

## **Symbiotic effect of *Aloe vera* juice on the growth of *Lactobacillus fermentum* and *L. helveticus* isolates *in vitro***

Lamiaa Al-Madboly, Amal Kabbash, Mona El-Aasr, Akira Yagi

Lamiaa Al-Madboly, Lecturer, PhD, Pharmaceutical Microbiology, Faculty of Pharmacy, Tanta University, Tanta, Egypt

Amal Kabbash, Professor, PhD, Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta, Egypt

Mona El-Aasr, Associate professor, PhD, Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta, Egypt

Akira Yagi, Editor-in-Chief of Journal of Gastroenterology and Hepatology Research, Emeritus Professor, PhD, Fukuyama University, Hiroshima, Japan.

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**Correspondence to:** Akira Yagi, PhD, Emeritus Professor, Fukuyama University; 2-10-1, Hanagaura, Kasuya-machi, Kasuya-gun, Fukuoka, 811-2310, Japan.

Email: [akirayagi@nexyzbb.ne.jp](mailto:akirayagi@nexyzbb.ne.jp)

Telephone: +81-92-938-2717

Fax: +81-92-938-2717

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### **ABSTRACT**

**AIM:** Long-term dietary intake of non-caloric fiber, such as *Aloe vera* gel influences the structural and activity of microorganisms in the human gut. The present investigation was executed with an innovative concept: symbiotic effect of probiotics that are used commercially as lactic acid bacteria i.e.

*Lactobacillus fermentum* and *Aloe vera* juice as prebiotics for a prospective prophylaxis.

**MATERIALS AND METHODS:** Fermentation of *L. fermentum* with *Aloe vera* juice certified by International Aloe Science Council was carried out and the quantification of short chain fatty acids (SCFAs) from the fermentation broth in large scale was determined by gas-chromatography-mass-spectrometry selective detection in the selective ion monitoring mode.

**RESULTS:** The growth of *L. fermentum* and *L. helveticus* with *Aloe vera* juice (AVJ) individually on MRS broth was continued to keep at pH 3.5 and 3.6, while pH of the negative controls changed to 4.3 and 4.0, respectively, during 24 hr incubation. The growth rate and the viability of *L. helveticus* incubated with different concentrations (5-25%) of *Aloe vera* juice were strongly reduced. However, the growth rate of *L. fermentum* was enhanced in a concentration dependent manner with emphasis on the use of 15% AVJ that resulted in two times more growth than that of the negative control. Continuation of *L. fermentum* growth at pH 3.6 in combination with AVJ during incubation for 24 hr suggests the durability of prebiotic potential by AVJ in *in vitro* fermentation. Acetic, propionic and lactic acid as SCFAs in the ether extract were identified from the fermentation culture medium.

**CONCLUSION:** The prebiotic activity of AVJ may be assessed by the participation of SCFAs during 24 hrs-incubation with *L. fermentum*. An innovative concept of symbiotics: a combination of AVJ and *L. fermentum*, is a perspective prophylaxis on future intestinal health claims. Due to tolerance to acid, *L. fermentum* may pass through the gastric barrier and colonize the intestine after oral administration.

**Key words:** Symbiosis; *Aloe vera* juice; *Lactobacillus fermentum*; Acetic; Propionic and Lactic acid; GC/MSD analysis

