RESEARCH ARTICLE

Short chain fatty acids enriched fermentation metabolites of soluble dietary fibre from *Musa paradisiaca* drives HT29 colon cancer cells to apoptosis

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Abstract

In this study, the prebiotic potential of soluble dietary fibre extracted from plantain inflorescence (PIF) was investigated. PIF demonstrated prebiotic potential by enhancing the growth of the probiotics under study and thereby hindered colon cancer development. The soluble dietary fibre from *Musa paradisiaca* inflorescence (PIF) was fermented using *Lactobacillus casei* and *Bifidobacterium bifidum*. The fermentation supernatants (LS and BS) were enriched with short chain fatty acids (SCFA) and were able to initiate apoptotic signalling in HT29 colon cancer cells leading to cell death. Both BS and LS exhibited cytotoxic effect; induced DNA damage and enhanced generation of reactive oxygen species in HT29 cells leading to apoptosis. The induction of apoptosis was facilitated by the reduction of membrane potential of mitochondria and ATP synthesis; enhanced delivery of cytochrome c and interference with the expression of pro/antiapoptotic proteins. BS, which exhibited better activity, was further analysed for the identification of differentially regulated proteins by performing two dimensional electrophoresis and MALDI-TOF mass spectrometry. Results emphasized on the fact that, the exposure to BS altered the HT29 proteins expression, particularly the upregulation of apoptosis- inducing factor-AIFM1 leading to apoptosis of HT29 cells.

1. Introduction

Colorectal cancer (CRC) is a leading communal health predicament, being the second most frequently identified cancer in females and the third in males globally. Increased incidence of environmental factors, in particular, lifestyle and diet alterations are reported to influence CRC epidemiology [1]. Various epidemiological studies have shown the healthy coalition between higher fibre consumption and a decreased incidence of inflammatory bowel disease...
and CRC [2]. Dietary fibre plays a protective role in the morbid physiology of CRC. Consumption of fibre rich grains and vegetables has been confirmed to have significant protective effect against CRC through various mechanisms [3, 4]. The main mechanism involves–reduction in transit time of faecal by enhancing the stool bulk; reduces the interaction of carcinogen with colon cells, and most importantly, the utilization of these dietary fibres by gut microbiome [5]. The human digestive system lacks enzymes that can digest dietary fibres, and these fibres are mainly fermented by colonic microbiota [6]. The gut microbiome contributes immensely to maintain a healthy intestine and alterations of this bacterial community can stimulate and lead to CRC progression [7].

The dietary fibre consumed is usually fermented by colon microbiota resulting in the production of short-chain fatty acids (SCFA) -acetate, propionate, and butyrate. These SCFA’s are endorsed with various health beneficial properties [8, 9]. Butyrate is readily absorbed by gut whereas propionate and acetate are absorbed into blood stream. Butyrate extensively nurtures colon stability and is reported for its efficacy against various diseases including cancer, chronic inflammation, diabetes etc. Butyrate induces apoptosis, and inhibits metastasis of colon cancer cells [10]; and suppresses inflammatory responses in ulcerative colitis and colon cancer [11]. Butyrate is involved in proliferation and differentiation of beta cells in pancreas and reduces diabetes associated complications [12]. Propionate lowers serum cholesterol and lipid levels [13]. Propionate and acetate reduces the risk of developing colon cancer by reverting expression of peptide YY [14, 15].

SCFA production lowers the intestinal pH which hinders pathogens and enhances the nutrient absorption [16]. Bifidobacteria have been reported to inhibit enteropathogens through acetate production [17]. Intestinal epithelial cells utilize butyrate as an energy source; and increases mucin production which influences bacterial adhesion [18] and enhanced tight-junctions integrity. SCFA, especially butyrate, has been studied for its part in preventing the CRC development [19]. Butyrate is known to enhance mobility of colon, decreases inflammation, induces apoptosis, and prevents the progression of tumor cells there by significantly preventing the risk of developing CRC [20]. Thus, the production of SCFA from the fermentation of dietary fibre appears to be a crucial player in the preservation of the gut health and reduces the threat of developing CRC. The activation of innate and adaptive immune systems as an outcome of microbiota disproportion usually results in chronic inflammation [21] and has been associated with an increased hazard of cancer occurrence. It has been observed that pathogens are able to endorse the inception and succession of CRC by the initiation of chronic inflammation, and hinders with the normal functioning of cell division, or origination of carcinogenic molecules that are of pro-diet origin [22].

