*atp*D expression of probiotic *Lactobacillus plantarum* 91 under acidic environment

Archana Chandran[1]*, Sunita Grover[2] and V.K. Batish[2]
[1]*College of Dairy Science & Technology, Kerala Veterinary and Animal Sciences University, Thrissur, Kerala, India.*
[2]*National Dairy Research Institute, Karnal, Haryana, India.*

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Abstract

Lactobacillus plantarum is a flexible and versatile microorganism that inhabits a wide variety of environmental niches, including the human gastrointestinal (GI) tract. Strains of *Lactobacillus plantarum* have proven ability to survive gastric transit and can colonize the intestinal tract of humans and other mammals. The ability of these microorganisms to grow in harsh environment prevailing in is linked to their ability to resist acidic conditions in the stomach of healthy humans. Changes in pH in the environment have been reported to influence the expression of many genes and atp operon is chiefly involved in the acid tolerance of probiotic lactobacilli in the gut. The *atp* genes are included in the category of housekeeping genes. However, the regulation of this pH-inducible phenotype has not been clearly established at the molecular level. In this study the influence of low pH on inducible gene expression in *Lactobacillus plantarum* 91 was investigated both *in vitro* and *in vivo*. Logarithmic phase cultures were exposed to pH 2.5, 2.0 and 1.5 for various time intervals and cultured for monitoring survivability. The cultures were able to survive at pH 1.5 to an appreciable level even after 1-3 h. *In vivo* study was carried out by feeding *L.plantarum* cultures to mice followed by isolation of bacterial RNA from stomach at different time intervals. The isolated RNA was reverse transcribed and the resultant cDNAs were subjected to RT-qPCR and the products were resolved by electrophoresis. The *atp*D gene was significantly up-regulated to 1.48, 2.04 and 3.05 folds after 15, 30 and 60 min. transit in the stomach of mice. This result clearly demonstrates that *atp*D gene expression is essential for survival of probiotic bacteria under acidic environment prevailing in the stomach.

Keywords: *Lactobacillus plantarum*, Probiotic, atpD, Acid tolerance

Introduction

Probiotics are defined as "live microorganisms that confer a health benefit to the host when administered in adequate amounts" (Fuller, 1989). Several health-related effects associated with the intake of probiotics have been reported in different animal models as well as in human studies (Roberfroid, 2000). However, the scientific evidence is still scarce and the mechanisms by which probiotics influence the host organism are only beginning to be explored. To fully understand the beneficial effects of probiotics, it is important to characterize the intestinal microbial community and examine the mechanisms by which probiotics exert their effects on human and animal health. Of particular relevance to probiotic research, RT qPCR can examine quantitative changes of specific members of the microbial community and the influence of probiotics on the structure and function of human and animal intestinal ecosystems. Lactobacilli are important residents of the microflora (Bengmark, 1998), and have been the subject of intense and growing interest because of their possible role in

*Author for correspondences: Phone - +91-9744975460, Email <archanac@kvasu.ac.in>.

the maintenance of gastrointestinal health. The ability of these microorganisms to grow in this environment is also linked to their ability to resist harsh conditions in the GI tract as the gastric pH is less than 2.0 in healthy humans (McLauchlan et al., 1998). Changes in pH in the environment have been reported to influence the expression of many genes (Olson, 1993), most of which are involved in maintaining the pH at values around 7.0.

For several microorganisms that inhabit the gastrointestinal tract, the F1F0-ATPase is an important element in the response and tolerance to low pH. In organisms with a respiratory chain, the primary role of this enzyme is to synthesize ATP from proton gradient of respiratory chain. Conversely, in bacteria that lack a respiratory chain, its role is to create a proton gradient and this process is then driven by ATP hydrolysis. In all these bacteria, the activity of the F1F0-ATPase increases as the pH of the growth media decreases. However, the regulation of this pH-inducible phenotype has not been clearly established at the molecular level. The *atp* genes are included in the category of housekeeping genes. In fact, their presence in the bacterial genome is considered essential for the survival of these micro organisms. Especially due to its ubiquitous distribution, functional constancy, and conservation, the gene encoding the â subunit of the *atp* operon ('*atp*D') is considered to be a suitable molecular marker for bacterial phylogenetic investigations (Ventura et al., 2004). In this respect, the study was conducted to monitor the atpD gene expression of *Lactobacillus plantarum* 91, an indigenous probiotic under acidic environment both *in vitro* and *in vivo* by real time quantitative PCR.

