

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.605.052>

Phenotypic Safety Assessment and Molecular Characterization of *Enterococcus faecium* MBTU-P1F1 (KF745071) from Infant feces

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ABSTRACT

Keywords

Enteric fever pathogens, *Enterococcus faecium*, Phenotypic safety assessment, Probiotic.

Article Info

Accepted:
04 April 2017
Available Online:
10 May 2017

LAB strain *Enterococcus faecium* isolated from infant feces was found to show desirable probiotic capabilities. The strain was designated as *Enterococcus faecium* MBTU-P1F1 and gene accession number was obtained as KF745071. The strain did not have plasmid, it was susceptible to Vancomycin and the Minimum Inhibitory Concentration of Ampicillin towards it was examined. Comparison of the growth profile of *Enterococcus faecium* MBTU-P1F1 with enteric fever pathogens supported early findings which had proved the higher auto aggregating and adhesion ability of the strain than the pathogens. *In vivo* studies were carried out in balb/c mice to examine the safety of oral administration of the test strain on the health status of animal model. The study further was focused on the safety of the strain for use as human and animal probiotic at phenotypic level.

Introduction

The protective role of probiotic bacteria against gastrointestinal pathogens and the underlying mechanisms has received special attention recently. The rapid emergence of antibiotic resistance in pathogenic strains and the adverse consequences of antibiotic treatment on the protective microflora have led to the development of alternative therapies based on bacterial replacement. Probiotic bacteria can form a natural barrier against pathogen and provide significant human health protection against infection. The clinical applications of probiotics have been related to the management of gastrointestinal infections caused by pathogenic microorganisms. It is assumed that a probiotic

will be most competitive *in vivo* if it has a short lag phase and a fast growth rate. Lag period and doubling time are the most appropriate criteria for the comparison of growth between probiotic strains and pathogens (Vine *et al.*, 2004). Therefore, screening of candidate probiotics preferably requires that growth characteristics be also considered with the other selective criteria such as antagonism, production of beneficial compounds and attachment.

Although it is a naturally occurring bacterium that grows in human and animal intestinal contents some strains of Enterococci are potential human pathogens. The emergence

and the increased association of Enterococci with human disease and multiple antibiotic resistances have raised concern regarding their use as probiotics (Foulquie *et al.*, 2006).

Safety assessment of Enterococcal strains based on the absence of any possible pathogenic properties is therefore essential. Carefully selected and researched strains of *Enterococcus faecium* are well-documented as safe and effective probiotics. Among the microbial additives currently used in animal nutrition, nearly one third contain strains of *Enterococcus faecium* (EFSA panel on additives and products or substances used in animal feed (FEEDAP), 2012). The present work has been conducted with the LAB strain isolated from infant feces, characterized for preliminary and desirable probiotic properties and identified as *Enterococcus faecium*, based on biochemical characterization and PCR with species specific primers. It showed higher auto aggregating property than enteric fever pathogens, co-aggregated the pathogens and adhered to intestinal mucosa more than the pathogens (Jacob *et al.*, 2010; Raghavan *et al.*, 2013).

The objectives of the present study includes, Molecular characterization of the *Enterococcus faecium* strain isolated from infant feces. Comparison of the growth pattern of the test strain with that of the enteric fever pathogens. To Study the susceptibility pattern of the test strain towards antibiotics. The effect of oral administration of the test strain on the health status of animal model. Safety assessment of the strain (at phenotypic level) for animal and human use.

Materials and Methods

Microorganisms used in the study

Test strain: The LAB strain isolated from infant feces characterized for preliminary and desirable probiotic properties and identified as

Enterococcus faecium. The enteric fever pathogens, *Salmonella typhi* (MTCC 734), *Salmonella paratyphi* A (MTCC 735) obtained from MTCC Chandigarh.

Molecular characterization by 16s rRNA sequencing

DNA isolation was performed and PCR was carried out using the primers in Agilent Technologies Sure Cyclor 8800. The sequence of the product was determined using the automated DNA sequencing service. The sequence was deposited in the National Centre for Biotechnology Information (NCBI) gene bank data base. The phylogenetic tree was constructed by the tree building software Mega 5.05 Version. ML Heuristic method was used for inferring the tree by Nearest Neighbor Interchange.

