

Persistence and safety assessment of novel probiotic strain *Lactobacillus plantarum* 1 strain Lp86 and Lp36 in *Salmonella typhi* infected mice

Muneera Naz Baloch¹, Roquya Siddiqui¹, Urooj Zafar¹, Fouzia Haider¹,
Naheed Mojmani² and Sana Eijaz¹

¹Department of Microbiology, University of Karachi, Karachi, Pakistan

²Department of Biotechnology, Razi Vaccine and Serum Research Institute, Agriculture Research Education and Extension Organization (AREEO), Karaj, Iran

Abstract: The species of Lactic acid bacteria are known to confer beneficial effects on the host by inhabiting in their gastrointestinal tract (GIT). They succeed in surviving the harsh conditions of the GIT by exhibiting strong tolerance against gastric acids, digestive enzymes and bile simultaneously antagonizing the pathogens by production of antimicrobials. This study has been conducted to elaborate these probiotic characteristics *in vivo* for which mice were intragastrically given a probiotic approved dose of 10^{11} cfu/ml for 4 days to assess the persistence of two probiotic candidates *Lactobacillus plantarum* Lp36 and *Lactobacillus plantarum* Lp86. The fecal count of the test probiotic candidates were seen to persist well in the GIT for 15 days with a count ranging between 10^4 - 10^8 cfu/ mg of feces ($p>0.01$). The safety assessment of *L. plantarum* Lp36 in healthy and *S. typhi* in infected mice showed an increase in cell count from (day zero of inoculation) 10^6 cfu/100mg of feces to 10^8 cfu/100mg ($p>0.01$) which was maintained till day six, suggesting the persistence in the GIT. The sections of the mice intestinal lining under scanning electron microscope revealed the adherence of Lp36 and Lp86 to the intestinal epithelia. The mice did not show any adverse effect on its health. These findings make our strains promising probiotic candidates to be used to promote health benefits after further assessments.

Keywords: *Lactobacillus plantarum*, Probiotics and Lactic acid bacteria.

INTRODUCTION

Probiotics are “Live microorganisms that when administered in adequate amounts, confer a health benefit on the host” (Zielinka and Kolozyn-Krajewska, 2018). A sufficient intake of these probiotics maintains a good health of the gut by improving the number of such beneficial bacteria (FAO/WHO, 2002).

The presence of high number of viable probiotic bacteria is considered to maintain a healthy gut. This function is manifested through excluding the pathogens by exhibiting strong adherence to the intestinal epithelial cells. Such beneficial bacteria succeed in competing with the incoming pathogens by attaining nutrients and occupying the receptors for attachment and inhibit them by producing a number of inhibitory agents including organic acids, hydrogen peroxide and bacteriocins (Vanbelle, 2001). Most commonly reported probiotic bacteria belong to genera *Lactobacillus* and *Bifidobacterium* (Leeber, 2010). It is prerequisite for an efficient probiotic to reach at their intestinal target sites alive and survive the harsh conditions of Gastrointestinal tract (GIT) (Veen, 2015).

The beneficial effect of *Lactobacillus acidophilus* M92, *L.*

plantarum L4 and *Enterobacter faecium* L3 is reported by their ability to sustain in high number under acidic conditions and in presence of bile *in vitro*. They are also able to provoke a non-specific immune response in mice raising a high level of IgA, being a key factor for the inhibition of pathogens in intestine (Frece, 2005). Similar work was done where *L. plantarum* Lp36 and Lp86 were found to efficiently survive at given physical constraints (Baloch *et al.*, 2019). Hence, a probiotic candidate that is fulfilling the essential criteria of a probiotic *in vitro* will necessarily display similar functional properties *in vivo*, as well. The present study was designed to observe the *in vivo* properties of *L. plantarum* Lp86 and Lp36.

Colonization of *L. plantarum* in the intestine depends on its ability to confront the mucous barrier by adhering to the host epithelial cells by means of mannose lectins (Tallon, 2006). Since it is established that all strains from a particular species do not display the same properties and careful selection based on their claimed beneficial effect is needed (Kechaou, 2013). The present study includes the *in vivo* persistence of *L. plantarum* Lp86 and Lp36 in mice gastrointestinal tract and evaluation of their safety and their ability to function in prevention of infection was also assessed.

