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Components Secreted by *Lactobacillus Fermentum* PC1 Inhibit *Salmonella Enterica* Serotype Typhimurium Induced IL-8 from Intestinal Epithelial Cells

Meera Esvaran[1, *], Patricia Lynne Conway[2, 3]

[1]Centre for Marine Science and Innovation, The University of New South Wales, Sydney, NSW, Australia

[2]School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, NSW, Australia

[3]Nanyang Food Technology Centre, School of Chemical and Biomedical Engineering, Nanyang Technology University, Singapore

Email address:

M.Esvaran@unsw.edu.au (M. Esvaran), pconway@ntu.edu.sg (P. L. Conway)

[*]Corresponding author

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Abstract: Lactobacillus strains have been shown to confer health benefits to the host including attenuation of intestinal inflammatory responses. However, the health benefits of lactobacilli are strain specific. The aim of this study was to determine whether *Lactobacillus fermentum* PC1 (PC1) cell wall extract (CW) and the spent culture supernatant (SCS) had the capacity to inhibit IL-8 production by *Salmonella enterica* serotype Typhimurium (*S*. Typhimurium) infected epithelial cells. Epithelial cell line, HT-29 was treated with CW or SCS of PC1 both pre- and post-infection with *S*. Typhimurium and the resultant levels of IL-8 protein was assayed. Both the CW and SCS of PC1 was shown to inhibit *S*. Typhimurium induced IL 8 production in HT-29 cells in the therapeutic and prophylactic models. Furthermore, a secreted molecule produced by PC1 responsible for this effect was identified and characterized. The molecule was produced in mid-stationary phase of growth. This active component was present in both the cell wall extracts and spent culture medium of PC1. The bioactive molecule(s) had a size of Mr 2-30KDa, was heat stable at 90°C for 30 min, insensitive to lipase, distinct from acetic and lactic acid, and optimal function at pH 4.5. The activity of the molecules was inactivated by Proteinase K, Na-metaperiodate and Trypsin indicating that the molecule(s) was a glycoprotein. The isolation of an immunomodulatory molecule that could be used in the treatment of *S*. Typhimurium infection would be of great value.

Keywords: Anti-inflammation, Epithelial Cells, *L. fermentum*, IL-8, S. Typhimurium

1. Introduction

Lactobacillus spp have a 'generally regarded as safe' status and are used extensively in foods. In addition, they have also been shown to confer protection to the host against several diseases. Specifically, their role in the protection of adults and infants against enteric bacterial infections has been known since the 1900s [1-3]. They can mediate protection via a variety of mechanisms including but not limited to enhancing the integrity of the epithelial barrier, colonisation resistance and production of antimicrobial molecules (reviewed in [4]).

Non-typhoidal *Salmonella*. serotypes (NTS) are a leading cause of acute food borne disease worldwide. One of the most common human clinical isolates, *Salmonella enterica* serotype Typhimurium (*S*. Typhimurium) is associated with gastroenteritis, a localized infection of the terminal ileum and colon that manifests as fever, diarrhea and intestinal cramping [5, 6]. It is estimated that 93.8 million cases of gastroenteritis due to *Salmonella* species occur globally each year, with 155,000 deaths [7]. *S*. Typhimurium invade the epithelium and the underlying resident immune cells, leading to the production of a distinct array of proinflammatory cytokines and chemokines such as IL-8, monocyte chemotactic protein-1, GM-CSF, TNF-α and neutrophin-3 [8, 9]. IL-8 plays a key role in the initiation and maintenance of intestinal inflammation by recruiting and activating other immune cells such as neutrophils, macrophages, dendritic cells and T and B cells into the inflamed intestinal tissue [10]. However, their

secretion must be tightly regulated, because if the inflammatory response becomes sustained and uncontrolled it can lead to chronic intestinal inflammation and, ultimately tissue damage. In many cases, infection with *S*. Typhimurium results in the production of uncontrollable levels of IL-8 [11].

Many *Lactobacillus* and *Bifidobacterium* strains have been shown to inhibit *Salmonella* induced IL-8 production by intestinal epithelial cells [12-15]. Carey et al [12] used different strains of *Bifidobacterium longum* (FRP 68 vs FRP 69) and *Bifidobacterium adolescentis* (FRP 61 vs FRP 335) to show that the ability to modulate *Salmonella* induced IL-8 production from epithelial cells is strain specific.

