

Dietary Cancer Prevention with Butyrate Fermented by *Aloe vera* Gel Endophytic Microbiota

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ABSTRACT

AIM: Diet is a natural source of butyrate through the fermentation of non-digestive fiber, such as acemannan in *Aloe vera* gel, is a strong appearing target for health and quality of life as an immune modulation and colorectal cancer prevention in aged people. In our

earlier research on fermentation by endophytic bacteria in *Aloe vera* gel, butyric acid was identified by GC/MSD analysis. Present investigation aims the identification of the microbiota.

MATERIALS AND METHODS: The endophytic microbiota of *Aloe vera* gel in the fermented media were examined by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry.

RESULTS: The following microbiota were identified: *Bacillus cereus*, *B. licheniformis*, *Lactobacillus paralimentarium*, *Yeast: Clavispora lusitaniae*. The safety pattern of the prepared *Aloe vera* gel was tested on normal non-cancerous cells and indicated the absence of any significant possible toxicity on the cells. Also, the extracted gels showed abilities to regulate the inflammatory responses in the inflammation cell models via the reduction in the amount of induced reactive oxygen species and both COX 1 and 2 enzymes.

DISCUSSION: Identification of butyrate-producing endophytic microbiota in *Aloe vera* gel fermentation and finding of inflammatory as well as antioxidant activities of butyrate in the fermented gel may help explain the known beneficial effects of butyrate in intestinal colon and on colitis. An innovative concept of symbiotics: a combination of *Aloe vera* gel juice and microbiota: *Bacillus cereus*, *B. licheniformis*, *Lactobacillus paralimentarium* and *Clavispora lusitaniae*, is a perspective on alleviation of cancer disease and improvement of gastrointestinal health by butyrate fermentation.

Key words: Butyrate; Endophytic bacterial fermentation/identification; Anti-inflammation

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INTRODUCTION

Histone acetylation is an epigenetic modification that is regulated through the opposing action of histone acetylases (HATs) and

deacetylases (HDACs). The ability for butyrate to inhibit HDACs was initially discovered by Boffia LC. group^[1] in 1978. HDAC has been linked to tumorigenesis and tumor angiogenesis, and its inhibitors induce cell cycle arrest, differentiation and apoptosis in a variety of cancer cells. Butyrate has been an essential agent for determining the role of histone acetylation in chromatin structure and function. The potential of butyrate and HDAC inhibitors in the prevention and treatment of cancer was presented by Davie JR^[2]. While inhibition of HDACs is a common mechanism to inhibit cancer growth, the effect of butyrate on the prevention of colorectal cancer (CRC) has been inconsistent. The discrepancy in findings can be attributed to differences in the energy status of cells used in studies because butyrate can be used as an energy source by nutrient-deficient cells to proliferate, but it inhibits cell proliferation when there is sufficient energy. Sengupta S. group^[3] proposed further study of the relationship between butyrate and CRC in human to focus on the development of strategies for butyrate delivery. Fung KY. group^[4] reviewed that butyrate inhibits proliferation and induces apoptosis of CRC cell lines at physiological concentrations. These effects are attributed to butyrate's ability to alter gene transcription by inhibiting histone deacetylase activity. However, recent discovery of G-protein coupled receptors that bind butyrate and other short chain fatty acid (SCFA) and data obtained from proteomic and genomic experiments suggested that alternative pathways are involved. Singh N. group^[5] showed the mechanisms involved in butyrate-induced apoptosis in CRC cells, and the butyrate may play an important role in mediating key processes in tumorigenesis including genomic instability, inflammation and cell energy metabolism. GPR 109A (a G-protein-coupled receptor for nicotinate) is a receptor for butyrate in the colon, and GPR 109A signaling promoted anti-inflammatory properties in colonic macrophages and dendritic cells and enabled them to induce differentiation of T_{reg} cells and IL-10-producing T cells. Thus, GPR 109A mediates the tumor-suppressive effects of the bacterial fermentation product butyrate in colon.