*Corresponding author: e-mail: mnbaloch@uok.edu.pk

MATERIALS AND METHODS

Identification

The LAB probiotic strains Lp36 and Lp86, isolated from tomato and milk on MRS (De Man Rogosa Sharpe media (Baloch *et al.*, 2019) were identified by DNA sequencing using two sets of primers; forward primer 1 for Hemolysin like protein (CGTATTATTAGTACAACAAG) and reverse primer 1 (GCTTGCGCAAATTCCACGAT) and forward primer 2 of Universal stress protein (CTACCGAATCACCAGTACTG) and reverse primer 2 (GGTTGATGATTCGGATGACG). Working solution 20 pmol/ μ l of each primers were prepared for PCR mixture using Gotaq master mix (Promega) for five reactions, amplifying at an initial denaturation at 94°C for 3 minutes followed by 30 cycles for 94°C for 3 seconds, for annealing at 56°C for 10 seconds, for elongation at 72°C for 30 seconds and another 72°C for 5 minutes for final extension and run through PCR cleanup system (Promega lot#301884). The purified products were then subjected for DNA analysis (3130 Applied Biosystem /Hitachi) for sequencing. The sequences of the samples were obtained using sequence scanner (version: 1.0, build: 20050914-ofc11 copyright Applied Biosystem 2005).

Animal care and treatment

Specific-pathogen-free BALB/c mice (females, 6 weeks of age, 25-30g each; Agha Khan University Hospital, Karachi) were maintained under normal husbandry conditions and all animal experiments were started after the animals were allowed 2 weeks of acclimation and were performed according to European Community rules of animal care (Animal Welfare in the European Union, 2017). The mice were kept in separate cages suitably designed for mice having free access to feed and drinking water where the animal care unit maintained a room temperature of 30°C and humidity between 30-70% (Ward *et al.*, 2009). The health and activity of mice were checked daily especially for the signs of weakness, weight loss and symptoms of diarrhea.

The work on mice models was conducted after approval from the Bioethical committee after signing a written informed consent. The consent procedure was in accordance with the standards defined by the Bioethical committee of the Department of Microbiology, University of Karachi.

In vivo persistence of *L. plantarum* in mice gastrointestinal tract

Preparation of probiotic dose and intragastric inoculation *Lactobacillus plantarum* Lp86 and *Lactobacillus plantarum* Lp36 were grown in de Man Rogosa Sharpe (MRS) broths (Oxoid) at 37°C. Cells were harvested by centrifugation (10,000 rpm for 10 minutes), washed twice with sterile PBS (pH-7.2) and finally resuspended in 0.2M Na₂HCO₃ buffer containing 1% Glucose (Pavan, 2003),

the number of cells were adjusted up to 10¹¹cfu/ml. Cell suspension (100 μ l) of 10¹¹cfu/ml was given intragastrically so as to achieve a dose size of 10¹⁰cfu/ml in mice. Prepared *L. plantarum* Lp86 and *L. plantarum* Lp36 cell suspension (100 μ l) each was given to mice intragastrically with the help of a sterile feeding needle. Each test mice was dosed once a day for 4 days, while they were fed with the normal mice feed and water for drinking routinely.

Enumeration of *L. plantarum* Lp86 and Lp36

The number of surviving *L. plantarum* Lp86 and *L. plantarum* Lp36 was estimated individually by collecting the fecal sample of the respective mouse daily for a period of 15 days. Enumeration of the viable cells was done after mechanically homogenizing 100mg feces sample in 1ml MRS broth and diluting it serially up to 10⁻¹⁰ in Ringer's solution. Last 4 dilutions were plated on MRS and LPSM agar plates and cfu/ml was determined by Miles and Misra technique or Drop count (Miles and Misra, 1938). The persistence of the probiotic strain (*L. plantarum* 86 and 36) in each mouse was also confirmed by observing the dissected section of the intestine under Scanning Electron Microscope.

