addition, *S. cerevisiae* has been found to be able to modulate the immune response against *S.* Typhimurium in porcine intestinal epithelial and dendritic cells through cytokines and IFN-γ is one of them (Roger Badia *et al*., 2012).

3.6.2 Interleukin-4; a T helper 2 Cytokine

Mice in group III that were treated with only *L. acidophilus* (39.23 ± 14.47 pg/ml) or S. *cerevisiae* (48.94 ± 17.55 pg/ml) demonstrated the highest level of IL-4, and the difference between the two probiotics was not significant, while among groups of each probiotic some significant differences were observed. In *L. acidophilus* groups, the lowest level of IL-4 was noticed in group II (treatment group) after 14 days (11.09 ± 6.41 pg/ml), and such decreased level was significant as compared to the other groups. For *S. cerevisiae*, group II also demonstrated the lowest level of IL-4, but after 21 days (7.07 ± 3.68 pg/ml), and such decreased level was significant as compared with other groups of both probiotics. It was also observed that preventive group after 21 days (group I) showed a significantly higher level of IL-4 in *L. acidophilus* groups than the corresponding group of *S. cerevisiae* groups (30.50 ± 10.21 *vs*. 9.82 ± 4.92 pg/ml) (Table 3-8).

Table 3-8: Level of IL-4 in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IL-4 Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	27.86 ± 11.13^{BC}	27.17 ± 5.05^{C}	N.S.
	21	30.50 ± 10.21^{AB}	9.82 ± 4.92^{E}	**0.001**
II	14	11.09 ± 6.41^{D}	16.39 ± 4.88^{D}	N.S.
	21	22.56 ± 7.11^{C}	7.07 ± 3.68^{E}	**0.01**
III	8	39.23 ± 14.47^{A}	48.94 ± 17.55^{A}	N.S.
IV	8	$32.64 \pm 8.24A^{B}$	32.64 ± 8.24^{BC}	N.S.
V	8	14.33 ± 3.48^{D}	14.33 ± 3.48^{D}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

IL-4 also showed variation between the investigated groups, and in some cases, the level was below the normal control level; so it showed increased and decreased levels. This was correlated with and dependent on the type of

treatment and the probiotic under investigation; therefore IL-4 might have importance in the pathology of Salmonellosis, although it is Th2 cytokine and the cellular immune response mediated by Th1 cytokines is the most effective action against *Salmonella*. In this context it has been commented that the potent inflammatory reaction against *Salmonella* species provokes host cell death, as well as apoptosis of both inflammatory and epithelial cells following nutrient deprivation and termination of bacterial replication (dos Santos *et al*., 2011). The inflammatory response of the Th1-dominant type is destructive for host cells and for bacteria; it attenuates progressively and coincides with increase of the Th2-immune response. Th2 cells produce IL-4, IL-10, IL-13 and transforming growth factor (TGF) that cause powerful protective effect on host cells (hepatocytes and inflammatory cells) through partial inhibition of cytokines associated with the Th1 response (Fàbrega and Vila, 2013). With respect to probiotics, a study carried out by Jain *et al*. (2008) to investigate the protective effect of probiotic dahi (curd) supplemented with *L. acidophilus* and *L. casei* against *S*. Enteritidis infection has demonstrated that pre-feeding with probiotic dahi significantly increased anti-*S*. Enteritidis sIgA (secretary IgA) antibodies and lymphocyte proliferation in *S*. Enteritidis infected mice. Also, IL-2, IL-6 and IFN-γ production were significantly increased in supernatant of cultured splenocytes collected from mice pre-fed with probiotic dahi, while IL-4 levels were not changed significantly; an observation that is not supported by the present study, and the strain of probiotic might have its effect.

3.6.3 Interleukin-10; a T regulatory (Treg) Cytokine

IL-10 showed a less variation between the groups in comparison with IFN-γ or IL-4; however, treating mice with *L. acidophilus* only recorded the highest significant level (group III: 15.05 ± 7.04 pg/ml), which was significantly higher than its level in the corresponding group of *S. cerevisiae* (8.48 ±7.06 pg/ml), followed by *S*. Typhimurium treatment (group IV: 12.98 ± 6.53 pg/ml). In contrast, group I at 21 days of *S. cerevisiae* showed a significantly higher level

than the corresponding group in *L. acidophilus* (9.06 ± 7.49 *vs*. 5.16 ± 2.63 pg/ml) (Table 3-9).

Table 3-9: Level of IL-10 in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IL-10 Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	5.16 ± 2.63^{C}	9.06 ± 7.49^{A}	**0.05**
	21	5.20 ± 2.50^{C}	5.73 ± 2.84^{B}	N.S.
II	14	$7.97 \pm 5.04B^{C}$	7.12 ± 2.99^{AB}	N.S.
	21	$6.53 \pm 333.B^{C}$	8.81 ± 7.24^{AB}	N.S.
III	8	15.05 ± 7.04^{A}	8.48 ± 7.06^{AB}	**0.05**
IV	8	12.98 ± 6.53^{AB}	12.98 ± 6.53^{A}	N.S.
V	8	8.20 ± 5.35^{BC}	8.20 ± 5.35^{AB}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

There is no direct evidence to support the present distribution of IL-10 in the investigated groups, but it has been found that the concentration of systemic IL-10 in patients with salmonellosis is significantly higher compared to control subjects (Montagne *et al*., 2013). In general, this anti-inflammatory cytokine is considered as an essential immunoregulator in the intestinal tract, and its main function is to counterbalance an overly zealous pro-inflammatory response to protect the host from its harmful side effects (Rennick *et al*., 1997). On the other hand, this activity may result in the persistence of bacteria due to the interference with the innate and adaptive protective immunity (Mege *et al*., 2006). In the case of *S*. Typhimurium infection, it has been demonstrated that IL-10 not only suppresses the bactericidal response of macrophages against the pathogen, but also ultimately causes infected macrophages to function as hosts for its replication (Lee *et al*., 2011). In addition, IL-10 knock-out mice have been shown to develop a chronic enterocolitis associated with apparently