Susceptibility to antibiotics

Determination of resistance of *Enterococcus faecium* MBTU-P1F1 to commonly prescribed antibiotics was performed by Kirby Bauer disc diffusion method.

Determination of MIC of ampicillin towards *Enterococcus faecium* MBTU-P1F1

Varying concentrations of the antibiotic ranging from 0.2 g/l to 4 g/l was employed.

Examination of plasmid profile in *Enterococcus faecium* MBTU-P1F1

Small-scale preparation of plasmid DNA was performed using rapid alkaline lysis procedure.

Growth curve analysis of *Enterococcus faecium* MBTU-P1F1 in comparison with enteric fever pathogens

The test was performed spectrophotometrically under standard

conditions for the test strain and the pathogens *Salmonella typhi* and *Salmonella paratyphi* A. Growth curves were plotted and from the graph, the growth rate and doubling time of the test strain and the pathogens were calculated.

***In vivo* studies to examine the safety of oral administration of *Enterococcus faecium* MBTU-P1F1**

Animal model and experimental design for *in vivo* studies

Male Balb/c mice (8 week old, (20±0.8) g) were used in the study. Mice were obtained from small animal breeding station under Kerala Agricultural University, Department of Veterinary and Animal Sciences University, Thrissur, India and the animals were kept in animal house of School of Biosciences, Mahatma Gandhi University.

Animals were housed in polypropylene cages and were given standard sterile dry pellet (Sai Feeds, Bangalore, India) and sterile drinking water *ad libitum*. The animals were maintained at a controlled condition of temperature of 26-28°C with a 12 h light: 12 h dark cycle. Bedding in cages was changed every day. Care and use of animals under study were followed according to the institutional guidelines of Mahatma Gandhi University.

The experiment consisted of two groups- group 1 (Test mice) and group 2 (Control mice) with six mice each. Suitable dilution of test strain *Enterococcus faecium* MBTU-P1F1 in saline containing 10⁸ viable cells per ml was chosen as sample for oral administration in animal models. The animals in the test group were given 1ml (containing 10⁸ viable cells) of test strain, *Enterococcus faecium* MBTU-P1F1 orally for 20 days. The animals in the control group received 1ml of normal saline.

Measurement of general health, growth and assessment of pathogenicity

The weight of each of the animal in the experimental and control group were measured on a Sartorius balance before the commencement of the treatment and after the completion of treatment. Feed and water intake, behavioural changes, difference in hair luster, treatment related illness (diarrhea) or death were monitored throughout the experimental period. Also the gross pathology and weight of the internal organs such as liver, spleen and kidney were measured and compared with the control animals after they have been sacrificed.

Histopathological evaluations (Wagner *et al.*, 1997)

Colonic sections of the test and control group animals were fixed in 10% formaldehyde in pH 7.4 phosphate buffered saline. Sections were stained with hematoxylin and eosin. The tissue sections were evaluated in a phase contrast microscope (Q capture Pro7™ Olympus BX 43) by a pathologist for evidence of infection, inflammation, accumulation of leukocytes, epithelial erosion, and mucosal thickness. Photomicrographs were produced with a Nikon automatic camera attached imaging software.

Phenotypic assessment of safety of the strain *Enterococcus faecium* MBTU-P1F1 for animal and human use

Safety assessment was based on the following tests

1. Determination of Biofilm formation by the test strain
2. Susceptibility to Vancomycin
3. Susceptibility to Ampicillin and Minimum inhibitory concentration of Ampicillin
4. Effect of oral administration of the test strain on the health status of animal model

Results and Discussion

Molecular characterization of the *Enterococcus faecium* strain

Amplification of the 16S rRNA gene of the test strain and sequencing of the PCR product with the forward primer 27 F produced 776 base pairs formed product of 928 bp size (Fig. 1).