Materials and Methods

Lactobacillus plantarum 91(MTCC 5690; Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India), the subject of this study, was a laboratory isolate of human origin whose probiotic and colonization potential in Caco2 and HT-29 cell lines were established *in vitro* (Duary et al., 2011) previously in our lab as per FAO/WHO guidelines (FAO/WHO, 2002). The purity of the *Lactobacillus* cultures was ascertained by Gram staining and microscopic examination. The culture was propagated and maintained in MRS (de Man–Rogosa–Sharpe broth, HiMedia, India) broth at 37°C for 18 h. The active bacterial cultures were maintained in litmus milk (4°C) and also as glycerol stocks (- 70°C).

In vitro acid tolerance study

MRS (de Man, Rogosa and Sharpe**)** broth was used to simulate acidic conditions of gut after adjusting to different pH values 2.5, 2.0 and 1.5 with 1N HCl. Another set of broth was adjusted to neutral pH (7.0) to serve as a control. The broth tubes adjusted at different pH values were inoculated with overnight grown cultures of Lactobacilli @ 1.0% and incubated at 37ºC for 24 to 48 h. One ml of culture was taken from each tube immediately (0 h) and 10-fold serial dilutions were prepared in 0.1% peptone water. Pour plating was done using BCP-Lac (Bromocresol purple- Lactose) MRS agar. Similarly, one ml of culture was taken from each tube after 1, 2 and 3 h respectively and plated. The plates were incubated at 37°C for 24 to 48 h and the results were recorded. At the same time optical density of the aforesaid samples were measured at 600 nm to correlate plate count.

In vivo atpD gene expression in mice model

The mice were fed by oral intubation with 1.5 ml of overnight grown *Lactobacillus* cultures containing 10^8-10^9 cfu/ml of probiotic bacteria dissolved in Phosphate Buffer Saline (PBS). PBS alone served as a negative control. Animals were sacrificed 15 min., 30 min. and 1h after oral intubation. The stomach was flushed with sterile PBS solution to collect the bacterial cells. Total RNA from bacteria was extracted by TRI Reagent™. RNA samples were then electrophoresed at 100V using Mini gel electrophoresis (Amersham Biosciences, USA) as per the protocol of Sambrook et al. (1989).

The purity and quantity of RNA were analysed by spectrophotometery. DNase treated RNA was transcribed into cDNA, using ImPromII reverse transcriptase kit (Promega, USA). Forward primer (5'gccaacctggttcgtatgtg3') and reverse primer (5'accacgtcgtcgatcttacc3') were designed using Primer3plus software and used in this study. Reverse transcription-quantitative PCR (RT-qPCR) was performed for gene expression in a Light Cycler 480 instrument (Roche), with Relative Quantification Software (version LCS4801.5.0.39, Roche) with fluorescence signal detection (SYBR Green) after each amplification cycle. The thermal cycle conditions included initial denaturation (95^{0}C for 5 min.), followed by amplification and quantification program of 40 cycles (10 s at 95^{0}C, 15 s at annealing temperature and 15 s at 72^{0}C with a single fluorescence measurement). Melt curve analysis was done at a temperature range of 60 - 95^{0}C. Fluorescence was measured once every cycle after the extension step using filters for SYBR Green (excitation at 492 nm and emission at 530 nm). The normalized fluorescence data were converted to a log scale and the threshold was determined to calculate the threshold cycle value (*Ct*; the cycle at which the threshold line crosses the amplification curve). Upon completion of real-time PCR run, data were automatically analyzed for melt curve and quantified.

