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ANTI-RHEUMATOID ACTIVITY OF CELL WALL CONTENTS OF LACTOCOCCUS LACTIS SUBSP. CREMORIS

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ABSTRACT

Rheumatoid arthritis(RA) is an autoimmune disease characterized by persistent synovitis, systemic inflammation, and autoantibodies. Toll like receptors (TLRs) plays an important role in the pathogenesis of RA. *Probioticsare widely used in the food industry and is considered a good candidate for production of heterologous proteins for developing nutraceuticals or new live vaccine strategies.*To isolate cell wall contents of *Lactococcuslactis* subsp. *cremoris* and investigate the protective effect in rheumatoid arthritis in rats.Isolation of cell wall contents of bacteria using phenol extraction.RA rat model was established by Complete Freund's Adjuvant and Rats were treated with cell wall content as prophylactic and therapeutic treatment from day 1 to 21 and 7 to 21 respectively.The present study suggests that cell wall contents has protective activity in experimentally induced RA and the activity might be attributed to presence of cell wall contents Lipoteichoic acid, peptidoglycans etc.

KEYWORDS: Rheumatoid Arthritis,*Lactococcuslactis* subsp. *cremoris*, Inflammation and Cell wall contents.

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INTRODUCTION

Rheumatoid Arthritis(RA) is a systemic debilitating disease whose primary symptoms consist of chronic inflammation, synovial hyperplasia, and destruction of cartilage and bone of numerous joints[1, 2]. RA is characterized by synovial inflammation and hyperplasia ("swelling"), autoantibody production (rheumatoid factor and anti–citrullinated protein antibody [ACPA]), cartilage and bone destruction ("deformity"), and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders[3].Established RA is persistent inflammatory synovitis, usually involving peripheral joints in a symmetric distribution. The potential of the synovial inflammation to cause cartilage damage and bone erosions and subsequent changes in joint integrity is the hallmark of the disease[4]. There is a long-standing hypothesis that microbial infection plays a role in RA and that the infection triggers inflammatory responses recognized by pattern recognition receptors (PRRs). These inflammatory reactions damage the host tissue, release endogenous alarmins that can activate PRRs constituting a positive feedback[5]. RA that affects approximately 0.5% of the adult population worldwide, and occurs in 20–50 cases per 100000 annually, mainly in women after their 40s. Life expectancy was reduced by 8 years in males and by 9 years in females. If one of a pair of monozygotic twins is affected, the other twin has a 30 times greater risk of developing the disease[6].Toll-like receptors (TLRs) are a family of transmembranereceptors,TLRs belong to a class of molecules known as pattern recognition receptors. The ligands for these receptors are components of pathogenic microbes and are often called pathogen associated molecular patterns (PAMPs). Expression of TLRs 2, 3, 4, and 7 is enhanced in (early) RA synovial tissue and exogenous as well as endogenous TLR agonists have been detected in the joints of patients with RA. In animal models of RA, TLRs, in particular TLR-4, drive the expression of inflammatory cytokines and determine the severity of joint inflammation and destruction[7].TLR2 preferentially induces IL-10, a cytokine that inhibits the synthesis of several proinflammatory cytokines, and that belongs to the Th2 response in the mouse[8]. Probiotics are micro-organisms proven to exert health-promoting influences in humans and animals. *Lactococcuslactis* is a generally regarded as safe (GRAS) microorganism that is widely used in the food industry. During the last two decades, more research work has focused on using *L. lactis* as a delivery vector for therapeutic proteins and antigens[9]. The established safety profile of *L. lactis* and its potential use via mucosal routes of administration are important motivating factors. *L. lactis* is non-commensal and does not colonize the gut, a situation which minimizes the risk of side effects upon oral administration.The fate of orally administered lactic acid bacteria (LAB), including *L. lactis*, has been described in an important review by Wells and Mercenier Upon oral administration, some of the LAB that reach the intestine are taken up by the M cells of Payer's patches where they are transported across the epithelium to the underlying antigen presenting cells (APCs), mainly dendritic cells. The antigens are then processed and appropriately presented by the APCs to elicit specific immune responses[10]. Management of RA with analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) can be helpful in relieving pain, where they are not contraindicated. Within the class of NSAIDs is a group of drugs that were originally developed with a safer gastrointestinal profile in mind. These are referred to as COX-II inhibitors. Conventional disease modifying anti-rheumatic drugs (DMARDs) include methotrexate, sulphasalazine, hydroxychloroquine, leflunomide and gold injections. These drugs can be helpful in slowing down the damaging component of the disease process[11].

