Screening, Isolation, Characterization and Evaluation of Safety of Potential Probiotic Strains Isolated from Indigenous Rice Sources.

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DECLARATION

I, **FATIMA KHAN**, declare that all work presented in this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been mentioned in the thesis. The work herein was carried out while I was postgraduate student at Atta-ur-Rahman school of Applied Biosciences,NUST under the supervision of Dr. Rumeza Hanif.

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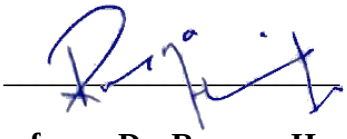
MS Thesis Work

Certificate

This is to certify that work in this dissertation has been carried out by Miss Fatima Khan and completed under my supervision in Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), H-12, Islamabad, Pakistan.

Supervisor: 

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Dedication

To my Father, who was a constant support to me throughout my studies and was always encouraging me to work hard to achieve my goals.

And Mother, who stood by me through all my hardships and because of her prayers I am able to accomplish my task work.

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The aforesaid project has been reviewed by Institutional Review Board (IRB) Committee, ASAB, keeping in view the following selection criteria:

- Qualification, Expertise and Scientific Caliber of the Principal Investigators
- Proposed Goals of the Study
- Subject Selection
- Selection Criteria of Subjects
- Informed Consent Process
- Potential Problems
- Research Design and Methods
- Potential Benefits of the Study
- Risks of the Study
- Management of Risks
- Assessment of Risk
- Confidentiality
- Conflict of Interest

The committee thus **APPROVES** the project on "**Safety Assessment of Probiotics on Mice models**" on the scales and criterion set by IRB.

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Abbreviations

AAD	Antibiotic Associated Diarrhea
CTX-30	Cefotaxime-30 µg
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GIT	Gastrointestinal Tract
GRAS	Generally Recognized as Safe.
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IL-6	Interleukin 6
LAB	Lactic Acid Bacteria
MRS Agar	de Man Rogosa Sharpe Agar
PBS	Phosphate Buffer Saline
SS Agar	Salmonella Shigella Agar
STEC	Shiga Toxin *Escherichia coli*
TGC-15	Tigecycline 15µg
WHO	World Health Organization

Abstract

Probiotics are living microorganisms which can change the microbiota of the host and ultimately have beneficial effects on the health of the host when present in significant numbers. Their applications are very diverse as they are used in foods, for the treatment and prevention of many diseases, maintaining the health of an individual and currently their applications as therapeutics are being discovered. The aim of my study was to isolate potential probiotics from indigenous sources, evaluate their characteristics and perform their safety assessment. Different varieties of indigenous rice were taken as source for the isolation of bacteria. Identification of the isolated strains was performed by various biochemical tests and 16S rRNA sequencing. The safety of isolated strains was assessed on wistar rats for 10 weeks including survival, adhesion, and colonization of the strains in gastrointestinal tract of rats. Antibacterial activity of isolated strains against five pathogenic strains namely Shiga toxin producing *Escherichia coli, Enterococcus faecalis, Salmonella enterica, Pseudomonas aeruginosa* and *Klebsiella Pneumoniae* was performed by well diffusion assay. Three strains were isolated whose biochemical testing and sequencing results revealed they were *Enterococcus faecium.* No significant antagonism was seen against *K. Pneumoniae* and *P. aeruginosa* whereas significant results were obtained against *STEC, E. faecalis* and *S. enterica*. Strains survived the GIT tract as they were re-isolated as rifampicin resistant colonies in the fecal samples of rats. They were able to adhere and colonize small intestine; large intestine and caecum of rats. The rats were gaining weight and no symptoms of disease were shown ensuring the safety of the strains. The results of the study suggest that these *E. faecium* strains can be used as potential probiotic strains.

1. Introduction:

The intestinal microflora of all the living organisms is composed of two types of bacteria; pathogenic and non-pathogenic. Some of the non-pathogenic bacteria have useful, beneficial effects on the host organism and are known as probiotics. Probiotics, as defined by Schrezenmeir and Vrese are, “living microorganisms present in sufficient numbers which can change the microbiota of the host and ultimately have beneficial effects on the health of the host” (Schrezenmeir & de Vrese, 2001). They beneficially influence human health. The useful physiologic effects are conferred to the host by microbial action. They particularly improve the intestinal microbial balance.

The common sources of probiotics are fermented food products like pickles, sauerkraut, miso, kimchi and particularly the dairy products like yogurt, kefir, fermented milk etc. They can also be obtained from the gut of different organisms, breast milk, fresh meat and fruits (Fontana, et al. 2013). Probiotics include different types of microorganisms like lactic acid bacteria, yeasts, fungi and *Bacillus* species. For an organism to be identified as a potential probiotic, it must have the following characteristics: (i) it should be safe and non-pathogenic (ii) should have beneficial effects on humans (iii) should remain viable throughout gastrointestinal tract (GIT) (iv) should have antibacterial activities against pathogens (v) should adhere to and colonize the intestinal walls and (vi) should maintain the microbial balance in the lumen of the host exerting beneficial effects. (Parvez *et al.*, 2006).

In the 20th and 21st century, many considerations have been given to the advantageous effects of probiotics. Their uses in different diseases have been identified and their additional benefits have been recognized. The positive effects they have on the overall health of an organism are numerous but the most prominent ones are listed below (Parvez, *et al*. 2006):

- Enhance the immune system
- Improve intestinal health
- Decrease the severity of certain intestinal diseases
- Help reduce lactose intolerance
- Decrease the effect of allergy
- Reduce the possibility of developing certain cancers, particularly colon cancer
- Synthesize and make nutrients bioavailable.

The exact mechanism of how probiotics exert these effects are mostly unknown but there are many propositions regarding it. For every effect, a different suggestion and explanation is given for example they release certain enzymes and vitamins in the intestine. The enzymes released hydrolyze certain proteins and fats making the nutrients bioavailable.

The applications of probiotics are very diverse., for instance they are used for the treatment of simple to complex diseases such as diarrhea and cancers, respectively. They are used for the treatment as well as for the prevention of many diseases. They provide protection against intestinal infections by forming a barrier on the intestinal walls and releasing toxins thus killing pathogenic bacteria (Song, et al. 2012). They

release and regulate inflammatory mediators like cytokines hence are used to treat inflammation (Isolauri, et al. 2001). They improve gut mucosal dysfunction, gut mucosal barrier and release anti-inflammatory cytokines therefore are used to treat hypersensitivity and allergic reactions (Ouwehand, et al. 2002). In lactose intolerant people they have shown to reduce the effects of the disease by secreting enzyme β-galactosidase (Ouwehand, et al. 2002). Their roles have also been highlighted in gastroenteritis and inflammatory bowel disease. Several studies have shown their antitumor activities as well (Perdigón, & Alvarez, 1992).

The applications of probiotics are not only confined to humans but they are also used for animals and plants. In animals, they are used in veterinary medicine and fortified feed. While in plants they are used as biofertilizers and biocontrol agents to improve plant growth and eradicate plant pathogens. Probiotics are also used in making functional foods that have added health benefits. So, the utilization of probiotics can be seen in all walks of life i.e from use in everyday life to their employment in industry.

The antimicrobial activity of probiotics against various food-borne pathogens has been reported in numerous studies. This property, as mentioned previously, is one of the main characteristics of a strain to be declared as a probiotic. The antimicrobial effect of the strain is due to the release of some toxins, organic acids like lactic acid, bacteriocins, H_2O_2, and CO_2 etc. Due to the frequent use of antibiotics a problem of antibiotic resistance emerged. The antimicrobial activity of probiotics provides a solution to the problem by treating many kinds of diarrhea associated with antibiotic resistant bacteria in an efficient and cost-effective manner (Suskovic, et al. 2010).

Probiotics are generally safe to use, have been used in fermented products throughout history. The safety of probiotics has been demonstrated in studies in which controlled clinical trials were performed involving different strains (Snydman, 2008). Lactic acid bacteria (LAB) have a long history of consumption. Among members of LAB, *Lactobacillus* and *Lactococcus* are generally regarded as safe while some other members of LAB including some species of *Streptococcus* and *Enterococcus* are opportunistic parasites. These opportunistic parasites are sensitive to many commonly used antibiotics and can easily be removed. The safety of newly discovered strains should, however, be carefully verified by performing experiments on animal models and if possible, clinical trials should also be performed. Their safety and efficacy should be prudently assessed before incorporating them into the pharmaceutical products (Salminen, et al. 1998).

