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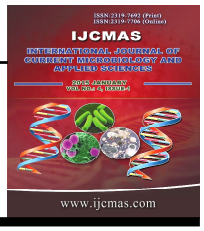

Original Research Article

Detection of anti-inflammatory activity of *Lactobacilli* producing bacteriocins

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Keywords

Lactobacilli, Probiotics, Inflammation, Cycloxigenase, Myeloperoxidase

A B S T R A C T

Inflammation is the complex biological response of vascular tissue to harmful stimuli like pathogens, damaged cells or irritant. Currently prescribed anti-inflammatory drug are non steroidal anti-inflammatory drugs (NSAIDS) and some steroids. The medical field is facing the challenge to discover safe and effective natural anti inflammatory drugs. The suitability of different bacteriocinogenic strains in the prevention and treatment of various diseases like gastrointestinal infections, skin infections, dental infections, vaginal infections etc have been conducted over the years, as in vitro studies, animal experiments as well as clinical trials. The goal of the present study was to determine whether bacteriocin producing *Lactobacilli* isolated from curd is having anti-inflammatory activity. Also, to detect whether there is any non-lactobacilli group of organism from curd possessing the similar anti-inflammatory activity, further the molecular identification of these anti-inflammatory strains was also done. Agar Well Diffusion method was used to identify bacteriocin production which involves 48 hour growth of *Lactobacilli* in MRS broth at 37 °C. Ten samples of *Lactobacilli* with antibacterial activity (bacteriocins) were purified and were subjected to anti-inflammatory activity testing in cell lines. The anti inflammatory effects of samples were determined by assessing the inhibition of COX, LOX, Myeloperoxidase (MPO) and nitrate levels spectrophotometrically. Our data support the role of MPO as key player in neutrophil extravasation and suggest that MPO alters chemokine and cytokine production which may govern inflammatory responses.

Introduction

In vitro studies on cell lines of ten (10) purified samples of lactobacilli showed that, only three of them *L. delbruecki*, *L. fermentum* and *L. paracesei* possess antiinflammatory activity and readings recorded. This study revealed the presence of *Bacillus subtilis*, Sporolactobacillus *dextrus*, *Bacillus amyloliquefaciens* and *Kurthia* with anti-inflammatory activity, all isolated from curd and their sequence obtained.

Materials and Methods

Chemicals and solvents were obtained from SRL, India and bacteriological media were obtained from Himedia, India respectively.

Screening of LAB and indicators

Organisms from stale foods were isolated and identified as indicators, based on the Bergey's Manuel of Systemic Bacteriology. *Lactic acid bacteria* were screened from dairy foods and also from non-dairy sources. de Man Rogosa and Sharpe (MRS) media (M641 Himedia, India) was used for the isolation of *Lactobacilli*, which was confirmed later by the biochemical reactions. Out of 750 dairy & non-dairy samples, 657 *Lactobacilli* were isolated. Only 522 *Lactobacilli* showed antibacterial activity.

Mueller Hinton Agar (MHA) was chosen for conducting agar well diffusion test in the detection of bacteriocinogenic LAB. This study was supported by the findings of Ouissal Bourouni *et al.* (2012). They found that MHA produce better inhibition in 48 hours than MRS and Trypticase Soy Agar (TSA) in 24 and 72 hours.

Bacteriocin production

Agar Well Diffusion method was used to identify bacteriocin production which involves 48 hour growth of *Lactobacilli* in MRS broth at 37°C. Cell free supernatants (CFS) were adjusted to pH 6.5 to avoid the interference of organic acids. Lawn culture of the indicator organisms were made in Mueller-Hinton agar and 50 µl of the neutralized CFS poured into the wells of 6 mm in the lawn and incubated for 48 hrs at 37°C. Results noted and zone of inhibition measured.

Ten samples of *Lactobacilli* with antibacterial activity (bacteriocins) were purified and were subjected to anti-inflammatory activity testing in cell lines.