MATERIALS AND METHODS

i. Animals

Five to six week-old healthy female wistarrats weighing 180 to 200 g were selected for present study. Animals were housed in 7 groups in polypropylene cages, consisting of 6 in each, under standard laboratory conditions, temperature(25±2)^{0}C and relative humidity

(55±10%). Animals had free access to standard pellet chow and water. Animals were acclimatized for a period of minimum 7 days and daily observed. The experiment protocol (KBIPER/2013/438) had been approved by Institutional (K. B. Institute of Pharmaceutical Education and Research) Animal Ethics Committee (IAEC) under the Committee for Purpose of Control and Supervision of Experiments (CPCSEA) on animals before carrying out the project.

ii. Drugs and chemicals

Dexamethasone was purchased from Cadila Pharmaceutical LTD., India. Probiotic*Lactococcuslactis*subsp*. cremoris* lyophilized vial purchased from Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology Sector 39-A, Chandigarh, India and complete Freund's adjuvant(CFA) was purchased from Sigma Aldrich, St.Louis, USA.

iii. Cell wall contents isolation using hot phenol aqueous extraction from L. lactis subsp.cremoris

Lactococcuslactis subsp*. cremoris* were been grown in nutrient broth medium at 20 – 25^{0}C and incubation period was 48 hrs. After incubation period bacteria were harvested by centrifugation (3,000 g, 10 min) in centrifugation tube and wet weight of bacteria was obtained. Bacterial cells were suspended in sodium chloride solution (0.9%, w/v) and shaken in a homogenizer for 15 minutes and These homogenized solution centrifuged at 16,000g for 30 minutes. The supernatant solution containing fragmented membranes was decanted. The supernatant containing the membranes was stirred with chloroform-methanol (250ml, 2:1, v/v), and then the residue were washed with chloroform-methanol. This procedure was repeated three times to ensure that all lipids had been removed. Traces of solvents were removed by drying the residue in air. The residue was suspended in water (125 ml) and stirred with an equal volume of 80% (w/v) aqueous phenol for 40 min at 65^{0}C. The resulting emulsion was centrifuged at 16,000g for 30 min. and the insoluble material at the interface and the lower phenol phase containing much of the protein were discarded. The volume of the solution was reduced to about 80 ml by evaporation at 30-40^{0}c, and the solution was mixed with an equal volume of 0.1M-Tris buffer, pH 8.0, containing 0.02 M magnesium chloride. Ribonuclease and deoxyribonuclease (10 µg/ml) were added, and the mixture was incubated at 37^{0}C under toluene in order to hydrolyse the nucleic acids. The incubation continued for 48-72 h, thereby removing alanine ester residues from the teichoic acid. Toluene (1 ml) was added to prevent microbial contamination, and the solution was incubated at 20 ^{0}C for 24 h. The enzymes were removed by a second phenol treatment as described above. After centrifugation, phenol will be removed from the water phase as mentioned before[12-15]. The yielded solution containing concentrated cell wall contents was identified using HPTLC.

