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Research Article

MODULATION OF GROWTH AND PROTON PUMPING ATPase ACTIVITY OF PROBIOTIC *Lactobacilli* BY DIETARY CUCURBITS

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Abstract: Gastrointestinal tract predominantly harbor probiotic *Lactobacilli* which exert beneficial effects on human health. Aqueous extracts from fruits of *Lagenaria siceraria (Ls), Luffa cylindrica (Lc)* and *Cucurbita maxima (Cm)* were prepared and lyophilized. Fruit extracts were investigated for their effects on *Lactobacillus rhamnosus* (*L. rhamnosus*), *Lactobacillus plantarum* (*L. plantarum)* and *Lactobacillus acidophilus* (*L. acidophilus*). Extracts were found to enhance growth of *Lactobacilli* without any toxic effect (up to 1000µg/mL concentration). Minimum concentration of extracts at which growth of probiotic strains were found to be enhanced significantly were determined (103.67 µg/mL-118µg/mL) and considered as effective concentration (EC) or growth stimulatory concentration (GSC). Proton pumping ATPase activity of *Lactobacilli* were examined and found to be enhanced significantly (29.89-61.96%) in extracts treated probiotics (*Lactobacilli*) as compared to the normal control. Inulin used as positive control and found to enhance the proton efflux activity (28.06-37.72%) with respect to the control. These dietary cucurbits enhance metabolic activity of probiotic *Lactobacilli* by modulating their proton pumping ATPase mechanism. This study suggested that the consumption of cucurbit fruits might be a natural source of enhancing the activities of probiotic *Lactobacilli* in the gut.

Keywords: ATPase; Cucurbits; H^+ efflux; *Lactobacilli* and Inulin.

Introduction

Probiotics inhabit the gut and have been implicated as a therapeutic agent for treatment of various ailments including cancer. *Lactobacilli* of probiotic nature confer health benefits by several mechanisms (Ciorba *et al*., 2012; Velez *et al.,* 2010; Ahrne *et al.,* 2011; Amdekar *et al.,* 2012; Segawa *et al.,* 2011). Probiotics suppress colitogenic and oncogenic bacterial activities in the gut (Patyar *et al.,* 2010). Altered microbial compositions in the gut with declined probiotics and increased population of enteropathogens have been evidenced in diseased persons (Othman *et al.,* 2011). In the present study three strains of *Lactobacilli* such as *L. rhmnosus*, *L. plantarum* and *L. acidophilus* were taken to evaluate the effect of cucurbits on these organisms. The hypothesis behind this study was based on an ancient quotation (2400 years ago) composed by the father of medicine (Hippocrates) that "Let food be thy medicine, and medicine be thy food" (Ahmad *et al*., 2011). *Lactobacillus rhamnosus* GG activate the epidermal growth factor receptor (EGFR) pathway, thereby suppressing cytokine-induced epithelial cell apoptosis and protecting against experimental colitis (Yan *et al.,* 2011). Furthermore, this bacterium attenuates enterohemorrhagic *Escherichia coli* O157:H7 (Johnson-Henry *et al.,* 2008). *Lactobacillus plantarum* has been reported to modulate the

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expression of GI epithelial tight junction proteins *in vivo* (Karczewski *et al.,* 2010). Gastroprotective efficacies of cell free culture supernatant of *Lactobacilli* have been studied. *L. acidophilus* have capacity to enhance total IgA titer significantly in the levels of IgA specific for *Salmonella enterica* subsp. *enterica* serovar Typhi (Link-Amster *et al.,* 1994).

Health benefits of cucurbits have been documented since time immemorial. Presence of secondary metabolites of medicinal values and essential nutritional supplements has recently been elucidated and reviewed (Habibur-Rahman, 2003; Ahmad *et al.,* 2011; Irshad *et al.,* 2013; Anamika *et al.,* 2007). Cucurbitacins, alkaloids, flavonoids, steroids, ribosome inactivating proteins and several other bioactive constituents of nutritional and pharmacological importance of cucurbits have been reported (Irshad *et al.,* 2010; Irshad et al., 2013). For example, *Lagenaria siceraria* (*Ls*) exhibited immune modulation, antioxidants and anticancer properties (Ahmad *et al.,* 2011). *Luffa cylindrica* (*Lc*) has been reported as effective antioxidants and anti-inflammatory while *Cucurbita maxima* (*Cm*) are reported for hepatoprotective and antioxidants activity (Irshad *et al.,* 2013; Jadhav *et al*., 2010). Therapeutic implications of these plants have not been extensively studied through probiotic bacteria. Dietary plant might be used as potential sitotherapeutic agent by targeting enteric probiotics through which host's physiology can be modulated. Therefore, the present study was designed to unveil the effects of dietary cucurbits on metabolic activities and modulation of the growth dynamics of probiotic *Lactobacilli in vitro.* Proton pumping ATPase activity of bacterial cells links the generation of ATP to the transmembrane proton motive force (PMF) by fermentative substrate-level phosphorylation. The PMF can facilitate the extrusion of protons from the cell cytoplasm. Lactic acid bacteria use H^+ ATPase pathway for generation of energy on which metabolic activities and cell viability depend. Furthermore, proton pump ATPase has been targeted for therapeutic delivery of fungicidal drugs of dietary nature (Shreaz *et al.,* 2011). Therefore, in the present study, aqueous extracts of cucurbit fruits were prepared and H^+ ATPase pathway was targeted to unveil the mechanism involved in extracts induced growth modulation of probiotics.