dysregulated production of Th1-type pro-inflammatory cytokines (Davidson *et al*., 2000). Furthermore, an administration of the probiotic *Bifidobacterium infantis* to IL-10 knock-out mice caused a reduced IFN-γ expression in the Peyer's patch of both wild type and IL-10 knock-out mice. Alterations in IFN-γ in the Peyer's patches of wild type mice (enhancement) versus IL-10 knock-out (reduction) were observed following *in vitro* stimulation with *Salmonella* (Sheil *et al*., 2006). Treatment of IL-10 gene-deficient mice with a probiotic containing *L. acidophilus*, *L. casei* resulted in normalization of colonic physiologic function and barrier integrity in conjunction with a reduction in mucosal secretion of TNF-α and IFN-γ and an improvement in histologic disease. *In vitro* evaluations showed that epithelial barrier function and resistance to *Salmonella* invasion could be enhanced by exposure to a proteinaceous soluble factor secreted by *L. acidophilus*, *L. casei* (Madsen *et al*., 2001). More recently, Chen *et al*. (2012) demonstrated that oral administration of a combination of select lactic acid bacteria strains reduce *Salmonella* invasion and inflammation of broiler chicks, and such reduction was associated with a higher level of IL-10 in the cecal tonsils of chicks. *In vitro* studies indicated that cells from human mesenteric lymph nodes that drain inflamed intestines secreted more anti-inflammatory cytokines (IL-10 and TGF-β) when stimulated with *Lactobacillus*, but more pro-inflammatory cytokines (TNF-α and IL-12) were produced when stimulated with pathogenic *Salmonella* (Mazmanian *et al*., 2008).

3.6.4 Interleukin-12

It was observed that IL-12 level was significantly increased in mice treated with *L. acidophilus* after 14 days in group I (8.69 ± 3.43 pg/ml), and then it was decreased to 2.17 ± 0.93 pg/ml in group II after 14 days. However, no such difference was observed in mice treated with *S. cerevisiae* in the corresponding groups (3.94 ± 1.84 and 4.91 ± 0.72 pg/ml, respectively), but the first mean was significantly lower than the corresponding mean in *L. acidophilus*. However, both probiotics (*L. acidophilus* and *S. cerevisiae*) shared a similar effect on IL-

12 level when they were admistited alone (7.31 ± 2.16 and 6.89 ± 1.55 pg/ml, respectively) (Table 3-10).

Table 3-10: Level of IL-12 in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IL-12 Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	8.69 ± 3.43^{A}	3.94 ± 1.84^{B}	**0.05**
	21	5.42 ± 0.88^{BC}	6.05 ± 2.71^{AB}	N.S.
II	14	2.17 ± 0.93^{C}	4.91 ± 0.72^{B}	N.S.
	21	2.31 ± 1.60^{C}	4.05 ± 2.12^{B}	N.S.
III	8	7.31 ± 2.16^{A}	6.89 ± 1.55^{A}	N.S.
IV	8	3.74 ± 1.17^{C}	3.74 ± 1.17^{B}	N.S.
V	8	5.96 ± 1.41^{BC}	5.96 ± 1.41^{AB}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

As in other investigated cytokines, IL-12 might have its role in the pathogenesis of salmonellosis, especially of observe that mice challenged with *S.* Typhimurium (group IV) were associated with a decreased level of it, while administrating *L. acidophilus* or *S. cerevisiae* alone or in a combination with *S.* Typhimurium was associated with a recovery of IL-12 level in the intestine of mice. In agreement with such findings, *Lactobacilli* isolated from human intestine were found to be potent stimulators of IL-12 production by human blood mononuclear cells or monocytes. In addition, it was shown that *Lactobacillus sakei* strongly induced IFN-γ and IL-12 secretion (Heyman and Ménard, 2002). In addition, strains of mainly lactobacilli were also found to induce IL-12 or IL-10, and more specifically *L. casei* induced production of IL-12 with a subsequent production of IFN-γ in murine splenocytes. Also, beneficial effects were also attributed to immunpotentiating effects by *Lactobacillus* strains. One specific effect was shown by heat-killed *L.* plantarum

L-137, which restored the inhibited IL-12 production in DBA/2 mice (Ljungh and Wadström, 2006)

3.6.5 Interleukin-17A; a T helper 17 Cytokine

In general, all groups of *L. acidophilus* showed a significant increased mean of IL-17A compared to the corresponding mean in groups of *S. cerevisiae*, although with different levels of significance. Group III, which involved mice treated with *L. acidophilus* only, showed the highest significant level of IL-17A (19.02 ± 4.27 pg/ml) among the other groups, while in *S. cerevisiae* groups, the pathogen *S.* Typhimurium (group IV) demonstrated the highest level (12.83 ± 6.95 pg/ml). A lowest level of IL-17A (4.45 ± 2.89 pg/ml) was observed in group I of *S. cerevisiae* after 21 days (Table 3-11).

Table 3-11: Level of IL-17A in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IL-17A Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	13.46 ± 4.13^{B}	7.21 ± 2.96^{B}	**0.01**
	21	11.91 ± 6.63^{C}	4.45 ± 2.89^{C}	**0.001**
II	14	16.33 ± 4.34^{AB}	$9.16 \pm 540.^{A}$	**0.05**
	21	12.22 ± 6.62^{B}	$5.92 \pm 259.^{BC}$	**0.001**
III	8	19.02 ± 4.27^{A}	12.83 ± 6.95^{A}	**0.01**
IV	8	14.48 ± 6.28^{B}	14.48 ± 6.28^{A}	N.S.
V	8	10.13 ± 6.80^{B}	10.13 ± 6.80^{A}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

IL-17A is a Th17 cytokine, and *S.* Typhimurium was demonstrated to have the ability to induce Th17 responses in the intestinal mucosa in mice (Raffatellu *et al*., 2007). Another study showed, as in the present study (group IV mice), that *S.* Typhimurium infection resulted in increased levels of IL-17A, IL-22 and

IL-23 in the intestine. These responses were suggested to be mainly driven by IL-23, which was not investigated in the present study (Godinez *et al*., 2009). In the setting of IL-12 deficiency, IL-23 dependent IL-22 was shown to be crucial in protection against disseminated infection with *S.* Enteritidis, while IL-17A was redundant in this model (Schulz *et al*., 2008). However, it has also been shown by the latter investigators that IL-17A deficient mice had slightly higher bacterial load in liver and spleen when compared to control mice in this model of disseminated *Salmonella* infection. During *S.* Typhimurium infection, IL-17A deficiency in mice resulted in impaired neutrophil recruitment to the intestinal mucosa, and IL-17A deficiency caused by Simian immunodeficiency virus (SIV) in macaques, which is the primate variant of HIV, resulted in increased translocation of *S.* Typhimurium (Raffatellu *et al*., 2008). Interestingly, *S. typhimurium* infection in SIV positive macaques caused significant less IL-17A and IL-22, whereas IFN-γ production was normal. These data indicate that Th17 responses play an important role in controlling mucosal host defense against *Salmonella* and protection against disseminated salmonellosis. In addition, *S.* Typhi can inhibit Th17 responses, which probably contributes to the higher virulence associated with this *Salmonella* spp. (Awoniyi *et al*., 2012).