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## INTRODUCTION

Studies showed that a number of species of intestinal microbiota plays an important role in maintaining normal physiological states of biological organisms with natural resistance, and intestinal microbiota perturbations may potentially lead to disease states. The gut microbiota have critical interactions with the host immune system and metabolism. The health benefits of *Lactobacillus* strains are specific and different strains display different beneficial effects as a useful nutritional adjunct. *Lactobacillus fermentum* is commonly found in fermenting animal and plant material. Examination of *L. fermentum* against different pH concentration solutions revealed that it has a strong pH tolerance by its ability of grow and survive for few hours after being incubated at a 3-pH level solution. Park JH. and his colleagues<sup>[1]</sup> found that ingestion of *L. fermentum* PL9005 in mice appeared safe and led to further support that the use of *L. fermentum* in food and drink is also safe. Lara-Viloslad F. and his colleagues<sup>[2]</sup> showed that safety assessment of *L. fermentum* CECT5716, a probiotic strain isolated from human milk, is non-pathogenic for mice even in doses 10,000 times higher (expressed per kg of body weight) than those normally consumed by humans. Young VB. and his colleagues<sup>[3]</sup> revealed that understanding how the gut microbiome contributes to intestinal health should lead to novel preventive strategies and therapies for a variety of gastrointestinal conditions. Ishibashi N. and Yamazaki S.<sup>[4]</sup> reported the status quo of isolation of probiotic bacteria from infections and reviewed each of the factors that have to be addressed in assessing the safety of probiotics, namely pathogenicity, infectivity, and intrinsic properties of the bacteria, and concluded that, with the exception of *Enterococci*, the overall risk of lactic acid bacteria infection is very low. Members of the genus *Lactobacillus* have been shown to inhabit the human gastrointestinal tract, and the long history of safe use of many species of *Lactobacillus* gave them generally-recognized-as-safe status. The influence of prebiotics, (isomalto-oligosaccharide and short-chain fructo-oligosaccharide) and probiotics (*Lactobacillus* and *Bifidobacterium* sp.) are widely investigated in symbiotic study. Effects of manno-oligosaccharide on the growth modulation of human intestinal microbiota were investigated by Dinoto A. and his colleagues<sup>[5]</sup>, and *Lactobacilli* were proliferated higher in the presence of manno-oligosaccharide than other carbon sources, showing pH decrease and short chain fatty acids (SCFAs)-lactic acid production. The effect of *Aloe vera* whole leaf extract on SCFAs production by *Bacteroides fragilis*, *Bifidobacterium infantis* and *Eubacterium limosum* was investigated by Pogribna M. and his colleagues<sup>[6]</sup>. The results of *in vitro* study under anaerobic condition suggest that non-starch polysaccharides treated with 4000 cellulase in the *Aloe vera* whole leaf extract altered the growth and SCFAs production of intestinal microbials.

A series of *Lactobacillus* strains were isolated from the natural fermented dairy products. Hu X. and his colleagues<sup>[7]</sup> suggested that *Lactobacillus plantanum* NS5 strain and *L. delbrueckii subsp. bulgaricus* NS12 strain showed better abilities to lower cholesterol in *in vivo* rats trials. Kumar M. and his colleagues<sup>[8]</sup> presented the anti-hypercholesterolemic effect of the probiotic *Lactobacillus rhamnosus* GG (LGG) plus *Aloe vera* gel in rats with reduced hypercholesterolemia, where serum cholesterol, LDL, VLDL, and TAG levels were found to be decreased significantly, and HDL cholesterol levels were observed to be increased significantly. Therefore, an optimized blend of the probiotic LGG and *Aloe vera* gel could be exploited as a potential bio-therapeutic remedy to decrease cholesterol levels and lower the risk of cardiovascular

disease. Five novel *Lactobacillus brevis* strains, isolated from naturally fermented *Aloe vera* leaf flesh, having pH 3.5-3.8, after fermentation of 20-30 days at room temperature, were identified by RAPD analysis and 16 rRNA sequence comparison. These strains named probiotics originating from *Aloe vera* leaf, POAL, exhibited high level of the glutamate carboxylase gene, which produces a beneficial neurotransmitter, r-amino butyric acid (GABA). Kim YW. and his colleagues<sup>[9]</sup> suggested that the five novel *Lactobacillus brevis* POAL strains may be resources for GABA production. Gullon B. and his colleagues<sup>[10]</sup> evaluated the prebiotic potent of *Aloe vera* mucilage or gel by *in vitro* fermentation using intestinal microbiota from six healthy donors as the inoculum. The prebiotic activity was assessed through the quantification of SCFAs and the evaluation of dynamic bacterial population in mixed fecal cultures by fluorescence *in situ* hybridization. *Aloe vera* mucilage or gel and inulin resulted in higher SCFAs production and moderate increases in butyrate level. Butyrate has been studied for its role to influence cell growth and differentiation, exert anti-inflammatory effects and has been linked to reducing colon cancer. These findings support that the possible incorporation of *Aloe vera* mucilage or gel in the development of a variety of food products known as prebiotics aimed at improving gastrointestinal health. *Aloe vera* mucilage or gel can be used as a bioactive ingredient in the formulation of novel functional foods, thereby increasing its array of application in food science. Nagpal R. and his colleagues<sup>[11]</sup> observed that *Aloe vera* juice (AVJ) was effective in promoting the growth of *Lactobacillus acidophilus*, *L. plantarum* and *L. casei*, as evident from the fall in pH and increased acidity. AVJ could promote the growth of probiotic *Lactobacillus* strains at particular concentrations, and could be used as a prebiotic for preparation of symbiotic therapeutic products. The use of AVJ in the probiotic foods can be a promising trend towards use of herb as well as functional ingredients in the dairy foods. *Aloe vera* fortified probiotic yoghurt was prepared and the effect of storage on syneresis, pH, *Lactobacillus acidophilus* count, *Bifidobacterium bifidum* count of *Aloe vera* fortified probiotic yoghurt was assessed for storage study. Panesar PS. and Shinde CS.<sup>[12]</sup> showed that the *Aloe vera* fortified probiotic yoghurt could be used as an adequate carrier of probiotic bacteria with bacterial counts more than suggested level. Basannavar S. and his colleagues<sup>[13]</sup> studied the effect of *Aloe vera* gel powder on angiotensin-converting enzyme inhibitory activity, extent of proteolysis during fermentation and survival of *Lactobacillus casei* NCDC19 during storage of fermented milk. *Aloe vera* powder addition led to an increase in viable counts of *L. casei* NCDC19 in fermented milk during storage for 7 days and the counts were maintained in sufficiently higher numbers, suggesting *Aloe vera* to be a good functional ingredient which can be further explored for different health attributes. Evaluation of substrate with AVJ for the *Lactobacillus plantarum* LB/103-1-5 strain growth was carried out and exhibited a pH drop denoting lactic acid production by Perez-Leonard H. and Hernandez-Monzon A.<sup>[14]</sup> *Lactobacillus helveticus* is an important industrial thermophilic starter that is predominantly employed in the fermentation of milk for the manufacture of several cheeses. Taverniti V and Guglielmenti S.<sup>[15]</sup> showed that *L. helveticus* had a number of health-promoting properties in interventional studies and clinical trials.