iv. CFA induced arthritis

Several experimentally induced rat models of autoimmune erosive arthritis are currently used to evaluate potential etiopathogenetic mechanisms in RA. To evaluate potential new therapeutic agents Rat models of erosive arthritis are also used[16].For inducing autoimmunity and autoimmune disease, Freund's adjuvants use the mechanisms as those by which they enhance specific immune responses to foreign antigens. In case of CFA, the heat-killed mycobacterial cells embedded in oily excipient persist for weeks or even months at the injection site and in phagocyte-rich organs such as lung and liver. As time elapses, they constitute an unabated stimulus for the production of monokines and Th1 lymphokines. These late-produced cytokines may play a role in bringing about the arrest of T-cell expansion and activation but also in the gradual build-up of myelopoiesis, most explicitly revealing itself in splenomegaly and disruption of spleen histologyTo explain induction of AIA by mycobacterium containing CFA, cross-reactivity of anti mycobacterial antibodies or T-cell receptors with epitopes of host proteins have been invoked[17]. The oil component and mycobacteria activate the MPC system, including the various categories of DCs. This results in overall enhanced phagocytosis of particulate material[18] and secretion of monokines[19], a correlate of this being the transient, increased aspecific

enhancement of resistance to infection[20]. The disease develops in females but is much more variable in onset and severity[21].Adjuvant can be injected at the base of the tail or in one of the foot pads[22]. If injection is into the footpad, it allows study of the acute inflammatory reaction in that local area as well as the immunological reaction that develops approximately 9 days later in the contralateral paw and various organs. Hind paw swelling is monitored from day 9 (onset of disease) to 15 or greater depending on duration desired. In the later stages of disease (day 12+), adjuvant arthritis rats are often relatively immobile due to severity of paw swelling and so require special care to insure that they have access to water and food. Treatments are initiated on day 0 (prophylactic model dosing) or day 8 (therapeutic model dosing)[21, 23]. Using this model easy to evaluate effect of drug in RA. On day 0, female Wistarrats (180-200 g)had injection on the sub plantar region of the left hind paw with 0.1 ml containing 1.0 mg dry heat killed Mycobacterium tuberculosis per ml sterile paraffin oil. A glass syringe with 26G needle was used for injection[24]. Animals were divided in to 7 Groups containing 6 animals in each group.

Group I Normal control groups were given only vehicle in volume equivalent to that of the cell wall contents.

Group II – model control group received 0.1 ml CFA (1mg/ml, subplantar),

Group III- standard control received 0.1 ml CFA and a dose of dexamethasone (0.7mg/kg, *S.C.*),

Group IV and Group VI received cell wall contents (2mg/kg, *S.C.*),

Group V and Group VII received cell wall contents(4mg/kg, *S.C.*).

Administration of cell wall contents in Group IV and Group V from day 0 to 21 as prophylactic treatment and in Group VI and Group VII from day 8 to 21 as therapeutic treatment.Dexamethasone was also given as therapeutic treatmentfrom day 8 to 21.

On day 0, 7, 14 and 21 evaluation of the following parameters Paw Edema[25], Gait test[26], Joint Stiffness test, Serum Rheumatoid factor(SRF), Serum C-reactive protein(SCRP)[27]were carried out. On day 14 and 21 secondary lesions were evaluated by Arthritis Index (AI) and on day 21 histopathology of synovial joint were carried out. The percent change in body weight of day 7, 14 and 21 as compared to day 0. Paw Volumes of both hind limbs were recorded before injection of CFA, using mercury plethysmometer. The paw volume of both the hind limbs were again measured on day 7, 14 and 21[28]. Arthritis index is the mean of the score/grade given to the severity of inflammation on the ears, nose, tail, fore paw and hind paw. All the animals were closely observed and scored. An Arthritis index for each animal was calculated and compared with respective normal control group.[28] Serum Rhematoid factor and Serum C-reactive protein estimation was done by turbidometry method[23].

v. Histopathology of synovial joint

Rats were sacrificed on 21st day and hind limbs were removed and fixed in 10% buffered formaline. Limbs were decalcified in 5% formic acid, processed for dehydration in ascending order of acetone solution (70%, 80%, 90%, 100%), xylene and then processed for paraffin embedding, sectioned of 5 µm in thickness and subsequently stained with haematoxyline-eosin for examination under a fluorescent microscope. Sections were examined for the presence of hyperplasia of synovium, pannus formation and destruction of the joint space[28].The Blue staining (Haematoxylin) for the nuclei and the pink (Eosin) for the cytoplasm.

vi. Statistical analysis

Results were expressed as the Mean ± SEM. The significant difference between parametric means was evaluated one-way ANOVA followed by Tukey's post hoc multiple comparison test for normal data. $P< 0.05$ was considered as significant.

