Lactobacillus casei reduces the Inflammatory Joint Damage Associated with Collagen-Induced Arthritis (CIA) by Reducing the Pro-Inflammatory Cytokines

Lactobacillus casei: COX-2 inhibitor

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Abstract This study evaluated the therapeutic efficacy of *Lactobacillus casei* in treating rheumatoid arthritis using collagen-induced arthritis (CIA) animal model. Healthy female Wistar rats (weight—180–200 g) were included in this study. Oral administration of *L. casei* was started on the same day. Indomethacin was used as standard reference drug. Serum level of IL-6, α-TNF, and IL-10 were observed. Four-point arthritis indexes were also assessed at the end of week for 28th day. *L. casei*-treated rats had shown normal histopathology without any synovial infiltration, pannus formation, cartilage, and bone destruction. Arthritis score was also lower for the group treated with *L. casei*. Oral administration of *L. casei* significantly decreased the pro-inflammatory cytokines. Present study suggests that *L. casei* has potent antiarthritic effect in CIA model. Inhibition of COX-2 via inhibiting the pro-inflammatory cytokines is an understanding of the complex interactions involved in these pathways.

Keywords Pro-inflammatory cytokines · anti-inflammatory cytokines · IL-10 · cyclocoxygenase-2

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Abbreviation

RA	Rheumatoid arthritis
CIA	Collagen-induced arthritis
CPCSEA	Committee for the Purpose of Control and Prevention of Experiments on Animals
NSAIDs	Non-steroidal anti-inflammatory drugs
DMARDs	Disease-modifying anti-rheumatic drugs
LAB	Lactic acid bacteria
IFA	Incomplete Freund's adjuvant
IL-6	Interleukin-6
IL-10	Interleukin-10
α-TNF	Tumor necrosis factor
NF-κB	Nuclear factor kappa

Introduction

Rheumatoid arthritis is a chronic autoimmune disease which leads to severe joints pain [1] and concomitant damage to both cartilage and bone [2]. A number of treatments of non-steroidal, steroidal, immunosuppressant,

and anti-inflammatory agents are being used as an alternative treatment for rheumatoid arthritis. All these drugs lower the symptoms, but they have undesirable side effects. Several studies were performed for antiarthritic properties of various natural and processed agents [3–8].

Chemotherapy with non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs) suppresses the immunological processes involved in the progression of rheumatoid arthritis [9], but they result in strong adversible effects like gastric damage and cardiovascular disease [10, 11]. Drugs having both NSAIDs and DMARDs are very scarce, and such drugs are more effective.

Various immunological phenomena are responsible for joint damage [12, 13]. RA is associated with hyperplasia, increased vascularity, and inflammatory cell infiltration. $CD4^+$ T cell, synovial cells, fibroblast, and macrophage release a large number of cytokines with synergistic and antagonistic effects. Cytokines included in pro-inflammatory cytokines group are IL-1, TNF-α, IL-8, IL-12, IL-15, IL-17, and IL-18 while IL-4, IL-10, IL-11, and IL-13 are included in anti-inflammatory cytokines that downregulate inflammation. Some cytokines have dual nature like IL-6, IFN-γ, and TGF-β [12]. Imbalance between pro-inflammatory and anti-inflammatory cytokines is an important factor in initiation of arthritis [14]. It has been found that TNF-α plays a pivotal role in progression of arthritis [15, 16].

Inflammation is a protective defense mechanism. It triggers an immune response to infection and various traumas which in turn guards the body to hasten the recovery process. Howsoever, sometimes, inflammation leads to deadly inflammatory disorders. Pro-inflammatory cytokines play an important role in such disorders. Previously, Lactobacillus species have been exploited for its property as probiotics. Many species of Lactobacillus species are used as probiotic agents like Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus delbrukii, Lactobacillus sporogenes, etc. These are nowadays used as a dietary supplement because of their immunomodulatory property [17–22].

The present study reports the protective efficacy of L. casei using a curative protocol (administered before the onset of arthritis) with special reference to role of pro-inflammatory and anti-inflammatory cytokines in disease symptoms. This would provide scientific use of L. casei for treating arthritic condition as well as provide an alternative approach for the drugs currently being used for controlling arthritis.

Materials and Methods

Chemicals

IFA was purchased from Genei, Bangalore, India. Cytokines assay kits were purchased from Ray Biotech, Norcross GA and DNA bio, Hyderabad, Andhra Pradesh, India. Bovine Tracheal cartilage type II collagen was purchased from Roche Diagnostics GmbH, Germany. Indomethacin was purchased from Recon, Banglore, India. All other chemicals used in this study were of analytical grade and purchased locally.

Bacterial Culture

L. casei (ATCC 334; Hi Media, Navi Mumbai, India) was used in this study. Lyophilized culture was streaked over De Mann Rogosa Agar (Hi Media, Navi Mumbai, India) at 37°C in anaerobic condition.

