Isolation & Characterization of Probiotic Bacteria To Fight Against Human Intestinal Infections

Dissertation submitted to Acharya Nagarjuna University in partial fulfillment of requirements for the award of the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted

by

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DECLARATION

I declare that the present work entitled **"Isolation & Characterization of Probiotic Bacteria To Fight Against Human Intestinal Infections** is a bonofide record of the research work carried out by me and no part of the discussion has been presented earlier for any degree, diploma and any other similar title.

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CERTIFICATE

This is to certify that the project work entitled **"Isolation &characterization of Probiotic Bacteria To Fight Against Human Intestinal Infections"** submitted by MARWAH ALI OUDAH for the degree of master of science in Biotechnology to Acharya Nagarjuna University,Nagarjuna Nagar, is a record of the bonfide project work actually carried out by her under my guidance in the Biotechnology department of A.N.U, NAGARJUNA NAGAR.The result embodied in this project have not been submitted for any other degree or diploma to any other University or institution.

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PREFACE

Probiotics are microorganisms that are believed to provide health benefits when consumed. The term probiotic is currently used to name ingested microorganisms associated with benefits for humans and animals. The term came into more common use after 1980. The introduction of the concept is generally attributed to Nobel laureate Élie Metchnikoff, who postulated that yogurt-consuming Bulgarian peasants lived longer lives because of this custom. He suggested in 1907 that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes. A significant expansion of the potential market for probiotics has led to higher requirements for scientific substantiation of putative benefits conferred by the microorganisms.

Some strains of lactic acid bacteria (LAB) may affect pathogens by means of competitive inhibition (i.e., by competing for growth) and some evidence suggests they may improve immune function by increasing the number of IgA-producing plasma cells and increasing or improving phagocytosis, as well as increasing the proportion of T lymphocytes and natural killer cells. Clinical trials have demonstrated that probiotics may decrease the incidence of respiratory-tract infections and dental caries in children. LAB products might aid in the treatment of acute diarrhea, and possibly affect rotavirus infections in children and travelers' diarrhea in adults, but no products are approved for such indications.

ABSTRACT

There is an ever-increasing interest in the field of characterization of new probiotics with possible purpose in health care and disease prevention. The aim of this study is to isolate and identify bacteria in human milk to analyze its possible probiotic potential. We isolated and identified five strains of bacteria in human milk. These strains were selected to evaluate their ability to survive *in vitro* simulated conditions of gastrointestinal stress, the antimicrobial effect, adhesion capacity and resistance to different pHs and temperatures. The results showed that three of the five selected strains, identified as *Lactobacillus reuteri, Ascococcus mesenteroides,* (Tsenkovskii 1878) and *Lactobacillus bulgaricus* GLB44, were resistant to digestive enzymes, showed resistance to low pH values (2.7 and 3.8) having adhesion capacity and viability at temperatures of 47 °C. Therefore, these bacteria may could be considered as potential probiotics for the pharmaceutical and food industry.

Keywords: Probiotics, isolation, human milk, *Lactobacillus reuteri,Ascococcus mesenteroides.*

Human breast milk consists of high volumes of essential nutrients for infants, including carbohydrates, essential fatty acids, proteins, vitamins and minerals, due to this it is recognized as the gold standard of infant feeding (Sherman). Breast milk plays an important role in supporting the survival and development of infants not just because of nutrient supply but due to transfer of microflora originated in breast milk. Several researches reported that human breast milk contains a wide spectrum of indigestible nutrients that are not utilized by the infants but put forth numerous potent bioactive functions on establishment of infants' native microflora .More than 200 different species are described in human milk. Breast milk has been shown to be a continuous source of commensal, mutualistic or probiotic bacteria to the infant gut, including staphylococci, streptococci, bifidobacteria, and, lactic acid bacteria. The genera Lactobacillus, Pediococcus and Lactococcus belong to the lactic acid bacteria (LAB), and the strains of these genera are frequently used on a large scale in the production and protection of many foods or as probiotics for humans and animals. LAB with probiotic activity are generally enteric flora and are believed to play a advantageous role in the ecosystem of the human gastrointestinal (GI) tract. There are many definitions of probiotics, but the prevailing one is that adopted according to an international scientific consensus in 2014 by the WHO and FAO. It states that probiotics are live

microorganisms which, when they are administered in sufficient amounts, provide anadvantage to the health of consumers.