The strain used in this study, *Lactobacillus fermentum* PC1 (PC1) was isolated from a human. This study examines the capacity of PC1 spent culture supernatant (SCS) or cell wall extract (CW) to suppress *S*. Typhimurium induced IL-8 in HT-29 cells. Here the ability of CW and/or SCS in regulating *S*. Typhimurium induced IL-8 production by HT-29 cells in both prophylactic and therapeutic models is examined. Furthermore, secreted molecule(s) in SCS mediating the anti-inflammatory effect was (were) identified and characterized.

2. Materials and Methods

2.1. Bacterial Strains and Culture Media

Lactobacillus fermentum PC1 (PC1; FII 511 400) was used in this study. It was grown in defined minimal media [16] in an anaerobic chamber maintained at 37°C for 18 h. *Salmonella enterica* serotype Typhimurium (*S*. Typhimurium; UNSW 078 300) was grown aerobically in Tryptone Soya Broth at 37°C for 12 h.

2.2. Cell line and Culture Conditions

This study was performed in vitro using a human adenocarcinoma-derived cell line, HT 29. These cells form junctional complexes and grow into a monolayer that mimics the intestinal epithelial barrier. HT-29 cells respond to bacterial invasion in a similar manner to freshly isolate intestinal epithelial cells. Therefore, this cell-line is a particularly good candidate to study bacterial-epithelial cell interactions in vitro. The HT 29 cell line was a kind gift from Dr Carmel Quinn (UNSW). HT-29 cells were cultured and maintained in McCoy's 5A medium supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 1.5 mM L-glutamine, 100 U.ml 1 penicillin and 100 $\mu g.ml^{-1}$ streptomycin (media). Cells were maintained in 5% CO_2 at 370C. The cells were maintained in 25 cm^2 tissue culture flasks (Greiner, USA) and re-seeded every 3 days in fresh medium. When needed for experiments, the cells were scaled up into 75 cm^2 flasks. All tissue culture reagents were purchased from Gibco (USA).

2.3. Preparation of Cell Wall Extracts (CW) from PC1

A mid-stationary phase bacterial culture of PC1 was centrifuged at 5,000 × g for 30 min at 40°C. The resulting bacterial pellet was washed twice with PBS, resuspended in PBS supplemented with the protease inhibitor, 1 mM phenyl methyl sulfonyl fluoride (PMSF) (Sigma, Australia) and then incubated at 37°C for 1 h. The bacterial suspension was vortexed with glass beads for 10 min using a tube mixer at room temperature, then centrifuged at 20,000 × g for 30 min at 4°C. The supernatants were filtered (0.2µm membrane, Pall Gelman, USA) and kept at -20°C until used. Protein concentrations of the CW extracts were determined using the Bio-Rad protein assay (Bio-Rad, USA). CW were used at a concentration of $0.5mg.ml^{-1}$.

2.4. Preparation of Spent Culture Supernatant (SCS) from L. fermentum PC1

PC1 at mid-stationary phase was centrifuged at 5,000 × g for 30 min at 4°C. The supernatant was collected, and 1 mM phenyl methyl sulfonyl fluoride (PMSF) (Sigma, Australia) was added. This spent culture supernatant (SCS) was filtered (0.2 µm membrane, Pall Gelman, USA) and then fractionated through Mr 30,000 and Mr 2000 centricon columns (Millipore, USA). The resulting SCS was concentrated 100-fold and kept at 4°C until used for all experiments. Heat treatment of SCS was carried out by placing the SCS in a water bath at 90°C for 30 mins. After the heat treatment the SCS was cooled to room temperature before use. The pH of the SCS was pH 4.5.