Probiotics is a term under the umbrella of which many kinds of microorganisms come. Each organism belongs to a different species and each species further contains different strains. The properties and activities of probiotics are strain-specific. Each and every strain of different species have different characteristics (Luyer *et al*. 2005; Canani *et al*. 2007;Kekkonen *et al*. 2008). The efficacy of each strain can vary so a property of one strain cannot be directly applied to the other. However, most probiotics share some common applications like the treatment of diarrhea and improvement of the health condition in irritable bowel syndrome (Weichselbaum, 2009).

These interesting strain-specific properties of probiotics make research in this field more fascinating. This has led researchers to an assumption that there may be many

unidentified strains present in nature with undiscovered potential. Keeping this in mind the present study intends to:

- Screen, isolate and characterize probiotic strains from indigenous sources.
- Assess their ability to remain viable through GIT tract and colonize in rat models.
- Assess the safety of the probiotics in *in vivo* model and their antibiotic susceptibility testing.
- Examine the antimicrobial activity of cell free supernatant of isolated probiotics against pathogens like *Shiga toxin-producing Escherichia coli, Enterococcus faecalis, Salmonella enterica, Pseudomonas aeruginosa,* and *Klebsiella Pneumoniae.*

2. Literature Review

Human gastrointestinal tract (GIT) has a microbiota that plays an important role in the overall health of the individual. There are more than 500 different species of bacteria present in the distal ileum. The population of the bacteria in the GIT increases from stomach to the colon thus colon having the largest numbers of bacteria which make up to 35-50% of the volume of the constituents present in the colon (Isolauri *et al.*, 2004).

The bacteria present in the GIT are either indigenous or transient. The indigenous ones contain certain types of bacteria that have growth promoting, protective and advantageous effects on the host health. These bacteria are known as Probiotics. Probiotics are living organisms that have beneficial effects on the host and restore the host's microbial balance in the intestines when administered in adequate amounts (Schrezenmeir & de Vrese, 2001). Probiotics play a significant role in improving the functions of digestive, respiratory and immunological systems. They also help in alleviating symptoms of many diseases and prevent other diseases from occurring (fao.org, 2017).

2.1. History of Probiotics

There is sufficient evidence supporting the fact that probiotics has been consumed throughout history. The oldest indication can be seen in the Persian version of the Old Testament (Genesis 18:8) which states that Abraham lived a long and healthy life due to drinking of sour milk (fermented milk). Their uses have also been observed in Roman

history, they used to prescribe fermented milk for the treatment of gastroenteritis (Schrezenmeir & Vrese, 2001).

Probiotics have been used for thousands of years in the form of various fermented products. People used to consume these fermented products to get benefits from them. One of the earliest civilization of Greco-Latin considered their diet and nutrition an important and essential part of natural medicine. So, the consumption of fibrous and functional foods was common (Tomasik & Tomasik, 2003). Consumption of buttermilk, fermented milk products, and yogurt as a valuable food has been seen throughout centuries. But it was only after the work of Metchnikoff that the reason behind the advantages of fermented food was recognized as probiotics (Otles *et al.*, 2003).

However, the grandfather of probiotics is Elie Metchnikoff (Behnsen, et al. 2013). He was a professor at Paster Institute, Paris, France. He studied about the apparent long life of Bulgarian peasants and hypothesized that it is because they consume fermented milk products which contain useful microorganisms like lactic acid bacteria. Lactic acid bacteria then prevent the harmful bacteria to release toxins and thus causing diseases (Singh *et al.,* 2011). He introduced this concept in 1908 and is consequently known as the founder of probiotics and probiosis. Probiosis is a phenomenon which explains that the consumption of fermented products containing cultures of live microorganisms will result in the positive beneficial effects on the equilibrium of intestinal microbiota (Tomasik, & Tomasik, 2003).

After the death of Metchnikoff, barely any attention was given to probiotics until after the second world war, when antibiotics were discovered. The main reason for the revival of interest in probiotics was that scientists wanted improved techniques to rear germ-free animals. In the 1950s the importance of gut microflora was realized, probiotics were given their due consideration and their beneficial effects were acknowledged. Thereafter the focus on the applications and uses of probiotics increased (Fuller, 2012).

2.2. Sources of Probiotics:

Generally, the fermented food products are considered as the rich source of probiotics. These foods include yogurt, cheese, fermented milk, sauerkraut, kefir, miso, kombucha, pickles and other fermented vegetables and olives (Borriello *et al.*, 2003). Traditionally fermented milk and milk products were considered as the best source of probiotics and they were used for the isolation of many probiotics (Fontana *et al.*, 2013). In certain regions of Africa and Mongolia, the milk was fermented to obtain its beneficial effects. In ancient Roman civilization, fermented milk was used for the treatment of gastric diseases (Schrezenmeir & Vrese, 2001). Many studies have been conducted on the composition of microorganisms present in the fermented milk products. It is largely composed of different species of LAB (Lactic Acid Bacteria). Fermented vegetables and pickles, also normally contain Bifidobacterium and LAB species (Fontana *et al.*, 2013).

Other than fermented products, probiotics can be obtained from breast milk, directly from the gut of animals, fresh fruits, vegetables and cereals. Fresh fruits, vegetables and

cereals have less quantities of probiotic bacteria until they are fermented, in that case the number of probiotic bacteria increase. Whereas the gut of animals proves to be a rich source of probiotic organisms as that is extensively inhabited by these growth-promoting bacteria. Therefore, a wide variety of microorganisms can be obtained from them. Probiotics can be isolated from any type of organism like in a recent study *Lactobacillus johnsonii* CRL 1647 was isolated from bee gut (Audisio & Benitez-Ahrendts, 2011). They have also been isolated from the gut and faeces of pigs, cattle, farm animals and poultry. Furthermore, probiotics are also isolated from different types of fish and shrimps (Fontana *et al.*, 2013).

Many fermented products in Asia are used for their beneficial properties along with the added benefit of long-term preservation caused by the addition of lactic acid bacteria (Swain *et al.*, 2014). Some of these fermented foods include pickles, gundruk, sinki, sauerkraut, kimchi, goyang etc. In a study, LAB were isolated from vegetables and fermented foods in India and their exopolysaccharide producing ability was checked (Patel *et al.*, 2014). In another study on the same food products, six *Weissella* strains were isolated (Patel *et al.*, 2013). 13 *Lactobacillus plantarum* species were isolated from fermented vegetables and were tested for their potential probiotic ability (Lapsiri, Nitisinprasert, & Wanchaitanawong. 2011). Similarly, many scientists have isolated various strains from many different types of foods.

In addition, another most exploited source of probiotics is human GIT as most of the probiotics being used today have been isolated from it. The main reason for this exploitation is that these probiotics are adapted to human intestinal environment and can

exert their beneficial effects more effectively. They have antimicrobial activity against many known food-borne pathogens (Varma *et al.,* 2010). Many strains have also been isolated from human fecal samples. *Lactobacillus gasseri, L. reuteri L. fermentum Bifidobacterium longum and L. acidophilus RY2* are some examples of probiotics of human origin (Fontana *et al.,* 2013).

2.3. Characteristics of Probiotic Bacteria:

In a meeting organized by Lactic Acid Bacteria Industrial Platform (LABIP) it was decided, in order for a bacterial strain to be characterized as a potential probiotic, it must possess certain characteristics. 1) It should have beneficial effects on the health of an individual. 2) It shouldn't be harmful, disease causing, virulent and non-toxic. 3) It must be present in the form of living cells particularly in large amounts. 4) It ought to be gut friendly, hence can easily colonize gut, adhere to the intestinal walls, be able to metabolize in the gut and survive through the GIT tract. 5) Also, it should be having long storage life and be stable during shelf life. 6) It should improve and enhance the immune system. 7) It should produce bacteriocins or antibacterial/ antimicrobial compounds against pathogenic organisms (Dunne *et al.,* 1999). In addition to these, it should be safe for humans to consume. The microorganism should be fully characterized and sequenced. Also if possible should be subjected to clinical trials (Singh *et al.,* 2011). Furthermore a probiotic should interact with host microbiota and must compete with pathogens in the gut (Song *et al.,* 2012).