The inflorescence from Musa paradisiaca, a widely cultivated plantain species in southern parts of India, has been selected for the study as there are ethno pharmacological reports that the inflorescence part serves as a remedy for gastrointestinal disorders [23]. Inflorescence is a major agro residue from plantain field as it is removed usually, at stage II, before harvesting from the stalk, for the better development of the fruits. The preliminary studies by our group reported that the inflorescence from Musa paradisiaca happens to be a rich source of dietary fibre [24]. We have also reported that the same is rich in polyphenols [24] and exhibits anticancer potential in HT29 colon cancer cells [25]. As the inflorescence of Musa paradisiaca stands rich in dietary fibre and following cues from our earlier studies, the present study was undertaken to delineate the prebiotic properties of the dietary fibre and to investigate the anticancer potential of the metabolites produced as a result of dietary fibrefermentation by known probiotics.
2. Materials and methods

2.1 Chemicals

General cell culture reagents (DMEM, FBS, trypsin, MTT), rhodamine 123 dye, bile salts, and pepsin (Sigma Aldrich Chemicals, St Louis, USA); Antibodies were acquired from Santa Cruz Biotechnology, USA. 3–10 pH gradient IPG strips and other materials for 2D electrophoresis and western blotting were obtained from Bio Rad Laboratories (Germany). Protease inhibitor cocktail was procured from Amresco (USA). Sucrose, calcium chloride, and starch were obtained from SRL (India). Microbiological media and L-cysteine were obtained from Hime-dia (India) and Merck chemicals (India) respectively.

2.2 Cell culture

HT29 cellswas acquired from NCCS, Pune, India and retained as reported earlier (12).

2.3 Microorganisms

The freeze-dried cultures of Lactobacillus casei (NCDC17), and Bifidobacterium bifidum (NCDC255) were supplied by National Dairy Research Institute, Karnal, Haryana, India. Lactobacillus casei (NCDC17) were cultured in MRS (de Man, Rogosa and Sharpe)broth, and Bifidobacterium bifidum (NCDC255) were cultured in MRS broth with L-cysteine (0.05%). Lactobacillus casei strainswere preserved in MRS broth containing 50% glycerol at -80˚C. The Bifidobacterium bifidum strains were maintained in MRS broth containing L-cysteine and 50% glycerol at -80˚C. Each bacterial strain was grown separately and sub-cultured in fresh media before use. E. coli (MTCC 2622) was obtained from the MTCC, Chandigarh, India and cultured in the Luria-Bertani medium.

2.4 Extraction of dietary fibre and preparation of fermentation supernatant

Inflorescence of Musa paradisiaca (PI), strictly Nendran variety (widely used plantain variety in Kerala, India), was gathered from a local plantain field (Thiruvananthapuram, Kerala, India). The inflorescence is a large and compact structure made up of spirally arranged reddish bracts under which there are two layers of flowers. At initial stage of development each bract will turn round towards the back to expose the outer layer of female flowers that eventually develop into fruits. Later bracts will show inner layer of male flowers. Once the fruits are developed from female flowers, the inflorescence is removed from the stalk before harvesting to get better fruit yield. At this stage the whole inflorescence without any female flowers were collected (S1 Fig) and is used for the present investigation. The extraction of soluble dietary fibre (PIF) from the inflorescence was done as per the protocol described by the authors in their previous publication [24], details given in S1 File.

2.5 Evaluation of prebiotic properties of SDF from PI

PIF was used as an additional carbon source for the growth of the probiotic strains Lactobacillus casei and Bifidobacterium bifidum. Inoculum (0.5 mL) of the probiotic cultures were added to the media containing 1% of PIF to obtain a final concentration of 1 x 10^6 cells/mL, mixed well and cultured for 72 h.5 mL sample was collected at an interval of 0, 24, 48 and 72 h and transferred to pre-weighed centrifuge tubes for various analyses. Inulin (1%) was used as positive control (reference standard) and the media without fibre/inulin as the control.

The prebiotic efficacy was assessed in terms of change in optical density, dry mass, decrease in pH and SCFA synthesis. After measuring the pH and OD (600 nm)at each time intervals (24 h, 48 h and 72 h), it was centrifuged at 10000 rpm for 10 min(Kubota Model No.7780,
Rotor AG-6512C, Japan) and the supernatant was collected. The residue obtained was freeze-dried (VirTis genesis, USA) and the dry weight of organisms was calculated. The supernatant was analyzed for SCFA.

SCFA content in supernatant collected at different time intervals was analyzed and quantified by HPLC following the protocol of Guerrant et al. [26] with some modifications. The HPLC specifications and other details were as described earlier in our publication [24].

As the prebiotic efficacy is also correlated with improved aggregation of probiotics, inhibition of pathogenic organisms and harmful enzymes produced by them in the colon, we also evaluated the efficacy of the fermentation supernatant to promote the aggregation of the probiotics under study, inhibit the growth of *E. coli* and β-glucuronidase enzyme.

### 2.6 Aggregation studies

Auto-aggregation and co-aggregation studies were done according to Pan et al. [27]. The auto aggregation was calculated as

\[
\% \text{ Auto Aggregation} = \left(1 - \frac{A_t}{A_0}\right) \times 100
\]

where \(A_t\) represented the absorbance at time \(t = 1\) or \(5\) h and \(A_0\) the absorbance at \(t = 0\).