SCS was treated with the enzymes trypsin, proteinase K and lipase A as well as with Na-metaperiodate. For the trypsin and proteinase K treatments, the pH of the SCS was increased to pH8 as these enzymes are not active in acidic pH. The SCS was treated with trypsin ($0.05\ mg.m^{-1}$) or proteinase K ($0.05\ mg.ml^{-1}$) or pancreatic lipase ($0.05mg.ml^{-1}$) for 90 min at 37°C. The MW of trypsin, proteinase K and Lipase A are Mr 24000, 28900 and 48000 respectively. Therefore, the SCS was subjected to size separation using a Mr 20,000 spin column (Ambicon) to remove the enzymes after treatment. Following this the SCS was returned to pH 4.5. Na-metaperiodate (0.01 M) in 0.1 M Na citrate buffer (pH 4.5) was also used as a treatment. HT-29 cells treated with 0.1 M Na citrate buffer and 0.1 M Sodium iodate were used as controls for Na-metaperiodate. All chemicals and enzymes unless stated otherwise were obtained from Sigma (USA).

2.5. Infection of HT-29 with S. typhimurium

After the 7th passage from liquid nitrogen, HT-29 cells were used for infection experiments (in the $75cm^2$ flask). The cells were washed twice with cold PBS (4°C) to remove all traces of medium. An aliquot (1 ml) of dissociation solution (0.25% trypsin (Sigma, USA), 1mM EDTA (Sigma, USA) in PBS)) was added to the flask and incubated at 37°C for 10 mins. Fresh medium (20 ml) was then added to the flask to inactivate the trypsin, and the cells were centrifuged at 1500 × g for 10 min. The cells were plated at a concentration of 5×10^3 cells per well (in 500 µl of medium) in 48-well tissue culture plates (Greiner, USA). Medium was changed every 3 days and flasks were used for experiments usually on day 7.

For the prophylactic model, HT-29 monolayers were first incubated with the CW or SCS (untreated and treated) at 37°C for 4h. The HT-29 cells were washed twice with warm medium

(37°C) prior to the addition of *S*. Typhimurium (5×10^3 cells per well; MOI of 1) in antibiotic free media to the HT-29 cells and incubation at 37°C for a further 4 h. The HT-29 cells were washed to remove all extracellular *S*. Typhimurium, then placed in fresh media at 37°C for 10 h to allow for the HT-29 cells to recover fully and produce IL-8 in response to *S* Typhimurium. At end of this incubation, HT-29 supernatants were collected and assayed for IL-8 using ELISA.

For the therapeutic model, the *S*. Typhimurium was added to the HT-29 cells prior to the addition of the CW extract or SCS. All other experimental conditions were as described for the prophylactic model.

2.6. Cell Viability Assay

The viability of the HT-29 monolayer was checked for each experiment to ensure that the SCS and CW of PC1 and the various treatments and procedures did not damage or kill the HT-29 cells. At the end of each experiment, cell viability was assessed using the Cell Proliferation kit 1 (Sigma, Australia).

2.7. Determination of IL-8 Levels

Cytokine IL-8 was assayed from HT-29 culture supernatants by ELISA using matched antibody pairs according to the manufacturer's instructions (Pharmingen, USA).

2.8. Statistics

Data are expressed as the means ± SEM of three independent experiments. Data for all assays were analysed by two-way ANOVA using XLSTAT statistical software. The differences between the mean of groups was determined using the REGWQ test. P-values of < 0.05 were considered significant. Results are visualized using GraphPad Prism 7.04.

3. Results

3.1. Effect of L. fermentum PC1 CW Extracts and SCS on Viability of HT-29

HT-29 monolayers were incubated with CW extracts or SCS for 4 h at 37°C, then tested for viability using modified MTT assay. Cell viability of HT 29 cells after exposure to CW extracts or SCS was routinely between 80-85%. Furthermore, viability assays were conducted for each experiment to ensure that the treatments did not result in cell damage and damage of the HT-29 monolayer.

3.2. Effect of PC1 Components on IL-8 Secretion by HT-29 Cells

It has been reported previously that a decrease in IL-8 protein levels could be due to degradation by bacterial proteases [17]. Therefore, purified IL-8 protein was incubated with SCS for 4 h at 37°C and then assayed for IL-8 level by ELISA. Incubation of IL-8 protein with SCS did not lead to any decrease in the protein (control wells; 1042 $U.ml^{-1}$ vs SCS; 1075 $U.ml^{-1}$ (Table 1).