2.3.1. Types of Probiotics:

Based on the characteristics of probiotics, many microorganisms have been identified with potential probiotic properties. These organisms belong to different classes of bacteria and fungi. A list of most commonly used probiotics is given below:

Table 2.1: Commonly used Probiotics

Lactobacillus Species	Bifidobacterium Species	Other Species
L. acidophilus	*B. adolescentis*	*Bacillus cereus*
L. amylovorus	*B. animalis*	*Clostridium botyricum*
L. brevis	*B. breve*	*Enterococcus faecalis*
L. casei	*B. bifidum*	*Enterococcus faecium*
L. rhamnosus	*B. infantis*	*Escherichia coli*
L. cellobiosus	*B. lactis*	
L. crispatus	*B. longum*	*Lactococcus lactis* subsp. *Cremoriss*
L. curvatus	*B. thermophilum*	
L. delbrueckii subsp.*bulgaricus*		*Lactococcus lactis* subsp. *Lactis*
L. fermentum		*Leuconostoc mesenteroides* subsp. *Dextranicum*
L. gasseri		*Pediococcus acidilactici*
L. helveticus		*Propionibacterium*

		freudenreichii
L. johnsonii		*Saccharomyces boulardii*
L. lactis		*Streptococcus salivarius* subsp. *Thermophilus*
L. paracasei		*Streptococcus cremoris*
L. plantarum		*Streptococcus diacetylactis*
L. reuteri		*Streptococcus intermedius*
L. salivarius		*Sporolactobacillus inulinus*
L. gallinarum		

Adapted from: (Prado *et al.*, 2008) (Parvez *et al.*, 2006; Song *et al.*, 2012)

2.3.2. Acid Bile Tolerance

Probiotics are taken orally so they must pass through various stress factors in order to reach the intestines. Their journey starts from the oral cavity exposing them to the various enzymes, then they reach the stomach where the pH is extremely low due to the release of HCl. As soon as they leave the stomach and enter the duodenum they encounter bile. Bile can destroy their cell membranes that are largely composed of lipids and fatty acids. So, the cellular stress is great which can influence their survival. For a probiotic to be able to benefit humans it must endure these extreme conditions and withstand the passage through upper GIT. This is a very important characteristic of a probiotic. *In vitro* and *in vivo* testing is done to ascertain this ability of a strain before declaring it a probiotic (Succi *et al.*, 2005). This property of probiotics is strain specific and have been demonstrated by Clark et al. (1993) and Lankaputhra and Shah (1995). Bifidobacterium are usually sensitive to low pH and cannot survive it, whereas most

strains of Lactobacilli have shown high resistance abilities to low pH (Fontana *et al.*, 2013).

2.3.3. Adhesion and Colonization of the Intestines

Adherence to intestinal mucosa is considered as one of the most important characteristic of a probiotic, because only after adherence these bacteria can colonize the intestines and exert their beneficial properties more efficiently. Colonization is a transient property of the strains nonetheless. They do not permanently colonize the intestines and bacteria cannot be found in the fecal samples after 2 to 3 weeks of cessation of the administration of the dose (Ouwehand & Salminen, 2003).

Every probiotic strain has a habitat preference. Like some Bifidobacterium strains adhere more effectively to human mucus then to any other site (Y.K. Lee & Salminen, 2009). Adhesion of the probiotic strains can be to the any of the four microhabitats in the GIT (Fuller, 2012) i.e.

1) The surface of the epithelium cells
2) The compartments of small intestine, caecum and colon
3) The mucus that glaze the epithelium
4) The lumen of the intestines

Adhesion is a pre-requisite to many of the important physiological benefits the probiotics exert. It is important for colonization, stimulation of the immune system (Tuomola *et al.*, 2001), making the nutrients bioavailable (Parvez *et al.*, 2006), and causing a competitive exclusion of enteropathogens (Y. K. Lee & Puong, 2002). It

increases their retention time in the gut thus giving them more time to exhibit their positive effects. For example, a previous study has reported that *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG are highly adhesive, prevent and even treat severe diarrhea in children (Gueimonde & Salminen, 2006).

Several models have also been developed to study the adhesion mechanism and ability of the probiotic strains. Caco-2 cell lines, HT-29 and HT-29 MTX are most widely used for adhesion assays. However, they cannot mimic the exact GIT environment and thus the exact capacity of adhesion cannot be determined except for the *in vivo* models. Furthermore, adherence to the biopsy samples of the intestines and colon can be considered as most similar to the real condition of the human GIT and can provide much valuable information regarding adherence. (Tuomola *et al.*, 2001).

2.3.4. Safety of Probiotics

The assessment of safety of a potential probiotic strain is a very essential requirement. Lactobacilli have been used safely in history and since have been used until now. Some probiotics have been tested for their safety but the long history of their safe utilization can be considered the perfect evidence of their safety. Many Lactobacillus and Bifidobacterium species have been generally regarded as safe (GRAS) while some strains of Enterococcus, Streptococcus, and few other species are opportunistic parasites (Salminen *et al.*, 1998). But there are a few considerations that are taken into account before regarding a probiotic as safe. These are as follows (Sanders, 2003):

1) History of the probiotics' safe use through the route of administration applied.

2) The health and the immune system condition of the consumer.
3) Any ability to transfer antibiotic resistance.
4) The occurrence of any association of the strain given with probable infections.
5) The sensitivity to antibodies given if infection develops.
6) The production of any harmful compounds either metabolic or mammalian toxins

In addition to this the source of isolation, complete taxonomic characterization of the microorganism, description of the final use, and complete information about the microbe should be gathered (Fontana *et al.*, 2013). A brief selection criteria for novel probiotic strains is given below in the table.

Table 2.2: Selection Criteria for novel Probiotics

Probiotic property/properties	**Target**	**Probiotic effect**
Adhesion (several methods: epithelial cell lines, target site mucus or target site intestinal segments)	Site or area of intestinal tract, colonization of the target site, impact on local microbiota	Balancing intestinal microbiota, intestinal permeability, local microecology, alleviation of inflammation, strengthening of barrier
Production of metabolites	Antimicrobial activity, local epithelial effects	Normalizing barrier

		function, strengthening of barrier, regulating bowel movements
Production of cytokines	Inflammatory deviations, reduction in risk of inflammatory development	Protecting against deviations in intestinal immune responses
Assessing toxin binding	Binding of specific toxins including mycotoxins, cyanotoxins, heavy metals and other contaminants from the diet and water	Protecting the intestinal integrity, reduction in the risk of contaminant induced deviations
Characterization of quorum sensing	Sensing and reacting to deviations in the diversity of microbiota in the intestinal tract	Balancing microbiota and immune response, fighting pathogens
Impact on gene expression	Gene activation or deactivation in target	Positive health effects locally on target tissues

	tissues in the epithelium	and reduction in risk of disease
Safety properties	Noninvasive in epithelial cell line models, production of anti-inflammatory rather than proinflammatory cytokines, no antibiotic resistance genes	Safety in food use with proved health promoting effects
Genomic information	Specific data on properties	Selection based on host properties

Adapted from (Gueimonde & Salminen, 2006)

2.4. Applications of Probiotics

Probiotics are the beneficial microorganisms whose positive effects on the host body are numerous and have been studied in various experiments. They can improve overall health of the individual and avert diseases. Even the host range for probiotics to exert their advantageous effects are diverse. They can benefit animals, humans, fishes, and plants. Some of the applications and benefits of probiotics on various organisms is given below.

2.4.1. Plant Health

Plant probiotics are different from animal probiotics. They are particularly the species that improve the plant growth namely Rhizobacterium, Azospirillum etc. The more these beneficial bacteria are present the more the conditions are excellent for the plant to grow. These microorganisms make the soil fertile for the plant by taking up the nutrients and breaking them down for the plant to absorb. They act as biocontrol agents to control plant diseases and as biofertilizers by making the nutrients available for the plant. They have the ability to promote plant growth and make it more healthy (Song *et al.*, 2012).

2.4.2. Animal Health

Probiotics in animals have been implied for various uses and their effects have also been studied in detail. They are not only used for their health benefits in animals but also for the prevention and treatment of diseases and increasing the production of animal products like milk, eggs etc. They are usually given to the animals in the form of fortified feed (addition to food supplements). Previous studies have reported increase in the weight of the animal and improvement of animal health. Different microbial species are used for different organisms like bacteria are usually used for calves, chickens and pigs whereas in ruminants fungi and yeasts have shown more effectiveness (Musa *et al.*, 2009).

From the last 30 years, consideration has been given to GIT microbial environment of the animals. At first antibiotics were given to improve the gut environment but due to

the development of antibiotic resistance the attention diverted to a more natural way of preventing diseases thus using probiotics (Durand & Durand, 2009). The modes of action of probiotics in animals is quite similar to that of the humans. They balance the intestinal microbiota, enhance the immune response, compete with and kill the pathogenic bacteria by secreting bacteriocins, provide nutrients to the animals, and help in the digestion (Song *et al.*, 2012).

2.4.3. Aquaculture:

In aquatic animals, the use of probiotics has shown significant results. The first use of probiotics in aquaculture was done by Kozasa in 1986 but the trend after that increased. The most common species of probionts used in aquaculture are gram negative, facultative anaerobes. In crustaceans, bivalves and marine fish *Vibrio* and *Pseudomonas* species are used while in fresh water aquatic animals *Enterobacteria, Aeromonas* and *Plesiomonas* are observed. They have shown similar results in aquatic animals as that of terrestrial animals. However, the stress in aquatic conditions is more as the water is continuously flowing through the gut of the animal so probiotics cannot colonize the gut of the animal but can stay there transiently. They are given to the animals in the form of feed or are directly mixed in the aquaculture ponds (Gatesoupe, 1999).