Results and Discussion

Acid tolerance is one of the most important criteria used for the selection of probiotic lactobacilli as they must survive harsh acidic environment in the gut to remain there in active viable form in a good number and express their health promoting functions. In the present study cultures were subjected to *in vitro* tolerance to different acid levels of pH 1.5, 2.0 and 2.5 for various time intervals at 37^{o}C to simulate conditions prevalent in the human gut. The cultures were able to survive at pH 1.5, 2.0 and 2.5 at 37^{o}C to an appreciable level even after 1-3 h. The results pertaining to acid resistance are given in Fig.1.The acid tolerance of *L. plantarum* 91 was relatively high with an optical density of 0.3902, 0.3801 and 0.4287 at pH 1.5, 2.0 and 2.5 after 3 h. As is quite evident from the data presented therein, the culture was able to survive at low pH. Our result in this regard is comparable with that of Mishra (2001) who also recorded high degree of acid tolerance in *Bifidibacterium* and *Lactobacillus* ssp. Almost similar observations were made by Jacobsen et al. (1999) who also recorded fairly high acid tolerance of probiotic Lactobacillus cultures such as Lactobacillus GG, C1 and Y strains. Our results on acid tolerance of Lactobacillus isolates are however inconsistent with the findings of Lankaputhra and Shah (1995) who in general recorded a decrease in the number of survivors of *L. acidophilus* strains during 3 h of incubation at all the pH conditions used in their study. This contradiction in results on acid tolerance could be attributed to varied tolerance of different probiotic Lactobacillus sp and strains towards acidic conditions.

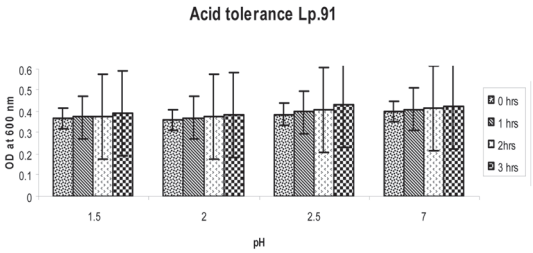

Figure 1. Expression of atpD gene of *L plantarum* 91 in mouse stomach

Lactobacilli possessing probiotic attributes are the important residents of the gut microflora and have been the subject of intense and growing interest because of their possible role in the maintenance of gastro intestinal health (Bengmark et al., 1998). However to express their functionality, most optimally, they must have the ability to endure the hostile acidic conditions of the GI tract particularly in the stomach. The survival of *L plantarum* 91 in the mouse stomach administered with *L plantarum* 91 after 15, 30 and 60 min. was determined after determining the viable counts in the flushed samples on MRS agar plates. The initial level of inoculum

that was administered intragastrically in mouse was 9.5 log counts per ml. Relative expression of the atpD along with the reference gene (Gapdh bac) in the surviving bacterial cells present in the stomach flushings was quantified by RT-qPCR. Specificity of primers for the target and reference genes used in RT-qPCR assay was confirmed by amplification of a specific single product of the desired amplicon i.e.,124 bp (atpD) and 147 bp (Gadph bac) and single product of specific melting temperatures of 82.01 and 80.92°C for atpD and Gadph bac, respectively(Fig.2). The target and reference genes were analyzed in three different sets of trials each conducted in duplicate to account for any intra and inter assay variation on the same 96 well plate. The atpD gene was significantly up regulated in mouse stomach at 15, 30 and 60 min. after oral administration. The increases in the expression of atpD gene in *L plantarum*91 were 1.48, 2.04 and 3.05 folds after 15, 30 and 60 min. transit, respectively (Fig. 3). Expression ratios were calculated using relative expression software tool (REST 2009). Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whisker-box plots represent the minimum and maximum observations.