The co-aggregation assay was similar as the auto-aggregation assay. *E. coli* was used as a representative pathogenic strain. Equal volumes (2 mL) of the three experimental groups and *E. coli* were mixed in combination and vortexed for 15 s. Samples were collected similarly as that of auto-aggregation protocol and co-aggregation was determined using the formula

\[
\% \text{ Co-aggregation} = \left[1 - \frac{(2A_{\text{mix}})}{(A_{\text{exp}} + A_{\text{coli}})}\right] \times 100
\]

where \(A_{\text{mix}}\) = Absorbance of mixed cell suspension, \(A_{\text{exp}}\) = Absorbance of the experimental group and \(A_{\text{coli}}\) = Absorbance of *E. coli*.

### 2.7 Inhibition of E. coli by fermented supernatant

The ability of fermented supernatant to inhibit *E. coli* was assessed by disc diffusion method. Three different volumes (10, 25 and 50 μL) of fermented supernatant from control, Inulin and PIF groups loaded in sterile paper discs were positioned on the *E. coli* containing nutrient agar plates; and stored at 37°C for 24 h. The diameter of zone of inhibition was taken after incubation time.

### 2.8 Inhibition of the β-glucuronidase enzyme

The ability of fermented supernatant to inhibit β-glucuronidase enzyme produced by *E. coli* was measured as described by Sekikawa et al. [28].

### 2.9 MTT assay for cytotoxic activity

The cytotoxic effect of different concentrations of the fermentation supernatant (obtained at 24 h) was performed in HT29 cell lines following the procedure mentioned in our previous publication [25].

### 2.10 Hoechst 33258 staining

Nuclear condensation or fragmentation happens to be one of the hallmarks of Apoptosis and this was visualized by Hoechst 33258 staining. The impact of fermentation supernatant on
nuclear fragmentation of HT29 cells were measured by the method described by Harada et al. [29].

2.11 Apoptosis assay by flow cytometry
We performed flow cytometry to examine the initiation of apoptosis by fermentation supernatant on HT29 colon cancer cells. The assay was performed using an apoptosis detection kit (Cayman Chemical Company, USA) [25].

2.12 Intracellular reactive oxygen production
The generation of intracellular reactive oxygen species was validated using the DCFH-DA fluorescent method [30].

2.13 Evaluation of mitochondrial membrane potential
Fluorescent dye Rhodamine 123 (Rh123) was employed for determining the impact of fermentation metabolites on the membrane potential of mitochondria of the HT29 cells. Flow cytometry was carried out to investigate whether the fermentation supernatant could decrease the mitochondrial membrane potential following the protocol described in our preceding report [25].

2.14 Estimation of ATP synthesis by HPLC
HPLC method was adopted [31] with slight modifications [25] to study the effect of fermentation supernatant on ATP synthesis.

2.15 Detection of release of cytochrome C
The release of cytochrome C was determined as described in previous reports [25, 32].

2.16 Apoptotic/antiapoptotic protein detection by western blot
The expression of major apoptotic/antiapoptotic proteins was performed according to the illustrated procedure from our earlier publication [25].

2.17 Proteome analysis by 2D electrophoresis
HT29 cells after the treatment with fermentation supernatant was analyzed for the expression of various proteins by performing two-dimensional electrophoresis. The detailed procedure is depicted in our previous report [25].

2.18 Statistical analysis
All data showed in this research article were expressed as mean ± standard deviation of triplicate measurements. The results were tested with one way analysis of variance (ANOVA) and the significance of differences between means were calculated by Duncan’s multiple range test. The statistical comparisons were performed using SPSS (standard version 7.5.1, SPSS Inc., USA). p ≤ 0.05 was appraised as statistically significant.
3. Results and discussion

3.1 Prebiotic efficacy of dietary fibre

The plantain inflorescence has been reported to contain 12.45 ± 1.14% (DW) of double dietary fibre [24]. The prebiotic potential of the PIF has been evaluated in terms of its potential to support the growth of two well-known probiotic species—*Lactobacillus casei* and *Bifidobacterium bifidum* when incorporated in the growth media. This was assessed in terms of lowering pH, increase in optical density, dry mass and production of SCFA. As can be seen (S2A–S2C Fig), the pH of the medium supplemented with the dietary fibres (PIF and inulin) decreased relevantly in comparison to the control indicating the utilization of this fibre by the organisms for their growth. The change in the pH of the media with PIF was significantly higher than that of the media with inulin. In the case of *L. casei* and *B. bifidum*, the pH of the media with PIF decreased to 4.06 and 4.0 after 72 h of fermentation, from an initial pH of 5.905 and 5.75, respectively, whereas the same for inulin was 5.955 to 4.345 and 5.88 to 4.285 respectively (S2A Fig). The turbidity measurement of the growth medium at 600 nm can be related to the growth of microorganisms indirectly. It was found that the optical density of the media with PIF was significantly higher than that of the inulin which indicates that PIF support the growth of probiotics (S2B Fig). The increase in the dry mass of the PIF incorporated media was considerably higher than the positive control and the control media (S2C Fig). The pH, OD, dry mass and colony count measurement indicates that SDF from PI promotes the growth of probiotic bacteria which was significantly higher than the inulin and control group. The SCFAs in experimental groups at different time intervals of incubation were identified and quantified by comparing with standards (S3 Fig and S1 Table). It could be seen that SCFA production increased when PIF was added to the growth media, both in the case of *L. casei* and *B. bifidum*. Butyric acid production by *L. casei* after 72 h incubation time in the media with PI and inulin were 37.60 and 29.28 μg/mL respectively. The same for *B. bifidum* was 45.60 and 37.28 μg/mL, respectively. As observed, *B. bifidum* produced maximum butyric acid which resulted in corresponding decrease in the pH of the media in correlation to SCFA production.