In the present study, we investigated symbiotic effect of AVJ on growth of *Lactobacillus fermentum* and *L. helveticus* *in vitro*. Acetic, propionic, and lactic acid were isolated and demonstrated from the symbiotic fermentation culture medium extract of *L. fermentum* as SCFAs by use of GC/MSD in the selected ion mode.

## MATERIALS AND METHODS

### Test microorganisms

*L. fermentum* and *L. helveticus* were previously isolated from dairy products and identified by API-50 CHL (Biomueirex, France).

### Fermentation of *L. fermentum* and *L. helveticus*

This was carried out according to de Man JD. and his colleague<sup>[16]</sup>. Briefly, the microbiota were individually inoculated into De Man Rogosa Sharp (MRS) broth and incubated at 37°C for 24 hrs. Bacterial cells were collected by centrifugation at 3000 rpm for 10 minutes, and washed with normal saline (0.9% NaCl). The microbiota were recultured after reasonable dilution (OD<sub>600</sub> 2) in the absence and presence of different concentrations of *Aloe vera* gel (5, 10, 15, 20 and 25%) and incubated at 37°C for 24 hrs under anaerobic conditions. Aliquots for viable counts were taken at 0, 2, 4, 6, 8, 24 hrs then counted on plate count agar with bromocresol purple (BCP). All the experiments were carried out in triplicates and the values presented are mean of three replicates.

### *Aloe vera* juice

Characterization of the ingredients in *Aloe vera* juice (Forever Living Products Japan, no: 12042D: AVJ) which was certified by International Aloe Science Council (IASC), was determined by Japan Food Research Laboratories. The analytic concentration (%) were shown as follows: water, 94.3; protein, 0.1; lipid, 0.1; ash, 0.2; carbohydrate, 5.1; plant fiber, 0.4; Na<sup>+</sup>, 0.007; PO<sub>4</sub><sup>3-</sup>, 0.001; Ca<sup>2+</sup>, 0.04; K<sup>+</sup>, 0.05; Mg<sup>2+</sup>, 0.006; ascorbic acid, 0.13;  $\alpha$ -tocopherol, 0.006; vitamin B<sub>6</sub> 4  $\mu$ g/100g; biotin 0.6  $\mu$ g/100 g; folic acid 2 $\mu$ g/100 g, and aloin content was less than 10 ppm<sup>[17]</sup>.

### Fermentation of *Lactobacillus fermentum* in large scale

Fermentation culture medium using MRS broth at 2°C was carried out in large scale and extracted with ether at pH 4.3, and the ether extract layer was evaporated to give ether extract.

### Separation of the ether extract

*Preparation of Sample C and J:* From ten A-J samples, samples C and J were picked up at random, and each sample C (21.13 mg) or J (30.45 mg) was dissolved in 2 mL hexane, sonicated for 10 min, and methylated by sodium methoxide, then clear hexane layer filtered through PTFE membrane was obtained. Samples were diluted 1: 50 with hexane before injection.

### Quantification of short chain fatty acids (SCFAs) by the gas-chromatography-mass spectrometry selective detection (GC/MSD) in the selected ion monitoring (SIM) mode

**Instrument used:** GC/MSD 5977A, Agilent, USA; column used: Agilent, HB 5ms -60°C-325°C(350°C) : 30m x 250  $\mu$ m x 0.25  $\mu$ m; Oven program: 25°C for 3.0 min, then 4°C/min to 225; Inlet: splitless mode, Liner Agilent 5190-2294: 990  $\mu$ L; Inlet temperature: 250°C; Auxiliary temperature: 250°C.