Determination of invitro antiinflamatory effect of bacteriocinogenic *lactobacilli* on cultured thp1 cell lines

THP1 (Human monocytic cell lines) was cultured in RPMI 1640 [HIMEDIA] media, supplemented with 10% heat inactivated FBS, antibiotics (Penicillin and Streptomycin) and1.5% sodium bicarbonate. The media was filtered using 0.2µm pore sized cellulose acetate filter (Sartorius) in completely aseptic conditions. The cells were then grown till 60% confluency followed by activation with 1µl LPS (1µ/ml). LPS stimulated THP 1 cells were exposed with 5µl, 10µl and20µl of samples and incubated for 24 hours. The anti inflammatory effects of samples were determined by assessing the inhibition of COX, LOX, Myleoperoxidase and nitrate levels spectrophotometrically. The isolation was done by spinning at 6000 rpm for 10 minutes. Supernatant was discarded and 200µl of cell lysis buffer (1MTris HCl, 0.25M EDTA, 2M NaCl, 0.5% Triton) was added .The incubation was done for 30 minutes at 4°C and enzymes assay was done in pellet suspended in a small amount of supernatant

Cox inhibitory assay

Cycloxygenase (COX) is an enzyme that is responsible for the formation of prostanoids. The three main groups of prostanoids -- prostaglandins, prostacyclins, and thromboxanes are each involved in the inflammatory response. Lipoxygenases are non-heme iron–containing enzymes that catalyze the stereospecific incorporation of molecular oxygen into polyunsaturated fatty acids with a 1,4-*cis, cis*-pentadiene motif leading to production of leukotrienes leading to inflammation

Assay of cycloxygenase

Reagents

100Mm Tris HCl (pH8), 5mM GSH, 5µM Hemoglobin, 200µm arachidonic acid, 10% TCA in HCl , 1% Thiobarbituric acid.

Procedure

The assay mixture contained Tris- HCl buffer, glutathione, hemoglobin & enzyme. Reaction was started by the addition of arachidonic acid and terminated after 20 min incubation at 37ºC by addition of 0.2ml of 10% TCA in 1N HCl, mixed and 0.2ml of TBA was added and contents heated in a boiling water bath for 20 min, cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 632nm for COX activity

Assay of 5-lipoxygenase

Reagents

0.2M sodium phosphate buffer (pH6.1), 50mM tris HCL buffer (pH7.4), Sodium linoleate 10mM

Procedure

70mg of linoleic acid and equal weight of tween 20 was dissolved in 4ml of oxygen free water and mixed back and forth with the a pipette avoiding air bubbles. Sufficient amount of 0.5N NaOH was added to yield a clear solution and then made up to 25ml using oxygen free water. This was divided into 0.5ml portions and flushed with nitrogen gas before closing and kept frozen until needed.

The reaction was carried out in a quartz cuvette at 25°C with 1cm light path. The assay mixture contain 2.75ml tris buffer of pH 7.4, 0.2ml of sodium linoleate and 50µl of the enzyme. The increase in OD was measured in 234nm.% inhibition was calculated using the formula given below.

% inhibition = (C-T/C) *100

C = Optical density of control

T = Optical density of Test

Estimation of Myeloperoxidase

Cultured sample was mixed in a solution containing 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 6 and 0.57 hexadecyl trimethyl ammonium bromide(HTAB).After freeze thawing 3 time, the samples were centrifuged at 2000g for 30 min a 4°C and resulting supernatant was assayed spectrophotometrically for MPO. Sample was mixed with 50mM phosphate buffer (pH 6) containing 1.67mg/ml guaicol and 0.0005% H_2O_2.The change in absorbance at 340 nm was measured. MPO activity was expressed in u/mg tissue .One unit of MPO activity was defined as degrading 1m mol peroxide degraded per minute at 25°C.

Estimation of Cellular Nitrite Levels

The level of nitrite level was estimated by the method of Lepoivre et al. To 0.5 mL of cell lysate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer and mixed well. To this, 530 µL of Griess reagent was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained

Molecular screening

Genomic DNA of the strains showing anti-inflammatory activities were identified by 16SrRNA gene sequence analysis using a commercially available QIAGEN's QIAamp^{TM} DNA mini kit. Amplification was carried using primers derived from highly conserved regions of the bacterial 16S rRNA gene 5-GGAGGAAGG TGGGGATGACG-3 and 5-ATGGTGTG ACGGGCGGTGTG-3.This primer is known as universal primer because of its ability to amplify a 241-bp product from any bacterial species. Later, sequencing of the PCR product was done and BLAST analysis done.