2.4.4. Human Health

Copious amount of research has been done on the beneficial effects of probiotics on human health so the advantages of probiotics on humans cannot be enumerated. Their applications vary from providing nutrients to treating various diseases. Most of these

effects are well tested and documented while others have shown promising results. Also the health benefits exerted by the probiotics are strain specific so the efficacy of every strain is different in different disease (Song *et al.*, 2012).

2.4.4.1. Health benefits of probiotics

Probiotics have shown the ability to increase the amount, improve the availability and increase the digestion of many dietary nutrients. They secrete enzymes and vitamins in the intestines. Thus, increasing digestion and alleviating malabsorption. These enzymes make the nutrients bioavailable by digestion of proteins and fats. LAB produce lactic acid which reduces the pH of the intestines hence preventing pathogenic invasion (Parvez *et al.*, 2006).

2.4.4.2. Immune System Stimulation

Immunomodulation is also one of the positive effects of probiotics on human health. They stimulate the effector T-cell thus activating cell mediated immunity. They cause phagocytosis mostly by granulocytes and sometimes by agranulocytes. Probiotics enhance secretion of interferon- γ and presentation of complement receptors on granulocytes. This is due to the phagocytosis of probiotics by macrophages which present them on their cell membranes resulting in the production of cytokines and cell-mediated immunity. They also act as adjuvants and modify the immune response by boosting the production of antibodies (Wold, 2016). Certain probiotics can also cause the stimulation of innate immunity in a local area of exposure by the production of

Tumor Necrosis Factor-α (TNF-α) and activation of NF-κB pathway (Pagnini *et al.*, 2010).

2.4.4.3. Alleviation of Lactose Intolerance

More than 70% of the world's population suffers from lactose intolerance. The symptoms of lactose intolerance differ from mild to severe. The people with severe lactose intolerance have very less amounts of β-galactosidase enzyme and cannot digest lactose present in the milk and dairy products. Probiotics provide bacterial lactase enzyme to the host which can digest lactose thus reducing the symptoms of lactose intolerance (Roberfroid, 2000).

2.4.4.4. Allergy

Probiotics have some effect in lowering the effect of hypersensitivity and thus reducing allergies. They are particularly used for food allergies and atopic eczema as they improve the mucosal barrier and intestinal inflammation with people suffering from allergies and atopic eczema. However, further investigations need to be performed in this regard (Majamaa & Isolauri).

2.4.4.5. Obesity

It has been observed that gut microbiota is altered in obesity and recent findings suggest that probiotics can restore the normal balance and alleviate the symptoms of inflammation and metabolic dysfunctions in the obese people (Yoo *et al.*, 2013).

Bifidobacterium have been found an important class of probiotics in this regard (Blaut & Bischoff, 2010). But further research in this context needs to be done.

2.4.4.6. Diabetes

A lot of research has proposed that probiotics have beneficial effects in people suffering from diabetes. They decrease the inflammatory response in the gut. They also reduce oxidative stress. In addition, they enhance the expression of adhesion proteins in the epithelium of the intestines causing them to be less permeable. All these factors cause the absorption of insulin to be more effective and reduction in autoimmunity. As a consequence the severity of diabetes is reduced (Gomes *et al.*, 2014).

2.4.4.7. Depression

There is direct relationship between the brain and the gut of human beings. The numerous benefits of the probiotics improve the gut health and in turn the overall health of the organisms which causes bidirectional communication between the brain and the gut. The gut microflora causes the activation of immune system and thus the activation of central nervous system by producing and delivering neurotransmitters like serotonin and γ-amino butyric acid. Many studies have been carried out in this regard (Huang *et al.*, 2016). Gut microbiota not only regulates the intestinal balance but also manages the extraintestinal homeostasis. Gut microflora is termed as a peacekeeper for the brain health as any disturbance in the functions or composition of microbiota are linked with neurophysiological disorders (Mu *et al.*, 2016).

2.4.4.8. Cancer

Cancer is a multifactorial disease and is usually caused by the accumulation of multiple mutations. The cancerous cells are usually recognized by the immune system and killed or their growth is halt by natural cellular mechanisms. But in some cases, these cells survive and continue to become a cancer. Probiotics are known to kill the cancerous cells, detoxify the chemical mutagens, decrease the population and metabolic activity of the microorganisms that might cause the production of carcinogenic compounds, and can produce compounds that cause the apoptotic death of abnormal cells (Parvez *et al.*, 2006).

2.4.5. Treatment and Prevention of Diseases

In addition to these health effects the applications of probiotics are well established in the treatment of numerous diseases like antibiotic associated acute diarrhea, irritable bowel syndrome, inflammatory bowel disease, vaginal infections, traveler's diarrhea, rotavirus associated diarrhea, hepatic encephalopathy, inflammation, arthritis, hypocholesteremia and prevention of HIV/AIDS (Noratto, 2014; Parvez *et al.*, 2006; Singh *et al.*, 2011).

2.4.6. Antimicrobial Activity of probiotics

The overuse of antibiotics has caused a problem of antibiotics resistance and the development of superbugs. A need for some innovative methods was needed to counter this rising problem. The antagonistic effects probiotics exert on pathogens provided a plausible solution to this problem. Probiotics in addition to forming a barrier on the

mucosal surface to inhibit the pathogens from attaching also release antimicrobial substances that kill the pathogens. It is also one of the characteristics of a probiotic which is important for its selection. All the probiotics must have some activity against the pathogens. These antimicrobial substances can be organic acids like lactic acid and acetic acid, low molecular mass antimicrobials like flavoprotein oxidases which can cause the production and accumulation of antibacterial substances like hydrogen peroxide, formic acid, acetaldehyde and acetoin etc. ethanol, diacetyl and other bacteriocins (Šušković *et al.*, 2010). The list of pathogenic organisms against which the antibacterial activities of probiotics is very diverse. Different kind of food borne pathogens like *Helicobacter pylori* (Lesbros-Pantoflickova *et al.*, 2007), *E. coli* (Mack *et al.*, 1999), *Clostridium botulism* (Rodgers *et al.*, 2003) and *Shigella* (Zhang *et al.*, 2011) etc. are usually used.

3. Materials and Methods

3.1. Chemicals

3.1.1. Luria Bertani Media (LB)

For the growth of pathogenic and antibacterial well diffusion assay, LB media was used. It was prepared by mixing 1% (w/v) sodium chloride (Merck, Germany), 1% (w/v) Tryptone (Merck, Germany), 0.5% (w/v) Yeast extract (CDH, India) and 1.5% (w/v) Agar (bioWorld, USA)) in distilled water and autoclaved.

3.1.2. Phosphate Buffer Saline (PBS)

1X PBS was used for various purposes during the experiments. It was prepared by mixing 0.8% (w/v) sodium chloride (Merck, Germany), 0.02% (w/v) potassium chloride (Merck, Germany), 0.144% (w/v) disodium hydrogen phosphate (Merck, Germany), 0.024% potassium dihydrogen phosphate (Merck, Germany) in distilled water. The pH was adjusted to 7.4 and autoclaved.

3.1.3. Rifampicin Solution

The stock solution of rifampicin was prepared by mixing 450 mg of rifampicin (Sigma Aldrich, Germany) in 45ml of Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) to make a solution of concentration 10mg/ml. The solution was syringe filtered and stored at 4°C.

3.2. Sample Collection

Six different varieties of rice namely Kainaat 1121, Super Basmati, Basmati 385, Super Kernel, KS 282 and motay chawal were obtained from local market. 25g of each variety of rice were soaked in 25ml distilled water separately in 6 screw tight sterile falcons. The falcons were then place in the incubator at 37°C for 24hrs to start fermentation. 1ml of water from each falcon was taken the next day, mixed in 14ml MRS (Man, Rogosa and Sharpe) (Merck, Germany) broth (pH 4) each and incubated for 24hrs. After 24hrs inoculating loop was dipped in each falcon and streaked on MRS agar plates and incubated for 24hrs again. Individual colonies were picked streaked further to obtain a pure colony.

3.3. General Characterization and Biochemical Testing of Probiotic Strains

3.3.1. Gram Staining

Isolated strains were streaked on MRS agar and given an overnight incubation. The next day colonies were picked and dissolved on a water droplet placed on a slide (Globe Scientific Inc. USA) to make a smear. The smear was air dried and heat fixed on the slide. Crystal violet dye (Merck Pvt Ltd. Pakistan) was added first to stain the cells after 1 min the stain was rinsed gently with tap water and smear was then flooded with Gram's iodine (Merck Pvt Ltd. Pakistan), allowed to stand for 1 min. The smear was then again rinsed with water followed by the addition of Ethanol to decolorize for 5 to 10 seconds. Safranin (Merck Pvt Ltd. Pakistan) was then added as a counter dye for 45

seconds and rinsed with water. The slides were then observed under light microscope (Optika, Itlay) with oil immersion.