**MS information:** Acquisition Mode; SIM/SCAN; Solvent delay 3.7/min; Scan Parameters, Low Mass: 27, High Mass: 550.00; **Environmental Condition:** Temp.: 18°C; Humidity: 51%.

## RESULTS AND DISCUSSION

*Aloe vera* juice certificated by IASC is considered as healthy soft drinks with nutritional qualities and tonics containing some amino acids, vitamins, bio-factors and minerals in world. In present experiment, effect of *Aloe vera* juice sample (AVJS) on growth and

acidity of probiotic *L. fermentum* and *L. helveticus* was studied *in vitro* incubation for 24 hrs. In the present investigation, *Aloe vera* juice affected the viability of *L. helveticus* isolate negatively at all the tested concentrations compared to the control as shown in Figure 1A. On the other hand, there was an improvement in the growth rate of *L. fermentum* isolate in a concentration dependent manner. Growth of *L. fermentum* with 15% AVJS showed two times more growth (7.511) than that (5.07) of the negative control sample after 24 hrs of incubation. However, 20% AVJS showed no change in the growth of the later isolate as compared to the untreated control (Figure 1B). A dramatic reduction in the growth was observed at 25% AVJS as noticed from Figure 1B. Nagpal R. and his colleague<sup>[18]</sup> reported that 5% *Aloe vera* juice promoted the growth of *L. acidophilus*, *L. plantarum* and *L. casei*, and this improvement was associated with pH fall. However, the use of 15-25% concentrations did not have a significant effect on the growth compared to the untreated cells. Cuvas-Limón and his colleague<sup>[19]</sup> reported similar results. It is tempting to speculate that the lactic acid-stressed cell surface proteins of *Lactobacillus spp.* may have a structural, physical function in combating against lactic acid stress and the higher concentrations above 20% of AVJ discourage the generation growth of *Lactobacillus spp.*, by attributing to the varied aspects in lactic acid concentration, pH and osmolarity.

Our work showed that pH values of AVJS with both microbiota were 3.5 and 3.6 for *L. helveticus* and *L. fermentum*, respectively, at 0 hr. These values were not changed after 24 hr of incubation suggesting that *Aloe vera* juice had no limiting effect on the viability and growth of *L. fermentum* isolate. On the other hand, pH of the negative controls containing the microbiota without treatment was 6.7 and 5.2 for *L. helveticus* and *L. fermentum*, respectively at 0 hr.

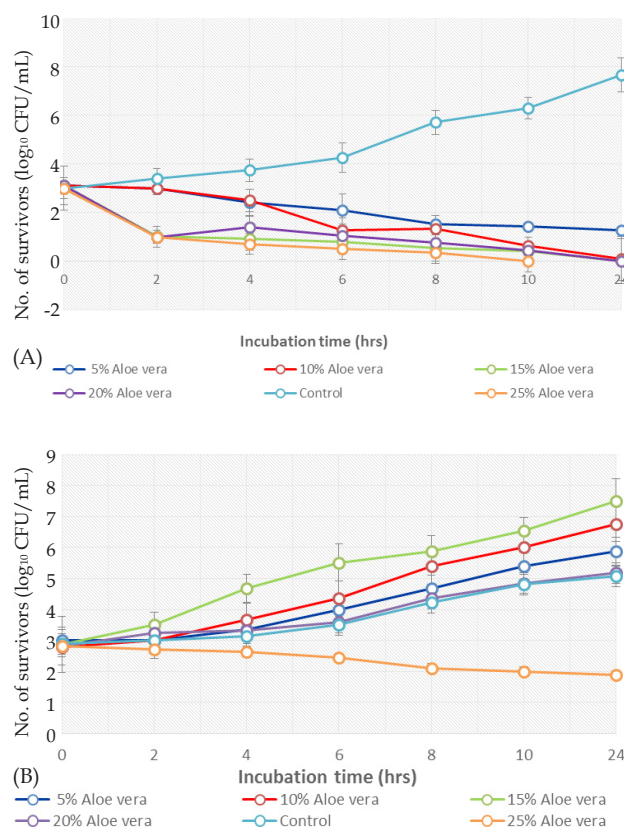


Figure 1 Time-kill curves of (A) *L. helveticus* and (B) *L. fermentum* isolates, showing the number of viable microbial cells in the absence and presence of different concentrations of *Aloe vera* juice.