Scanning electron Microscopy of *L. plantarum* Lp86 and Lp36 in the intestinal tissues

Small sections intestine were mounted on a cover slip and subjected to lyophilization carried out at -40°C in a vacuum pressure of 140 Torr. The specimen was warmed to temperature slightly higher than room temperature to prevent surface condensation of water prior to breaking the vacuum. For the histological examination of intestinal tissues the specimen was attached to specimen stub and processed for scanning electron microscopy (SEM; JSM 6380a, Jeol, Japan). For SEM the lyophilized tissue specimen was gold coated and observed under high magnification to observe the adherence of probiotic to the intestinal mucosa and the histology of the intestinal tissues.

Safety assessment of *L. plantarum* Lp 36 in healthy and *Salmonella typhi* infected mice

Lactobacillus plantarum Lp36 showing good persistence in mice was selected for safety assessment. It was grown in MRS broth and washed twice with sterile PBS pH-7.2 thus giving a standard plate count of 10¹¹cfu/ml.

Salmonella typhi was grown in nutrient broth and the cells were washed twice with PBS pH-7.4 by centrifugation at 10,000 rpm for 10 minutes and resuspended in the same buffer. The turbidity of the cells suspension was adjusted to give a cell count of 10¹¹cfu/ml for intrarectal inoculation. The washed cells of *L. plantarum* Lp36 were resuspended in 1ml Na₂HCO₃ buffer containing 1% glucose. A dose of 100 μ l (10¹¹cfu/ml) was given intragastrically to the test mice for 4 successive days

whereas the control mouse was given 100 μ l of the same buffer without the test strain.

In order to demonstrate probiotic effect, the mice were given the selected probiotic strain for four days, while on the 5th day the same mice were challenged with 100 μ l cell suspension of *S. typhi* (cell concentration of 10¹¹cfu/ml) by inoculation via catheter intrarectally whereas the control mice were not given *S. typhi*. Each mouse was monitored daily for 15 days for any change in activity or behavior. Also the general health status was checked for any weakness or symptoms of infection.

Selective isolation of test strain was done by collecting 100mg feces/ml MRS and LPSM (*Lactobacillus plantarum* selective medium) daily until the day of sacrifice of mice, while for *S. typhi* (from challenged mice) Bismuth Sulphite Agar was used. The cfu/ml was determined by Miles and Misra Technique (Miles and Misra, 1938; Munsch-Alatossava et al., 2007).

STATISTICAL ANALYSIS

Bacterial enumeration was subjected to one way ANOVA with Post Hoc Tukey test for the statistical analysis. P value greater than 0.01 was considered as significant.

RESULTS

Identification

Lactobacillus plantarum Lp36 and *Lactobacillus plantarum* Lp86 identified on the basis of protein hemolysin like sequence and universal stress like protein sequence with the product size of 591bp and 366 bp and sequence submitted to NCBI with a Genbank Accession number KX230689 and MK599338 respectively.

In vivo persistence of *L. plantarum* Lp86 and Lp36 in the mice gastrointestinal tract

Enumeration of *L. plantarum* Lp86 and Lp36

Lactobacillus plantarum Lp86 and *Lactobacillus plantarum* Lp36 showed significant survival in the mice GIT estimated by cell counts from the feces samples, as they gave yellow colored colonies on LPSM (Lick et al., 2001). Both the test probiotic candidates were seen to persist well in the GIT for 15 days with a count ranging between 10⁵-10⁹cfu/mg of feces (p>0.01). An increase in cell count of Lp36 was noticed from (day zero of inoculation) 10⁶cfu/100mg feces to 10⁹cfu/100mg till day 12, while the count never dropped down the initial probiotic count. Simultaneously, Lp86 also showed a rise in cell count from an initial 10⁶cfu/100mg feces to 10⁹cfu/100mg on day 8 which gradually maintained its initial inoculum number. However, in the control mice the count of 10⁴-10⁵cfu/100mg of feces was noticed throughout the experiment, representing the normal inhabitant LAB in the mice GIT (fig. 1). The adhesion of the probiotic strains to the intestinal mucosa under

scanning electron microscope gave an account of those that remained colonized to the intestinal mucosa of mice (figs. 3-4).