Phylogenetic tree and sequence similarities of the isolated strain

The phylogenetic tree was constructed by the tree building software Mega 5.05 and is displayed in figure 2. The test strain clusters with 100% Bootstrap support with the strain *Enterococcus faecium* H2 (Gene accession number EU 887814) as given in table 1. According to the guidelines of FAO and WHO for the evaluation of probiotics in food, the first consideration is to identify and characterize the organism to the genus and species level with internationally accepted methods, such as sequencing of DNA encoding 16s rRNA. The second consideration for particular strains that are being targeted for probiotic use is to have clear and consistent strain designation. This will allow physicians and consumers to track publications associated with that strain which has probiotic effects (FAO/WHO, 2002; Reid *et al.*, 2003).

Susceptibility to antibiotics and MIC of ampicillin

The antibiogram of *Enterococcus faecium* MBTU-P1F1 is given in table 2. It was found that the test strain was resistant to Erythromycin, Gentamicin, Kanamycin, Streptomycin, Amikacin and also to the β lactam drug, Methicillin. But the test strain was moderately sensitive to Ampicillin and was sensitive to Tetracyclin, Chloramphenicol, Ciprofloxacin and most importantly towards

the glycopeptide, Vancomycin. Susceptibility of *Enterococcus* sp to Ampicillin is of considerable significance especially when they are to be characterized as probiotic for animal or human use. Here we found that our probiotic strain showed moderate susceptibility towards Ampicillin and hence determination of minimum inhibitory concentration of Ampicillin became essential. The result of MIC summarized in table 3 reveals that the growth of *Enterococcus faecium* MBTU-P1F1 has been completely inhibited by all the different concentrations of Ampicillin starting from 0.4g/l to 4 g/l.

Examination of plasmid in *Enterococcus faecium* MBTU-P1F1

Presence of plasmid was not detected in the test strain (result not shown).

Growth curve analysis

The growth curve pattern of *Enterococcus faecium* MBTU-P1F1 was compared with the growth pattern of the pathogens *Salmonella typhi* and *Salmonella paratyphi* A and the result is summarized in figure 3. Even though the lag phase of *Enterococcus faecium* MBTU-P1F1 is found to be slightly longer than the pathogens, it is evident from the graph that the growth rate of the test strain is much higher than the pathogens. The doubling time of *Enterococcus faecium* MBTU-P1F1 can be determined (time taken for the OD value to double) from the graph as 2.5 h where as that of the pathogens can be seen as 5 h. The test strain had lesser doubling time and greater growth rate and biomass than the pathogens, *Salmonella typhi* and *Salmonella paratyphi* A. Also it can be learned that the test strain has a longer stationary phase extending more than 24 h. To remain within their host, probiotics must either attach to the intestinal tract or grow fast enough to prevent them from being flushed out by the movement of food through the

digestive tract. The lesser doubling time and the greater growth rate and biomass will definitely be an advantage for our candidate probiotic strain to establish itself in the gastrointestinal tract of the host and to out compete the enteric fever pathogens *Salmonella typhi* and *Salmonella paratyphi A* during the onset of an infection. Similar study was conducted by Balakrishna and Keerthi (2011) where they compared the growth curve pattern of potential probiotic strains from the major flora of *Poecelia reticulata* with fish pathogens. It has been reported (Monaghan *et al.*, 1999) that candidate probiotic bacteria may only produce antimicrobial metabolites during the stationary growth phase. Here the longer stationary phase of the test strain can be related to production of various metabolites, most of which can add to the antimicrobial property of the probiotic candidate. The higher biomass will also facilitate enhanced production of antimicrobial metabolites.

It has been found in previous study that the test strain had higher auto aggregation property and higher ability to adhere to intestinal mucosa than enteric fever pathogens. The ability of the test strain in co-aggregating the pathogens was also established in the previous findings. Combining the above results with that of the growth profile study it can be suggested that the test strain *Enterococcus faecium* MBTU-P1F1 when used as a probiotic can prevent pathogenic invasion by these pathogens.

