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Expression of the *atpD* gene in probiotic *Lactobacillus plantarum* strains under *in vitro* acidic conditions using RT-qPCR

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Abstract

F_1F_0-ATPase has been identified as an operon directly involved in the tolerance of probiotic bacteria towards a hostile acidic environment encountered in the stomach. Expression of *atpD* (a key part of the F_1F_0-ATPase operon) gene of the two putative probiotic *Lactobacillus plantarum* isolates (Lp9 and Lp91) under different *in vitro* pH conditions which closely mimic the physiological environment prevalent in the human gut was investigated by quantitative real-time PCR (RT-qPCR). A battery of housekeeping genes, i.e. *gapB*, *dnaG*, *gyrA*, *ldhD*, *rpoD* and 16S rRNA, were evaluated using geNorm 3.4 Excel-based application for normalizing *atpD* gene expression in Lp9 and Lp91. The most stably expressed genes were found to be *gapB*, *gyrA* and *ldhD.* Although both putative probiotic *L. plantarum* isolates investigated in this study were able to survive acid stress under *in vitro* conditions, amongst the two, Lp91 exhibited relatively greater acid tolerance, as revealed by 4.7-fold upregulation of the *atpD* gene as well as higher log counts at pH 2.5 after 90 min These results clearly demonstrate that expression of the '*atp*' operon was chiefly instrumental in *in vitro* survival and tolerance of test cultures at acidic conditions encountered in the stomach.
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Keywords: *Lactobacillus plantarum*; F_1F_0-ATPase; Probiotics; Real-time PCR (RT-qPCR); geNorm

1. Introduction

Lactic acid bacteria (LAB) have been traditionally used as starter cultures in fermentation industries for many food products over the past decades. Amongst these, *Lactobacillus* and *Bifidobacterium* are the two key members which are being used extensively as probiotics because of their GRAS (Generally Recognized As Safe) status and their ability to exhibit several health-promoting functions in the human gut (Alvarez-Olmos and Oberhelman, 2001; Shanahan, 2002; Guarner and Malagelada, 2003). Lactobacilli are natural inhabitants of healthy human gastrointestinal (GI) tract and also have a long history of safe use in foods and fermented products. However, one of the essential prerequisites for optimal expression of their physiological functions in the gut is that they must overcome the host's physiological barrier, including acidic conditions in the stomach, in order to reach the gut (Morelli, 2000; Barmpalia-Davis et al., 2008) with extended transit time. The ability of probiotic organisms to survive in an acidic environment is important for *in vivo* functionality as well as stable fermentation characteristics. Therefore, mechanisms contributing to the ability of a microorganism to tolerate acidic pH are essential in the production and functionality of a probiotic culture. In a recent investigation, *Lactobacillus acidophilus* NIT (isolated from infant feces) was reported to exhibit fairly strong tolerance to acidic pH ranging from 2 to 4 (Pan et al., 2009).

Recent advances in genomics and proteomics have evoked considerable interest in whole genome sequencing of several lactic acid bacteria which also include a few probiotic *Lactobacillus* strains. The entire genome sequences of well established probiotic *Lactobacillus* and Bifidobacterium strains are now available in the public domain. Amongst

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probiotic *Lactobacillus plantarum* strains, WCFS1 is the only one which has been completely sequenced at present (Kleerebezem et al., 2003). The generation of nucleotide and amino acid sequences of probiotic strains has led to the identification of important genes encoding desirable probiotic attributes such as F_1F_0-ATPases associated with acid stress in probiotic lactobacilli (Kullen and Klaenhammer, 1999). The multi-subunit F_1F_0-ATPase ('*atp*' operon) which facilitates the extrusion of protons from the cytoplasm by proton motive force (PMF) is one of the main proton pumps utilized by Gram-positive bacteria to survive or tolerate low pH (Cotter and Hill, 2003). The '*atp*' operon, one of the key components, is mainly associated with pumping of protons from the bacterial cytoplasm to outside and hence helps in maintaining neutral pH in the bacterial cytosol. Bron et al. (2004) were able to identify 72 genes expressed by *L. plantarum* WCFS1 in mouse GI tract by using resolvase-based *in vivo* expression technology (R-IVET). Among these, copper-transporting ATPase (*copA*) was found to be induced in response to acidic stress. Using microarray technology, Wall et al. (2007) reported the expression of 72 differentially expressed genes at pH 2.7. Amongst these, early induction of the *clpL* (ATPase with chaperone activity) gene of *Lactobacillus reuteri* ATCC 55730 was found to be involved in their survival capacity at pH 2.7, which was close to human stomach conditions. The role of ATPase in regulating the acidic condition has also been reported in *Lactobacillus casei* (Chen et al., 2009) and *L. acidophilus* NCFM (Azcarate-Peril et al., 2009). However, there is very little information available on the expression of such genes induced in probiotic *L. plantarum* strains subjected to *in vitro* and *in vivo* hostile acidic environments. This gap leaves ample scope for extending similar studies in *L. plantarum* strains as well.