Certain quality requirements have been established in order to ensure competence, efficiency and advantage to the host from those microorganisms, including among those features are non pathogenic or have toxic effects, contact stability and bile acid and adhesion to the intestinal mucosa . Probiotics may be one of the most effective therapies for the prevention of several diseases in the new born. At birth, an infant's gastrointestinal tract is germ-free and colonization of the gastrointestinal tract starts immediately after birth with the initiation of feedings and is well established within the first few days of life. It have been considered that in breastfed infants, bacteria of the genus Bifidobacterium and Lactobacillus predominate, with other enteric organisms being present less frequently. Conversely, in formula-fed infants, it have been reported that coliforms, enterococci, and bacteroides predominaely colonize the intestinal tract. Moreover, preterm infants are particularly susceptible to abnormal colonization. A combination of antibiotic use, delayed initiation of feedings, and exposure to the unusual microorganisms that populate the neonatal intensive care unit may lead to abnormal patterns of colonization. Gewolb et al, reported that the gastrointestinal tract of extremely low birth weight infants is colonized by fewer than 3

bacterial species by the tenth day of life and that species of Bifidobacterium and lactobacillus could be found in the stool of fewer than 5% of patients studied within the first month of life. Feeding oral probiotic bacteria may be an effective way to change this pattern of colonization. On the other hand, it has been postulated that introducing probiotics to preterm infants might be beneficial to avoid overgrowth of pathogenic organisms. Probiotic supplementation has been proposed to enhance feeding and prevent diseases and nosocomial infections in preterm infants. The proposed beneficial effects of probiotic administration come from potentially competing with other organisms for binding sites and substrate in the bowel, which increases the production of antiinflammatory cytokines, decreases the production of proinflammatory cytokines, reduces intestinal permeability, and enhances enteral nutrition On the other hand, there is increasing interest in some lactic acid bacteria which are considered as potentially probiotic species present in breast milk such as L. gasseri, L. salivarius, L. rhamnosus, L. plantarum and L. fermentum.

It has been shown that human milk from healthy women contains approximately 103-104 CFU/mL representing a continuous source of potential commensal bacteria for the infant, and that some of the lactic acid bacteria strains isolated from this biological fluid have the ability to inhibit the growth of a wide spectrum of pathogenic bacteria by competitive exclusionor through the

production of antimicrobial compounds such as bacteriocins, organic acids or hydrogen peroxide. Therefore, the aim of this work was to isolate and identify bacteria in human colostrum and mature milk to analyze its possible probiotic potential.

Lactobacillus reuteri is a Gram-positive bacterium that naturally inhabits the gut of mammals and birds. First described in the early 1980s, some strains of L. reuteri are used as probiotics.

Though the species Lactobacillus reuteri has been recognized for some time, knowledge of its probiotic properties did not come until much later.As early as the turn of the 20th century, L. reuteri was recorded in scientific classifications of lactic acid bacteria, though at this time it was mistakenly grouped as a member of Lactobacillus fermentum. In the 1960s, further work by German microbiologist Gerhard Reuter – for whom the species eventually would be named – began to distinguish L. reuteri from L. fermentum. Reuter reclassified the species as "Lactobacillus fermentum biotype II". L. reuteri was eventually identified as a distinct species in 1980 by Kandler et al. This group found significant differences between L. reuteri and other biotypes of L. fermentum, and thus proposed it be given formal species identity. They chose the species name "reuteri", after discoverer Gerhard Reuter, and L. reuteri has since been recognized as a separate species within the Lactobacillus genus.