Safety of viable oral administration of *Enterococcus faecium* MBTU-P1F1 on the health status of Balb/c mice

The dosage for oral treatment was selected as 10^8 viable cells per ml of normal saline for each mouse per day. Probiotic concentrations ranging between 10^8 and 10^9 CFU/mouse/day are sufficient to efficiently colonize the

intestinal mucosa of rodent. This dosage is biologically relevant since it is based on a daily intake of about 3,600 billion bacteria for an adult human weighing 70 kg (Bassaganya *et al.*, 2012). Immunomodulatory effect of the potential probiotic strain *Bacillus subtilis* MBTU-PBBM1 in mice was more on treatment with 10^8 viable cells for 20 days (20). Mice in both the groups exhibited almost similar growth and body weight (Fig. 4). Also no change was observed in the general behaviour or food and water intake by the animals.

No significant difference was observed in the gross pathology and weight of the vital organs (Kidney, Spleen, Liver) indicating absence of hepatomegaly or splenomegaly (Fig. 5). All these observations give information on the safety of viable administration of the test strain *Enterococcus faecium* MBTU-P1F1 as a probiotic. Assessment of pathogenicity is one of the important components of probiotic safety studies, the indicators for which include splenomegaly and hepatomegaly (Zhou *et al.*, 2000).

Histopathological evaluation

Figure 6a, 6b, 6c and 6d represent the photomicrographs of the intestinal sections of the test group mice and the control group mice. Increased colonization by bacteria was observed in the treatment group with no signs of infection, inflammation, epithelial erosion, or abscesses.

Thickness of the mucosal layer was unaffected indicating absence of infection after oral administration of the potential probiotic strain *Enterococcus faecium* MBTU-P1F1 for 20 days. Also increased neutrophil infiltration was observed in the treatment group than the control mice. The results further support the safety of test strain for use as a probiotic.

Phenotypic assessment of safety of the strain *Enterococcus faecium* MBTU-P1F1 for animal and human use

In the present study safety of the test strain, *Enterococcus faecium* MBTU-P1F1 was assessed based on the following phenotypic observations.

Determination of MIC of Ampicillin clearly indicated that *Enterococcus faecium* MBTU-P1F1 was completely inhibited by Ampicillin at a concentration as low as 0.4g/l, which is much lower than 2g/l and this is considered crucial in assessing the safety of *Enterococcus faecium* strains. According to the recommendation by the European Food Safety Authority, any strain of *Enterococcus faecium* with MIC of Ampicillin \geq 2g/l is unsafe for use as probiotic. Strains with Ampicillin MICs < than 4g/l have the *pbp5* – S form of the gene encoding Penicillin binding protein which is characteristic of community associated non-pathogenic *Enterococcus faecium* isolates. The result clearly proves the non-pathogenic nature of our test strain.

From the disc diffusion assay to detect the antibiotic susceptibility of the strain, it was found that the strain was sensitive to Vancomycin. Sensitivity to Vancomycin is an important criterion to be studied while considering *Enterococcus faecium* as probiotic. Extensive use of Vancomycin has steadily raised the percentage of invasive nosocomial Enterococci displaying high level Vancomycin resistance. Vancomycin resistant Enterococci are resistant to all standard anti-enterococcal drugs and form a serious risk group. Vancomycin resistance is associated with the genetic element IS16, which flanks the transposon Tn1547, a virulence marker associated with hospital strains. This confers resistance to Vancomycin in *Enterococcus faecalis*. Sensitivity to Vancomycin observed

for the test strain in the study can be associated with the absence of this genetic element.