The present results also highlighted the role *L. acidophilus* or *S. cerevisiae* in monitoring the intestinal level of IL-17A. In this regard, it has been argued that as probiotics are able to modulate both Th1 and Th2 mediated responses, attention has also been drawn to their potential use in modulating Th17 cells. Several studies have focussed on probiotic modulation of IL-17A and IL-23. Paolillo *et al*. (2009) found that *L. plantarum* treatment with LPS-activated Caco-2 epithelial cells reduced IL-23, and such finding has been supported by Ghadimi *et al*. (2012), who observed a reduction in IL-17A and IL-23 in peripheral blood monocytes co-cultured with human intestinal cells and treated with *B. breve* and *L. rhamnosus* GG. Evrard *et al*. (2011) further demonstrated that *L. rhamnosus* can increase CD86 and DC-SIGN expression on human

dendritic cells (DCs); suggesting the effects of *Lactobacillus* on Th17 activation to be mediated through modulation of DC function. These co-culture system studies have been suggested to be more applicable to *in vivo* settings; in which case particular probiotic strains may have a role in inducing Th17-mediated immunity through modulation of DCs affecting down-stream pro-inflammatory cytokine expression (Donkor *et al*., 2012). It has also been demonstrated that a consumption of *L. casei* fermented milk prevented *Salmonella*-induced reactive arthritis in mice by modulating IL-23/IL-17 expression (Noto Llana *et al*., 2013).

3.6.6 Interleukin-21

Mice treated with *L. acidophilus* only (group III) demonstrated the highest level of IL-21 (22.69 ± 5.28 pg/ml), and the difference was significant compared to all groups of both probiotics. However, in *S. cerevisiae*, group II and after 21 days was observed with the highest mean (17.25 ± 5.61 pg/ml). In terms of which probiotic was better in this regard, almost *L. acidophilus* enhanced a better response in IL-21 production than *S. cerevisiae* with the exception of group II after 14 days, in which the opposite picture was augmented (Table 3-12).

Probably, this is the first evaluation of IL-21 in salmonellosis especially in a combination with probiotic treatment in mice. IL-21 belongs to a family of cytokines that exclusively binds IL-21R (Spolski and Leonard, 2008). CD4+ T cells and NKT cells produce IL-21 (Coquet *et al*., 2008). Within the CD4+ T cell subset, IL-21 is expressed at the highest levels by T follicular helper (Tfh) cells and Th17 cells (Nurieva *et al*., 2008). Its receptor is expressed on T cells, B cells, NK cells, macrophages and DCs (Bauquet *et al*., 2009). IL-21 promotes antibody production, plasma cell differentiation, and switching to IgG1 in the context of thymus-dependent responses, and a requirement of IL-21 for Tfh cell differentiation has also been reported (Johnston *et al*., 2009); in naive T cells, IL-21 leads to up-regulation of Bcl-6, the transcriptional regulator of Tfh cells

(Yu *et al*., 2009). The defect in Tfh cell formation in the absence of IL-21 signaling has been proposed to explain the reduced numbers of germinal center B cells in the absence of IL-21 (Nurieva *et al*., 2009). Given these evidences of the effects of IL-21 on B and T cells, it is worth to ask whether the reported effects of IL-21 are affected by *Salmonella* infection and probiotics, especially when we observe that its level was 1.5 and 2.2 times higher in *S.* Typhimurium infected mice (group IV) and *L. acidophilus* group III mice than in normal mice (group V). No direct evidence to support such observation, but in *Salmonella*-infected colitis, it was demonstrated that IL-21 triggers T-cell activation and induces pro-inflammatory cytokine secretion, implying that it may be directly associated with the development of mucosal inflammation (Liu *et al*., 2009). With respect to probiotics, searching PubMed revealed that IL-21 has not be investigated in the context of *L. acidophilus* or *S. cerevisiae*.

Table 3-12: Level of IL-21 in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IL-21 Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	$9.40 \pm 1.83B^{C}$	12.14 ± 4.42^{B}	N.S.
	21	12.63 ± 5.17^{B}	7.20 ± 4.56^{C}	**0.05**
II	14	$7.60 \pm 233.^{C}$	$15.52 \pm 272.^{A}$	**0.01**
	21	19.11 ± 3.11^{A}	17.25 ± 5.61^{A}	N.S.
III	8	22.69 ± 5.28^{A}	10.22 ± 3.32^{B}	**0.001**
IV	8	15.63 ± 6.09^{B}	15.63 ± 6.09^{A}	N.S.
V	8	$10.46 \pm 510.^{BC}$	$10.46 \pm 510.^{B}$	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

3.6.7 Interleukin-1β

Mice treated with *L. acidophilus*, *S. cerevisiae* or *S.* Typhimurium (groups III and IV) shared an approximated mean of IL-β1 (10.63 ± 2.47, 13.12 ± 3.48 and 11.43 ± 6.01 pg/ml, respectively). However, in group I mice of *L.*

acidophilus, the level of IL-β1 was significantly increased in day 21 compared to the corresponding group of *S. cerevisiae* (14.07 ± 4.06 *vs*. 3.72 ± 1.54 pg/ml), and in group II, a similar obserbation was made (11.77 ± 3.11 *vs*. 1.96 ± 108. pg/ml) (Table 3-13).

Table 3-13: Level of IL-β1 in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IL-β1 Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	8.53 ± 1.94^{BC}	8.82 ± 2.10^{B}	N.S.
	21	14.07 ± 4.06^{A}	3.72 ± 1.54^{C}	**0.001**
II	14	5.41 ± 1.24^{C}	7.77 ± 1.60^{B}	N.S.
	21	11.77 ± 3.11^{AB}	$1.96 \pm 108.^{C}$	**0.001**
III	8	10.63 ± 2.47^{AB}	13.12 ± 3.48^{A}	N.S.
IV	8	11.43 ± 6.01^{AB}	11.43 ± 6.01^{AB}	N.S.
V	8	5.22 ± 1.94^{C}	5.22 ± 1.94^{C}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

IL-1β is an important cytokine in activating the release of other pro-inflammatory cytokines such as TNF-α and IL-6, and induces a Th17 bias in the cellular adaptive responses. *In vivo*, it is largely responsible for the acute phase response, and cytokines of the IL-1 family are a crucial component of the host defense against infections including *Salmonella* (Netea *et al*., 2010), and *S*. Typhimurium has been found to induce the synthesis of pro-IL-1β through the initial signaling involving the extracellular TLR4 and TLR5 receptors, and this may explain that IL-1β level was almost doubled in *S*. Typhimurium infected mice (group IV) than that in controls (group V). Signaling through the inflammasome by *S*. Typhimurium also plays a role in the activation of caspase-1, although the molecular mechanisms of this induction are unknown (Miao and Rajan, 2011). However, the activation of IL-1β during *Salmonella* infection is a

complex process employing several different pathways and it is difficult to discern the contribution of each process to the net systemic concentration of IL-1β, but overall, *S.* Typhimurium has been suggested as a potent inducer of IL-1β (Ktsoyan *et al*., 2013). *L. acidophilus* and *S. cerevisiae* have also doubled the level IL-1β in group III mice; an observation that suggests their role in promoting IL-1β release in the intestine. There has also been no evidence to confirm or contradict such observation, and certainly further investigations are required to shed light on this subject.