Probiotic microorganisms restore the balance of the gut flora, while prebiotics provide energy for these beneficial bacteria. In combination, they sustain the proliferation of vital bacteria in our bowels and encourage their activity and chances of survival. Dietary fibre can be termed as a prebiotic only if it could alter the intestinal microbiome towards a better configuration by controlling the adverse pathogens, and in turn stand favourable to gut health [33]. The optical density, pH and dry mass showed that the selected probiotic species—*Lactobacillus casei* and *Bifidobacterium bifidum* grows better in the media containing PIF than the media without PIF and positive control inulin. The optical density and dry mass showed direct correlation with the bacterial mass in the media [34] that increased significantly in the sample in comparison with the positive control, in the present study. The decrease in the pH value could be connected to the SCFAs, as they are important metabolites formed during the fermentation of prebiotic by probiotics [20]. As the number of viable organism increases, further will be the production of SCFA which lower the pH. Probiotic strains metabolize undigested carbohydrates to reclaim energy which in turn produces acetate, propionate and butyrate [20] that exert positive effects on human health by various mechanisms [35]. Acetate, propionate and butyrate are the important SCFAs and butyrate being the most important one [9]. The HPLC analysis showed that the production of all the three SCFAs in the media incorporated with PIF increases significantly, after the incubation period.

3.1.1 Aggregation studies. Bacterial auto-aggregation and co-aggregation properties help in maintaining the gut homeostasis by colonization of the probiotic strains that help in excluding the pathogens. In our study, we performed this experiment to prove that the PIF enhances
the growth of the probiotics and hence they have better aggregation properties when compared to other experimental groups. The results from the study indicated that the auto and co-aggregation properties of \textit{L. casei} and \textit{B. bifidum} increases when PIF was included in the growth media.

After 5 h incubation, the auto-aggregation increased from 56 to 93.6% and 47.05 to 93.66% respectively for \textit{L. casei} and \textit{B. bifidum} incorporated with PIF after 5 h incubation, which is better than Inulin included media (Fig 1A). Auto-aggregation triggers the development of biofilm in the gastrointestinal tract which preserves the intestine by forming a barrier and enhancing the immunity [36], and prevents colonization of harmful bacteria [37]. Moreover, the high auto-aggregation potential of probiotic strains helps them to colonize effortlessly in the intestinal walls [37]. This will inhibit the adhesion of pathogens and hence prevent pathogen translocation and subsequent infection [38]. The co-aggregation percentage with pathogenic \textit{E. coli} increases from 19.85 to 94.2% and 22.75 to 97.1% respectively for \textit{L. casei} and \textit{B. bifidum} incorporated with PIF after 5 h incubation, which is better than Inulin included media (Fig 1A). The efficacy of antimicrobial metabolites synthesised by live probiotic happensto be enhanced as co-aggregation minimizes the intercellular distances between probiotics and pathogens[38]. Even though the aggregation properties are highly strain
dependent, the presence of prebiotics multiplies the probiotic number which indirectly enhances the aggregation properties.

### 3.1.2 Inhibition of E. coli by fermented supernatant

One of the important mechanisms by which the probiotics promote gastrointestinal health is by preventing the growth of pathogenic strains—by the antimicrobial action of secondary metabolites produced by fermenting undigested carbohydrates. Hence we analyzed the antimicrobial effect of supernatant from the fermentation of PIF on the growth of E. coli.

From the results (Fig 1B, S4 Fig) it was evident that the fermentation supernatant obtained from the media with PIF inhibited E. Coli much effectively than that of the known prebiotic Inulin. For 50 μL fermentation supernatant with PIF, the zone of inhibition was 1.9 ± 0.141 and 2.3 ± 0.141 cm for L. casei and B. bifidum, respectively. However, the corresponding zone of inhibition for Inulin group (50 μL) was found to be only 1.45 ± 0.07 and 1.4 ± 0.141 cm. The zone of inhibition was found to increase with the amount of supernatant which possibly could be correlated to the increase in the amount of short chain fatty acid present in the supernatant.

### 3.1.3 Inhibition of the β-glucuronidase enzyme

Reddy and Wynder [39] had correlated high content of β-glucuronidase with increased colon cancer risk and one of the primary factors in the etiology of colon cancer. Formation of β-glucuronidase and its activity is reduced by high-fiber diet, probiotics, and low meat diet that lower the colonic pH. Therefore, in the present study, we analyzed the effect of fermented supernatant (10, 25 and 50 μL) of different experimental groups, in inhibiting β-glucuronidase produced by E. coli. The inhibition of β-glucuronidase (%) for the supernatant (50 μL) from PIF and Inulin was found to be 42.36 and 21.55 for L. casei, and 53.78 and 28.55 for B. Bifidum respectively (Fig 1C) which demonstrate the potential of PIF in the enzyme inhibition.