3.3.2. Catalase Test

Using a sterile wooden stick some colonies were transferred to slide (Globe Scientific Inc. USA). A drop of 3% H_2O_2 was placed on the colonies and observed for effervescence.

3.3.3. Acid Production

To check if the isolated strains released acid and hydrolyze calcium carbonate the strains were streaked on the MRS media supplemented with 1% calcium carbonate. Formation of clear zones will indicate a lactic acid producing bacteria.

3.4. 16S rRNA Gene Sequencing

The isolated strains after proper biochemical characterization were sent for 16S rRNA gene sequencing to Macrogen, South Korea.

3.5. In vivo Probiotic Potential Testing

3.5.1. Rifampicin Tagging

To differentiate the selected strains from the natural microflora of Rats, the selected strains were tagged with rifampicin resistance. Rifampicin resistance was introduced in the strains by culturing them in the increasing concentrations of the antibiotic (25ug/ml, 50ug/ml, 100ug/ml and 200ug/ml). The strains were first grown in MRS broth for 24hrs

which was then replaced with MRS broth having 25ug/ml of rifampicin and was again incubated for 24hrs at 37°C. The next day bacteria from the broth was streaked on MRS agar plate having concentration of 50ug/ml of rifampicin. Colonies were picked from 50ug/ml MRS agar plate and inoculated in 100ug/ml rifampicin containing MRS broth for 24hrs. Inoculum from the broth was then streaked on MRS agar plate having 200ug/ml concentration of rifampicin. The resistant strain colonies were then picked form this plate and checked for stability by repeated culturing on MRS agar plate containing 200ug/ml of rifampicin for at least 20 generations.

3.5.2. Animal Model

Permission for experiments regarding animal models was obtained from the internal board review (IRB). Approval form is attached. 20 female rats, aged 8 months were taken and divided into groups of 5 each. The fecal samples of all the rats were initially tested for any rifampicin resistant strain by spreading the first three serial dilutions of the samples on MRS agar plates containing 200ug/ml of rifampicin. All the rats negative for growth on the plates were proceeded for the experiments. Each group was kept in a separate cage under standard conditions i.e. 12h light/12h dark cycle, 20-30°C, water availability and non-sterile diet (standard animal feed). One group was kept as a control and three groups were taken as experimental.

3.5.3. Probiotic Dosage

Each rat was given a total probiotic dosage of 2 billion CFU/ml/day. The probiotic dosage was administered to the animals through drinking water. The control group was

given plain drinking water. The bacterial culture was grown for 18hrs and optical density was measured together with the spreading of the dilutions made. The culture was grown till 24hrs. The optical density was noted and sample was collected every 2hrs for making the dilutions. The time interval at which 2 billion CFU were obtained was noted together with the optical density of the growth media at that time. Fresh dose was prepared 1 day before giving it to the rats by growing the bacterial culture to the same optical density.

3.5.4. Gastrointestinal transit

To check the survival of the probiotic strains through the gastrointestinal tract including tolerance to salivary amylases, acidic environment, gastric juices, enzymes and pancreatic juice, the fecal samples of the rats were collected after 24 hours of giving the first probiotic dose. After that the fecal samples were collected every 3rd day of the feeding chart to check for the bacterial counts. The fecal samples were collected in 10ml of PBS and dissolved by thorough vortexing. Serial dilutions were prepared from these samples and viable bacterial cell counts per gram of fecal samples were checked through spread plate method. The last six dilutions for each sample were spread on MRS agar plates containing 200ug/ml of rifampicin.

3.5.5. Tissue and Blood Collection

The animals were euthanized using ketamine/xylazine combination injected intraperitonially to check for the adhesion of the probiotic strains to the GIT tract. Sections of duodenum, large intestine (at least 2cm) and caecum were harvested and

placed in the sterile PBS solution immediately. Blood was also collected by direct cardiac puncture and complete blood count was performed by diagnostic lab ASAB.

3.5.6. Microbiological analysis of Tissues collected

The tissues collected in the PBS solution were mixed through rigorous vortexing and then serially diluted. The serial dilutions were plated on MRS agar plates containing 200ug/ml of rifampicin and incubated at 37°C for 72hrs to check for viable bacterial cell counts.

3.6. Antibiotic Susceptibility Test:

The isolated probiotic strains were each grown separately for 24hrs in MRS broth at 37°C. After 24hrs 100ul of the broth with growth was spread on MRS agar plate each. 11 antibiotic discs namely Amitacin-30ug (AK-30), Cefotaxime-30ug (CTX-30), Ciprofloxacin-5ug (CIP-5), Gentamicin-10ug (CN-10), Imipenem-10ug (IPM-10), Levofloxacin-5ug (LEV-5), Ofloxacin-5ug (OFX-5), Piperacillin tazobactam-110ug (TZP-110), Sulbactam/Cefoperazone-105ug (SCF-105), Sulphamethoxazole trimethoprim-25ug (SXT-25) and Vancomycin-30ug (VA-30), were placed on the plate to evaluate the susceptibility of the strains against these antibiotics. All the antibiotic discs were purchased from Oxoid Ltd. Basingstoke. Hampshire, England.

3.7. Preparation of Cell Free Supernatant

To prepare cell free extract the probiotic strains were inoculated separately each in 15ml MRS broth anaerobically at 37°C for 24hrs. After 24hrs the media was centrifuged at 6000 rpm for 15 mins. The supernatant was collected and syringe filtered using a 0.2um

syringe filter (Corning, USA). The supernatant was further treated and the rest was stored at 4°C.

3.7.1. Heat Inactivation of CFS

10ml of the prepared CFS was heat treated at 15atm pressure, 121°C for 15 minutes.

3.7.2. Proteinase K Treatment

From the heat-treated samples 4 ml of each were taken and were treated with proteinase K. 5µl of proteinase K enzyme was added in the each sample and they were kept at 55°C for 24hrs.

3.7.3. pH Treatment

The proteinase K treated samples were further neutralized to a pH of 7 by using 2M NaOH.

3.8. Collection of Pathogenic Strains

The clinical isolates of *Shiga toxin producing E. coli*, *Pseduomonas aeruginosa*, *Klebisella pneumoniae* and *Salmonella enterica* were attained from Pakistan Institute of Medical Sciences (PIMS). *Enterococcus faecalis* was obtained from Virology laboratory, ASAB, NUST. *STEC* and *S. enterica* were tested for confirmation on Salmonella Shigella agar (SS agar) (Oxoid Ltd. Basingstoke. Hampshire, England).

3.9. Antimicrobial Activity

3.9.1. Agar Well Diffusion Assay

The antimicrobial activity of the isolated probiotic strains was tested against the pathogenic strains by agar well diffusion. Wells of diameter 9mm were bored in LB agar plates. Pathogenic strains were mixed in 2 ml PBS solution such that the final turbidity of the solution was 0.5 McFarland. 100ul of this solution was spread on the LB agar plate with the wells. 100 ml of each CFS of probiotic strains was added in the properly labelled well and the plates were incubated at 37°C for 18-24hrs. Together with the CFS of probiotic strains a negative MRS control, a positive control antibiotic Cefotaxime-30ug (CTX-30) (Oxoid Ltd. Basingstoke. Hampshire, England) was used. For STEC instead of CTX-30, TGC-15 (Tigecycline-15ug) (Oxoid Ltd. Basingstoke. Hampshire, England) was used. The diameter of clear zone was measured around the well as zone of inhibition. the experiment was performed three times.

For Statistical Analysis, all the graphs were generated using GraphPad Prism.

4. Results:

4.1. Bacterial Isolation and Identification

From the rice sample varieties, fourteen strains were isolated. Out of these fourteen strains, three strains had probiotic characteristics, which were determined by biochemical tests, and were named as FK1, FK2 and FK3. These strains were further sequenced (Macrogen, South Korea) to confirm their identity. All the three strains were identified as *Enterococcus faecium*.

Table 4.1: List of isolated Strains

Name Given	Source of Isolation	Strain name
FK 1	Kainat	*Enterococcus faecium*
FK 2	Irri 6	*Enterococcus faecium*
FK 3	Basmati 386	*Enterococcus faecium*

4.1.1. Morphological analysis:

The colonies of the three isolated strains were round, smooth, white in color and pin pointed. The bacteria were round shaped in all three isolates.