RESULTS

1. Chromatographic evaluations of cell wall contents

Chromatographic results of cell wall contents revealed the presence of the lipoteichoic acid, peptidoglycans, Lipoic acid ~~*etc*~~ these results were compared with standard chromatogram of individual respective content of cell wall.

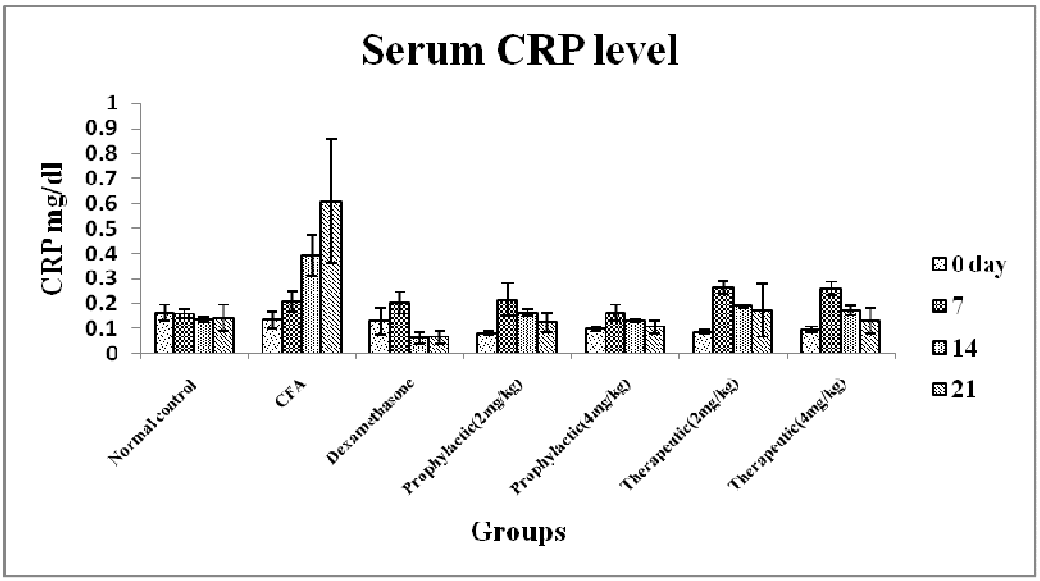
2. CFA induced Arthritis

Paw volume on day 21 was significantly lower in cell wall contents treated rats than CFA treated rats (P<0.05). There was not significant difference in paw volume between cell wall contents treated rats (prophylactic 2mg/kg and 4mg/kg, therapeutic 4mg/kg) and dexamethsone treated rats on 21st day.

Serum CRP level

Graph 1

Effect of cell wall contents of Lactococcuslactis subsp. cremoris on Serum CRP level



Each bar represents Mean ± SEM, n=6 , *Shows significant difference as compared to normal control group,at p<0.05 , #Shows significant difference as compared to disease control group, at p<0.05 , (One way ANOVA followed by Turkey' test)

Body weight in CFA treated rats was not significantly reduced compared to normal control (P<0.05). The body weight was reduced in dexamethsone treated rats as compared to cell wall contents treatment groups. There was no difference of percentage change in body weight in prophylactic treated groups as compared to therapeutic treated groups. (Table 1) Arthritic score, gait score, joint stiffness score, Serum Rheumatoid factor, Serum C-reactive protein level was significantly increased in CFA treated rats compared with normal control group (P<0.05) and all parameters were significantly decreased in Prophylactic (4mg/kg), Serum Rheumatoid factor, paw volume, arthritis index were significantly decreased in prophylactic (2mg/kg) and therapeutic (4mg/kg) treated rats (P<0.05).