In the early 1980s, shortly after its recognition as a distinct species, scientists began to find L. reuteri in many natural environments; it has been isolated from many foods, especially meat and milk products. Interest in L. reuteri began to increase as scientists began to find it colonizing the intestines of healthy animals. Gerhard Reuter first isolated L. reuteri from human fecal and intestinal samples in the 1960s, and this work was later repeated by other researchers. The same experiments – attempting to isolate L. reuteri from feces and intestine of healthy animals – were also done for nonhuman species, proving that L. reuteri seems to be present almost universally throughout the animal kingdom. For example, L. reuteri was discovered to be present naturally in the intestines of healthy sheep, chickens, pigs, and rodents. Furthermore, a study searching for 18 major species of gut flora, including Lactobacillus acidophilus, in a variety of animals found L. reuteri was the only bacterium to constitute a "major component" of the Lactobacillus species present in the gut of each of the host animals tested. It is now well-established as one of the most ubiquitous members of the naturally occurring gut bacteria.In a related discovery, each animal host seems to have a host-specific strain of L. reuteri, e.g. a rat strain for rats, a pig strain for pigs, etc. The universality of L. reuteri, in conjunction with this evolved host-specificity, has led scientists to make inferences about its importance in promoting the health of the host organism.

In the late 1980s, Walter Dobrogosz, Ivan Casas, and their colleagues discovered L. reuteri produced a novel broad-spectrum antibiotic substance via the organism's fermentation of glycerol. They named this substance reuterin, also after Gerhard Reuter. Reuterin is a multiple-compound dynamic equilibrium (HPA system, HPA) consisting of 3-hydroxypropionaldehyde, its hydrate, and its dimer. At concentrations above 1.4 M, the HPA dimer was predominant. However, at concentrations relevant for biological systems, HPA hydrate was the most abundant, followed by the aldehyde form. Reuterin was found to inhibit the growth of some harmful Gram-negative and Gram-positive bacteria, along with yeasts, fungi, and protozoa. Naturally, a gut organism capable of fighting off other, harmful gut organisms was of great interest. Researchers found L. reuteri can indeed secrete sufficient amounts of reuterin to cause the desired antimicrobial effects. Furthermore, since about four to five times the amount of reuterin is needed to kill "good" gut bacteria (i.e. L. reuteri and other Lactobacillus species) as "bad", this would allow L. reuteri to remove gut invaders while keeping normal gut flora intact.

One of the most well-documented effects of L. reuteri is in the treatment of diarrheal diseases in children, where it has been shown to significantly decrease the duration of symptoms. Treatment of rotaviral diarrhea by consumption of L. reuteri significantly shortens the duration of the illness

as compared to placebo. Furthermore, this effect is dose-dependent: the more L. reuteri consumed, the faster the diarrhea stops. L. reuteri is also effective as a prophylactic for this illness; children fed it while healthy are less likely to fall ill with diarrhea in the first place. With regard to prevention of gut infections, comparative research has found L. reuteri to be more potent than other probiotic organisms. It has also been found in animal research to reduce motor complexes and thus intestinal motility. L. reuteri may be effective in the treatment of necrotizing enterocolitis in pre-term infants. Meta-analysis of several randomized studies suggests that L. reuteri can reduce the incidence of sepsis and shorten the required duration of hospital treatment in this population. L. reuteri is also an effective treatment against infant colic. Studies suggest that colicky infants treated with L. reuteri experience a reduction in time spent crying compared to those treated with simethicone, or to those receiving placebo. However, colic is still poorly understood, and it is not clear why or how L. reuteri ameliorates its symptoms. One theory of colic, though, holds that affected infants cry because of severe gastrointestinal discomfort; if this is indeed the case, it is quite plausible that L. reuteri somehow acts to lessen this discomfort, since its primary residence is inside the gut.Growing evidence indicates L. reuteri is capable of fighting the gut pathogen Helicobacter pylori, which causes peptic ulcers and is endemic in parts of the developing world.