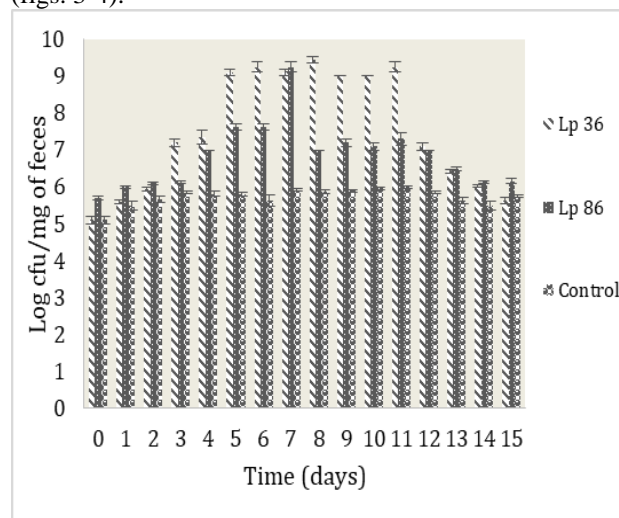


Fig. 1: Enumeration of *L. plantarum* Lp86 and *L. plantarum* Lp36 in feces of Test mice and Lactic acid bacteria flora in control mice.

Scanning Electron Microscopy (SEM)

The scanning electron photomicrographs of the small section of intestinal mucosa of mice given *L. plantarum* Lp86 and *L. plantarum* Lp36 showed good adherence to the intestinal epithelia indicating the ability of each of them to persist in the intestine of mice (figs. 3-4) without abnormally influencing the histology of intestinal tissues of the mice.

Health of test mice

Daily monitoring of the weight of mice showed that there was no gain in weight of the mice as the average weight loss was not very significant (fig. 2).

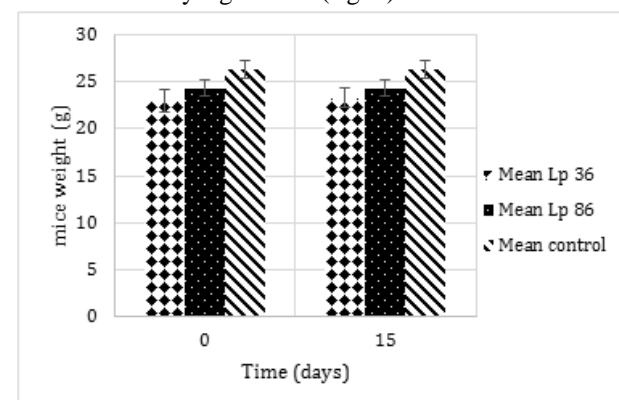


Fig. 2: Change in total body weight of mice given Lp36, Lp86 and control mice

Safety assessment of *L. plantarum* Lp36 in healthy and mice infected with *S. typhi*

The cell count of Lp36 sustained between 10⁷cfu/100mg to 10⁹cfu/100mg feces throughout the 7 days of

assessment ($P>0.01$), while the count never dropped down the initial probiotic count. However, in the control mice the count 10^4 - 10^5 cfu/100mg of feces was noticed throughout the experiment, representing the normal inhabitant LAB in the mice GIT.

