Ain Shams University
Faculty of Pharmacy
Microbiology & Immunology dept.

"Isolation and Characterization of Some Potential Probiotic Candidates for the Control of *Clostridium difficile* Infection"

A Thesis

Submitted in Partial Fulfillment of the Requirements for the

Master degree

In Pharmaceutical Sciences
(Microbiology and Immunology)

By
May Mohamed Awad Bahr
Bachelor of Pharmaceutical Sciences
Faculty of Pharmacy, Ain Shams University, 2010

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May Bahr

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LIST OF ABBREVIATIONS

AAD	Antibiotic associated diarrhea
A. fecalis	*Alcaligenes fecalis*
API	Analytical profile index
ATCC	American Type Culture Collection
B. bifidium	*Bifidobacterium bifidium*
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BP	Base Pair
B. subtilis	*Bacillus subtilis*
Caco-2	Colon adenocarcinoma cells
CDAD	*Clostridium difficile* associated diarrhea
CDI	*Clostridium difficile* infection
C. difficile	*Clostridium difficile*
CFU	Colony Forming Unit
CLSI	Clinical and laboratory standards institute
Co.	Company
DMSO	Di methyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
E. faecium	*Enterococcus faecium*
E. fecalis	*Enterococcus fecalis*
ELISA	Enzyme linked imuunosorbent assay
FAO	Food and agriculture organization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FMT	Fecal microbiota transplant
GIT	Gastro intestinal track
GRAS	Generally recognized as safe
h	hours
IC 50	Inhibitory concentration 50
Ig	Immunoglobulin
IL	Interleukin
K. pneumoniae	*Klebsiella pneumoniae*
L. acidophilus	*Lactobacillus acidophilus*

L. delbrueckii	*Lactobacillus delbrueckii*
L. rhamnosus	*Lactobacillus rhamnosus*
LAB	Lactic acid bacteria
LB	Lurai Bertani
M. luteus	*Micrococcus luteus*
MRS	Man Rogosa Sharpe
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
NRRL	The Northern Regional Research Laboratory
OD	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
q PCR	Quantitative polymerase chain reaction
QPS	Qualified presumption of safety
RNA	Ribonucleic acid
RPMI	Roswell Park memorial Institute Medium
rRNA	Ribsomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
SCFA	Short chain fatty acid
SD	Standard deviation
sIg	Secretory immunoglobulin
S. thermophiles	*Streptococcus thermophiles*
TAE	Tris acetic ethylene diamine tetra acetic acid
TLRs	Toll like receptors
TNF	Tumor necrosing factor
U.K	United Kingdom
U.S.A	United states of America
WHO	World Health Organization

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ABSTRACT

This study is aimed at the isolation, identification, and characterization of potential probiotic strains capable of inhibiting *C. difficile* infection in *vitro* and in *vivo*.

Twenty isolates were isolated from breast fed infant fecal samples and screened against *C. difficile* using their cell-free supernatant. Only three isolates showed maximum inhibition from 56.05% to 60.60% thus they were characterized for probiotic properties and safety. The results revealed their tolerance to the GIT conditions and safety profile. They were identified by sequencing 16S rRNA as *E. faecalis* NM815, *E. faecalis* NM915 and *E. faecium* NM1015. Their sequences were submitted to GenBank as KU365166, KU365167 and KU365168 respectively.

For in *vivo* evaluation, a viable mixture of the three strains (10^9 CFU/ml) was administrated to group of mice (treated group) in daily dose for 14 days, then followed by a challenge with viable *C. difficile* (10^5 CFU/ml) in daily dose for 7 days, then a second administration of a viable mixture of the three strains was done daily for 10 days. For control two mice groups were used; control group which was administrated PBS only, untreated group which received PBS instead of the probiotic mixture before and after the challenge with *C. difficile*. The results obtained from histological analysis in addition to assessment of the toxin A and toxin B gene copies within the mice fecal samples from each group, confirmed the effectiveness of the three potential probiotic strains which expressed as inhibition of *C. difficile*, reduction in the toxin A and toxin B gene copies and maintenance of the structural integrity of the examined intestinal and liver cells compared to the untreated groups.