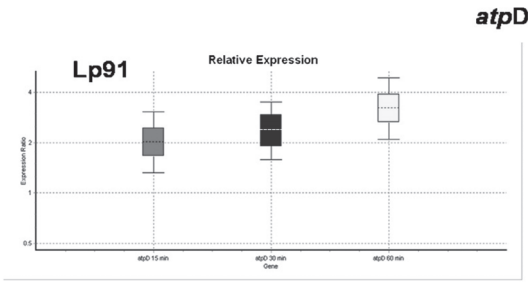

Figure 2. Confirmation of specificity of primers for target and reference genes

Surviving the host's physiological barrier i.e. high acidic conditions in stomach and bile salts' toxicity due to excessive accumulation in intestine are the two key pre-requisites along with other functional properties, to confirm probiotic status of a particular organism (Zhang et al., 2011). The level of gene

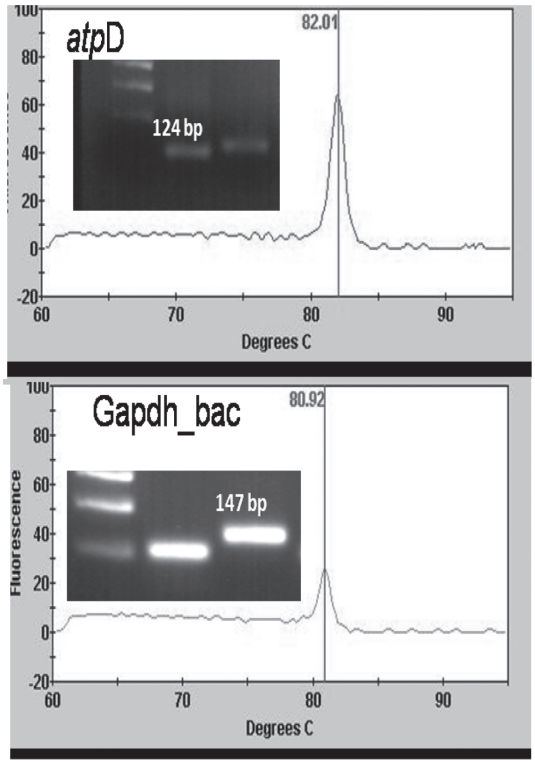

Figure 3. Relative expression of *atp*D in *L. plantarum* 91 in mouse stomach.

expression in the probiotic cultures at low/acidic pH in the stomach may vary from strain to strain. The pH changes in the environment have been reported to influence expression of many genes in bacteria most of which are involved in maintaining the pH at neutral values around 7.0 (Kullen and Klaenhammer, 1999; Jacob et al., 2007). In many bacteria, the activity of F1F0ATPase increases as the pH of growth medium decreases. However, the regulation of pH inducible phenotypes particularly under *in vivo* conditions prevalent in the gut has not been clearly elucidated as yet at molecular level. This is perhaps one of the few *in vivo* studies conducted in mouse model to demonstrate up regulation of *atp*D gene in lactobacilli in the mouse stomach on oral administration. Our results indicate unequivocally that *atp* operon is essential for the growth and survival of probiotic lactobacilli under acidic conditions which is in agreement with the observation that activity of F1F0ATPase in some related bacteria was enhanced at low external pH as described previously (Kullen and Kleanhammer

1999; Koebmann et al., 2000). The genes encoded on *atp* operon particularly *atp*D had all the desirable features to serve as appropriate molecular markers such as high genetic stability and wide distribution as reported in previous studies (Ludwig et al., 1993; Ludwig and Schleifer,1999). In one of the recent studies carried out under *in vitro* conditions, *L. plantarum* 91 exhibited high tolerance to acidic stress conditions by significant increase in the relative expression of *atp*D gene by 4.7 fold at pH 2.5 after 90 min (Duary et al., 2010).

Study concludes that the indigenous probiotic *L. plantarum* 91 is quite robust and has the potential to survive the hostile acidic environ-ment with extended transit in the gut with optimal functionality. Hence this probiotic can be applied extensively in the production of fermented foods.

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