The present investigation was intended to study the expression of the *atpD* component of F_1F_0-ATPase in *L. plantarum* Lp9 and Lp91 in comparison to the reference strain *L. plantarum* CSCC5276 by quantitative real-time PCR (RT-qPCR) to establish a relationship between the activity of membrane-bound F_1F_0-ATPase and tolerance of lactobacilli cultures to the acidic conditions. For accurate gene quantification, the geNorm application was used to select the most stable reference gene from amongst the six housekeeping genes, i.e. *gapB*, *dnaG*, *gyrA*, *ldhD*, *rpoD* and 16S rRNA. The expression ratio of the *atpD* gene over different acidic conditions was tested for significance by a randomization test implemented in the relative expression software tool (REST).

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. plantarum Lp9 and *L. plantarum* Lp91, selected as the subjects of this study, were laboratory isolates recovered from buffalo milk and human feces respectively. The probiotic attributes of these cultures were tested under *in vitro* conditions as per FAO/WHO (2002) guidelines. Their colonization potentials were also ascertained on Caco 2 and HT-29 cell lines. Standard *L. plantarum* CSCC5276 (also designated as NCDO 82 or VTT E-71034) (Crittenden et al., 2002; Kaushik et al., 2009) was used as a reference culture for comparative analysis. The *Lactobacillus* cultures were grown in MRS (de Mann–Rogosa–Sharpe broth, pH 6.8) at 37 °C for 18 h aerobically and activated cultures were maintained in both litmus milk (4 °C) and as glycerol stocks (−70 °C).

2.2. Survival curves of probiotic strains at acidic pH

The overnight cultures were pelleted by centrifugation at 5000 rpm for 5 min, and washed twice in sterile PBS (pH 7.4). The washed cells were resuspended in MRS broth set at different pH values, i.e. 6.5, 4.5, 3.5 and 2.5, by setting the OD_{600} of cultures to 1.8 ($\approx 2 \times 10^9$ cfu/ml). The suspended cultures were incubated at 37 °C and samples drawn at 30-, 60- and 90-min intervals were serially-diluted and plated on MRS agar. Surviving bacteria were counted as colony-forming units per milliliter (cfu/ml) after incubating the plates at 37 °C for 48 h and survival curves were plotted for each strain to determine their survival. The experiment was repeated twice and each reading represents the mean of three observations.

2.3. Acid tolerance

The overnight cultures were transferred to fresh MRS broth with an OD_{600} of 1.8 and incubated for 4 h at 37 °C. The cultures were pelleted by centrifugation at 5000 rpm for 5 min and washed twice in sterile PBS (pH 7.4). The washed cells were resuspended in MRS broth set at different pH values by setting the OD_{600} of culture to 1.8 ($\approx 2 \times 10^9$ cfu/ml). Both test cultures along with the standard culture *L. plantarum* CSCC5276 were grown separately in MRS broth pre-adjusted to different pH values, i.e. 4.5, 3.5 and 2.5 to monitor their expression profile of the *atpD* gene. The overnight cultures grown in MRS at normal pH 6.5 were taken as experimental controls. The suspended cultures were incubated at 37 °C and samples were drawn for further analysis at 30-, 60- and 90-min intervals effectively covering the expected transit period of these cultures in the gut.

2.4. Total RNA isolation and cDNA synthesis

The cellular pellet obtained from a 1.5 ml sample of each culture was resuspended in 200 μl of Tris–HCl buffer (10 mM, pH 8.0) and mixed thoroughly by vortexing. The cells were lysed by addition of an aliquot of 6 μl of lysozyme (50 mg/ml of 10 mM Tris–HCl. pH 8.0) (Ramiah et al., 2007) and incubated at 37 °C. The mixture was processed for total RNA extraction with TRI Reagent (Sigma, USA) as per the manufacturer's instructions. RNA was suspended in 50 μl of DEPC-treated water. Purity of the total RNA extracted was determined as the 260/280 nm ratio and integrity was checked by electrophoresing on 1% agarose gel. Residual DNA was removed by treating RNA with RNase free DNase I as per the manufacturer's instructions (Promega, USA). RNA was stored at −80 °C until further use. An aliquot of 1 μg of the DNase-treated

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