3. Histopathology of synovial joint (Figure 2) (X100)

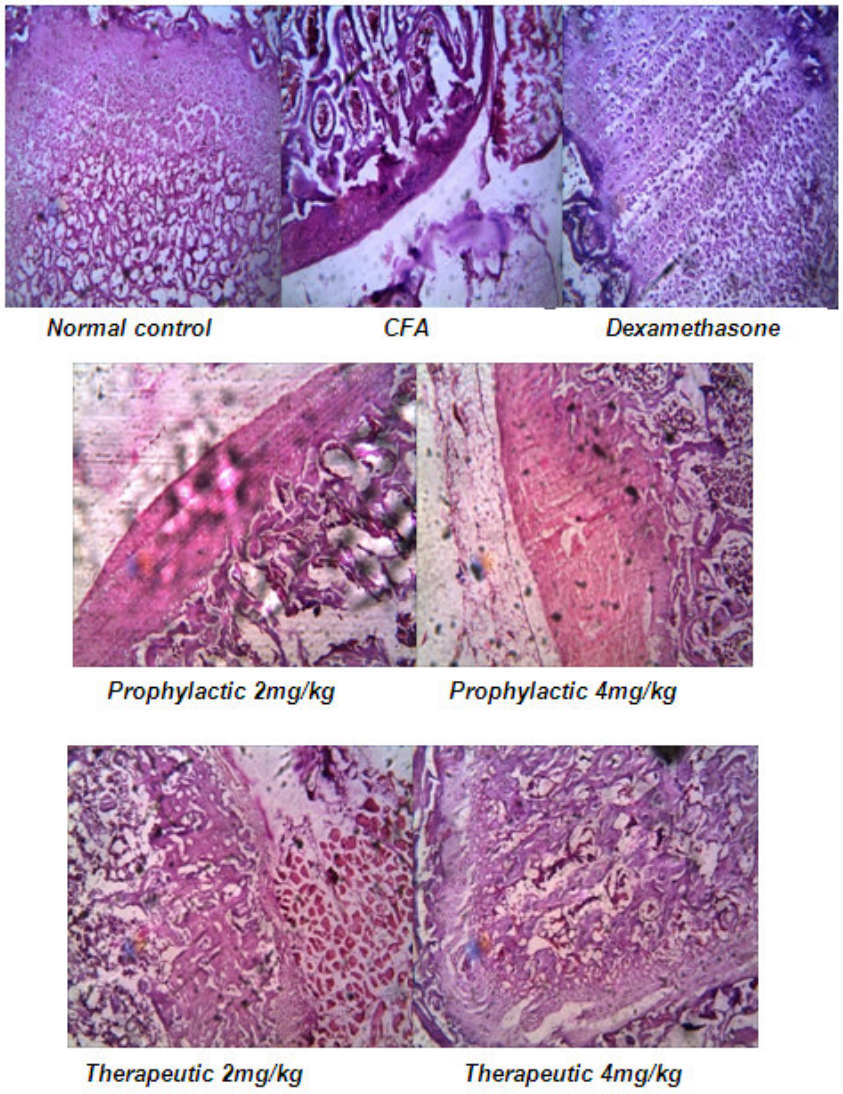


Figure 1

Histology slides showing haematoxylin and eosin stained chondrocyte cells. Normal and dexamethasonetreated rats showed normal chondrocyte and CFA treated rats showed increase synovial lining of synovial joint, Inflammation, inflammatory cell proliferation and chondrocytes migration.Prophylactic (4mg/kg) treated group showed less migration of chondrocytes and protection of cartilage destruction.

Table 1

Effect of cell wall contents of L. lactis subsp. cremoris on an average body weight (Mean±SEM)

Treatment Groups	Average body weight(g)			
	0 day	7th day	14th day	21st day
Normal control	234.0±14	242±13.56	256.6±12.15	250±13.42
Disease control	221.7±18.87	231.7±18.15	255.8±18.46	245±17.46
Dexamethasone	195.8±10.03	204.5±7.41	173.0±9.73	173.3±9.1
Prophylactic 2mg/kg	205.8±13.69	219.2±15.3	241.7±11.67	231.7±12.76
Prophylactic 4mg/kg	226.7±7.60	223.3±9.97	243.3±8.82	240.0±9.31
Therapeutic 2mg/kg	233.3±7.60	220.0±5.77	225±12.04	218.3±9.46
Therapeutic 4mg/kg	218.3±5.43	191.7±7.92	215±8.47	181.7±7.49