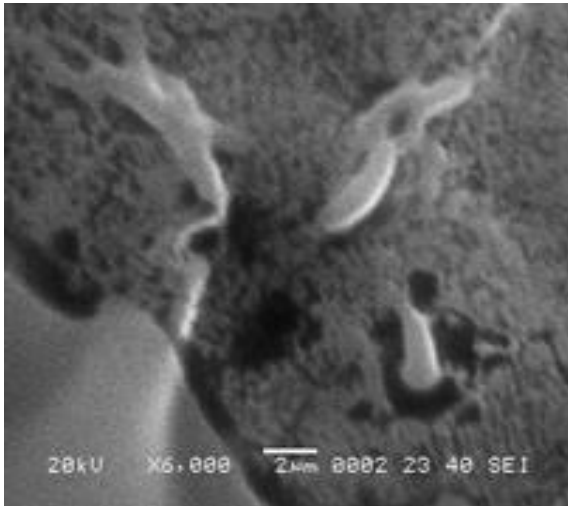


Fig. 3: Adherence of *L. plantarum* Lp36 to the intestine of mice

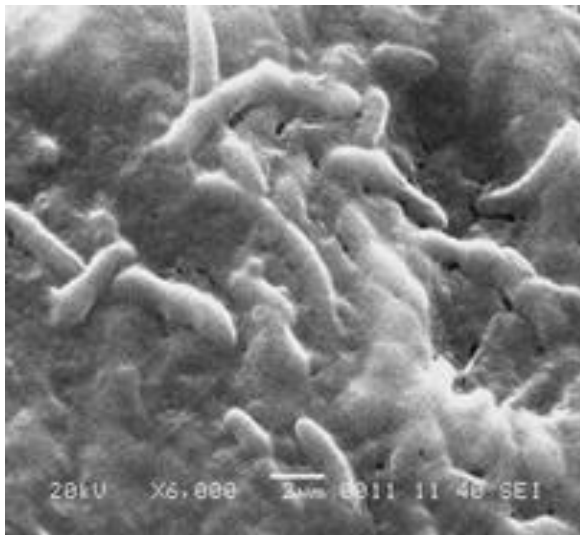


Fig. 4: Adherence of *L. plantarum* Lp86 to the intestine of mice

After 48 hours of inoculation the count of *S. typhi* was 100 folds less (10^6 cfu/ml) than the infective dose (10^8 cfu/ml). Feces from positive control mice given only *S. typhi*, on the 5th day showed a significant rise representing the infective dose (fig. 5). It was noticed that there was not much difference between the numbers of colonies on both the media. Hence, this is a clear indication that amongst the total recovered LAB, *L. plantarum* Lp36 showed maximum recovery. None of the mice (test and control) showed any symptom of illness or even weakness as there was no significant weight loss (Fig.6). The count of *L. plantarum* 36 in intestinal tissues and scrapping was 10^4 cfu/100mg.

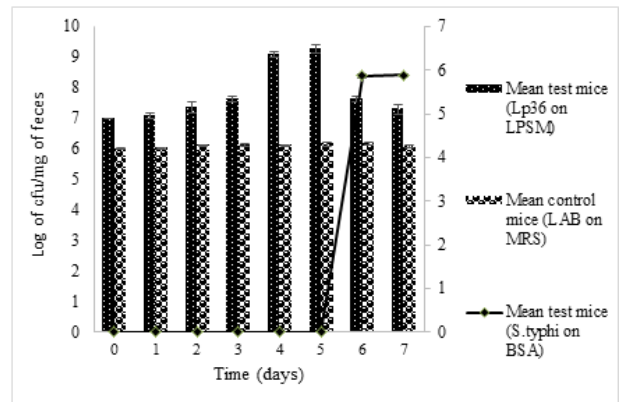


Fig. 5: Enumeration of probiotic *L. plantarum* Lp36 in healthy and mice infected with *S. typhi*.

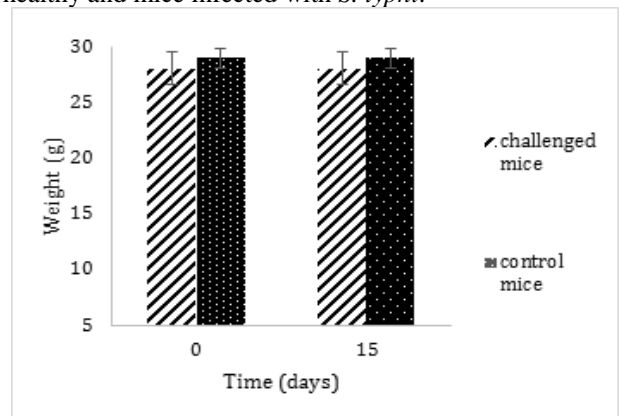


Fig. 6: Change in total body weight of challenged and control mice

DISCUSSION

The presence of surviving probiotic candidates passing out in the mice feces led us to the conclusion that both the strains were able to persist in the mice GIT for more than a week in significant number. Also during the 2nd week till the last day of assessment, the number did not go lower than the initial count i.e. 10^4 cfu/100mg of feces. This indicates that the test probiotics are able to show high level of persistence even after the probiotic administration period (rest period). These results correlate with the observations made by Daniel *et al.*, 2006 and Pavan *et al.*, 2003, who also reported a high number of probiotic bacteria, *L. plantarum* NCIMB8826 after the intake was stopped. Both strains demonstrated a high rate of excretion in the feces of mice even at post administration period (rest period) which in turn is directly proportional to their survival percentage in the GIT of mice.