3.6.8 Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF)

The cytokine GM-CSF responded almost similarly in both groups of probiotics (*L. acidophilus* and *S. cerevisiae*) and no significant difference was observed in the level of GM-CSF between them. Furthermore, treatment of animals with the probiotic only (group III) elevated its mean to the highest level (21.74 ± 5.59 and 19.87 ± 564. pg/ml, respectively), and the difference was significant compared to other groups in each probiotic. Group I after 21 days came second in such increase in both probiotic (16.08 ± 3.57 and 16.35 ± 3.42 pg/ml, respectively) (table 3-14).

GM-CSF is a cytokine produced by NK cells and T cells in response to a variety of stimuli; for instance IL-15 and IL-18. It activates monocytes and enhances their bactericidal activity. Moreover, monocytes pre-stimulated with GM-CSF secrete increased quantities of TNF and IL-1β when stimulated with lipopolysaccharides. Furthermore, GM-CSF induces differentiation of human monocytes into a macrophage type that is capable of producing IL-23 (Carryn *et al*., 2004). It has also been demonstrated that GM-CSF or IFN-γ could prime monocytes directly for enhanced IL-23 production in response to stimulation with heat killed *Salmonella*. In addition, inflammation caused by infections such as *Salmonella* drives the production of GM-CSF in target tissues and may therefore be involved in directing DC differentiation under these conditions (van de Wetering *et al*., 2009). Such demonstrations support the current significant

increased level of GM-CSF in *S.* Typhimurium infected mice (group IV), and possibly in a similar pathway. With respect to probiotics, no similar experimental design has been established to explain the significant increase of GM-CSF, but immature DC has been suggested to be activated and matured by commensal bacteria; for instance, lactic acid bacteria, and these activated mature DCs produced cytokines (TNF-α and IFN-γ) that are able to activate NK cell cytotoxicity and induce their proliferation. At the same time, the early release of IFN-γ by NK cells interacting with lactic acid bacteria-activated DC, most likely in secondary lymphoid organs such as the mesenteric lymph nodes, is critical for shaping the following adaptive immune response toward a type 1 T cell response (Rizzello *et al*., 2011). Therefore, investigating GM-CSF in the ground of probiotics and salmonellosis may shed further light on the polarization of different subsets of T helper cells.

Table 3-14: Level of GM-CSF in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	GM-CSF Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	7.64 ± 3.04 ^{D}	9.14 ± 4.27 ^{BC}	N.S.
	21	16.08 ± 3.57 ^{BC}	16.35 ± 3.42 ^{AB}	N.S.
II	14	8.18 ± 313.^{D}	10.16 ± 4.45 ^{B}	N.S.
	21	14.32 ± 6.53 ^{C}	13.14 ± 4.37 ^{B}	N.S.
III	8	21.74 ± 5.59 ^{A}	19.87 ± 564.^{A}	N.S.
IV	8	12.03 ± 5.03 ^{C}	12.03 ± 5.03^{B}	N.S.
V	8	7.09 ± 317.^{D}	7.09 ± 317.^{C}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

3.6.9 Interferon-γ inducible protein-10 (IP-10); a Chemokine

Group III mice that were received *L. acidophilus* or *S. cerevisiae* shared a similar level of IP-10 (5.67 ± 3.51 and 5.48 ± 3.71 pg/ml, respectively);

however, when mice administrated with a probiotic and then challenged with *S.* Typhimurium, the level was increased, especially at day 14 of group I and at day in *S. cerevisiae* (17.08 ± 7.51 pg/ml), and similar increase was observed in group II but at day 21 (17.60 ± 7.06 pg/ml). The latter value was also significantly higher than the corresponding value in *L. acidophilus* (8.79 ± 3.72 pg/ml) (Table 3-15).

Table 3-15: Level of IP-10 in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	IP-10 Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	10.31 ± 5.52 ^{A}	17.08 ± 7.51^{A}	0.05
	21	10.10 ± 343.^{A}	10.74 ± 410.^{B}	N.S.
II	14	8.88 ± 2.45^{A}	12.52 ± 6.69^{AB}	N.S.
	21	8.79 ± 3.72^{A}	17.60 ± 7.06^{A}	**0.01**
III	8	5.67 ± 3.51^{BC}	5.48 ± 3.71^{CD}	N.S.
IV	8	6.20 ± 377.^{AB}	6.20 ± 377.^{BC}	N.S.
V	8	3.50 ± 195.^{C}	3.50 ±195.^{D}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference (P ≤ 0.05) between means of columns.

As in other investigated cytokines, IP-10 responded positively to *L. acidophilus*, *S. cerevisiae* or *S.* Typhimurium in mice of the present study. IP-10 is a member of the CXC subfamily of chemokines expressed by monocytes, and a pivotal role in the immune system development. It is induced in monocytes, fibroblasts, and endothelial cells by IFN-γ, also known to be induced by IL-1β, TNFα and IL-12. Its pleiotropic functions include the stimulation of monocytes, NK cells and T-cell migration, as well as the regulation of T-cell and bone marrow progenitor maturation (Hassanshahi *et al*., 2007). Such chemokine has also been investigated in the ground of *Salmonella* and tumor, and IP-10 has been found to recruit activated effector cells within the tumor after *Salmonella* treatment *in vivo*, and the suggestion was that *Salmonella* involved in

upregulation of IFN-γ and IP-10, which may be responsible for recruiting peripheral NK and T cells to the tumor (Chang *et al*., 2014). A further demonstration revealed that immunodeficient mice engrafted with human fetal liver hematopoietic stem and progenitor cells were able to support S. Typhi replication and persistent infection, and four weeks after infection, a large proportion of the infected animals showed increased levels of the human cytokines TNFα, IL-8, IL-10, IL-12, IFN-γ, MIP-1α, and IP-10 (Song *et al*., 2010). In addition, by using a gnotobiotic C3H mouse model with a background microbiota of eight bacterial species (SIHUMI), the impact of the mucin-degrading commensal bacterium *Akkermansia muciniphila* (SIHUMI-A) on inflammatory and infectious symptoms caused by *S*. Typhimurium was investigated. Presence of *A. muciniphila* in *S*. Typhimurium-infected SIHUMI mice caused significantly increased histopathology scores and elevated mRNA levels of IFN-γ, IP-10, TNF-α, IL-12, IL-17 and IL-6 in cecal and colonic tissue (Ganesh *et al*., 2013). These results came to confirm the present results and it may be concluded that *S*. Typhimurium alone leads to a considerably weaker gut inflammation and cytokines may encounter such effects through probiotics.