Furusawa Y. group^[6] showed that a large bowel microbial fermentation product, butyrate, induced the differentiation of colonic T_{reg} cells in mice, and ameliorated the development of colitis induced by adoptive transfer of CD4(+)CD45RB(hi) T cells in Rag1(-/-) mice. Treatment of naive T cells under the Treg-cell-polarizing conditions with butyrate enhanced histone H3 acetylation in the promoter and conserved non-coding sequence regions of the Foxp3 locus, suggesting a possible mechanism for how microbial-derived butyrate regulates the differentiation of Treg cells. The findings by Furusawa's group provided new insight into the mechanisms by which host-microbe interactions establish immunological homeostasis in the gut. It is suggested that gut microbiota-derived SCFAs absorbed in the blood play a role in regulation of systemic inflammation by inducing apoptosis of neutrophils as well as chemotaxis of regulatory T cells. Colonic crypts protect stem/progenitor cells from microbial metabolite butyrate anti-proliferative activities. In healthy colonic environment, epithelial cells from the top of crypts metabolize specially butyrate (but not acetate, propionate) through Acyl-CoA dehydrogenase leading to reduced levels of butyrate at the bottom of crypt. Decreased levels of butyrate do not display its anti-proliferative activities on residing stem/progenitor cells at the bottom of crypt. The colonic injury or dysbiosis condition (absence of crypts) leads to uncontrolled exposure of metabolites consisting of butyrate suppress the proliferation of stem/progenitor cells through blocking activities of histone deacetylases. Vemula PK. and Jala VR^[7] demonstrated that butyrate acts on stem/progenitor cells to acetylate histones and induces a Foxo3-dependent suppression of proliferation as the

negative cell-cycle regulator.

Fermentation by endophytic bacteria in *Aloe vera* leaf gel provided butyric acid and suggested that the daily ingestion may be beneficial to prevention for health and QOL as an immune modulator. Probiotic potential of *Aloe vera* leaf gel was evaluated by *in vitro* fermentation with human fecal microbiota by Gullon B. group^[8]. Synthetic oligonucleotide probes targeting specific regions of the 16S rRNA molecule was utilized, and the evaluation of dynamic bacterial population in mixed fecal cultures was assayed by FISH. After the fermentation, SCFAs in the media were assessed by HPLC. Bacterial composition of intestinal human microbiota are following: *Faecalibacterium prausnitzii*, *Lactobacillus-Enterococcus* group, *Bifidobacterium* genus, *Bacteroides-Prevotella* group, *Atopobium* cluster, *Clostridium histolyticus* subgroup, and *Ruminococcus-Eubacterium- Clostridium* cluster. The maximum butyrate concentration was coincident with the highest *C.coccodes-E.rectale* and *F. prausnitzii* numbers.

Bacillus licheniformis and *B.subtilis*, isolated from Daqu, was inoculated to ferment a liquid culture of Daqu. Growth of bacteria and organic acid production during the fermentation were investigated, and the production of SCFAs, including butyric acid, was observed by Yan Z. group^[9]. A two extraction procedure for concentrating acidic organic volatiles in a aqueous solution prior to GC-head space analysis was carried out. The method's usefulness for trace analysis of SCFAs was exemplified in a study on broth cultures of *Bacillus cereus*, where acetic, iso-butyric, iso-valic acid could be detected after 12 h incubation by Larsson L. group^[10]. *Lactobacillus paralimentarius* was identified as a psychro-trophic lactic acid bacteria from chicken carcasses with inhibitory activity against *Salmonella* spp. and *Listeria monocytogenes* by Sakaridis I. group^[11].

The function of the Sir2 ortholog Hs1 in the yeast *Clavispora lusitaniae*, was investigated by Froyd CA. group^[12]. Deacetylase of yeast Sir2 is a defining member of a large family of protein deacetylases in organisms ranging from bacteria to humans. Sir2's activity was established as a NAD-dependent protein deacetylase to regulate life cycle progression and life span in response to nutrient availability. Sir2 proteins regulate genome stability by chromatin silencing in yeast. Regulation of gene expression seems to play an important role in Sir2 functions, since increasing the dosage of Sir2 gene increases genome stability in yeast. Kyrlylenko S. group^[13] reported that the modification of histone/protein acetylation status by several class I and II HDAC inhibitors induces differential changes in gene expression profiles of seven SIRTmRNAs in cultured neuronal cells. SIRT2, SIRT4 and SIRT7 were upregulated, whereas SIRT1, SIRT5 and SIRT6 were downregulated by butyrate and trichostatin A (TSA). Chemically different type of HDAC inhibitors, such as TSA and butyrate, induced remarkably similar responses in SIRT1-7 mRNA expression patterns. Differential responses in SIRTmRNA expression profiles indicate that the expression of the Sir2 family of gene is selectively regulated and dependent on histone/protein acetylation status.