Quantitative analysis followed by SEM analysis indicated the absence of biofilm formation by test strain *Enterococcus faecium* MBTU-P1F1 isolated from infant feces (Raghavan *et al.*, 2013). Enterococci in biofilms are highly resistant to antibiotics and biofilms form an important factor in the pathogenesis of enterococcal infection. Production of biofilm can in some cases be associated with *esp* gene which is part of a large pathogenicity island in *Enterococcus* sp. A strong association between the presence of an *esp* gene and greater levels of biofilm formation in *Enterococcus faecalis* has been reported (Heikens *et al.*, 2007; 2011; Mohamed *et al.*, 2004). Other investigators have reported that *Enterococcus faecalis* (95%) isolates produced a biofilm more often than *Enterococcus faecium* (29%). The result of biofilm formation in our study can be explained by the less pathogenic trait of our isolate which can be attributed to its human origin. The result can hence be related to the absence of the *esp* gene indicating the safety of the strain for use as probiotic

The *hyl* genes have shown to increase lethality in murine peritonitis model and according to Rice *et al.*, (2003) this gene is never present in community associated clade. The *hyl_{efm}* gene is predominantly seen in Vancomycin and Ampicillin resistant strains which come under hospital associated clade. Vancomycin sensitivity and the MIC of Ampicillin has proved that the strain belong to the community associated clade. Absence of plasmid and safety assessment in the *in vivo* studies would further rule out the possible presence of the virulence marker *hyl_{efm}*. No adverse effects or signs of infection or morbidity were found in the test mice during or after oral treatment with the test strain characterized in the study.

Table.1 Molecular characterization of the selected LAB isolate

Isolate	Amplified gene region	Gene Bank accession number	Bacterial genus	Organism
MBTU-P1F1	16s rRNA gene	KF745071	<i>Enterococcus</i>	<i>Enterococcus faecium</i> MBTU-P1F1

Table.2 Study of susceptibility to antibiotics of *Enterococcus faecium* MBTU-P1F1 by Kirby Bauer Method. (R: resistant, S: sensitive, MS: intermediate)

Antibiotics and disc potency	Zone size in mm	Interpretation
Ampicillin (10mcg)	13	MS
Amikacin (30mcg)	8	R
Ciprofloxacin (30mcg)	19	S
Chloramphenicol (30mcg)	18	S
Erythromycin (10mcg)	8	R
Gentamycin (10mcg)	8	R
Carbenicillin (100mcg)	7	R
Methicillin (5mcg)	7	R
Kanamycin (30mcg)	9	R
Tetracyclin (10mcg)	19	S
Streptomycin (25mcg)	9	R
Vancomycin (30mcg)	18	S

Table.3 Evaluation of Minimum Inhibitory Concentration of Ampicillin towards *Enterococcus faecium* MBTU-P1F1

Concentration of Ampicillin in g/l	Turbidity (OD at 600nm)
0.4	0.008
0.8	0.001
1.2	0.012
1.6	-0.004
2	-0.006
2.4	-0.006
2.8	-0.016
3.2	-0.030
3.6	-0.028
4	-0.024

Fig.1 Agarose gel electrophoresis of the PCR product obtained after the amplification of the 16S rRNA genome of *Enterococcus faecium* isolated in the study from infant feces