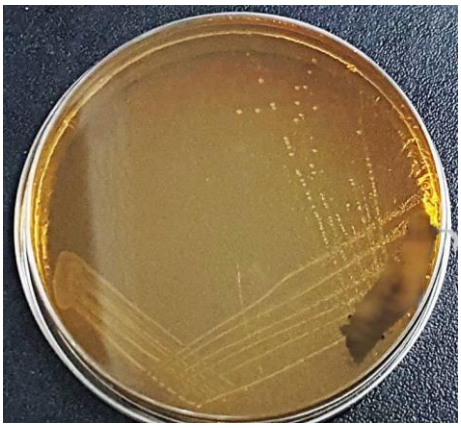

Fig 4.1 Representative image of Bacterial Isolates

4.1.2. Biochemical Tests:

4.1.2.1. Gram Staining:

The seven isolated strains were gram stained, among them six strains were gram positive, which were proceeded for further biochemical testing.

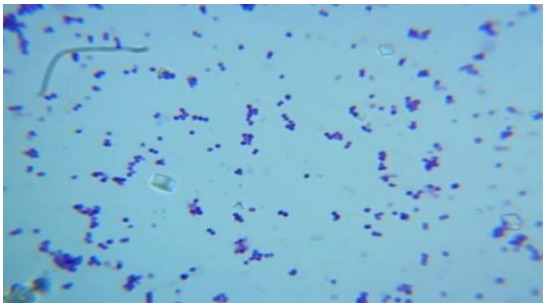

Fig 4.2 Representative image of Gram Staining

4.1.2.2. Catalase Test:

From the six isolated strains, three strains gave no effervescence indicating them to be catalase negative.

4.1.2.3. Lactic Acid production:

Lactic acid bacteria produce lactic acid which reacts with calcium carbonate present in the media forming calcium lactate thus clear zones are formed. The three catalase negative strains gave clear zones on $CaCO_3$ supplemented MRS media indicating acid production.

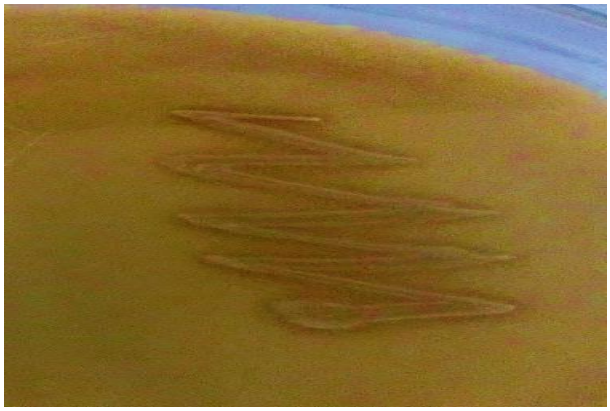

Fig 4.3 Representative image of Lactic acid production by bacterial isolates

A complete list of the isolated strains together with their identification and biochemical testing is given below.

Table 4.2: Complete list of isolated strains with their biochemical characterization

Name	Source	Biochemical Characterization			
		Morphological Analysis	Gram Staining	Catalase Test	Acid Production
PB 1	Kainat	Small, white, round	Purple, round	No bubbles	Zone Formation

		colonies	shaped		
PB 2	Irri 6	White, large, round colonies	Purple, round shaped	No bubbles	Zone Formation
PB 3	Super Basmati	Small, white, round colonies	Purple, round shaped	No bubbles	No zone formed
PB 4	Super Kernel	Small, pink round colonies	Pink rods	No bubbles	No zone formed
PB 5	Super Kernel	White, smooth colonies	Purple rods	Bubbles Formed	No zone formed
PB 6	Super Kernel	White, rough colonies	Purple rods	Bubbles Formed	No zone formed
PB 7	Basmati 386	Small, white colonies	Purple, round shaped	No bubbles	Zone Formation
PB 8	Basmati 386	Small, white, round colonies	Pink, round shaped	No bubbles	No zone formed
PB 9	Basmati 386	Large, milky white colonies	Purple, round shaped	No bubbles	Zone Formation
PB 10	Basmati 386	Large, translucent colonies	Pink, round shaped	No bubbles	No zone formed
PB 11	Irri 6	Small, translucent colonies	Purple, round shaped	No bubbles	Zone Formation
PB 12	Irri 6	Large, white colonies	Purple rods	No bubbles	No zone formed
PB 13	Super	Small, pink colonies	Purple rods	Bubbles	No zone formed

	Kernel			Formed	
PB 14	Super Kernel	Small, translucent colonies	Purple rods	No bubbles	No zone formed

The highlighted isolates had probiotic characteristics. Out of these five only three were selected for further testing. These strains were PB 1, PB 2, And PB 7. PB 1 was renamed as FK 1, PB 2 was renamed as FK 2 and PB 7 was renamed as FK 3 respectively.

4.2 *In vivo* Probiotic Potential Testing:

One of the important characteristic of a probiotic is the ability to adhere to intestinal walls and survive the extreme conditions present in the GIT tract. The GIT endurance, adhesion and colonization of the intestines was checked in wistar rat models. The overall health of the rats improved during the experiments and no infections or deaths were noted.

4.2.1. Gastrointestinal Transit

All the strains survived the GIT and were obtained in the fecal samples even in the 7th week of the experiment. The strains were observed in the fecal samples even after two days of giving the dose. Colonies were obtained in all the fecal samples collected twice a week showing viability of the strains through the GIT indicating that not only the strains were able to survive the harsh conditions but were also able to somewhat colonize the GIT. In the control group, no colonies were obtained in the fecal samples throughout the experiment.

The number of bacteria in the fecal samples for all the strains increased till the 9th day and after that a stationary phase can be observed till the 49th day which may indicate that the probiotic strains have adhered and colonized the GIT.

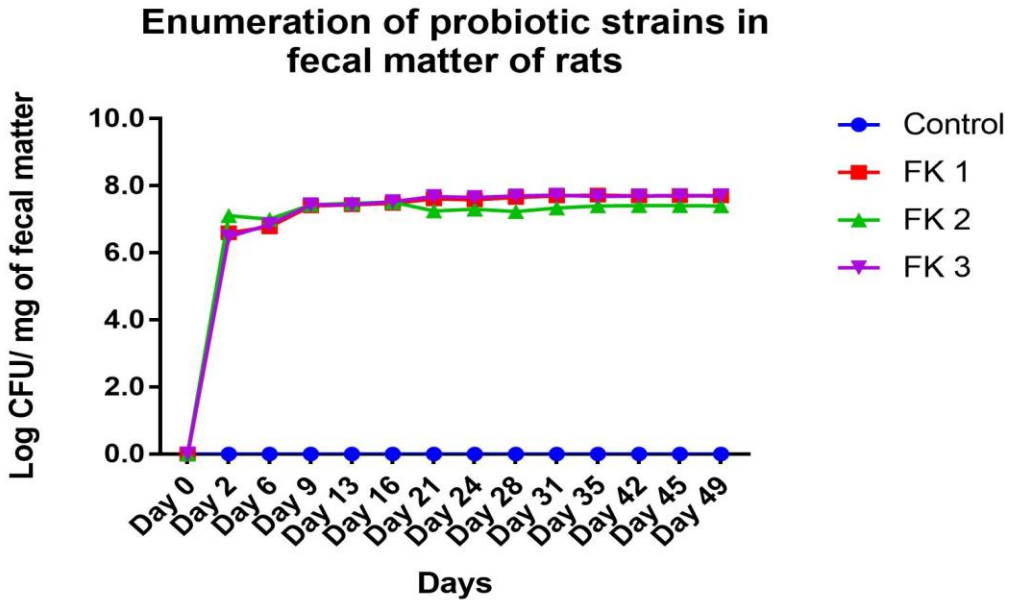

Fig 4.4 Viable cells of *Enterococcus faecium* in rat faeces at different days

4.2.2. *In vivo* Adhesion and Colonization Assay

The adhesion and colonization preference of the rifampicin resistant isolated strains was tested in the tissues of small intestine, large intestine and caecum collected from the rats. Colonies were obtained in the homogenates of all the samples collected and a variance in the colonization preferences of the strains can be seen. Strain FK 1 had poor colonization as compared to the strain FK 2 and FK 3. It showed colonization preference in the small intestine whereas FK 2 and FK 3 both showed a preference for large intestine.