Table 2
Effect of cell wall contents of L. lactis subsp. cremoris on Biochemical and Physical parameter (Mean±SEM) at 21st day

Groups	Rheumatoid factor (IU/ml)	Serum CRP level (mg/L)	Paw volume (ml)	Arthritis index (Score)	Gait test (Score)	Joint stiffness (score)
Normal	13.22±0.63	0.14±0.05	0.78±0.06	0.00±0.00	2.0±0.0	2.0±0.0
CFA	34.13±0.94*	0.67±0.07*	1.51±0.61*	1.83±0.17*	1.0±0.0*	0.17±0.17*
Dexamethasone	16.41±1.28#	0.13±0.02#	0.91±0.05#	0.17±0.17#	1.83±0.17#	1.83±0.17#
Prophylactic(2mg/kg)	27.70±1.92*#	0.26±0.04	0.94±0.03#	0.5±0.22#	1.17±0.17*	1.5±0.22
Prophylactic(4mg/kg)	22.52±1.77*#	0.21±0.03#	0.94±0.03#	0.17±0.17#	1.83±0.17#	1.67±0.21#
Therapeutic(2mg/kg)	32.78±1.36*	0.38±0.1	1.21±0.04*#	0.5±0.22#	1±0.00*	1.33±0.21
Therapeutic(4mg/kg)	23.16±1.63*#	0.28±0.05	1.03±0.02*#	0.33±0.21#	1.33±0.21*	1.5±0.22

shows significant difference as compared to control group, at p<0.05
#shows significant difference as compared to disease control group, at p<0.05

DISCUSSION

Rheumatoid arthritis (RA) is an autoimmune disease ofchronic polyarticular inflammation that leads to joint swelling, stiffness, deformity and loss of joint function with systemic manifestations[2].Various animal models of inflammation are used extensively in research on pathogenesis of inflammatory arthritis. Important criteria to select a model include: 1) capacity to predict efficacy of agents in humans, 2) Ease of performing, reproducibility of data, reasonable duration of test period and 3) similar pathology and/or pathogenesis to that of human disease. The animal model in the present study is subjected to through critical appraisal and validated as animal models for rheumatoid arthritis[(28)]. For inducing autoimmunity and autoimmune disease, Freund adjuvants use the mechanisms as those by which they enhance specific immune responses to foreign antigens. However, in the case of CFA, embedded heat-killed mycobacterial cells in oily excipient persist for weeks or even months at the injection site and in phagocyte-rich organs such as lung and liver. As time elapses, they constitute an unabated stimulus for the production of monokines and Th1 lymphokines[17].Mycobacterium-induced arthritis stimulated the expression of IL-2 in the splenocytes, which was reduced in the probiotic-treated mice. probiotic treatment ameliorated the disease outcome in the Mycobacterium butyricum model of rheumatoid arthritis in mice, showing systemic immunomodulatory properties[29]. A probiotic L. lactis possesses potent capacity to activate both DCs and NK cells and subsequently promote T cell immunity. Oral administration of LcFC enhanced the production of IFN-γ and IL-10 from splenocytes of treated mice. These suggest that this LAB strain is an efficient activator of protective cellular immunity via stimulation of myeloid cells including DCs. IL-10 is a cytokine with potent anti-inflammatory activity that has been demonstrated to be able to inhibit the synthesis of several cytokines including IL-10 and IL-12. Dendritic cells (DCs) stimulated for 5 h by TLR2 agonist released a higher amount of IL-10 than did cells stimulated with TLR4 or TLR7 agonists[30]. In present study the cell wall contents of L. lactissubsp.cremoriswas evaluated for their effect in arthritic condition in rats. Treatment with different doses of cell wall contents of L. lactissubsp.cremoriswas found to be beneficial in CFA induced RA. All animals tolerated the experimental procedures well, and showed no evidence of drug toxicity and death up to completion of study. So, it was found to be safe.Body weight is normally reduced in most of the autoimmune disorders, which may be due to systemic or local action of cytokines resulting from chronic inflammation. Administration of CFA resulted into reduction in body weight in therapeutic treatment groups. Further decrease in body weight was observed in Dexamethasone treated group whereas treatment with cell wall content of L. lactissubsp. cremoris did not affected body weight significantly. Interaction of glucocorticoid with leptin might be the reason behind reduction in body weight of