Table 1. *Effect of L. fermentum PC1 SCS at pH4.5 on the stability of IL-8. SCS was incubated with IL-8 protein for 4 h at 37°C, and then the sample was assayed for IL-8 protein levels by ELISA. IL-8 protein was also incubated in pH7.2 and pH4.5 to determine if the pH affected its stability. Data are represented as mean ± SEM. Errors indicate the SEM of triplicate cultures. These results are representative of one of two separate experiments protein.*

Groups	IL-8 levels
SCS + IL-8 in pH4.5 buffer	1042.67 ± 33.07
IL-8 in pH4.5 buffer	1075.60 ± 20.98
IL-8 in pH7.2 buffer	1089.92 ± 31.54

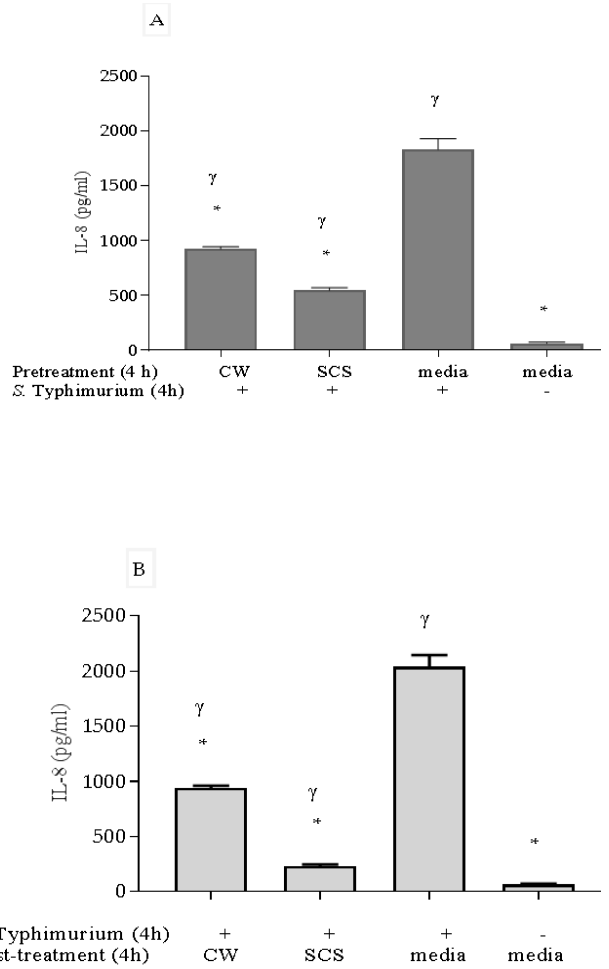

Figure 1. *Prophylactic (A) and therapeutic (B) effect of CW and SCS on S. Typhimurium induced IL-8 by HT-29 cells. For prophylactic model (A), HT-29 monolayers were first incubated with CW or SCS at 37°C for 4 h. The HT-29 cells were washed and S. Typhimurium (at a MOI of 1) was added to the HT-29 cells and incubated at 37°C for a further 4 h. The HT-29 cells were washed again and placed in fresh media at 37°C for a further 10 h. At end of this incubation, supernatants were collected and assayed for IL-8 protein by ELISA. For the therapeutic model (B), the HT-29 cells were stimulated with S. Typhimurium, then post treated with CW or SCS. All other experimental conditions were the same. Data are represented as mean + SEM of triplicate wells. Results are representative of one of three separate experiments. *, $P < 0.05$ against S. Typhimurium treated wells. γ, $P < 0.05$ against medium control wells.*

3.3. Prophylactic and Therapeutic Effect of CW and SCS of PC1 on S. typhimurium Induced IL-8 by HT-29 cells

In the prophylactic model, HT-29 monolayer was first incubated with either CW ($0.5mg.ml^{-1}$) or SCS (concentrated

100-fold), then infected with *S*. Typhimurium. As shown in Figure 1A, both the CW (P = 0.05) and SCS (P = 0.002) exhibited significant suppression of IL-8 levels compared to the *S*. Typhimurium group. In addition, the SCS showed significant reduction in the IL-8 level compared to the CW group (P = 0.007).

In the therapeutic model, HT-29 monolayer was first infected with *S*. Typhimurium, after which CW and SCS were added to the wells. As shown in Figure 1B, both the CW (P = 0.03) and SCS (P = 0.001) exhibited significant suppression of IL-8 levels compared to the *S*. Typhimurium group. In addition, the SCS showed a greater reduction of IL-8 levels compared to the CW group (P = 0.003).