Materials and Methods

Procurement of bacterial strains and their culture conditions

Some standard strains of probiotic *Lactobacilli* (Table 1) were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Organisms were cultured and maintained in MRS growth medium (De Mann Rogosa and Sharpe, 1960).

Table 1
Probiotic Lactobacilli used in this study

Strains with MTCC Code	*Growth medium*	*Temperature (^{0}C)*	*pH*
L. rhamnosus 1408	MRS	36.5	6.5 ±.02
L. plantarum 1407	MRS	36.5	6.5±.02
L. acidophilus 447	MRS	36.5	6.5±.02

Preparation of Extracts

Fresh fruit of cucurbits, *Lagenaria siceraria, Luffa cylindrica* and *Cucurbita maxima* were purchased from local market of Jamia Nagar, New Delhi and identified. Fruits were washed, homogenized in double distilled water and filtered by muslin cloth. At the end juice was centrifuged at 6000 rpm and supernatant was lyophilized. Samples were kept in vacuum desiccators for further studies.

Growth Stimulatory Concentration (GSC)

Microtiter Assay

Cells from mid log phase were diluted in 10 mL of MRS growth medium, adjusted its optical density to 0.1 (OD at 600nm) or ~10^4 colony forming unit per mL (CFU/mL) and dispensed in round bottomed 96-well microtiter plates (100 µL/well) containing equal volumes of medium and different concentrations of test compounds (25µg/mL to 1000 µg/mL) (Irshad *et al.,* 2011). Extract free controls were also taken and plate was incubated for 24h at 36^{0}C in incubator shaker at 150 rpm. At the end OD at 600nm was read in a microplate reader (BIO-RAD, *i*Mark, USA). GSC was evaluated by comparing the OD obtained for

extract treated and extracts free control. Same procedure was adopted for inulin (100µg/mL) treated and untreated organisms. GSC was considered as pronounced effective concentration (EC).

Growth Curve Studies

All the three strains of probiotic *Lactobacilli* were subjected for their growth dynamics in the presence and absence of extracts. Briefly, 100µl from overnight fresh culture (~10^8 CFU/mL) was inoculated in respective flasks containing 100mL of MRS growth medium with and without extracts, incubated at 37^0C in incubator shaker and optical density (OD_{600}nm) was read at the interval of every 2h for 24h using LaboMed Inc. spectrophotometer (California, USA). Extracts used were at the rate of their GSC concentration. Inulin at the concentration 100 µg/mL was also supplemented in the growth medium and inoculated with the test organisms as positive control.

Proton (H^+) Efflux Assay

Acidification of medium due to proton (H^+) efflux by *Lactobacilli* was monitored by measuring the pH (Rashid *et al.,* 2004). Briefly, cells from mid-log phase were washed twice with distilled water and 0.2 g cells were suspended in 10 mL solution containing 0.1MKCl, 0.1mM$CaCl_2$. Isotonic condition was maintained by adding KCl and $CaCl_2$. Suspension was kept in a double-jacketed glass container with constant stirring connected to a water circulator at 25^0C. Initial pH was adjusted to 7.0 using 0.01 M HCl/NaOH. Extracts to be tested were added to achieve the desired concentrations in 10 mL solution. 5 mM glucose in total volume of suspension was used for glucose stimulation experiments. Experiments were also performed with 450µg/mL inulin the commonly used prebiotic for stimulating growth of probiotics. At the end H^+ extrusion rate was calculated from the volume of 0.01 N NaOH consumed.

Statistical Analysis

All the experiments were performed three times and results were expressed as Mean ± Standard Deviation (SD).