In our study, a relatively higher fecal count of *L. plantarum* Lp36 and Lp86 was achieved as 5.1-9.4 and 5.6- 9.2 log of cfu/100mg of feces in mice respectively than recovered in one such study where the count was 4.6-5.3 log of cfu/gm of feces of pig (Tsuda *et al.*, 2008) , while the recovery of *L. casei* at a rate of 2.9-4.7 log of cfu/gm of feces of pig was reported (Ohashi *et al.*, 2001).

Nevertheless, it could be concluded that both our test strains persisted for more than a week at post administration period which correlates well with the results reported by Gardiner *et al.*, (2003) where *L. murinus* strains showed good fecal persistence for 5 days of post administration in pigs and for 3-10 days (Pedersen *et al.*, 1992; Rogelj, 2002). A high rate of excretion of viable strain in the feces could be said to be directly proportional to good survival in the GIT of mice which in turn indicates good adhesion (Fujiwara *et al.*, 2001). Although some strongly oppose this phenomenon to be taken as a criteria for determining the survival of strains in GIT, as Murphy *et al.*, reported in 1999, his strain demonstrating high affinity for intestinal tissues without being recovered in the feces.

The mechanism of adhesion of Lactobacilli in human gastrointestinal tract is multifactorial and their adherence has been established in many studies. As *L. reuteri* 1063 and *L. acidophilus* NCFM attach to the mucus by means of extracellular mucus binding proteins (Mub), *L. plantarum* WCSF1 displays adhesion by its lectin-like mannose specific adhesion (Msa). Whereas, other Lactobacillus strains mediate adhesion to various extra cellular matrices (made up of laminin, collagen and fibronectin) of the intestinal mucosa by their surface proteins. Thus by adhering to the target sites in the GIT, these Lactobacillus strains lead to competitive exclusion of pathogens by engaging their binding sites (Velez *et al.*, 2007). Another study reports the adhesion of LAB in mice is to the squamous stratified epithelium of mice GIT (Walter, 2008), where the attachment is facilitated by large surface proteins (Lsp) and this adherence and cell aggregation is augmented by the extra cellular polysaccharides (Peterson *et al.*, 2007).

Although, the adhesion of Lactobacillus in pigs is well explained by its association with the intestinal cells by means of heterogeneous surface determinants i.e. the bacterial external appendages covered with lectins. Similarly *Lactobacillus fermentum* 104R exhibits adhesion by means of a growth promoting protein weighing 29 kDa and *Lactococcus johnsonii* La 1 strain by its lipoteichoic acid. While a protease tolerant component on the surface of *Lactococcus acidophilus* strain LB and BG2FO has been reported to aid its adhesion to the intestinal cells (Servin and Coconnier, 2003) also that 26 genes have been identified in *Lactococcus acidophilus* that encode for cell surface proteins functioning for binding to the mucus and fibrinogen (Pfeiler and Klaenhammer, 2007). Due to structural dissemblance, the persistence phenomenon of Lactobacilli in mice does not correlate with that in humans yet it is commonly documented that the adherence ability of Lactobacilli to the epithelia augments its persistence in the GIT of humans (Walter, 2008). The probiotic while growing in the intestine utilizes the available micronutrients and glucose (Brudnak, 2008) and in turn reduces their absorption

ultimately leading to no weight gain (Duangjitchareon *et al.*, 2008).

On the other hand, it is frequently reported that a Probiotic bacteria can yield beneficial effects on the general health of the host only if it exhibits good survival in the GIT of the host by passing the hostile conditions of the gut. Such a property was observed in an indigenous isolate *Lactobacillus plantarum* Lp9 being able to resist pH-2 demonstrated very insignificant decrease in cell count while passing through the host gut along with showing good persistence, thus having great potential as a probiotic for promoting health benefits (Kaushik *et al.*, 2009). This report correlates with persistence pattern of the two probiotic candidates (*L. plantarum* Lp36 and *L. plantarum* Lp86) tested in our study. Hence, both of these tested isolates could be considered prime candidate for being used as probiotic for human consumption, but only after further *in vivo* investigations on human subject.