In the prophylactic model, CW and SCS showed a 49.5% and 70.12% reduction in IL-8, respectively, compared to *S*. Typhimurium. In contrast, in the therapeutic model, CW and SCS showed a 54.01% and 88.66% reduction in IL-8, respectively. Therefore, therapeutic administration of CW and SCS mediated greater suppression of *S*. Typhimurium induced IL-8.

3.4. Effects of Fractionation and pH of SCS on Suppression of S. typhimurium Induced IL-8 by HT-29 Cells

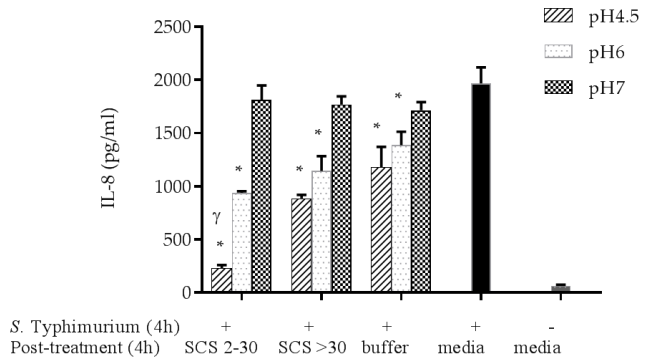

Figure 2. *Effect of pH of the L. fermentum PC1 SCS fractions (Mr 2-30KDa or >30KDa) on S. Typhimurium induced IL-8 by HT-29 cells. The HT-29 cells were infected with S. Typhimurium (MOI of 1) for 4 h at 37°C. The cells were washed, and the fractions at the differing pH were added to the wells for 4 h. The HT-29 cells were washed and placed in fresh media for 10 h. Supernatant was collected at end of incubation and assayed for IL-8 protein by ELISA. Data are represented as mean + SEM of triplicate wells. Results are representative of one of three separate experiments. *, P < 0.05 against S. Typhimurium alone treated wells; γ, P < 0.05 against buffer at same pH.*

The concentrated SCS was separated into > Mr 30,000 and 2,000-30,000 KDa fractions using spin columns. SCS had a pH of 4.5. Aliquots of the fractions were adjusted to pH 6 and 7. As shown in Figure 2, both fractions at pH 4.5 and pH 6 significantly decreased IL-8 compared to the *S*. Typhimurium control group (in medium). However, only the 2-30KDa fraction at pH 4.5 displayed significant inhibition of IL-8 compared to the buffer at the same pH (P=0.001). The presence of acetic and lactic acids in the SCS giving a false decrease in IL-8 can be discounted as they would have been removed by passing the SCS through the spin column. This result shows that the active component(s) functions best at the pH of the untreated SCS.

3.5. Characterization of the Bio-active Compound

The impact of SCS which was pre-treated with either heat, enzymatic or periodate on *S*. Typhimurium induced IL-8 levels was examined. As shown in Figure 3, both heat and lipase treatment did not reduce activity (P = 0.436 and P = 0.208 respectively, compared to the SCS control). Proteinase K, Na-metaperiodate and trypsin led to complete destruction of activity (P = 0.001, 0.002 and 0.005 respectively), compared with SCS control and the iodate control (not shown). Therefore, the component(s) which affected the IL-8 is most probably a heat stable glycoprotein.