3.6.10 RANTES; a Chemokine

RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) showed no much variation in the investigated groups of, and almost no significant difference was observed. The exception was mice treated with probiotics (*L. acidophilus* and *S. cerevisiae*) in group III demonstrated the highest level of RANTES (25.40 ± 976. and 20.41 ± 724. pg/ml, respectively), which was significantly different compared to untreated controls in group V (9.73 ± 5.79 pg/ml) (Table 3-16).

Table 3-16: Level of RANTES in small intestine wash fluid of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	RANTES Mean Level ± S.D. (pg/ml)*		P ≤
		L. acidophilus	*S. cerevisiae*	

I	14	13.67 ± 4.59 ^{B}	10.24 ± 5.91 ^{B}	N.S.
	21	10.92 ± 3.86 ^{B}	9.31 ± 2.50 ^{B}	N.S.
II	14	7.77 ± 3.11 ^{B}	11.32 ± 6.63 ^{B}	N.S.
	21	10.94 ± 6.28 ^{B}	13.91 ± 5.79 ^{B}	N.S.
III	8	25.40 ± 976.^{A}	20.41 ± 724.^{A}	N.S.
IV	8	13.60 ± 2.26 ^{B}	13.60 ± 2.26 ^{AB}	N.S.
V	8	8.73 ± 5.79 ^{B}	8.73 ± 5.79 ^{B}	N.S.

*The mean was based on values of six mice.
Different superscript letters: Significant difference ($P \leq 0.05$) between means of columns.

In RANTES, an important observation was made in mice fed with *L. acidophilus* or *S. cerevisiae*, in which RANTES was dramatically increased. RANTES is a CC chemokine that binds CCR1, CCR3, CCR4, and CCR5 and is produced by epithelial cells, lymphocytes, and platelets, and acts as a potent chemoattractant for monocytes, NK cells, memory T cells, eosinophils, dendritic cells and basophils. In addition, RANTES and other chemokines can selectively activate their corresponding lymphoid cell targets (Lillard *et al*., 2001). Due to these potentials, its observed might be expected, and RANTES may be able to potentiate antigen-specific mucosal immune responses, and Impairment of this response may also account for the reduction in splenic macrophages and NK cells that were observed by Jansen *et* al. (2011) in *S.* Typhimurium infected mice. In addition, Dongol *et al*. (2012) incubated individually live cells of four *Lactobacillus* strains (*L. casei*, *L. rhamnosus*, *L. plantarum* and *L. reuteri*) and two *Bifidobacterium* strains (*B. longum* and *B. bifidum*) with human peripheral blood mononuclear cells from healthy older subjects and revealed that all of strains increased the production of IL-1β, IL-6, IL-10, TNF-α and GM-CSF. Although such findings were based on in vitro experiments, but they support the GM-CSF increase or other cytokines that was observed in mice fed with *L. acidophilus* or *S. cerevisiae*.

3.6.11 Cytokine Ratios

For a further understanding of the effects of the two investigated probiotics alone or in a combination with *S.* Typhimurium on the cytokine profile of the small intestine in mice, ratios between some cytokines were inspected, which were IFN-γ/IL-4, IFN-γ/IL-10, IL-4/IL-10 and IL-17A/IL-10. The four involved cytokines belong to important subsets of T lymphocytes, which are Th1 (IFN-γ), Th2 (IL-4), Th17 (IL-17A) and Treg (IL-10) cells that normally direct the adaptive immune response against pathogens, and their polarizing shapes effective humoral or cellular immune response (Luckheeram *et al*., 2012).

For *L. acidophilus* groups, IFN-γ/IL-4 ratio was 0.2 in group I mice at 14 days, and then increased to 0.3 in the same group but at 21 days. A further increase (0.5) was observed in group II at 14 days, and the ratio was maximized to reach 1.0 on day 21. In control groups (III, IV and V) the ratio was leveled at 0.2 – 0.3. In the case of IFN-γ/IL-10 ratio, group I mice at days 14 and 21 shared approximated ratio (1.8 and 1.6, respectively), but in group II mice, it was 2.2 at 21 days and then increased to 5.2 at day 21, while control groups maintained a ratio level of 0.8 – 1.2. Inspecting IL-4/IL-10 ratio revealed a higher variation, in which group I at 14 days and group II at 21 days were observed with the highest ratios, which were 4.9 and 6.4, respectively. The variation continued to involve IL-17A/IL-10, but group II at 14 and 21 days scored the highest ratios, which were 4.9 and 3.7, respectively (Figure 3-3).

Experimental groups of *S. cerevisiae* showed either less or similar pattern of cytokine ratio distributions, but it was exceptionally to note that mice treated administrated with only *S. cerevisiae* recorded IL-4/IL-10 ratio that reached 8.0, which was the highest observed ratio, while the same ratio or other cytokine ratios did not exceeded 4.2, which was for IFN-γ/IL-4 ratio in group I mice at 14 days (Figure 3-4).