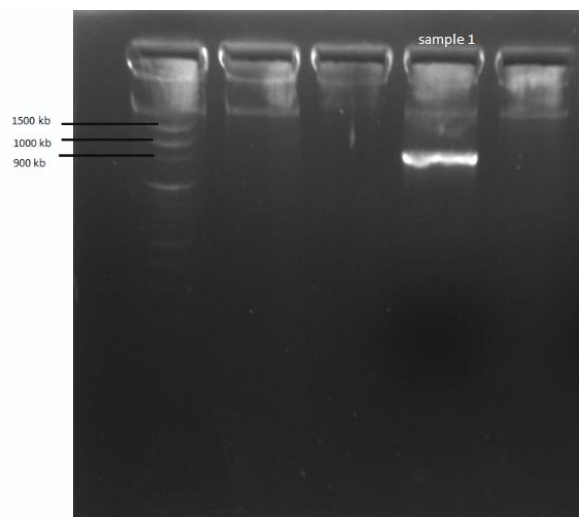


Fig.2 Phylogenetic tree of *Enterococcus faecium* MBTU- P1F1

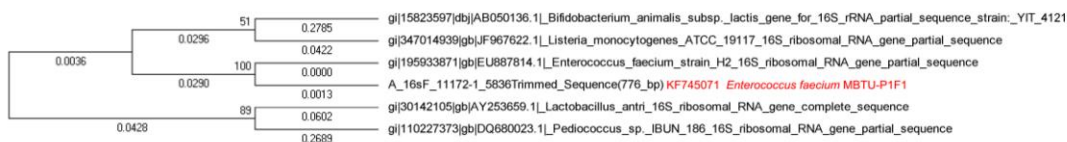


Fig.3 Comparison of the growth curve pattern of *Enterococcus faecium* MBTU-P1F1 and the pathogens *Salmonella typhi* and *Salmonella paratyphi* A

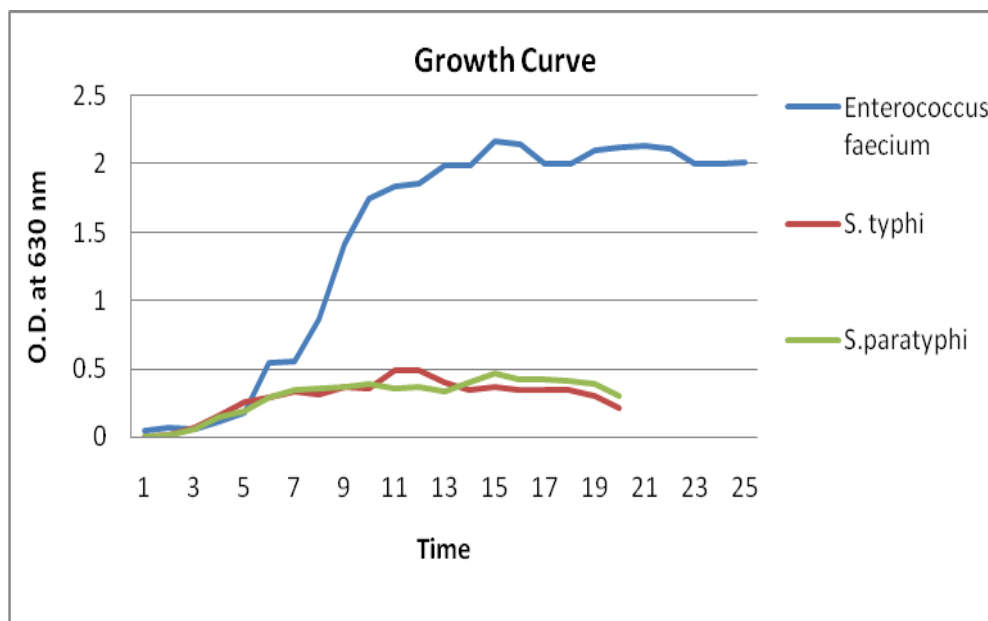


Fig.4 Body weight of test mice during the three weeks of oral administration of *Enterococcus faecium* MBTU-P1F1 in comparison with the control group. Bars represent mean \pm standard deviation of three determinations