Following 24 hrs, pH of both negative control was changed into 4.0 and 4.3, respectively. This is because of the ability of lactic acid bacteria to decrease the pH of the surrounding environment by producing organic acids preventing the growth of gastrointestinal pathogens. Suskovic J. and his colleague<sup>[20]</sup> reviewed the lactic acid bacteria as starter cultures lowering pH due to lactic acid or acetic acid produced by probiotic bacteria in the gut have a bactericidal or bacteriostatic effect.

Continuation of *L. fermentum* growth at pH 3.6 in combination with AVJS during incubation for 24 hr suggests the prebiotic potential of 5-15% AVJS by *in vitro* fermentation. The prebiotic activity of AVJS may be assessed through the participation of SCFAs including lactic acid, and the incubation of *L. fermentum* with AVJS was highly tolerated to acid, surviving in pH 3.6 for 24 hr. Due to tolerance to acid, *L. fermentum* may pass through the gastric barrier and colonize the intestine after oral administration. The AVJ can possibly be used as a prebiotic, because of its nutritional composition, particularly acemannan and its oligo-saccharides, vitamins and bio-factors<sup>[17]</sup>. Symbiotic combination study of AVJ and *Lactobacillus fermentum* exhibited the production of acetic, propionic and lactic acid as SCFAs by GC/MSD analysis under SIM mode. Acetic, propionic and lactic acid in sample C showed 91.42, 88.64 and 87.41% library matching with an authentic sample at retention time: 3.530, 6.353 and scan range: 13.1: 17.7, respectively. Acetic and lactic acid in sample J showed 88.96 and 96.94% of library matching with an authentic sample at retention time: 3.539 and scan range: 13.1: 15.5, respectively (Table 1).

Wang T. and his colleague<sup>[21]</sup> reported that *Lactobacillus fermentum* NS9 strain administration reduced the anxiety-like behaviour and alleviated the ampicillin-induced impairment in memory retention in rats. It was suggested that *L. fermentum* NS9 may be beneficial to the host, because it restores the physiological and psychological abnormalities induced by antibiotic ampicillin in rats. SCFAs such as acetic, propionic and butyric acid which are produced by gut microbial fermentation of dietary fiber, are recognized as essential host energy sources and act as signal transduction molecules via G-protein coupled receptors (FFAR2, FFAR3, OLF78, GPR109A) and as epigenetic regulators of gene expression by the inhibition of histone deacetylase. Kasubuchi M. and his colleague<sup>[22]</sup> summarized the roles of gut microbial SCFAs in the host energy regulation and presented an overview of the current understanding of its physiological functions.

The present investigation pointed out an innovative concept of symbiotic effect merging *Lactobacillus fermentum* into AVJ to determine lactic acid, acetic acid and propionic acid with library

**Table 1** Short chain fatty acids identified by GC/MSD under SIM conditions.

Sample C			
Compound	Retention times	Library matching	SIM ion matching
Acetic acid	3.53	91.42	28, 43, 44, 60
Propionic acid	6.353	88.64	Yes (29, 41, 45, 57)
Butyric acid	10.774	No	No
Lactic acid	Scan range(13.1: 17.7) V high concentration	87.41	Yes (27, 29, 43, 45)
Sample J			
Acetic acid	3.539	88.96	28, 43
Propionic acid	6.444	No	Yes (29, 57)
Butyric acid	10.8	No	Yes (43, 74)
Lactic acid	Scan range(13.1: 15.5) V high concentration	96.94	Yes (27, 29, 43, 45)

matching percent of 87.41, 91.42 and 88.64, respectively, by GC/MSD with SIM mode in the fermentation extract.

## CONCLUSION

Present *in vitro* investigation clearly demonstrated that AVJ certificated by IASC could promote the growth of probiotic *Lactobacillus fermentum*. The ether extract from SCFAs fermentation medium of *L. fermentum* with AVJ revealed acetic, propionic and lactic acid as main SCFAs by GC/MSD analysis. In our earlier fermentation study<sup>[23, 24]</sup> of *Aloe vera* gel with endophytic bacteria, *Lactobacillus paralimentarium*, *Bacillus licheniformis*, *B. cereus* and *Clavispora lusitanae* in *Aloe vera* gel, only butyric acid was identified in *in vitro* fermented broth extract. The difference in SCFAs fermentation indicates the significant importance of probiotics comparing to prebiotics in present experiment. An innovative concept of symbiosis; a combination of AVJ, containing polysaccharide; acemannan and its oligo-saccharides, with *L. fermentum*, is perspective for future intestinal health claims to target general health and well-being of human in daily AVJ intake.

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