From the above studies, it has been established that the soluble dietary fibre from plantain inflorescence exhibits significant prebiotic potential as it effectively promotes the growth of selected probiotic strains (L. casei and B. bifidum) and enhances the production of SCFA in the corresponding fermentation medium. The inhibition of the growth and β-glucuronidase production by E. Coli shown in the present study stands very crucial in maintaining the homeostasis of the colon. The SCFA has been reported to exhibit anticancer potential especially against CRC [40]. Since SCFA is one of the important secondary metabolites which was found to be increased in fibre incorporated medium, we were interested to study further the anticancer effect of fermentation supernatant from PI (PIF) and the possible mechanism behind it. Hence the anticancer potential of fermented supernatant (after 72 h incubation) was investigated for its anticancer activity on HT29 colon cancer cells.

### 3.2 Anticancer potential of PIF fermentation supernatant containing SCFA

#### 3.2.1 Cytotoxic effect of PIF fermentation supernatant

The anticancer studies were carried out using the fermentation supernatant after 24 h of incubation. It was found from the MTT assay that the fermentation supernatant exhibited cytotoxicity (Fig 2[I]). The IC₅₀ value for the fermentation supernatant of L. casei with PIF (LS) was found to be 1510.88 μL, whereas the same for B. bifidum (BS) was 905.75 μL. BS exhibited better cytotoxic effect, may be due to the presence of higher content of SCFAs. 500 μL of LS and BS were used for further studies (concentration below IC₅₀ value). The HPLC quantification of SCFAs indicated the presence of 27.265 μg of acetic acid, 15.65 μg of propionic acid and 10.66 μg of butyric acid in 500 μL of LS whereas the same for BS were 30.925, 19.785 and 15.155 μg respectively. The higher concentration SCFA in BS may be correlated with better utilization of PIF by Bifidobacterium bifidum. H₂O₂-250 μM was employed as positive control. The cytotoxicity of SCFAs on colon cancer cells has been reported earlier [41].
3.2.2 Fermentation metabolite induces DNA condensation. The DNA condensation/fragmentation is a trademark of cells moving towards apoptosis [42]. Apoptotic cells are characterised with compressed and fragmented DNA [43]. The effect of LS and BS on the nuclear changes of HT29 colon cancer cells was studied by the DNA-binding fluorescent dye (Hoechst 33342 stain). As shown in Fig 2[I], cells treated for 24 h with LS, BS, and H$_2$O$_2$ showed notable changes in nuclear structure when compared to the untreated sample. In contrast, the untreated cells remained uniformly stained. BS was found to induce more DNA condensation as compared to LS. The morphological analysis indicated that LS and BS induced DNA condensation HT29 cells which is an indication of apoptosis in. However, the activity of the supernatant was less when compared to the positive control (H$_2$O$_2$). We counted number of cells with condensed nuclei in ten different fields and the average percentage was plotted (Fig 2[III]). The results showed that BS induced condensation in 41.33 ± 2.08% cells which is significantly better than LS (24 ± 3%).

3.2.3. Apoptosis induction by fermentation supernatant. Apoptosis is a tightly controlled programmed cell death contributing to the exclusion of unwanted cells in order to retain the equilibrium between survival and death of cells. In the incidence of cancer, apoptosis fails to ensue which results in generation of malignant cells that are immortal. The apoptosis system is multifaceted and abnormalities in apoptosis play important roles in tumor pathogenesis. Therefore, cancer research essentially concentrates in the induction of apoptosis in cancer cells.
Since LS and BS demonstrated cytotoxicity and DNA condensation effect, its ability to induce apoptosis was further investigated. As can be seen from the flow cytometry studies (Fig 2[IV] and 2[V]), it was clear that the BS had a better effect in inducing apoptosis than LS, particularly in late apoptosis stage. But, the activity is not significant when compared with standard camptothecin as it has better activity than LS and BS, particularly in early apoptosis stage. However, these results confirmed that the supernatants, obtained from fermentation of PIF by the probiotic bacteria, induce apoptosis in HT29 cells. These experimental outcomes are in line with the earlier reports explaining the apoptotic effect of fermentation supernatant from various sources [44, 45].

### 3.2.4 Determination of intracellular reactive oxygen production.

The apoptotic inducing capacity of the LS and BS prompted us to investigate further on its mechanism. A variety of stimuli can initiate apoptosis. ROS and oxidative damage have been associated in the signaling of apoptosis. Hence we analyzed whether ROS production showed increase after the treatment by supernatant in HT29 cells. The level of reactive oxygen species in HT-29 cells was estimated using DCFH-DA using flow cytometry. The comparison with untreated control cells showed that the ROS levels in treated HT-29 cells increased significantly. Among the supernatant, BS was found to induce more ROS production than LS (Fig 3I). Various anticancer drugs are known to upregulate ROS production in cancer cells and induce apoptosis [46]. Butyrate is reported to trigger apoptosis in HT29 cells [47]. Hence the apoptotic effect of fermentation supernatant may be related to the presence of butyrate. The increased production...
of ROS in cells pre-treated with BS leading to apoptosis may be linked to the higher butyrate content in BS.