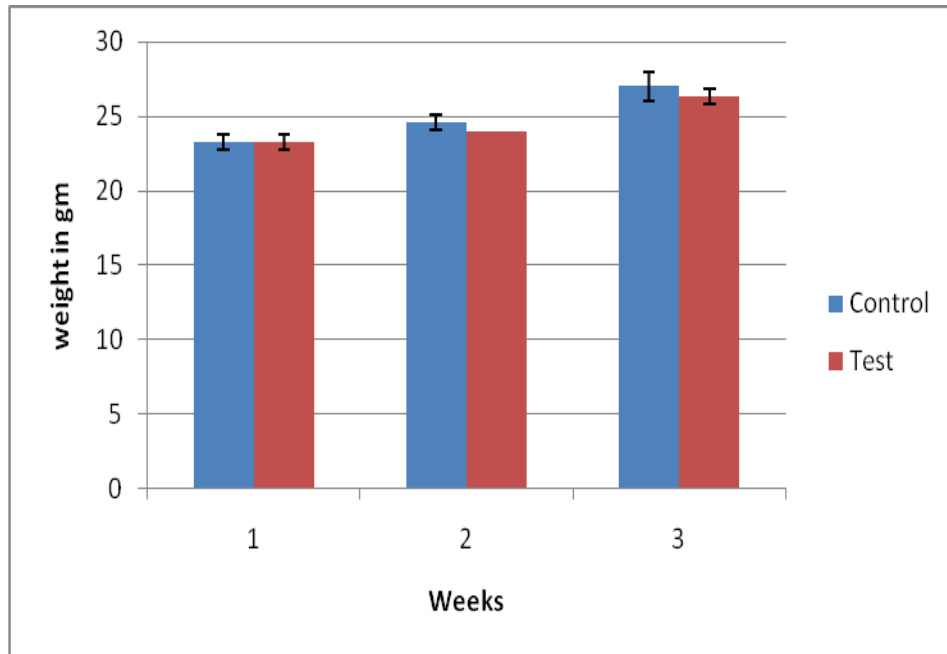


Fig.5 Effect of oral administration *Enterococcus faecium* MBTU-P1F1 as probiotic on the weight of the vital organs of test mice when compared to control mice. Bars represent mean \pm standard deviation of three determinations

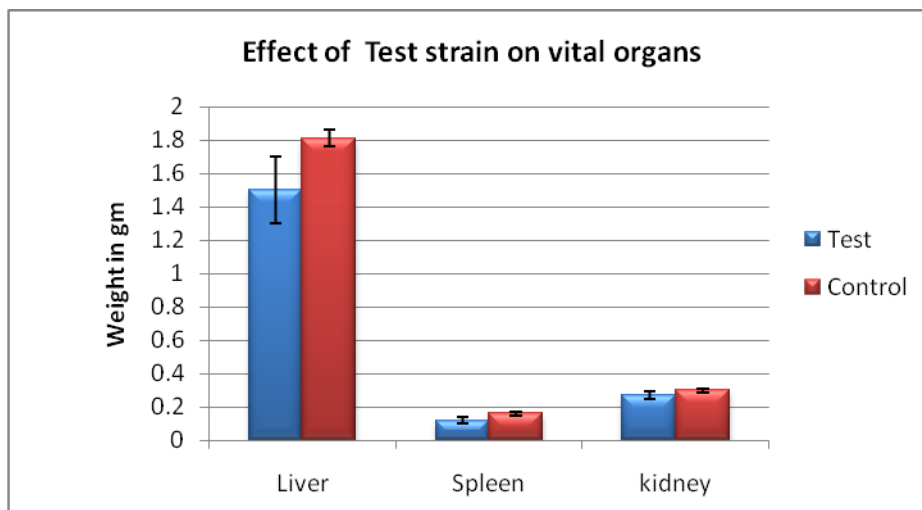


Fig.6a and b Histopathological evaluation of the intestine of the test group mice which received 10^8 viable cells of *Enterococcus faecium* MBTU-P1F1 as probiotic
(A) Enhanced bacterial colonization of intestinal mucosa
No sign of epithelial erosion, inflammation or mucosal thickening

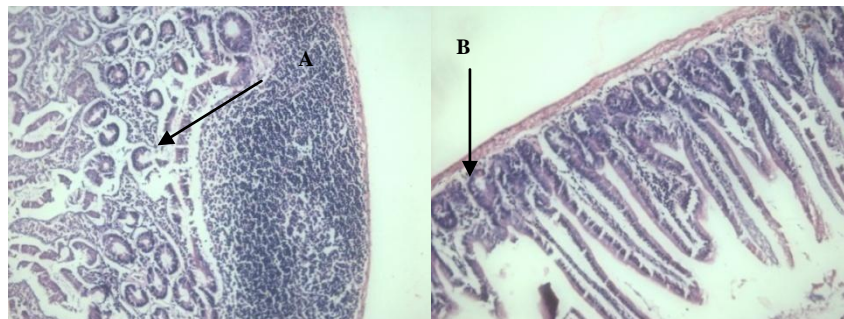
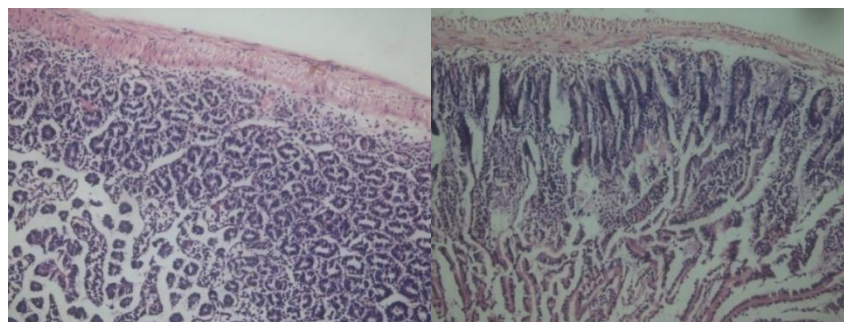