Results and Discussion

Evaluation of growth Stimulatory Concentration (GSC) of Extracts

The aqueous extracts of cucurbits have been reported to contain anti-oxidant compounds and secondary metabolites of therapeutic importance in our previous findings (Irshad *et al.,* 2010; Irshad *et al.*, 2013). In the present study we intended for growth stimulatory effects of the aqueous extract of cucurbit fruits on three strains of probiotic bacteria. *Ls* and *Lc* were found to enhance the growth of all the three strains of *Lactobacilli* at the minimum concentration 103.67±6.11 and 106.33±7.02 whereas *Cm* exhibited the same effect at the minimum concentration 113.00±6.00 (Table 2). Plant polysaccharides such as inulin and fructo-oligosaccharide have been implicated as therapeutic and prophylactic agent due to their prebiotic effects. *L. siceraria* fruit has been reported to contain a water-soluble polysaccharide such as methyl-á-D-galacturonate, 3-O-acetyl methyl-á-D-galacturonate, and *â*-D-galactose residue in equal proportions (Kaushik *et al.,* 2009). Our results of GC-MS (data not shown) of *Ls, Lc* and *Cm* were found to contain 2-Deoxy-D-galactose. Therefore, it can be concluded that the growth stimulatory effect on probiotic *Lactobacilli* might be due to the presence of this or other compounds present in the extract.

Table 2
Growth Stimulatory Concentration of Extracts (*Ls, Lc* and *Cm*) Evaluated on three Strains of Probiotic *Lactobacilli*

	L. rhamnosus 1408	*L. Plantarum 1407*	*L. Acidophilus 447*
Ls	103.67 ± 6.11	110.67± 4.04	113.33 ± 10.41
Lc	106.33 ± 7.02	111.33 ± 5.51	117.67 ± 7.51
Cm	113.00 ± 6.00	121.00 ± 3.61	118.00 ± 7.55

Unit of concentration denoted as µg/mL (103-118 µg/mL) and results were expressed as Mean ± Standard Deviation (SD) of three independent experiments.

Studies on Growth Dynamics (turbidometric measurement)

The effect of different extracts on all the three strains of probiotic *Lactobacilli* were evaluated by

the growth curve studies. Significant and pronounced effects were observed on all strains with lag phage of 4-8h (*L. rhamnosus* and *L. plantarum*), 6-9h (*L. acidophillus*), and active exponential phase 8, 9-18h before attaining stationary phase (Figure 1). Concentration of the extracts used for growth curve studies were their respective GSC (Table 2). Inulin (100µg/mL) was used as positive control. Significantly enhanced growth pattern were observed for all the three extracts on *L. rhamnosus* 1408, *L. plantarum* 1407 and *L. acidophilus* 447 in comparison to normal and positive controls. The growth patterns of probiotics used in these studies suggested that the extracts have more potent prebiotic effect than inulin. Our studies display a pronounced evidence of the therapeutic implications of cucurbits by direct effects through their antioxidant properties (Irshad *et al.,* 2013) and by prebiotic effects. Therefore, cucurbits may be recommended for daily intake in adequate amount to validate the hypothesis described above based on Hippocrates.

Proton (H^+) Efflux Measurement

The proton pumping ATPase activities link with the production of ATP and metabolic status of respiring organisms. Lactic acid bacteria utilize proton pump ATPase and GAD (glutamic acid decarboxilase/glutamate decarboxilase) system for nutrient uptake (Zuniga *et al.,* 2002; Higuchi *et al.,* 1997). In the present study H^+ ATPase system of probiotic *Lactobacilli* was targeted to understand the nutrient uptake mechanisms while supplying extracts of dietary cucurbits. *Lactobacilli* depleted of carbon source when exposed to glucose, rapidly acidify the extracellular medium to generate PMF for nutrient uptake. Probiotic *Lactobacilli* were investigated for their capacity to efflux intracellular proton (H^+) to the external medium (as monitored by the alteration of pH of the external medium) in the presence and absence of extracts (Table 3). Proton pumping ATPase activity was found to be enhanced by 61.96, 57.36 and 59.62% in *L. rhamnosus*, *L. plantarum* and *L. acidophilus* respectively with 450µg/mL of *Ls* whereas 48.78%, 42.13% and 40.85% with 450µg/mL of *Lc* and 33.71%, 35.36%, 29.89% with 450µg/mL of *Cm* as compared to untreated control