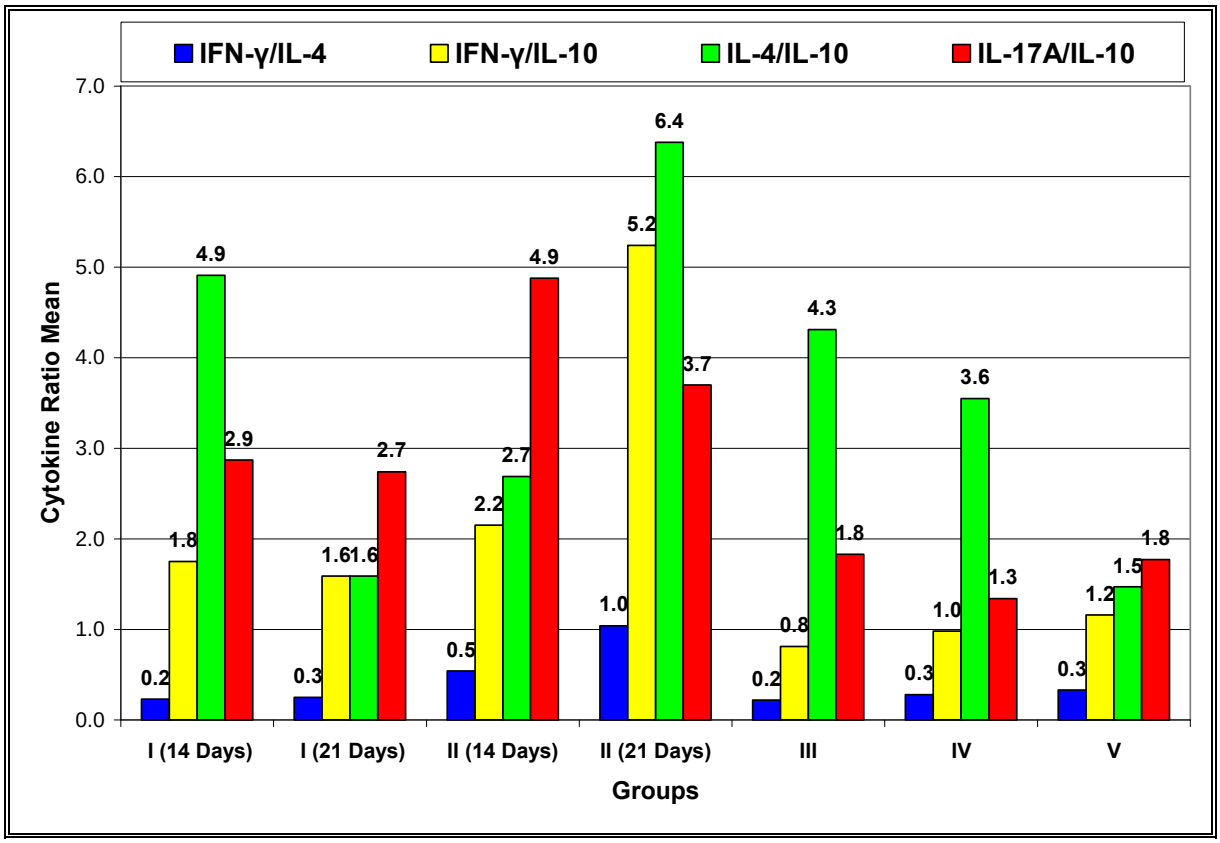

Figure 3-3: Cytokine ratios in small intestine wash fluid of mice treated with *L. acidophilus* and infected with *S.* Typhimurium.

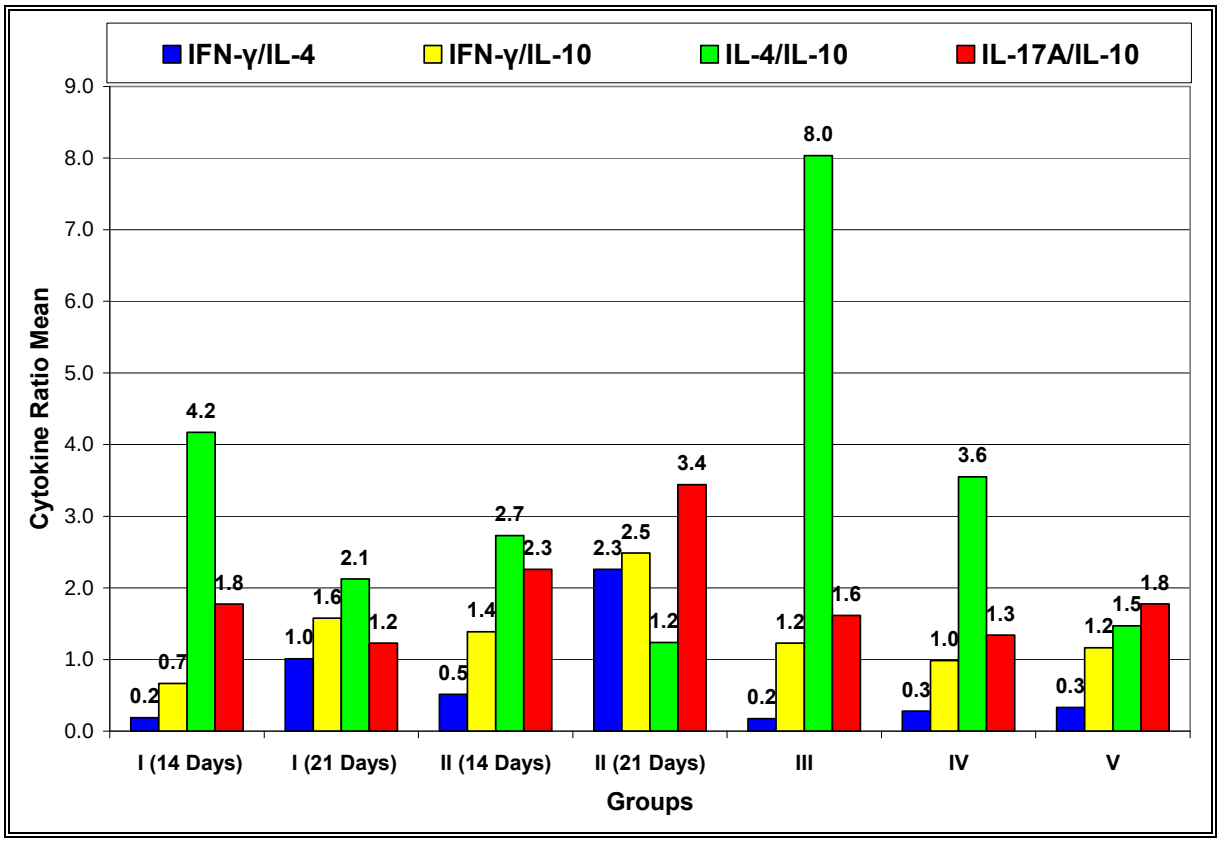

Figure 3-4: Cytokine ratios in small intestine wash fluid of mice treated with *S. cerevisiae* and infected with *S.* Typhimurium.

These results suggest that the investigated ratios were deviated and the deviation was dependent on the type of treatment in each group of mice. Such deviation was a reflection of the change in the profile of investigated cytokines in the small intestine. However, the effects of *L. acidophilus* or *S. cerevisiae* were obvious in shaping the immune response against *S.* Typhimurium, and inducing gut immunity. In probiotic-fed mice, both probiotics produced a diverse cytokine profile causing increased production of IL-4, IL-17A, IL-21, GM-CSF and RANTES, while the rest of cytokines, especially IFN-γ and IL-10 maintained a slightly increased level. Such variation contributed to increased IL-4/IL-10 and IL-17A/IL-10 ratios (i.e. on the expenses of IL-10). IL-10 is the main cytokines produced by Treg cells that play important roles in regulating immune responses, and down-regulating such cells may enhance Th2 and Th17 responses in the gut mucosa of fed mice (Awoniyi et al., 2012; Fàbrega and Vila, 2013). However, it has been reported that commensal bacteria appear to down-regulate IL-17A production by Th17 cells via up-regulation of IL-25, which selectively suppresses IL-23 secretion by intestinal dendritic cells with resultant reduction of IL-17A and inflammation (Noto Llana *et al*., 2013).