Dexamethasone treated animals[31-33]. Arthritic index is summation of scores obtained by giving ranks to extent of inflammation, formation of nodules and extent of spread of disease at non-injectable sites. Arthritis index is secondary immune response to administration of CFA. A rise in Arthritic index is due to fibrin deposition, fibrosis, and necrosis. Hyperplastic synovial tissue (pannus) may erode cartilage, articular capsule, and ligaments. Arthritic index was found to be increased significantly in disease control group, which proved that inflammatory arthritis was developed in the animals. Treatment with different doses of cell wall content of L. lactissubsp.cremorisreduced the score of arthritis index. Observational tests like Gait test and Joint stiffness test were also accessed for evaluation of arthritis. The scores in disease control group were higher as compared to normal control group and treated animals scored less as compared to disease control group. Serum Rheumatoid factor and serum C-reactive protein levels were analyzed to confirm the arthritis biochemically. RF, an antibody is secreted by certain normal cell populations. The highest level of rheumatoid factor are usually found in rheumatoid arthritis. Serum rheumatoid factor level in CFA treated rats was increased significantly in Disease control group as compared to normal control. The prompt decrease in rheumatoid factor level after treatment with cell wall content of L.lactissubsp.cremoris and dexamethasone indicates their anti-arthritic potential.C-reactive protein is a marker of inflammation, and its level rise significantly during inflammatory processes. In the present study the serum CRP level was significantly reduced as compared to disease control, which direct prohibits level of various cytokines released. Treatment of inflammation tend to reduce the CRP level[16, 34]. Serum C-reactive protein were decreased significantly by the treatment with dexamethasone and with cell wall content ofL. lactissubsp. cremoris[29].Histology of normal control rats showed intact morphology of synovium and synovial lining of synovial joint. No inflammation, chondrocytes migration and inflammatory cell proliferation was observed (Figure 2). CFA treated rats showed increase of synovial lining of synovial joint. inflammation, inflammatory cell proliferation and chondrocytes migration observed. Dexamethasone treated rats showed protection against cartilage distruction and less chondrocytes migration. Histology of Prophylactic (2mg/kg) treated rats showed increase space of synovial lining of synovial joint. Inflammation, inflammatory cell proliferation and chondrocytes migration observed. Prophylactic (4mg/kg) treated rats showed Comparatively normal space of synovial lining of synovial joint. Less inflammation, inflammatory cell proliferation and chondrocytesmigration ,No cartilage destruction was observed. Therapeutic (2mg/kg) treated rats showed increase space of synovial lining of synovial joint. Cartilage destruction and chondrocytes migration was observed. Therapeutic (4mg/kg) treated rats showed less inflammation, inflammatory cell proliferation, less cartilage destruction and chondrocytes migration was observed.Dexamethasone is one of the most successfully used anti-inflammatory agents. The biochemical changes of rheumatic arthritis patients in histopathological studies are partially restored by dexamethasone treatment. These arthriric changes, such as increase in synovial joint space, more number of neutrophils infiltrations, degradation of cartilage in the histopathological section were restored in prophylactic and therapeutic(4mg/kg) treatment groups. Probiotic supplementation given comparatively similar effects as dexamethasone in CFA treated rats. Thus, supplementation of probiotics prevents progression of rheumatic arthritis by increasing anti-inflammatorycytokines and decreasing pro-inflammatory cytokines which prevents synovial bone and cartilage damage.

CONCLUSION

From the present study, we concluded that treatment with cell wall contents of *Lactococcuslactis* subsp.*cremoris* was beneficial in treating the inflammatory conditions related to Rheumatoid arthritis in rats. Prophylactic treatment was more effective than therapeutic treatment and 4mg/kg dose is more effective than 2mg/kg dose.

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