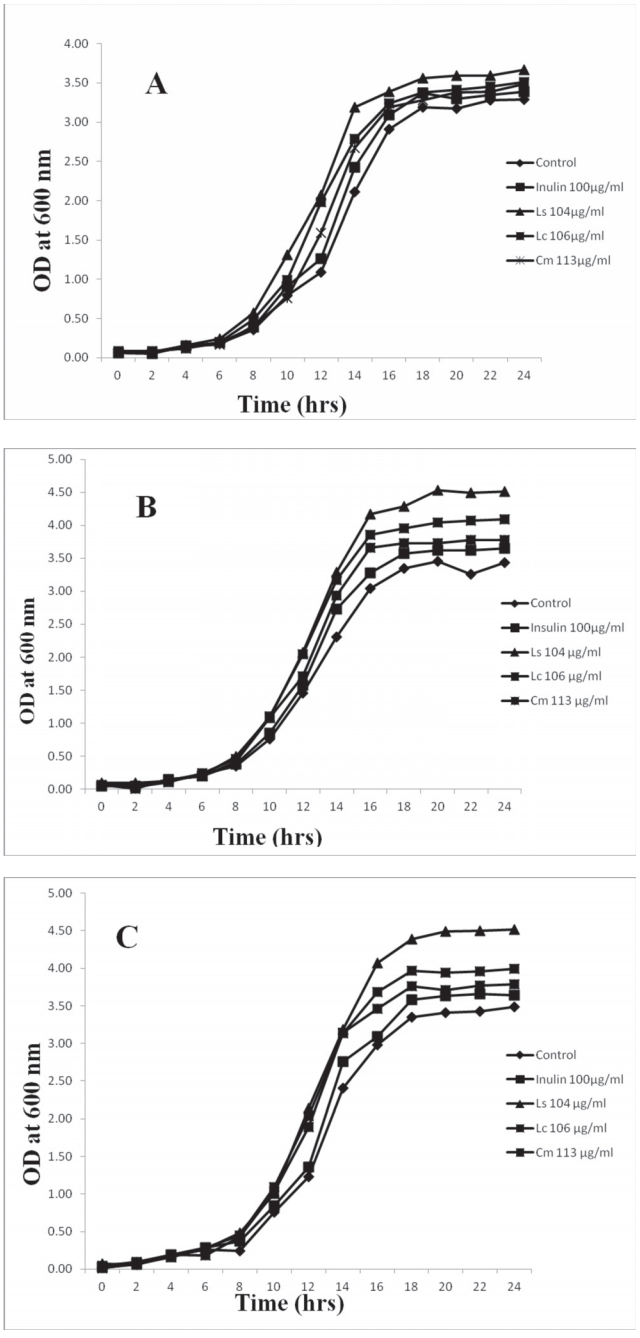

Figure 1: **Effect of *Ls*, *Lc* and *Cm* on growth of *L. rhamnosus* 1408, *L. plantarum* 1407 and *L. acidophilus* 447. Inulin was used as positive control.**

(A: *L. rhamnosus* 1408; B: *L. plantarum* 1407; and C: *L. acidophilus* 447)

(Table 3). Inulin used as positive control and found to enhance the proton efflux activity by 28.06, 31.98 and 37.72% as compared to untreated control. Glucose (5 mM) stimulated proton efflux in all the strains by ~2 folds with respect to the normal control. This study provides us the

Table 3
Effect of aqueous extracts (lyophilized) from fruits of *Ls*, *Lc* and *Cm* at concentration 450µg/mL on the rate of proton efflux by probiotic *Lactobacilli* at pH 7. Cells were suspended in 0.1M KCl and 0.1mM $CaCl_2$ at 25 °C

	Range of relative proton efflux rate (nmol min^{-1} mg $cells^{-1}$)		
Incubation with	*L. rhamnosus*	*L. plantarum*	*L. acidophillus*
Control	1.77±0.15	1.97±0.15	2.13±0.15
Ls	2.87±0.15 (61.96)	3.10±0.10 (57.36)	3.34±0.10 (59.62)
Lc	2.63±0.25 (48.78)	2.80±0.10 (42.13)	3.00±0.10 (40.85)
Cm	2.37±0.21 (33.71)	2.67±0.06 (35.36)	2.77±0.15 (29.89)
Inulin (450µg/mL)	2.27±0.12 (28.06)	2.60±0.10 (31.98)	2.93±0.21 (37.72)
Glucose (5mM)	3.47±0.25 (95.86)	3.93±0.15 (99.66)	4.1±0.20 (92.49)

Values were expressed as mean ± SD (n=3). Values in parentheses give the enhanced %-tage of H^+-efflux with respect to the untreated control.

mechanism involved to stimulate growth of probiotic *Lactobacilli* when cultured with various extracts of cucurbits used.

Conclusion

Dietary cucurbits investigated here under the present study explicitly revealed that these group of plants have remarkable growth stimulatory effect on probiotic *Lactobacilli*. These bacterial genera have potential to augment enteric health hence; their population can be maintained in the gut through dietary intervention of cucurbits in adequate amounts. Our findings provide the first scientific evidence targeting proton pumping ATPase activity of *Lactobacilli* through dietary cucurbits to reveal the mechanism involved in stimulating the growth of organisms investigated in this study. Therefore, dietary cucurbits can be of high sitotherapeutic values to regain or rehabilitate antibiotic/chemotherapeutic degraded enteric microbial ecosystem. However, formulation of any prophylactic and therapeutic agent would require *in vivo* evaluations.

Abbreviations

Ls, Lagenaria siceraria; Lc, Luffa cylindrica; Cm, Cucurbita maxima; MRS, De Mann Rogosa and Sharpe; GC-MS, gas chromatography and mass spectroscopy; PMF, proton motive force.

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