INTRODUCTION

Probiotics are defined as 'live micro-organisms, which when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Various bacterial species have been identified as probiotics according to their beneficial effects and contributions, including the most familiar two genera *Lactobacillus* and *Bifidobacterium* in addition to some species of *Enterococcus* and yeast especially *Saccharomyces boulardii*. The research on this field had a clear conclusion confirmed that the health benefits conferred by any probiotic bacterium are strain specific and cannot be global to other strains from the same species. The individual strains possess different specific abilities and characteristics thus make the differentiation and specificity for each strain. For example, *L. rhamnosus* GG is a specific bacterial strain which demonstrates a probiotic effect in the prevention of AAD (McFarland, 2006), other strain of *L. rhamnosus* species may not have this effect, and likewise other species in the genus of *Lactobacillus* may not act as probiotics. This is because individual strains exhibit different specific characteristics (Jacobsen, *et al*., 1999).

C. difficile infection (CDI) is considered a dominant health problem in hospitals causing an acquired diarrhea in adults (Bauer, *et al*., 2009). The consequence of CDI varies from mild diarrhea to fulminant colitis, toxic mega colon and death. *C. difficile* showed colonization in low percentage in healthy adults up to 5% (Parkes, *et al*. 2009; Hautmann, *et al*. 2011; Hell, *et al*. 2012; Moudgal and Sobel, 2012), while in patients at hospital, the colonization reached high percentage up to 39% (Hickson, 2011; McFarland, 2011). It is recognized as the major antibiotic associated infection which causes morbidity and increases health care overheads. Antibiotics cause disorder of the original gut microbiota and generate other conditions within the intestine that stimulates circumstances for *C. difficile* from spore germination, vegetative growth and toxin production, causing epithelial damage and colitis. Several studies and scientific reports demonstrated the possible ability of indigenous microbiota to inhibit growth and persistence of *C. difficile*. Although the specific mechanisms

of these processes are not known, they are likely to interfere with key aspects of the pathogen's physiology, including spore germination and competitive growth. Increasing our understanding of how the intestinal microbiota manages *C. difficile* could lead to better means of controlling this important nosocomial pathogen.

Aim of this study includes isolation and characterization of potential probiotic strains that able to adhere to the intestinal mucosa and control *C. difficile* pathogenesis. This is a principle of using probiotics for therapeutic and prophylactic manipulation of the intestinal pathogens such as *C. difficile*.

Aim of the work:

Protocol of the present study includes the following:

1. Isolation of Gram positive isolates from infants' fecal samples.
2. In *vitro* evaluation of probiotic isolates against *C. difficile* pathogen.
3. Characterization of the probiotic properties of the promising probiotic isolates.
4. Molecular identification of the promising probiotic isolates.
5. Evaluation of the safety of the selected probiotic isolates.
6. In *vivo* evaluation of the promising probiotic isolates.

LITERATURE REVIEW

1. Probiotics

1.1. Definition

The term "Probiotic" is elaborated from Greek language which means “for life” and it is opposite to antibiotic "for death". Probiotics are defined as the live micro-organisms that when administrated exert a health benefit on the human body (FAO / WHO, 2001); in addition there are certain principles for probiotics which showed to be accomplished.

1.2. History

The discovery of beneficial microbes or probiotics refers to the olden days in 1907, when the Russian Nobel laureate Elie Metchnikoff mentioned his idea that ingestion of certain beneficial microbes could benefit human health and he endorsed the longevity of Bulgarian peasants to their yogurt drinking. Then Metchnikoff established a theory that aging is begun by toxic bacteria in the gut and that lactic acid resulted from yogurt bacteria possibly would prolong life. He supported the potential life lengthening properties of lactic acid bacteria in particular *L. delbrueckii subsp. Bulgaricus* (Podolsky, 2012; Mackowiak, 2013). Metchnikoff explained that aging process could be due to putrefaction in the large intestine and he suggested restoring this problem by replacing the proteolytic micro-organisms in the colon with saccharolytic strains that produced lactic acid (Hamilton-Miller, 2008). In this way, the gut environment becomes more favorable to the growth of beneficial micro-organisms and less favorable to harmful micro-organisms.