Fig.6c and 6d Histopathological evaluation of the intestine of the control group mice



Taken together, the phenotypic assessment based on the results of biofilm formation, MIC of Ampicillin, susceptibility to Vancomycin and the *in vivo* studies favour the safety of the strain *Enterococcus faecium* MBTU-P1F1. The various bacterial communities in the gut have many functions including metabolic, barrier effect, and trophic and immunological functions. Many recent researchers have highlighted the critical role of intestinal microbes on health. The gut microbiota performs a large number of important roles that define the physiology of the host. Characterisation of probiotic bacteria with diverse beneficial roles from human gut microflora can definitely pave way towards development of probiotic preparations of human origin. These

preparations can become useful in therapeutic strategies practised for management of gastrointestinal diseases.

Enterococci are an essential part of the endogenous gut microbiota of humans and animals, where they are believed to play a key role in the balance of the microbiota and thereby showing great potential as probiotics (Izquierdo *et al.*, 2008). Enterococci are also promising for the biopreservation of food, especially by means of bacteriocin production. Despite the concerns for Enterococci as opportunistic pathogens, they have long been used as human and animal probiotics (Franz *et al.*, 2007). The ability of the test strain *Enterococcus faecium* MBTU-P1F1 to colonize the gastro intestinal

epithelial wall of mice model was confirmed when appreciable no of colony forming units were obtained in selective media from the intestinal contents of the experimental animals. The result was further supported by histological evaluation of the intestinal wall, where enhanced colonization of the gastrointestinal mucosa with no signs of infection or inflammation was observed. The survivability and colonization in the digestive tract are considered critical factors to ensure optimal functionality and expression of the beneficial health effects by probiotics. The growth curve pattern of the isolate in comparison with the enteric fever pathogens project the advantage of the strain with its short doubling time and fast growth rate in outcompeting the pathogens. The final part of the study explains the safety assessment of the strain *Enterococcus faecium* MBTU P1F1 isolated from infant feces for use in animal and human nutrition. A critical or important criterion to examine while using *Enterococcus faecium* as probiotic for human or animal use is the absence of resistance to the antibiotics Vancomycin and Ampicillin. Our research strain is sensitive to vancomycin, and the MIC of Ampicillin was found to be less than 2mg/l. Also *Enterococcus faecium* MBTU-P1F1 do not form biofilm in *in vitro* tests and no mortality or lethality was observed in balb/c mice after colonization of the gastro intestinal tract with the strain. These phenotypic assessments support the absence of potential virulent factors associated with the strain and its safety for use as probiotic. Future studies can therefore be focused on genotypic evaluation of the safety assessment of the strain and clinical trials for its use as animal and human probiotic.

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How to cite this article:

Anjali Anne Jacob, T.R. Keerthi and Resmi, S.S. 2017. Phenotypic Safety Assessment and Molecular Characterization of *Enterococcus faecium* MBTU-P1F1 (KF745071) from Infant feces. *Int.J.Curr.Microbiol.App.Sci*. 6(5): 449-460.
doi: <https://doi.org/10.20546/ijemas.2017.605.052>