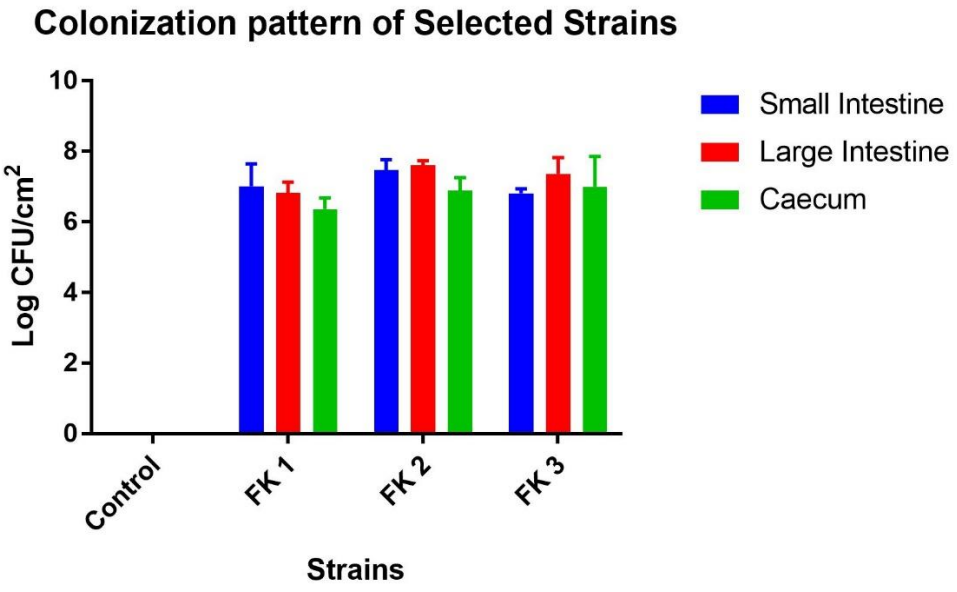

Fig 4.5 Bacterial count in small intestine, large intestine and caecum of rats after 11 weeks of feeding *E. faecium*. (n=3) (Error bars represent standard deviation from mean from three samples)

4.2.3. Weight Increase in Rats

Weight increase was considered as a standard for the health of rats. There was increase in the weight of all the rats during the 10 weeks experiment. Significant increase in the weight gain can be seen from the chart below. There was a noticeable difference in the weight increase in the rats given probiotics as compared to the control group.

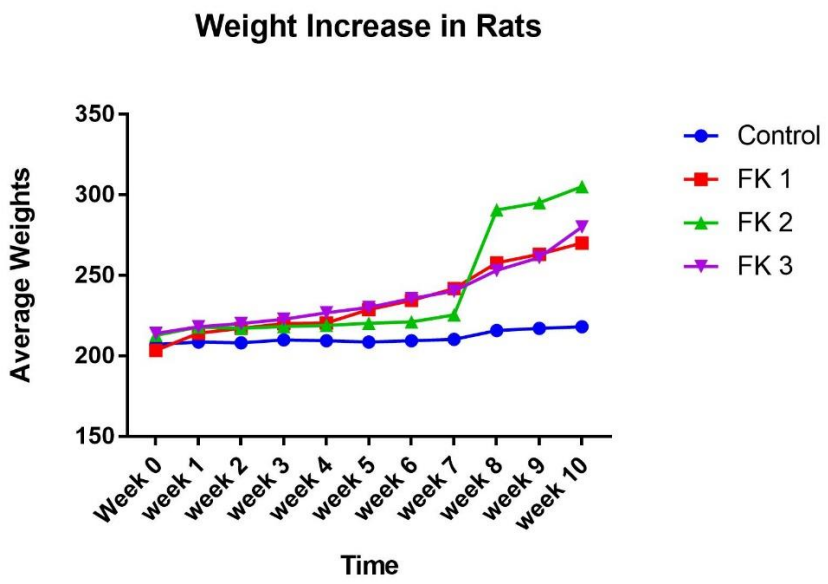

Fig 4.6 Average weight increase in rats (n=5)

4.2.4. Complete Blood Count (CBC) of the Rats

To ensure the safety of the isolated strains, CBC of the rats was done. The table below shows that all the blood parameters of the rats is in the normal range indicating the strains to be safe and noninfectious.

Table 4.2 Complete Blood Count of Wistar rats

Test Name	Normal Range	Control Group	Strain FK 1	Strain FK 2	Strain FK 3
RBC Count	(7.16-9.24) $10^6/\mu L$	6.55 ± 0.35	7.47 ± 0.25	7.13 ± 1.12	6.63 ± 0.15

Hematocrit	38.5-49.2%	37.35 ± 1.77	41.27 ± 0.95	39.43 ± 0.81	37 ± 0.76
Hemoglobin	13.7-17.2 g/dL	12.35 ± 0.49	13.37 ± 0.25	13.1 ± 0.26	12.3 ± 0.36
MCV	50.3-57 fL	56.65 ± 0.35	55 ± 1.05	54.87 ± 0.47	55.4 ± 1.25
MCH	17.6-20.3 pg	18.75 ± 0.21	17.77 ± 0.42	18.23 ± 0.47	18.4 ± 0.1
WBC Count	(1.98-11.06) 10^3/µL	5.25 ± 0.92	3.6 ± 1.23	6.57 ± 2.87	4.76 ± 1.72
Lymphocytes	48.9-88.1%	79 ± 0	83.83 ± 1.86	80.03 ± 2.5	78.47 ± 2.7
Platelets	(599-1144) 10^3/µL	1043 ± 24	1135.7 ± 93.38	1026 ± 28.99	940.67 ± 296

4.3. Antibiotic Susceptibility Test:

The isolated strains were tested for their sensitivity against 11 different antibiotics. The results obtained are presented in the form of a table below.

Table 4.3 Antibiotic Susceptibility of Isolated Strains

Antibiotics	**Probiotics Strain**		
	FK 1	FK 2	FK 3
Amikacin (AK-30)	±	R	R
Cefotaxime (CTX-30)	±	S	±
Ciprofloxacin (CIP-5)	S	S	S
Gentamicin (CN-10)	±	±	R
Imipenem (IPM-10)	S	S	S
Levofloxacin (LEV-5)	S	S	±

Ofloxacin (OFX-5)	R	±	±
Piperacillin tazobactam (TZP-110)	S	S	S
Sulbactam/ Cefoperazone (SCF-105)	S	S	S
Sulfamethoxazole (SXT-25)	±	±	±
Vancomycin (VA-30)	S	S	S

R: resistant, S: sensitive, ±: marginally susceptible

4.4. Antibacterial Activity

Antimicrobial activity of the isolated strains was tested against *Shiga toxin producing Escherichia coli, Salmonella enterica, Enterococcus faecalis, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae.* No significant antimicrobial activity was seen against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* while clear zone of inhibition was observed against the other three pathogenic strains. Cefotaxime-30 µg (CTX-30, Oxoid, Hampshire, England) was used as a positive control. For *STEC* Tigecycline 15 µg (TGC-15, Oxoid, Hampshire, England) served as a positive control. MRS was used a negative control and no zone of inhibition was detected against the wells with MRS. The result of antimicrobial activity of the isolated strains is shown below in the tables against different pathogenic strains together with the results of treated cell free supernatants.

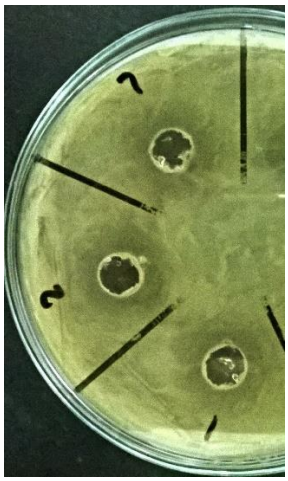

Fig 4.7 Representative image of antibacterial activity of the isolated strains

Table 4.4 Antibacterial activity against STEC

Probiotic Strain	**Shiga Toxin producing *E. coli* (STEC)**		
	Cell Free Supernatants	Heat Treated Cell Free Supernatants	Proteinase K Treated Cell Free Supernatants
***Enterococcus Faecium* (FK 1)**	18.5 ± 0.58	20 ± 0.5	17 ± 1.5
***Enterococcus Faecium* (FK 2)**	18 ± 1.5	19.5 ± 0.58	18 ± 0.82
***Enterococcus Faecium* (FK 3)**	17 ± 1.26	19.75 ± 1.89	17.5 ± 1
TGC-15 (Positive Control)	20	21	20
MRS (Negative Control)	0	0	0

Average zone of inhibition (in mm) given by normal and treated probiotic cell free supernatants against STEC (n=4) (well size: 9mm) ± Standard Deviation of Mean

All the strains had given almost same zones of inhibition before and after heat and Proteinase K treatment.