In addition and due to the diverse profile of cytokine levels in the groups of investigated mice throughout the small intestine, it is possible to speculate that the employed probiotics induced a balance between Th and Treg responses in intestinal mucosa, because mucosal immune system normally keeps a state that promotes tolerance to itself, showing a slight deviation in Th1 over Th2 polarization, which characterizes a physiologically healthy state. Therefore, an adequate balance between pro-inflammatory (Th1, Th2 and Th17) and regulatory responses (Treg) is important to preserve healthy mucosa (Zaph *et al*., 2008). Thus, probiotic supplementation can correct dysbiosis and restore intestinal homeostasis by immunomodulatory mechanisms induced by these probiotics. Therefore, *L. acidophilus* and to less extent *S. cerevisiae* were shown to be an interesting candidate as probiotic strains to modulate the level of a set

of anti-inflammatory (IL-10) and pro-inflammatory cytokines (IL-12, IL-17A and IFN-γ) and may contribute to a proper balance between different subtypes of polarized CD4+ T cells in intestinal mucosa (Steinberg *et al*., 2014). However, such balance might be disturbed by the complications of *S.* Typhimurium challenge, but the investigated probiotics were able for some degree to counteract such disturbance through modulating the profiles of cytokines. Cytokine level data in intestinal mucosa therefore showed that a aprobiotic-treatment in challenged animals showed a reduced level of important cytokines that are required for fighting the infection, such as IL-10, and did not increase the expression of other cytokines involved in protection against infection, such as IL-12, because activation of IL-12:IL-10:IFN-γ axis is considered as one of most important mechanisms in the fight against *Salmonella* infection (Reid *et al*., 2011). Lipopolysaccharides and certain lipoproteins of the cell wall of *Salmonella* have been regarded as an inducer of a strong local inflammatory response in intestinal tissue leading to increase of TNF-α, IL-1β, IL-6 and IL-12 secretion. Furthermore, IFN-γ production is a major milestone in immune responses against *Salmonella* and its increased production is often associated with protection against infection (Kupz *et al*., 2014). In recent years, it has also been reported that responses involving IL-17A/Th17 appear to complement the response of IFN-γ/Th1 axis that is characteristic of the combat against infection caused by *Salmonella* (Olivier *et al*., 2013).

Therefore, according to the obtained data of present study, it is safe to say that *L. acidophilus* and *S. cerevisiae* treatments modulated the immune response against the MDR *S.* Typhimurium isolate through different and interrelated cytokines, and might have provided a therapeutic option against infection by this pathogen.

Conclusions and Recommendations

Conclusions and Recommendations

Conclusions

1. Diarrhea in children still represents a major health concern, in which *Salmonella enterica* Serovar Typhimurium is an important causative agent, especially in children younger than five years old and the percentage of infection was 7.03%.
2. Out of the nine isolates of *S.* Typhimurium, one was resistance to three antibiotics (amoxicillin, clavulanic acid and nalidixic acid).
3. *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* proved to be important probiotics in reducing the *S.* Typhimurium load in liver and spleen of the experimentally infected mice, and a continuous treatment with these probiotics, especially *L. acidophilus*, gave better results.
4. Both the probiotics were also efficient in modulating the small intestine immune response against *S.* Typhimurium via up-regulation and down-regulation of some cytokines.
5. These findings together with findings of present study demonstrate that *L. acidophilus* and *S. cerevisiae* were an effective agent in controlling bacterial growth of MDR *S. Typhimurium* and might be able to prevent hepatic injury that can be induced by *Salmonella* infection in a mouse model.

Recommendations

1- Further investigations are required to detect the causative agents of diarrhea in children by using an epidemiological approach and assess their antibiotic susceptibility.

2- The genetic basis of multi-drug resistance *S.* Typhimurium needs to be more investigated at the molecular level.

3- Studying other kinds of Probiotics and cytokines is a fruitful area of research if they are considered together in controlling infectious pathogens such as

Salmonella Typhimurium and even other pathogens which have wide spread and cause health problems in the community and this can be done by using an *in vivo* approach.

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Appendices

Appendix I: General Equipment

Equipment	Company	Origin
Anaerobic jar	Rodwell	UK
Autoclave	Koskun	Japan
Centrifuge (Cooled)	Gallenkamp	UK
Centrifuge (Portable)	Beckman	USA
Disposable Syringes	CMP	Turkey
ELISA system	Human HS	USA
Incubator	Fischer	USA
Laminar air flow hood	Heraeus	Germany
Micropipette	Brand	Germany
Micropipette tips	Walter	Germany
Millipore filters	Serious membrane	Germany
Pasteur pipettes	BioMeraux	France
Precision	Eppendorff	Germany
Sensitive balance	Delta Range	Switzerland
Swab collectors	BDH	UK
Vacuum oven	Fischer	USA
Water distiller	Glf	Germany

Appendix II: Chemical and Biological Materials

Material	Company	Origin
Ethanol	BDH	U.K.
Glycerol	BDH	U.K.
H_2O_2	Himedia	India
KOH	BDH	U.K.
Methanol	BDH	U.K.
Normal saline	ADWIC	Egypt
Peptone	Himedia	India
Phosphate buffer saline	BDH	U.K.
Skimmed milk	Biolife	Italy

Appendix III: Kits

Kit	Company	Origin
Anti-sera for identification of *S.* Typhimurium	BioRad	USA
API 20 E Kit	BioMerieux	France
Murine GM-CSF Mini ELISA Development Kit	Peprotech	USA
Murine IFN-γ Mini ELISA Development Kit		
Murine IL-10 Mini ELISA Development Kit		
Murine IL-12 Mini ELISA Development Kit		
Murine IL-17 Mini ELISA Development Kit		
Murine IL-1β Mini ELISA Development Kit		
Murine IL-21 Mini ELISA Development Kit		
Murine IL-4 Mini ELISA Development Kit		
Murine IP-10 Mini ELISA Development Kit		
Murine RANTES Mini ELISA Development Kit		