Nagpal R. group^[14] reported that *Aloe vera* gel or juice could possibly be used in combination with probiotic *Lactobacillus* strain (s) as a combinational therapy for gastrointestinal disorders and cardiovascular diseases. In an early our *in vitro* study, the fermentation production of butyric acid was confirmed by endophytic bacteria within *Aloe vera* gel^[15] and possible prophylaxes of *Aloe vera* gel ingestion to butyrate metabolism was suggested^[16].

Present investigation shows the identification of *Bacillus cereus*, *B.licheniformis*, *Lactobacillus paralimentarium* and *Clavispora lusitaniae* by use of MALDI-TOF/MS, a rapid, reliable and cost-

effective identification technique, and presents the regulation of the inflammatory responses in the inflammation cell models via the reduction in the amount of induced ROS and both COX1 and 2 enzymes with butyrate fermented and discussion on modulation of life span by HDAC inhibition of butyrate in yeast.

MATERIALS AND METHODS

Aloe vera sample preparation

Aloe barbadensis Mill. (*Aloe vera*) leaves collected from the medicinal farm of the department of pharmacognosy, Faculty of Pharmacy, Tanta University. A voucher specimen was deposited at the international herbarium of Faculty of Science, Tanta University, Egypt. *Aloe vera* leaves were rinsed with water, the rind is removed by hand, to produce gel fillets. These fillets were kept at room temperature (28-30°C) for 6 weeks in presence of 40% W/W sucrose.

Bacterial identification and MALDI TOF/MS analysis

Aliquots of fermented *Aloe vera* gel were cultured on different culture media including; Sabouraud agar, nutrient agar, mannitol salt agar, and MacConkey agar. The plates were incubated at 37°C for 24hrs. Pure isolated colonies were subjected to Gram staining technique as well as MALDI-TOF/MS analysis for identification of endophytic bacteria. MALDI-TOF/MS experiment and analysis were done in the Faculty of Medicine, Alexandria University, Egypt, using Bruker Daltonik MALDI Biotyper, Germany. The score value of the direct bacterial identification by MALDI TOF/MS is interpreted as follows: 2.300-3.000: means highly probable species identified, 2.000-2.299: secure genus identification, probable species identification, 1.700-1.999: probable genus identification.

Mammalian cell lines

Normal noncancerous cells: human peripheral blood mononuclear cells (PBMCs) were cultured on RPMI-1640 media. Human dermal fibroblast cells (FB) were cultured on DMEM media. All media were supplemented with 200 mM L-glutamine and 10% fetal bovine serum (FBS, Gibco-BRL). Peripheral blood cells (PBMCs) were isolated by gradient centrifugation, as reported by Lohr HF. group^[17].

Cytotoxicity assay

The safety pattern of the prepared *Aloe vera* gel was tested quantitatively on normal PBMC and FB using neutral red assay protocol. Briefly, fermented *Aloe vera* gel was subjected to sterile filtration and the filtrate was freeze dried then a stock solution (10 mg/mL) was prepared in media. Aliquots of serially diluted gel in media was incubated with pre-cultured (seeding intensity 6×10^4 cell/mL) cell on 96-well plates. After 48 hours, dose response studies

were quantified in order to assess the nontoxic suitable dose for further experiments using neutral red assay protocol as described by Borenfreund E. and Puerner JA^[18]. The IC50 was calculated using Graphpad prism program (version 7).

Immunomodulatory activities of the prepared gel

About 2×10^5 cells/mL of PBMC were grown in RPMI medium in rounded bottom 96-well plates for 24 hrs. Cellular inflammatory responses were induced with 100 μ l of 10 mg/mL *E. coli* lipopolysaccharides (LPS) for 24 hrs in the absence or presence of *Aloe vera* gel. At the end of incubation, the concentrations of COX 1 and 2 were quantitatively measured using COX 1 and 2 kits (Chongqing Biospes Co.) according to the manual instructions.