Result and Discussion

In this study, we have investigated the action of inflammatory mediators on *Lactobacilli* producing bacteriocins.

In vitro studies on cell lines of ten (10) purified samples of *Lactobacilli* showed that, only three (3) of them possess antiinflammatory activity and the readings were recorded.

From our results it can be observed that addition of extracts produced a dose dependent increase in COX inhibition. Bacteriocin from sample 7 was more effective with a 45% inhibition in total COX activity. COX inhibition can further limit the progression of inflammation by down regulating synthesis of prostaglandins the major factor for inflammatory response.

Agar-well diffusion assay is considered as the best method for the detection of bacteriocins, because it is a simple and low-cost method (Tagg and McGiven,1971). Mueller Hinton Agar (MHA) was chosen for conducting agar well diffusion test in the detection of bacteriocinogenic *LAB.* This study was supported by the findings of Ouissal Bourouni *et al.* (2012). They found that MHA produce better inhibition in 48 hours than MRS and Trypticase Soy Agar (TSA) in 24 and 72 hours.

MPO is an early biomarker of inflammation and plays an important role in initiation and progression of acute and chronic inflammation (Josune *et al.* 2012). It is and enzyme most abundantly expressed in neutrophils and to a lesser extent in monocytes (Van der Veen *et al.,* 2009) generating reactive oxygen species that contribute to the destruction and killing of the engulfed pathogens (Vand der Veen *et al.,* 2009). It has been demonstrated that MPO is involved in the cellular homeostasis and plays an important role in initiation and progression of acute and chronic inflammatory disease.

Thus our results add further support to the concept. MPO has proved to be an active mediator of endothelial dysfunction in cell culture and animal models (Eiserich *et al.,* 2002). Mycloperoxidase (MPO) is a heme enzyme released by activated neutrophils from its azurophilic granules, into extracellular milieu, where it uses H_2O_2 and chloride anion (Cl^-) forming hypochlorus acid (HOCl) (Klebanoff, 2005).

HOCl is a powerful oxidant and it reacts with amines to form chloramines. Nitrite (NO_2^-), a major product derived from NO, may be oxidized by MPO to nitrogen dioxide (NO_2); which may be further react with HOCl to form the highly reactive compound nitryl chloride (NO_2^-Cl) (Bian, 2006).Our data support the role of MPO as key player in neutrophil extravasation and suggest that MPO alters chemokine and cytokine production which may govern inflammatory responses Inflammatory

bowel disease (IBD) are generally comprised of either ulcerative colitis or Crohn disease, which are considered immune mediated functional gastro intestinal disorder. It is characterized by relapsing or chronic abdominal discomfort with varying frequency or consistency of feaces. The pathogenesis of the condition is influenzed by genetic(y (Anderson *et al.,* 2011; Franke *et al.,* 2010) and environmental factors.

Clinical data and experimental studies revealed that the immune response to the intestinal microflora plays a major role in the progression of the chronic intestinal inflammation (Artis, 2008; Howarth, 2008). In addition, the intestinal microbiota in inflammatory bowel disease patients has been shown to differ from the one in healthy individuals with respect to the number and variety of micobiota present (Manichanh *et al*., 2006; Qin *et al*., 2010).

Milk enriched with *Lactobacillus acidophilus* has been suggested to help patients with IBS. It is believed to act by correcting the imbalance of the intestinal microflora. It is tolerated better by lactase-deficient subjects by providing bacterial lactase in the small intestine (Kocian, 1994). *Lactobacilli and Bifidobacteria* which are natural components of the colonic microbiota, and as probiotic agents, they have been tested in the prevention and treatment of IBD (Gionchetti *et al*., 2003). One particular strain of *Lactobacillus casei*, DN-114 001, has been shown to reduce the secretionof TNF-α from the inflammed ileum of Crohn's disease patients (Borruel *et al.,* 2002; Borruel *et al.,* 2003). A lack of bacteria with anti-inflammatory properties in the dysbiosis accompanying inflammatory bowel diseases (IBD) could be a key factor in the persistence of inflammation (Sokol *et al*., 2008).