Appendix IV: Culture Media

Medium	Company	Origin
Brain heart infusion agar	Himedia	India
Brain heart infusion broth	Himedia	india
MacConkey agar	Himedia	India
MRS agar	Oxoid	UK
MRS broth	Oxoid	UK
Muller Hinton agar	Oxoid	UK
Nutrient agar	Himedia	India
Nutrient broth	Himedia	India
Sabaroud dextrose broth	Oxoid	UK
S-S agar	Himedia	India
TSI agar	Oxoid	UK
XLD	Himedia	India

Appendix V: Antibiotic Discs

Antibiotic disk	Symbol	Dose (µg)	Company (Origin)
Amoxicillin/Clavulanic acid	AMC	20/10	Bioanalysis (Turkey)
Ampicillin	AMP	10	
Azithromycin	AZ	15	
Ciprofloxacin	CIP	5	
Nalidixic acid	NAL	30	
Trimethoprim/Sulfamethoxazole	SXT	1.25/23.7	

AppendixVI:Probiotics

Probiotics	Source
Lactobacillus acidophillus	Nahrain university- biotechnology department
Lactobacillus casei	
Sachromyces cereviseae	
Sachromyces boulardi	

الخلاصة

هدفت هذه الدراسة الى تقدير تكرارية تواجد بكتريا السالمونيلا والعزلات متعددة المقاومة للأدوية (م م د) منها باعتبارها الممرض المسبب للأسهال في الأطفال دون سن الخامسة من العمر، بالأضافة الى دراسة دور اربع معالجات حيوية في السيطرة على مثل هذه الأصابة شملت هذه الدراسة جمع 128عينة برازلمرضى (76 ذكور و52 اناث) يمثلون اطفال بأعمار تراوحت من ستة ايام الى خمس سنوات ممن يعانون من الأسهال والحمى المراجعين لمستشفى الطفل المركزي في بغداد خلال الفترة من نيسان الى ايلول 2012. تمت دراسة التأثيرات المناعية للمعالجات الحيوية و بكتريا السالمونيلا م م د في الفئران المستحثة تجريبيا و المعاملة بالمعالجات الحيوية وذلك بتقدير مستوى عشرة حركيات خلويه (IL-4وIL-1βو10 IP وIFN-γو RANTES) وGM-CSFو IL-21 وIL-17A و IL-12 وIL-10و

في غسيل الأمعاء الدقيقة.

قسمت فئران التجارب المستخدمة الى خمس مجموعات: الأولى، ضمت فئران مجرعة بالمعالجات الحيوية لسبعة ايام متعاقبة ومعرضة للسالمونيلا في اليوم الثامن والتي تم تشريحها في اليومين 14 و 21. والمجموعة الثانية مشابهة للأولى ولكن باستمرار المعالجات الحيوية لمدة 14 يوما. واعطيت المجموعة الثالثة معالجات حيوية فقط، فيما عوملت المجموعة الرابعة بالسالمونيلا، اما الخامسة فاستخدمت كمعاملة سيطرة.يمكن تلخيص النتائج التي تم الحصول عليها في هذه الدراسة بالأتي :

1- من مجموع 128عينة براز اطفال، امكن عزل وتشخيص 9 (7.03%) عزلات تعود الى بكتريا السالمونيلا(*S.*Typhimurium). وباستثناء عزلة واحدة التي كانت مقاومتها معتدلة، فقد كانت جميع هذه العزلات مقاومة كليا للمضاد الحيوي حامض نالديسك. ومن جهة اخرى، قاومت عزلة واحدة (رمزها)Bثلاث مضادات (امبسيلين، اموكسيلين/كلافيولينك، حامض نالديكسك). لهذا السبب فقد تم اعتبار هذه العزلة متعددة المقاومة للأدوية وأختيارها في التجارب اللاحقة للدراسة.
2- لدى اخضاع العزلة Bلأختبار حساسيتها لأربعة معالجات حيوية (*Saccharomyces cerevisiae, S. boulardii, Lactobacillus acidophilus and L. casei*)، اظهرت النتائج ان العزلتين *S.cerevisiae* , *L.acidophilus* اعطت اوسع منطقتي منع نمو (12.6 ملم للخميرة و 16.3 ملم للبكتريا)، لذا فقد تمت دراسة تأثيريهما على بكتريا السالمونيلا في الزجاج وفي حيوانات التجارب.
3- وجد لدى استخدام رواشح هذين المعالجين الحيويين غير المركزة والمركزة لمرة ومرتين وثلاث مرات، ان الرواشح المركزة لثلاث كانت الأكفا في تأثيرها المضاد وذلك عندما بلغت منطقتي منع النمو (25 و31 ملم) بالتعاقب.
4- لوحظت زيادات معنوية بقيم معامل الكبد (Liver index) في الفئران المعاملة ببكتريا *L.acidophilus* فيالمجموعة 1 بعد 21 يوم (10.73%) مقارنة بنفس المجموعة المعاملة بخميرة *S.cerevisiae*(7.41%)او المجاميع الأربعة الأخرى. وبالنسبة للطحال، فقد كان قيم معامل الطحال للمحاميع المعاملة بالبكتريا اعلى من المعاملة بالخميرة. اما الفئران المعاملة بمعالج حيوي لوحده او بكلا

المعـالج الحيـوي مـع البكتريـا الممرضـة، فقد ازدادت هذه القيم معنويالكل المجـاميع باسـتثناء المجموعـة الخامسة.

5- اظهر كلا المعالجين الحيويين كفاءة في خفض اعداد بكتريا السالونيلا في الكبد والطحال. ففي الكبد، سجلت المجموعة الرابعة ما معدله 224.4 وحدة مكونة للمستعمرة للطبق والتي كانت اعلى معنويا من جميع المجاميع لكلا المعالجين الحيويين. وأعطت المجموعة الثانية نتائج افضل من المجموعة الأولى، فيما سجل اوطا عدد من الوحدات المكونة للمستعمرة بعد 21 يوم والتي بلغت 21.6 للبكتريا و 27.8 للخميرة.

6. اعطت السايتوكينات العشرة المستخدمة مستويات متباينة في غسيل الأمعاء الدقيقة وذلك اعتمادا على مجموعـة الفئـران التـي تضـمنتها الدراسـة ونـوع المعـالج الحيـوي. كمـا ولـوحظ تبـاين فـي النسـب الآتية:IFN-γ/IL-4 وIFN-γ/IL-10 وIL-4/IL-10 وIL-17A/IL-10 .

جمهورية العراق
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التأثيرات المعدلة-مناعيا للمعززات الحيويه ضد بكتريا *Salmonella enterica* Serovar *Typhimurium* المعزولة من حالات الإسهال لدى الأطفال في ذكور الفأر الابيض

أطروحة

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