The safety assessment of any probiotic candidate is a prerequisite before human consumption. Hence, the two *Lactobacillus plantarum* strains Lp36 and Lp86 characterized *in vitro* for their ability to resist the hostile conditions of the host GIT (Baloch *et al.*, 2019) also showed good persistence in the gut *in vivo* where they proved to be the best surviving strains exhibiting prolonged colonization in the intestine of mice. In the present study, *L. plantarum* Lp36 was given in sufficient number i.e. 10^{10} cfu/ml (Drakoularakou *et al.*, 2003) for the provision of necessary health benefits to the host (mice). One such research reported this number to be in the range of 10^8 - 10^{11} cfu/day to promote good health (Vanderhoof and Young, 1998). Studies on adhesion of LAB to the GIT of mice revealed that a sufficiently high number of non-adhered LAB is required to be kept controlled so as to avoid the dissociation of LAB from the intestinal cells by the pathogens because the *Lactobacillus* being unable to divide rapidly in the GIT were replaced by *Enterobacteria* (Lee, 2000).

In vivo experimental studies by Ramiah, (2008) on *L. plantarum* 423 in mice revealed it as one of the dynamic probiotic candidate on the basis of its firm adhesive capability, along with *Enterococcus mundtii* ST4SA also displayed great potential in arresting Enteropathogens. Collectively, there have been many studies which have concluded that the incidence of infections by intestinal coliforms and members of family Enterobacteriaceae have been reduced upon the uptake of Lactic acid bacteria (Gardiner *et al.*, 2003).

Similarly the potential of *Lactobacilli* as a proficient probiotic is established by its ability to manifests deep prophylactic effect in resisting *Clostridium difficile* causing *Clostridium difficile* associated diarrhea (CDAD) (Parkes, 2009). *Lactobacillus plantarum* is previously documented as being safe for human consumption and can be used as a probiotic for the prevention of infection

by entero pathogens (De Vries, 2005). It has also been reported that, *L. plantarum* NCIMB 8826 when given to mice displayed good persistence for more than a week in its digestive tract, without demonstrating inflammation in the intestinal tissues and abnormal dissemination in other organs thus indicating the safety of adhesion (Pavan, 2003). Similarly a variety of LAB assessed for their safety in mice did not cause any detrimental effect leading to no weight loss and colon inflammation (Daniel, 2006). All these reports mutually relate and strongly support our experimental observations while studying the safety profile of *L. plantarum* Lp36 in mice. This preventive capability of *L. plantarum* Lp36 was exploited when given intragastrically for 4 consecutive days followed by an intrarectal inoculation of *Salmonella typhi*. Although *S. typhi* is the causative agent of typhoid fever leading to inflammation of the intestine resulting in diarrhea, and fever and on gaining access to the bloodstream the infection may disseminate to other organs like liver and spleen (Gerichter, 1960; Levison, 2008), but the uptake of *L. plantarum* Lp36 as a probiotic bacterium in sufficient number proved to be quite beneficial as it prevented the onset of infection by *S. typhi* by causing the exclusion of this pathogen from the GIT.

We reached the above mentioned conclusions by observing the fact that *S. typhi* were not recovered in the feces in numbers being significant enough to bring about the manifestations of *S. typhi* infection in mice. Some *Lactobacillus* strains are known to prevent the pathogen from adhesion to the intestinal mucosa by co-aggregating with the pathogen they interfere with their adhesion ultimately leading to their exclusion. As *Lactobacillus salivarius* have been reported to have a relatively high potential to exclude *Salmonella* from the intestine by demonstrating strong adhesion to the mucin along with showing good co-aggregation mechanism with these pathogens (Olivares, 2005).

It has been reported by many workers that as soon as the consumption of probiotic bacteria is ceased the persistence of non-adherent probiotic in the GIT is also terminated, hence this accounts for a continuous uptake of probiotic for a prolong beneficial effect (Tsuda, 2008). *Lactobacillus plantarum* Lp36 recovered in sufficient numbers even after its administration was halted led us to report it as being one of the adherent bacteria exhibiting survival for an extended period of time. Its adhesive nature has also been exploited when the intestinal tissues were subjected to scanning electron microscopy.

CONCLUSION

These *in vivo* assessments in animal models leads us to report the significant persistence of the two probiotic candidates *Lactobacillus plantarum* Lp36 and Lp86. Although their use in humans would require more evaluations to exploit their beneficial potential in alleviating a number of clinical conditions.

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