3.2.5 Mitochondrial membrane potential ($\Delta \psi_m$) was decreased. The $\Delta \psi_m$ of cancer cells are more hyperpolarised than normal cells. The $\Delta \psi_m$ is reported to have a great influence in the genesis of colon cancer. The elevation in the mitochondrial membrane as well as the structural abnormalities and function of the membrane has been related to colon carcinoma [48]. During apoptosis the proapoptotic BAX and/or BAK translocation from the cytosol to the mitochondria which results in dissipation of $\Delta \psi_m$ further releasing cytochrome c and other elements [49]. These events drive the recruitment and processing of pro-caspase-9, leading to terminal apoptosis [50]. Degeneration of $\Delta \psi_m$ has been reported to decrease or inhibit apoptosis that can be correlated with the elevated mitochondrial membrane potential levels in colonic tumors [51].

The histograms obtained from flow cytometry analysis showed that the $\Delta \psi_m$ was reduced when treated with LS and BS. The activity of fermentation supernatant was less when compared to the positive control ($H_2O_2$) used in this assay. It was found that the loss of mitochondrial membrane potential was 61.8 ± 0.1% in $H_2O_2$ (250 μM) treated cells whereas the same for LS and BS treatment were 30.37 ± 2.84 and 55.27 ± 0.115% respectively (Fig 3I). The reduction in the $\Delta \psi_m$ as evident from current experiment remains interesting, as the production of ATP is linked to mitochondrial membrane potential and thus can lead to initiation of apoptotic events e.g., release of Cytochrome c.

3.2.6 Determination of ATP production. Cancer cells divides uncontrollably and therefore have high demand for ATP as it is the main energy bank for the cells. ATP plays vital role in the processes that mediates all types of cell death—apoptosis, autophagy, and necrosis. Late-stage apoptosis is associated with decreased levels of ATP due to loss of mitochondrial function and consumption by ATP-dependent proteases. Therefore an increased production of ATP is essential for the persistence of cancer cells. In this perspective, one of the focuses of anticancer studies could be to evaluate the production of ATP by the cells.

The ATP synthesis in HT29 cell lines after incubation with fermentation supernatant was analysed by HPLC as reported earlier [25]. The results pointed out that the ATP synthesis was diminished after the treatment of HT29 colon cancer cells with LS and BS. The control group was estimated to have an ATP content of 0.929 ± 0.004 μg/mL, which was abridged to 0.4675 ± 0.002 μg/mL, when treated with $H_2O_2$. It can be noted that the ATP production when treated with LS and BS were 0.709 ± 0.033 μg/mL and 0.619 ± 0.031 μg/mL respectively (Fig 4). The decreased production of ATP cells treated with BS and LS, in comparison with the control cells could be correlated with the loss of mitochondrial potential. The production of ATP is influenced by the enzymes of the glycolytic pathway. Therefore, the reduction in ATP production may also be due to the interaction of fermentation supernatant with these enzymes. The results demonstrated that BS has better activity than LS in reducing the ATP production.

3.2.7 Determination of cytochrome C release. The cytochrome c release from mitochondria is the initial foot step of apoptosis. The cytochrome c gets fixed to APAF-1 protein further activates caspases that are crucial for the execution of apoptosis. The antiapoptotic BCL2 proteins are usually overexpressed in cancers including colorectal cancer [52], which prevents cytochrome c release and thereby preventing the cells from entering apoptosis.

Hence we analyzed the effect of fermentation supernatant LS and BS on cytochrome c release by HPLC as reported earlier [25]. As can be noted, the release of cytochrome c was enhanced in experimental groups [1.074 ± 0.023 μg/mL (LS) and 1.292 ± 0.027 μg/mL (BS)] when compared to the untreated cells (0.771 ± 0.012 μg/mL) (Fig 5). The same for the positive control was 1.813 ± 0.031 μg/mL. The activity of BS was significantly better than LS.
3.2.8 Western blot analysis. The results from the above assays evince that the fermentation supernatants, LS and BS, have significant anticancer effect on HT29 colon cancer cells by inducing apoptosis. To ascertain this, we analyzed the expression of some of the key proteins involved in the apoptosis after treating the HT29 cells with LS and BS.

The BCL-2 protein family consist of anti/pro-apoptotic proteins that mainly control the events of apoptosis. The apoptotic process is inhibited by the anti-apoptotic members of this group, such as BCL2, either by averting the release of cytochrome c or by sequestering the caspases. On the contrary, pro-apoptotic members, such as BAX, elicit the release of cytochrome c and leads to the caspase activation. The cytochrome c initiates breaking of caspases which
leads to the cleavage of poly(ADP-ribose) polymerase (PARP) and cleaved PARP is mainly assayed as a marker for apoptosis [53].