1.3. Probiotic Species

The common bacterial genera that are known as probiotics are Lactic Acid Bacteria and bifidobacteria. LAB group are generally recognized as safe (GRAS) status and approved by the qualified presumption of safety (QPS) for food production and human consumption (Leuschner, *et al.*, 2010). Furthermore they have a long history in

manufacturing food and dairy products. LAB members are classified as probiotics among species of *Lactobacillus*, *Enterococcus* and *Streptococcus*. In addition to these common genera, there are other genus members which are proved as probiotic such as: strains belonging to *Propionibacterium*, *Bacillus* and *Escherichia coli*. Examples of commercial probiotic products in the market are listed in Table (1).

Table (1): Examples of commercial probiotic products in the market

Probiotic product	Manufacturer	Strains
1. Accuflora	Northwest Natural Products	*L. acidophilus*, *L. rhamnosus*, *B. bifidum*, *L. salivarius*, *S. thermophiles*
2. Acidophilus Complex	Puritan's Pride	*L. acidophilus*, *B. bifidum*
3. Acidophilus XTRA	Sundown Naturals	*L. acidophilus*, *B. lactis*, *L. bulgaricus*, *S. thermophiles*
4. ACTIFlora	Kendy	*L. bulgaricus*, *L. acididophilus*, *B. ssp*, *S. thermophiles*
5. Active Balance High Potency Probiotic	Active Balance	*L. acidophilus*, *B. bifidum*
6. Adult Probiotic	CVS Pharmacy	*B. breve*, *B. longum*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. plantarum*, *L. lactis*, *S. thermophiles*
7. Advanced Acidophilus Plus	Solgar	*L. acidophilus*, *B. lactis*

1.3.1. *Enterococcus* genus

The genus *Enterococcus* consists of non-spore forming, Gram positive cocci bacteria which are common commensal inhabitants of the gut microbiota in human and animal, in addition to their impact in the food and cheese industries, such as black pickled olives (Franz, *et al*., 1996) and specific type of cheeses (Coppola, *et al*., 1990; Litopoulou-

Tzanetaki and Tzanetakis, 1992; Olasupo, *et al*., 1994; Giraffa, *et al*., 1995). They are also known for production of a wide group of bacteriocins (enterocins), a family of ribosomally synthesized antimicrobial peptides and proteins with potential antibacterial activity against food borne pathogenic bacteria so they became attractive compounds in the food and dairy industries. They are widely used as supplementary starter cultures for the bio-preservation of vegetables and cheeses (Nunez, *et al*., 1997; Sarantinopoulos, *et al*., 2002; Moreno, *et al*., 2003).

Enterococci are proved as probiotics (Holzapfel, *et al*., 1998) such as, the *E. faecium* SF 68 which has been investigated and established for the cure of antibiotic associated diarrhea (Marteau, *et al*., 2001) and modifies the immune responses (Sun, *et al*., 2010; Bybee, *et al*., 2011). In addition, *E. faecium* MMRA recognized as enterocin producer with a strong activity against the pathogenic *Listeria* (Rehaiem, *et al*., 2010). Actually, there are some strains presently in usage as therapeutic treatments such as: 1- Cylactins (Hoffmann-La Roche, Basel, Switzerland) for antibiotic associated diarrhea, 2- Fargo 688s (Quest International, Naarden, The Netherlands) to alleviate the symptoms of irritable bowel syndrome, 3- Ecoflor (Walthers Health Care, DenHaag, The Netherlands) for diarrhea, 4- Symbioflor 1 (Symbio Pharm, Herborn, Germany) for bronchitis (Moreno, *et al*., 2006).

1.3.1.1. Safety of *Enterococcus* members

Even though *Enterococcus* species are members in LAB and included in dairy and food fermentation, in addition to arise of many strains as probiotics, many researches have observed that some *Enterococcus* could contain virulence genes (Weckx, *et al*., 2010; Leisner, *et al*., 2012). In fact, such species do not own strong virulence factors or toxins, but they may have some structural and metabolic traits in addition to multiple antibiotic resistances, which could be considered as virulence factors from the view of some researchers (Cebrián, *et al*., 2012). The existence of one or more virulence determinants does not certainly sort a strain pathogenic (Frans, *et al*., 2011).

Enterococci are well known for their capability to exchange genetic information by conjugation (Clewell, 1990), and this exchange is recognized to occur in the gastrointestinal tract (Huycke, *et al*., 1992). Transmissible plasmids carrying antibiotic resistance, virulence factors such as hemolysin - cytolysin production and the capacity for