Animals

Healthy 20 female Wistar rats, 8 weeks old (180–200 g) were used for the experiment. Animals were randomly divided into four groups of five animals in each group. These were purchased from the rat colony at Indian Institute of Toxicological Research, Lucknow, India and maintained in the animal house at Institute of Biomedical sciences, Bundelkhand University, Kanpur Road, Jhansi, Uttar Pradesh. Animals were acclimatized for 7 days before the experiment started. Animals were housed in standard stainless steel cages and were fed with balanced diet purchased from Amrut Feed Ltd, India, given water ad libitum (Principles of Laboratory Animal Care NIH publication no, 85–23, revised 1985) and maintained under laboratory conditions (temperature 22±2°C, relative humidity 60–70%, and 12–12 h light–dark cycle). Ethical committee of the University approved the use of these animals for experimental purposes. All procedures and techniques used in these studies were in accordance to CPCSEA guidelines.

Experimental Protocol

Animals were randomly divided into four groups ($n=5$):

Group I: Serve as control (No Arthritis + No treatment)
Group II: Arthritis induced rats orally administered with 500 μl of distilled water
Group III: Arthritis induced rats orally administered with 2×10^8 CFU/ml L. casei in 500 μl distilled water
Group IV: Arthritis induced rats orally administered with Indomethacin at 10 mg/Kg of body weight in 500 μl distilled water

Dose Selection The dose was selected on the basis of previous study [23]; 2×10^8 CFU/ml of L. casei was

suspended in 500 μl distilled water and vortexed on cyclomixer. *L. casei* was administered orally to Group III animals. All treatments were performed with the help of syringe cannula from 1st to 28th day.

Induction of Arthritis

The method applied by Remmers et al. [24] was used for induction of collagen-induced arthritis. Rats were injected intraperitoneally with collagen (bovine tracheal cartilage type II; dissolved in 0.2 N acetic acid 2 mg/ml) and mix with equal volume of incomplete Freund's adjuvant on day 1st on several locations on the back. Booster dose of 0.1 ml same mixture was injected after 7th day on tail. These treatments were given to all groups except Group I (Control). In Group II, 500 μl of distilled water was given daily from 1st to 28th day. Indomethacin, standard anti-inflammatory drug, was administered in Group IV animals with the dose of 10 mg/kg body weight from 1st to 28th day [25]; 2×10^8 CFU/ml of *L. casei* suspended in 500 μl distilled water was given orally to Group III animals.

Sample Collection

On day 29th, animals were killed with cardiovascular bleeding with the help of diethyl ether according to the guidelines of CPSCEA committee. Blood was collected for the cytokines assay. Blood was collected from cardiac puncture and left to coagulate for room temperature for 60 min. Blood sample was then centrifuged at 1,500×*g* for 15 min, and crude serum was kept in new tube. Serum was harvested and stored at −20°C until use. Right hind paw knee joint was amputated for histopathological analysis.

Arthritis Evaluation

The clinical symptoms of arthritis were evaluated on a four-point scale by visual appearance [13]. The score was given for each knee joint on days 7th, 14th, 21st, and 28th. The scores were calculated on a scale of 0–4-point scale: 0=no swelling or erythema, 1=slight swelling and/or erythema, 2= low to moderate edema, 3=pronounced edema with limited joint usage, and 4=excess edema with joint rigidity. Arthritis score was given for each foot of collagen-induced arthritis rat. The total score for each animal was then calculated and used as an articular index with a maximum value of 16.

Cytokines Assay

IL-6, TNF-α (pro-inflammatory cytokines), and IL-10 (anti-inflammatory cytokines) in picogram per milliliter was estimated with the help of ELISA Reader (Lisa Plus, Germany). Serum sample were used. IL-6 and TNF-α

(Ray Bio®) and IL-10 (DNA bio) ELISA kits were used. Assays were performed according to the manufacturer's recommendations.

Tissue Processing

Right hind knee joint were examined for histopathological analysis. Method as described previously by [26] was used with some modifications. It was preserved in 10% formalin and then decalcified for 21 days in 10% EDTA followed by dehydrated in increasing percentages of ethanol. These tissues were than cleared in xylene for 3 h and embedded in paraffin blocks. The embedded organs were sectioned using microtome and stained with hematoxylin–eosin to study the histopathological changes associated with collagen induced arthritis and *L. casei* treatment.

Statistical Analysis

The results of cytokines are expressed as a mean ± SEM of five rats per group. The results obtained from different groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Data were considered statistically significant if $P < 0.0001$.