We have analyzed the expression levels of cleaved PARP (c-PARP), cleaved caspase (c-caspase), BCL2 and BAX after the treatment of HT29 cells with LS and BS. The results suggested that LS and BS do not affect BCL2 expression (Fig 6IA). However, pro-apoptotic protein BAX showed an increased expression after the treatment with LS and BS (Fig 6IB) which was higher for BS as compared to LS. The fermentation supernatant LS and BS were able to manifest the establishment of c-caspase 3 as well as c-PARP (Fig 6IC and 6ID). Therefore, it may be hypothesized that the activation of BAX initiated the release of cytochrome c which in turn lead to breaking of caspase 3 and PARP that sequentially initiated the cell death. Both LS and BS were less active compared to 5-Fluorouracil (50 μM), the standard used in the assay.

The fermentation of dietary fibre by the colon microbiome leads to the synthesis of SCFAs (butyrate, propionate, and acetate) in the gut. Butyrate ceases proliferation and induces apoptosis in colon cells [54]. It is also reported that butyrateinitiatescaspase3 activation and lead to cell death in HCT116 colon cancer cells [55]. In Caco2 cells, butyrate is reported to induce apoptosis through the mitochondrial pathway [56]. Summarizing the role of SCFA, especially butyrate, from these reports we can assume that the apoptotic effect of LS and BS fermentation supernatant is due to the presence of SCFA. BS exhibited better activity than LS which may be correlated with SCFA.

3.2.9 Influence of pre-treatment with BS fermentation supernatant on differential expression of proteins in HT29 cells. Based on the above anticancer assays it was noted that the supernatant obtained from the fermentation of PIF by B. bifidum (BS) is better than that of L. casei (LS). Therefore, to discern the effect of BS on colon cancer cells HT-29, Proteomic profiling by 2D electrophoresis and protein fingerprinting was carried out.
After treatment with BS, total proteins were isolated from HT-29 cells and separated by 2D gel electrophoresis. The protein spots on the gels were scanned, and analysed using the PDQuest 8.0 software as reported earlier [25]. The up/downregulated proteins were selected and cut out physically from the gels followed by identification of proteins using peptide mass fingerprinting. As can be seen from the two dimensional map (Fig 6II), one protein is upregulated and four proteins are downregulated on treatment with BS. The details of these proteins are given in Table 1. An upregulation of apoptosis-inducing factor-mitochondria associated 1 isoform 6 (AIFM1) was observed with a fold difference of 2.315 ± 0.105. Annexin A2 isoform 2, Solute carrier family, 25 members, 35, Heat shock cognate 71 kDa protein isoform 2 and Triose phosphate isomerase isoform 3 are found to be down regulated. Among the selected downregulated proteins, significant fold change (0.514 ± 0.017) has been demonstrated by heat shock cognate 71 kDa protein isoform.

Apoptosis-inducing factor, mitochondria-associated 1(AIFM1) protein serves as proapoptotic as well as antiapoptotic factor. In normal cell it maintains mitochondrial respiration and redox metabolism. On the other hand, in response to the apoptotic stimuli, AIFM1 enhances DNA condensation and fragmentation, inhibits protein synthesis and triggers mitochondria to release other apoptotic proteins. The 2D profiling demonstrated that the treatment with BS upregulates AIFM1 isoform 6 expression which can be correlated with the apoptotic effect of BS in HT29 cells. Annexin A2 is up-regulated in different tumors and controls angiogenesis, proliferation, apoptosis, cell migration, invasion, and adhesion. The in vitro metastatic effect of Caco-2 cells was correlated with the overexpression of Annexin A2 [57]. In the present study BS pre-treatment downregulates Annexin A2 which may be one of the reasons for the anticancer efficacy of BS.

The solute carrier family 25 member 35 (SLC25A35), a mitochondrial carrier protein facilitate transport of solutes across the inner mitochondrial membrane [58]. The membrane potential of mitochondria was dropped after the treatment of BS which might have lead to the downregulation of SLC25A35 as obvious from the proteome data.

Heat shock cognate 71 kDa protein isoform 2 helps in cell survival under stress conditions and the same has been reported to be overexpressed in various colon cancer cell lines [59]. Proteomic data suggest the pre-treatment with BS downregulates heat shock cognate 71 kDa protein, and thereby HT29 colon cancer cells fail to survive.

Triosephosphate isomerase converts dihydroxyacetone phosphate to glyceraldehyde 3-phosphate in glycolysis. Cancer cells particularly enhance glycolytic trail ("Warburg effect") to meet the ATP requirement [60, 61]. Glycolytic enzymes including triose phosphate isomerase were found to be upregulated in colon cancer cells and tissues [62, 63]. BS treatment downregulates triose phosphate isomerase isoform 3 and there by prevents HT29 cells depending on the glycolytic pathway for ATP requirement.