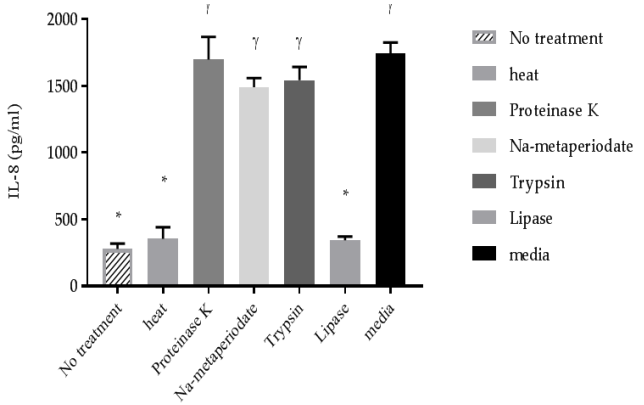

Figure 3. *Characteristics of the SCS activity which suppressed S. Typhimurium induced IL-8 production in HT-29 epithelial cells. The HT-29 cells were infected with S. Typhimurium for 4 h at 37°C. The cells were washed, and the SCS (treated prior with the various enzymes) was added to the wells at 37°C for 4 h. The HT-29 cells were again washed and placed in fresh media for a further 10 h. Supernatant was collected at end of incubation and assayed for IL-8 protein by ELISA. Media group was infected with S. Typhimurium and served as the positive control. No treatment group was infected with S. Typhimurium and then treated with SCS and served as the SCS control. Data are represented as mean + SEM of triplicate wells. Results are representative of one of three similar but separate experiments. *, P < 0.05 against S. Typhimurium treated group; γ, P < 0.05 against no treatment (SCS control) group.*

4. Discussion

The intestinal epithelium consists of a monolayer of epithelial cells and are the first line of defence against invading enteropathogens. The intestinal microbiota plays a pivotal role in maintenance of homoeostasis in the gut mucosal immune system. Indigenous microbiota have been shown to assist in the defence of intestinal tract in several ways. They can co-agglutinate bacteria, facilitate phagocytosis of other bacteria, prevent adhesion of enteropathogens to the intestinal epithelium and produce a variety of bioactive molecules with wide-ranging functions (reviewed in [18]). However, the effects of the indigenous microbiota are strain specific and the mechanisms by which they exert effect vary.

In this study, we found that both the SCS and CW extracts of *L. fermentum* PC1 were effective in attenuating the production of IL-8 from HT-29 cells infected with *S*. Typhimurium. Probiotic strains have been shown to down-regulate IL-8 expression by HT-29 following a stimulation by *S*. Typhimurium in a strain specific manner [13,

14, 19, 20]. In contrast, other probiotic strains can increase IL-8 production in TNF-α pre-treated HT-29 cells [21]. This highlights the need for careful strain selection in the use of probiotic bacteria in treating inflammation as some strains can exacerbate inflammation in patients with a background of chronic inflammation. Furthermore, the anti-inflammatory effect of the CW and SCS from PC1 was observed in both the therapeutic and prophylactic models with the therapeutic effect being more potent. Other strains have demonstrated only prophylactic effect [12, 22]. Therefore, the capacity of PC1 to mediate both preventive and therapeutic setting effects of *S.* Typhimurium will allow for greater applications.

In general, commensal Gram-positive bacteria do not induce IL-8 in resting IECs [14, 23, 24]. However, Ohkusa et al identified a few Gram positive commensals that invaded and induced pro-inflammatory cytokine secretion [24]. In this study, it was noted that the SCS and CW of PC1 did not induce IL-8 secretion beyond basal levels whilst *S.* Typhimurium induced significantly elevated levels of IL-8 by HT-29 cells.

Probiotic bacteria can secrete a wide range of bioactive molecules including but not limited to bacteriocins, enzymes, oligosaccharides, exopolysaccharides, short chain fatty acids and immune-modulators compounds (reviewed in [25]). Other studies have shown secreted anti-microbial compounds of probiotic strains to inhibit inflammatory responses by intestinal epithelial cells [26, 27]. The bioactive molecules secreted from *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0389 were sensitive to pH, resistant to nucleases and <10KDa in size were shown to modulate inflammatory cytokine production from intestinal epithelial cells [26]. Secreted bioactive molecules isolated from three *Bifidobacterium* strains of molecular size less than 3KDa that are neither proteins nor nucleic acids isolated were found to be responsible for the strains' capacity to attenuate inflammatory responses from TNF-α stimulated epithelial cells [27]. The bioactive molecule isolated and characterized from the SCS of PC1 appears to be a heat-stable glycoprotein of molecular weight between 2-30KDa with optimal activity at pH 5.

5. Conclusion

The findings of this study are that both cell wall extracts and the SCS of *L. fermentum* PC1 are very potent suppressors of *S.* Typhimurium induced IL-8 production by HT-29 cells. This effect appears to be mediated by a secreted molecule(s). Further studies into the mechanism(s) by which the bioactive molecule(s) suppress IL-8 is recommended. In conclusion, the ability of the bioactive molecule(s) to function both as a prophylactic and therapeutic agent makes it a very attractive agent that can be used to modulate intestinal inflammation caused by enteropathogens.

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