Table 4.5 Antibacterial activity against *S. enterica*

Probiotic Strain	***Salmonella enterica***		
	Cell Free Supernatants	Heat Treated Cell Free Supernatants	Proteinase K Treated Cell Free Supernatants
***Enterococcus Faecium* (FK 1)**	22 ± 1.63	21 ± 3.2	23.5 ± 1.29
***Enterococcus Faecium* (FK 2)**	20 ± 3.6	21 ± 1.5	22 ± 2.8
***Enterococcus Faecium* (FK 3)**	23 ± 2.2	22 ± 1.4	23.25 ± 0.95
CTX-30	30	30	31
MRS (Negative Control)	0	0	0

Average zone of inhibition (in mm) given by normal and treated probiotic cell free supernatants against *S. enterica* (n=4) (well size: 9mm) ± Standard Deviation of Mean

The zones of inhibition are almost similar for all the strains before and after the treatment with Proteinase K and heat treatment.

Table 4.6 Antibacterial activity against *E. faecalis*

Probiotic Strain	***Enterococcus faecalis***		
	Cell Free	Heat Treated Cell	Proteinase K Treated

	Supernatants	Free Supernatants	Cell Free Supernatants
***Enterococcus Faecium* (FK 1)**	14.5 ± 1	16 ± 0.5	20.5 ± 2.1
***Enterococcus Faecium* (FK 2)**	16.5 ± 2.1	17 ± 0.61	19.5 ± 0.71
***Enterococcus Faecium* (FK 3)**	14.5 ± 0.71	15 ± 1.1	22.5 ± 3.5
CTX-30	25	26	25
MRS (Negative Control)	0	0	0

Average zone of inhibition (in mm) given by normal and treated probiotic cell free supernatants against *E. faecalis* (n=4) (well size: 9mm) ± Standard Deviation of Mean

Here it can be seen that zones of inhibition after treatment with proteinase K has significantly increases which might indicate the fact that there was some heat sensitive protein or compound which was inhibiting the effect of antibacterial compound which after heat treatment was denatured.

5. Discussion

The aim of the present study was the isolation of probiotic strains from the indigenous non-dairy sources like rice grains and the characterization and safety profiling of the isolated strains in *in vivo* models. The inhibitory effect of the strains against pathogenic strains was also evaluated. Three strains were isolated from different varieties of rice and sequenced. The strains were identified as *Enterococcus faecium*. *Enterococcus faecium* has established probiotic properties. They are generally safe to use and have many beneficial properties. They are used for the treatment of many diseases like diarrhea, irritable bowel syndrome and lowering of blood cholesterol etc (Franz *et al.*, 2011).

The probiotic potential of a strain can be confirmed if it survives the GIT and adheres to the intestinal walls. Adherence is necessary not only for bacterial attachment but also for its persistence, and colonization. It prevents bacteria from being swept away (Adlerberth, 2000; Parvez *et al.*, 2006). For this purpose, the isolated strains were tested on rat models which is more similar to the real conditions and give more reliable results. Mice and rats are considered as a good model to study gut microbiota and the interaction of different bacteria in the gut to that of the host health as it is the most studied animal. Rats are monogastric animals like humans although anatomical differences exist the microbiota of the two is quite similar so rats can be used effectively to perform experiments related to probiotics (Tannock, 1999). The results of the experiment showed that the strains were able to survive the GIT, were able to

compete with the already established microbiota of the host and were obtained in the fecal samples after giving dose for as less as 2 days.

To differentiate the given probiotic strains from the indigenous microbiota of the rat the isolated strains were tagged with rifampicin resistance. Rifampicin tagging lasts for at least 20 generations and is nontransferable so it is an efficient method for differentiating tagged bacteria from other bacteria. Antibiotic tagging can be seen as a competent method to discriminate desirable bacteria form the normal microflora by many researchers (Bouhnik *et al.*, 1992; Frece *et al.*, 2005; Fujiwara *et al.*, 2001). The bacteria endured the harsh GIT conditions and were obtained in fecal samples through the entire experimental period. During the first nine days, the bacterial count was less, indicating that the bacteria were adapting to the environment, which the increased and remained constant till the 49th day showing the successful adhesion and colonization of the GIT. Similar method was adopted in a study in which three strains were rifampicin tagged and further tested on mice for in vivo adhesion, colonization and survival through the GIT for 14 days (Frece *et al.*, 2005).

In vivo adhesion and colonization of the strains was examined in the samples of small intestine, large intestine and caecum obtained from the experimental rats. It is a strain specific property as different strains show different colonization preferences. It is also evident from our results. It can be clearly seen that FK 1 preferred small intestine while FK 3 had an inclination towards large intestine. FK 2 had mixed preferences for small and large intestine. Caecum was seen to be least colonized by all the three strains. In another study *Enterococcus faecium* showed preference for large intestine as compared

to small intestine which does not comply with my study as all the three isolated strains showed mixed preferences (Frece *et al.*, 2005). *Enterococcus faecium* has been shown to have less adhesion and colonization ability as compared to other LAB strains (Collado *et al.*, 2007) this is also observed in the current study as very less number of colony forming units were obtained on dilution plates.

In this study, the rats were fed with the probiotic dosage for 10 weeks to determine the safety of the administered probiotic strain (*Enterococcus faecium*) as they are known to cause nosocomial infections. Throughout the experiment the rats were gaining weight which is an indicator of good health and after the 11^{th} week, rats were sacrificed and complete blood count was determined. All the blood parameters lied in the normal range indicting no disease. Weight gain has been shown as an indicator of normal health in many studies (Bhardwaj *et al.*, 2010). Particularly in a study involving young children *Enterococcus faecium* was given to check its effect on bodyweight and salivary IgA, all the children gained weight with no side effects (Surono *et al.*, 2011). As the dose was administered through the drinking water so there was a chance of infection as enterococci genera is notorious for causing various diseases when entering the body through any route other than the mouth (Higuita & Huycke, 2014), the bacteria might have entered the in the nose of the rats and might have had contact with the skin but overall health parameters of the rats were normal and no abnormalities in the CBC of the rats were observed indicating the strains to be safe for use.

In order for a probiotic to be safe for use it should not carry genes for antibiotic resistance that can be transferred to other bacteria, as transfer of such genes can result in

the development of antibiotic resistant bacteria (Morelli & Wright, 1997; Saarela *et al.*, 2000). For this purpose, the three isolated strains were tested against 11 antibiotics, all of them were broad spectrum antibiotics. The strains were found to be sensitive to majority of the antibiotics except for Amikacin. This again confirms the safety of the isolated strains for use.

One of the most important characteristic of a probiotic is to exert antibacterial effect against pathogenic strains. This activity can be due to various reasons that is either the production of bacteriocins or some other compounds like organic acids, hydrogen peroxide etc. (Šušković *et al.*, 2010). The three isolated strains were tested for their antimicrobial activity against five clinical isolates of pathogenic strains namely *Shiga toxin producing E. coli, Pseudomonas aeruginosa, Klebisella pneumoniae, Salmonella enterica* and *Enterococcus faecium.* Antibacterial activity was obtained against *STEC, S. enterica* and *E. faecium* whereas no significant results were obtained against *K. pneumoniae* and *P. aeruginosa*. Similar results have been proposed in different studies (Belgacem *et al.*, 2010; Mansour *et al.*, 2014).

Different treatments were applied on the cell free supernatants (CFS) of the isolated strains to determine the possible antibacterial agents. The CFS were first heat treated to rule out all the heat sensitive compounds and proteins. Then these samples were further treated with Proteinase K. to denature all the proteins and in the last the pH of these CFS was neutralized to exclude all the organic acids. Results were obtained for all the three strains after heat treatment and proteinase K. treatment but no clear zones were

formed after the pH neutralization of the samples indicating the possible antibacterial agent to be organic acids.

Organic acids lower the pH of the environment thus preventing many bacteria to grow (Puupponen-Pimiä *et al.*, 2005). Organic acids are intentionally added to many foods as preservative to prevent the growth of pathogenic organisms, hence they are used as natural antimicrobial agents (Davidson *et al.*, 2013). The CFS of three isolated *Enterococcus faecium* strains had a pH of 4 hence representing the amount of organic acids. So, it can be evidently suggested that the antimicrobial activity of the strains was due to the organic acids.

6. Conclusion

It is concluded from the current study that the three isolated Enterococcus faecium strains are potential probiotics. They are safe to use as they have shown no symptoms of infections or sepsis in rat models. Weight increase was observed and CBC picture of the rats lied in the normal range. They have strong abilities to survive the harsh GIT environment and were able to adhere and colonize the GIT, however, there was poor colonization. They had shown antibacterial property against three established food borne pathogens, STEC, *E. faecium*, and *S. enterica*, which was due to the organic acids released by the probiotic strains.

7. Future Implications

Further clinical trials need to be done on these strains before using them on human subjects as FDA has declared probiotics as drugs. Several approaches can be used in this regard. Also, their additional applications in different diseases can be tested and hence they can be used for their benefits.

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