The intracellular induced reactive oxygen species (ROS) was assessed by Rao's method^[19] using the fluorescent membrane permeable probe 2,7-dichlorofluorescein diacetate (Molecular Probes, Sigma Aldrich) with final concentration of 20 μ M. The resulted fluorescence was measured using flow cytometry.

RESULTS AND DISCUSSION

Culture of *Aloe vera* gel on different culture media revealed bacteria as well as yeast that were Gram stained and presented in Figure 1. Identification of the recovered microbes was done by MALDI-TOF/MS Biotyper and the results were recorded in Table 1: Firmicutes; Bacilli: *Bacillus licheniformis*, *B. cereus*, and *Lactobacillus paralimentarius*. yeast: *Clavispora lusitanae*. Similar results were reported by Gullon B. group^[8], Yan Z. group^[9], Larsson L. group^[10], and Sakaridis I. group^[11].

Table 1 Score values of endophytic bacterial isolates identified by MALDI-TOF/MS Biotyper.

Identification	Score value ^a
<i>B. cereus</i> (n=4)	2.440; 2.192; 1.909; 1.848
<i>B. licheniformis</i> (n=4)	2.149; 2.162; 2.000; 1.729
<i>L.paralimentarius</i> (n=1)	2.440
<i>C. lusitanae</i> (n=5)	2.210; 1.901; 1.783; 1.747; 1.738

^a Score value: 2.300-3.000: highly probable species identified, 2.000-2.299: secure genus identification, probable species identification, 1.700-1.999: probable genus identification.

The cytotoxicity of *Aloe vera* gel was carried out using neutral red assay. The obtained data confirmed the safety use of the gel on both PBMC and fibroblast cells (FB). The maximum used concentration of the gel recorded toxicity percentage 27.2 and 0.11 on FB and PBMC, respectively (Figure 2a). The calculated IC50 reached up to 2058 mg/mL on PBMC indicating the safe use of the fermented *Aloe*

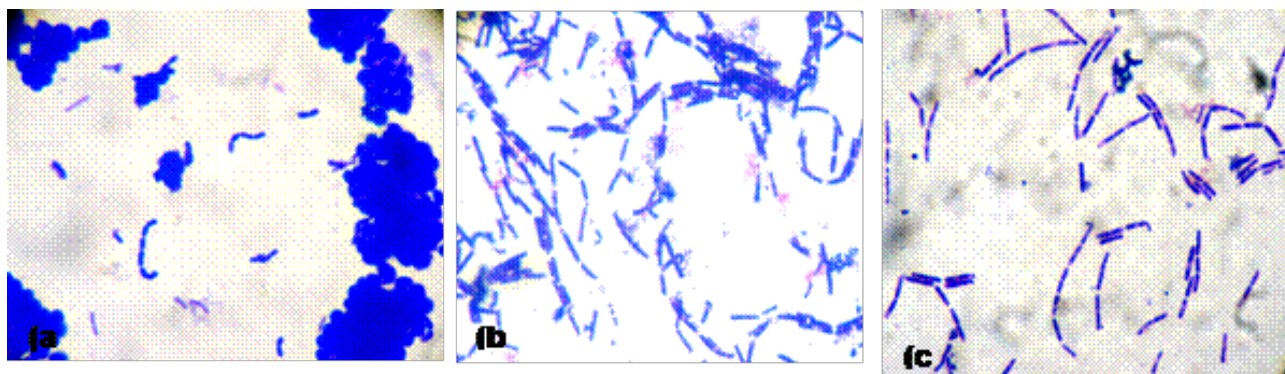


Figure 1 *Aloe vera*, (a) *C. lusitanae* and *L. paralimentarium* (b) *B. cereus* (c) *B. licheniformis*.