Table 1. Differentially expressed proteins as recognized by two dimensional electrophoresis.

| Proteins                                           | Molecular Mass (kDa) | Isoelectric point (pI) | Fold change (treated/control) |
|----------------------------------------------------|----------------------|------------------------|------------------------------|
| **Upregulated proteins**                           |                      |                        |                              |
| P1 Apoptosis inducing factor, mitochondria associated 1 isoform 6 | 26.03                | 9.26                   | 2.315 ± 0.105                |
| **Downregulated proteins**                         |                      |                        |                              |
| P2 Annexin A2 isoform 2                            | 40.41                | 8.53                   | 0.787 ± 0.025                |
| P3 Solute carrier family 25 member 35              | 32.43                | 9.21                   | 0.929 ± 0.018                |
| P4 Heat shock cognate 71 kDa protein isoform 2     | 53.52                | 5.61                   | 0.514 ± 0.017                |
| P5 Triose phosphate isomerase isoform 3            | 17.958               | 5.39                   | 0.579 ± 0.019                |

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The proteomedata of HT29 cells after exposure to BS showed that it had great potential to alter the expression of different proteins which may eventually lead to the death of HT29 cells.

The *in vitro* studies carried out as discussed in the present study suggests the fermentation of the plantain inflorescence dietary fibre by selected probiotic species leading to the production SCFA that demonstrate anticancer potential by inducing apoptosis. Several randomised blinded studies were performed in animal models to study the effect of dietary fibre against colon cancer. The anticancer effect of dietary fibre is mainly attributed to the production of SCFA in the colon, especially butyrate, due to the fermentation of dietary fibre by the beneficial microbioata in the colon [64–67]. Researchers had reported the increased colon motility in guinea pig and rats after administration of 100 mM and 5 mM butyrate respectively [68, 69]. On the other hand there are many *in vitro* reports stating the efficacy of SCFAs, butyrate in particular against colon cancer cell lines with concentration ranging up to 5 mM [70–72]. The concentration of butyrate in the fermentation supernatant we investigated for the present study falls in this range. To the best of our knowledge this is the pioneer report claiming the anticancer efficacy of fermentation supernatant of dietary fibre against colon cancer. The results from our *in vitro* experiments will definitely lay concrete basis for *in vivo* studies. The authors in their previous study has reported similar activity for the methanolic extract of plantain inflorescence against HT29 cells. The PI extract was shown to induce apoptosis, arrest cell cycle and modulate expression of pro/anti apoptotic proteins [25]. Though the anticancer properties of the dietary fibre fermentation metabolites of PI is less compared to the previously reported study, the experimental results from the earlier report and findings from the current study suggest that the consumption of PI may be helpful in maintaining better colonic biome and hence could be a potential candidate in combating colorectal cancer.

**4. Conclusion**

The soluble dietary fibre from PI exhibits potential prebiotic property as it promotes the growth of *L. casei* and *B. bifidum* which indirectly inhibits the growth of pathogenic strains and also protects the intestine. The supernatant obtained from the fermentation of PIP by *L. casei* and *B. bifidum* (LS and BS respectively) is rich in SCFA particularly that obtained from fermentation by *B. bifidum*. The schematic representation of anticancer effect demonstrated by SCFA against HT29 cells, based on the experimental results, has been described in Fig 7. HT29 cells treated with fermentation supernatant resulted in the over production of ROS; reduces ΔΨm; diminishes ATP synthesis; and enhances the release of cytochrome c. All these events ultimately leads the HT29 cells towards apoptosis. Since BS exhibited significantly better activity, the effect of BS on protein expression level was analyzed by western blotting and 2D electrophoresis. The results showed specified alteration in some of the protein expression which are known players in induction of apoptosis. The study particularly identified up-regulation of one of the key apoptotic inducing protein—Apoptosis-inducing factor, mitochondria-associated. Thus it is evident from the experimental results that the fermentation supernatant contains SCFA which induces ROS mediated apoptosis in HT29 cells. In our preceding reports, it was established that the presence of bioactive phytochemicals in PI and its health potential regarding antidiabetic, cardiovascular protection and anticancer properties. As the functional food market is one of the leading food markets globally and dietary fibre markets is on a boom, value addition of PI (a major part of the produce otherwise is discarded) for food, nutraceutical, and functional food application has immense socio-economic relevance.
Supporting information

S1 Fig. Photographic image of inflorescence from *Musa paradisiaca*.

S2 Fig. Prebiotic potential of PIF determined by the change in (A) pH, (B) optical density, and (C) dry weight for *L. casei* and *B. bifidum*. Values plotted are the average of triplicate experiments. *p* < 0.05 corresponds to significant change when compared to control and inulin (A–C). *p* ≤ 0.05 was considered statistically significant.

S3 Fig. Chromatogram of standard acetic acid (1), propionic acid (2) and butyric acid (3).

S4 Fig. Photographic images showing inhibition zone against *E. coli* by fermentation supernatant from different experimental groups.

S1 Table. Quantification of short chain fatty acids.

S1 File. Supporting information on methods.

S1 Dataset.

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