Table.1 Assay of cycloxygenase

Sample volume (µl)	**Average OD 632 nm**	**% inhibition**
Control	0.1092	
Sample-9		
5µl	0.0874	20.0
10µl	0.0842	22.89
20µl	0.0694	36.44
Sample-7		
5µl	0.0793	27.38
10µl	0.0713	34.70
20µl	0.0600	45.05
Sample-3		
5µl	0.0799	26.83
10µl	0.0750	31.31
20µl	0.0734	32.78

Table.2 Assay of 5-lipoxygenase

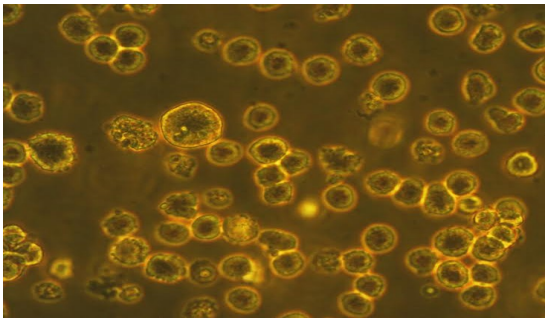
Sample volume (µl)	OD 234 nm	% inhibition
Control	0.117	
Sample-9		
5µl	0.114	2.5
10µl	0.097	17.9
20µl	0.052	55.55
Sample-7		
5µl	0.107	8.54
10µl	0.048	58.97
20µl	0.027	76.83
Sample-3		
5µl	0.077	34.18
10µl	0.039	66.66
20µl	0.030	74.35

Same as that of COX but here if we inhibit LOX it down regulates the progression of synthesis of leukotrienes

Table.3 Estimation of Myeloperoxidase

Sample volume (µl)	Average ΔOD 340 nm	Enzyme (U/ml)
Control	0.095	0.1263
Sample-9		
5µl	0.070	0.0931
10µl	0.062	0.0824
20µl	0.018	0.024
Sample-7		
5µl	0.023	0.030
10µl	0.021	0.027
20µl	0.014	0.018
Sample-3		
5µl	0.074	0.098
10µl	0.063	0.083
20µl	0.051	0.067

Fig.1 Representative Phase contrast images of THP cells used for anti inflammatory studies (20X)



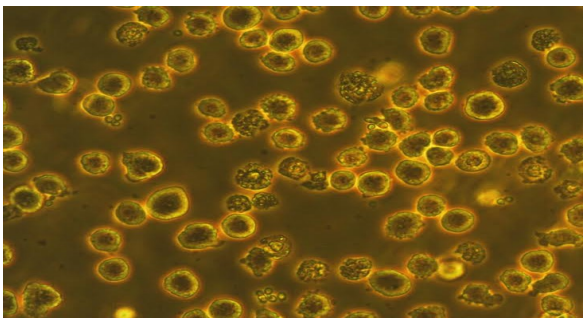


Table.4 Estimation of cellular nitrite levels

Sample volume (µl)	Average OD 540 nm	Concentration (µg)
Control	0.1225	608
Sample-9		
5µl	0.105	521
10µl	0.098	487
20µl	0.079	392
Sample-7		
5µl	0.075	372
10µl	0.072	357
20µl	0.070	347
Sample-3		
5µl	0.118	586
10µl	0.112	556
20µl	0.103	511

Molecular studies

Lactobacilli producing bacteriocins which produce anti inflammatory property, subjected to PCR and sequencing showed the presence of *Lactobacillus delbreuki* , *Lactobacillus fermentum* and *Lactobacillus paracasei* and their sequence obtained. This study revealed the presence of *Bacillus subtilis*, *Sporolactobacillus dextrus*, *Bacillus amyloliquefaciens* and *Kurthia*, all isolated from curd, with anti-inflammatory activity and their sequence obtained.

On the basis of the experiments performed and results obtained in the study, it is clear that, bacteriocinogenic *Lactobacilli* possessed anti inflammatory activity. This study has shown that lactobacilli isolated from dairy sources can be applied, in natural form, modified or as combinations, to save the mankind from the irritating diseases like Hemorrhoids, Inflammatory Bowel diseases, etc.

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