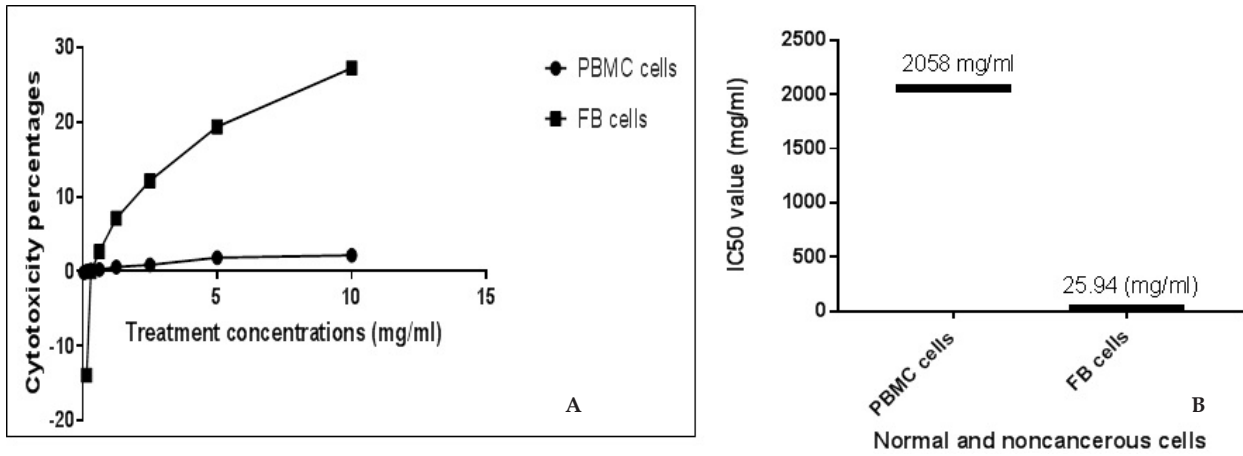


Figure 2 Cytotoxicity assay of the fermented *Aloe vera* gel on normal human FB and PBMCs showing; (A) cytotoxicity below 30% for the maximum concentration (10 mg/mL) used of the gel, and (B) the calculated IC50 from Graphpad prism program.

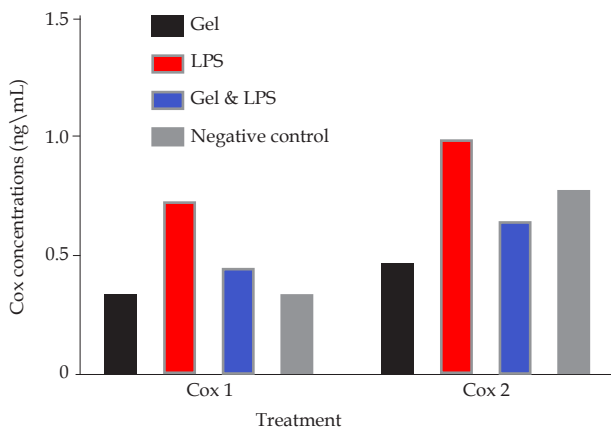


Figure 3 Anti-inflammatory activity of the fermented *Aloe vera* gel on PBMC.

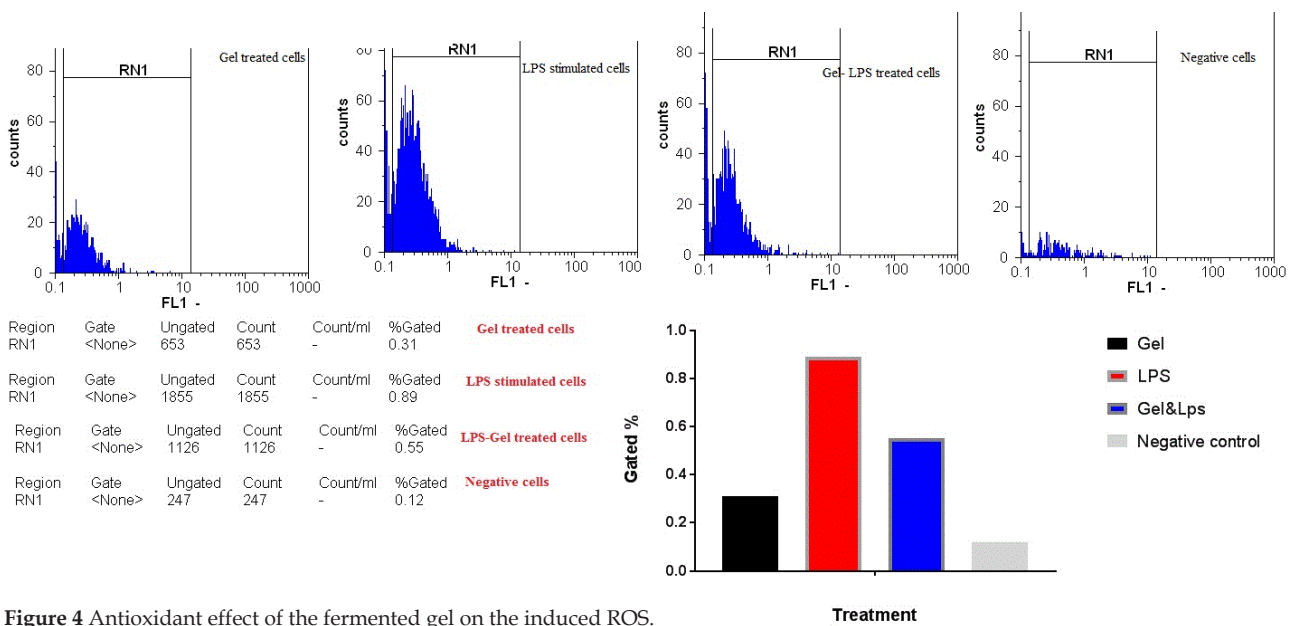


Figure 4 Antioxidant effect of the fermented gel on the induced ROS.

vera gel (Figure 2b). du Plessis L.H. and Hamman J.H.^[20] reported that *Aloe vera* gel was safe at 10 mg/mL and the cell viability only decreased above this concentration. Atiba A. group^[21] reported that *Aloe vera* oral administration had a protective effect on the fibroblast proliferation that was previously exposed to radiation. Yao H. group^[22] evaluated different concentrations of a polysaccharide fraction, extracted and isolated from *Aloe vera* leaves, on the viability of the human fibroblasts *in vitro*. They documented gradual increase in the cell viability that was directly proportional to the increase in the polysaccharide concentrations. This effect was significant at a concentration ranged between 100 to 400 µg/mL. Furthermore, Gontijo SML. group^[23] reported a similar effect to *Aloe vera* sponges on the cell viability.

The anti-inflammatory activity of the fermented gel was studied. The ability to reduce the induced COX 1 and 2 enzyme and ROS in PBMC induced cells were quantified using ELISA and flow cytometry. Generally, PBMC treated cells with the fermented gel showed abilities to reduce the concentration of the induced COX1 enzyme from 0.733 to 0.44 (ng/mL) and COX2 enzyme from 0.992 to 0.649 ng/mL. In addition, the treatment reduced the induced ROS from 0.89 to 0.55 with inhibition percentage 38.2% (Figure 3 and 4). Similarly, Bhise PP. and Zodape GV.^[24] and 't Hart LA.^[25] reported that supplementation of *Aloe vera* juice resulted in a significant improvement in the hematological parameters due to its antioxidant properties. In addition, there were several other reports by Chakravorty D. group^[26], Usami M. group^[27], and Fukae J. group^[28] documented that butyrate, recorded in our prepared fermented gel, was able to suppress the LPS-stimulated production of the pro-inflammatory mediators including nitric oxide (NO), nuclear factor-κB and tumor necrosis factor-α (TNF-α), respectively. Butyrate also enhanced the release of the anti-inflammatory cytokine IL-10. The latter effect was reported by Cox MA and Budai M.M groups^[29-30].

Identification of butyrate-producing endophytic microbiota in *Aloe vera* gel fermentation and finding of anti-inflammatory as well as antioxidant activities of butyrate in the fermented gel may help explain the known beneficial effects of butyrate in intestinal colon and on colitis. An innovative concept of symbiotics: a combination of *Aloe vera* gel juice and microbiota: *Bacillus licheniformis*, *B.cereus*, *Lactobacillus paralimentarium*, and *Clavispora lusitaniae* is a perspective on alleviation of cancer disease and improvement of gastrointestinal health by butyrate fermentation.

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