Results

L. casei has shown the persuasive protective effect against the chronic inflammation and arthritis induced by collagen-induced arthritis.
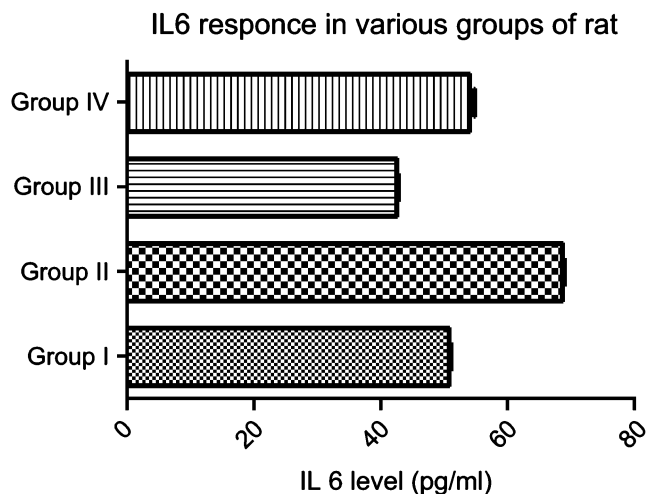
Arthritis Score

The result of oral administration of *L. casei* on arthritis score is shown in Fig. 1. Signs of arthritis appeared after 11 to 12 days. Swelling and reddening were the prominent symptoms shown by Group II animals. These animals were unable to walk. Group I animals were near to normal without any classical symptoms of arthritis. Groups III and IV animals were near to normal in comparison to Group II animals. Arthritis score of group II animals on 7th day was (7.225± 0.109), and it was significantly highest than all other group animals on 7th day. However, Groups III and IV animals have arthritis score of (4.275±0.130) and (4.25±0.112; $P < 0.0001$), respectively, which were also significantly different when compared with the group I animals. Arthritis score was significantly increased in case of Group II animals from 14th and 28th day. It was found to be (9.625±0.432) on the 28th, while for Group III animals, the score was significantly lowest (3.125±0.228). Group IV animals were showing arthritis score of (3.50±0.255) which was also significantly different at the level of $P < 0.0001$ (Fig. 1).

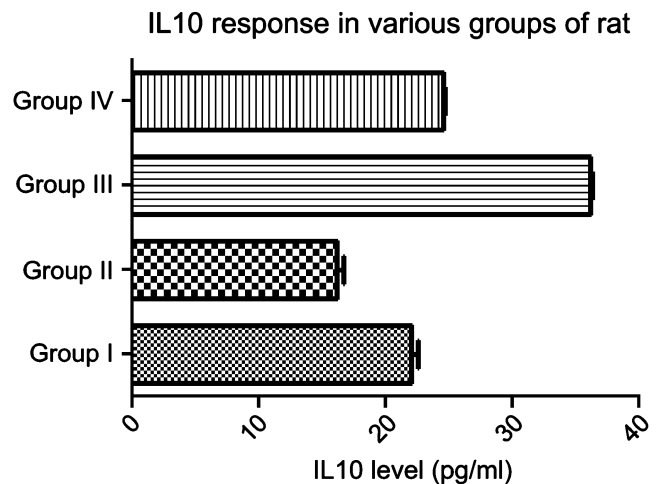## Response of various group of rats
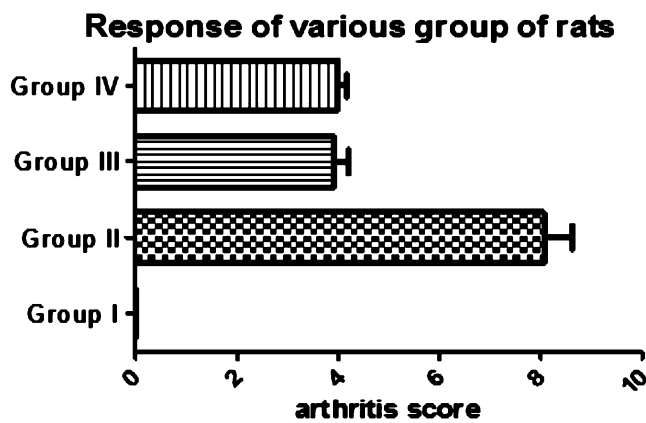
Fig. 1 Arthritis score at the end of the last week of experiment. *Group I* No arthritis induction and no treatment; *Group II* Arthritis induction and distilled water; *Group III* Arthritis induction and orally adminstered *L. casei*; *Group IV* Arthritis induction and standard drug indomethacin administered orally *P<0.0001*. Data are expressed as mean ± standard error of five rats per group

Cytokines Expression

Serum level of IL-6 and TNFα were highest in Group II animals i.e. 68.525±0.415 and 695.873±3.634 pg/ml, respectively. On the contrary, Group III animals were showing significantly lowest concentration of IL-6 (42.477± 0.264 pg/ml) and α-TNF (430.473±1.4777 pg/ml) at *P<* 0.0001. Concentration of IL-10 was 36.185±0.195 pg/ml for Group III animals which was significantly higher than Group II (16.145±0.934 pg/ml). These values were significant at level of *P<0.0001* in comparison with Group I animals (21.99±0.887 pg/ml). Howsoever, Group IV was showing

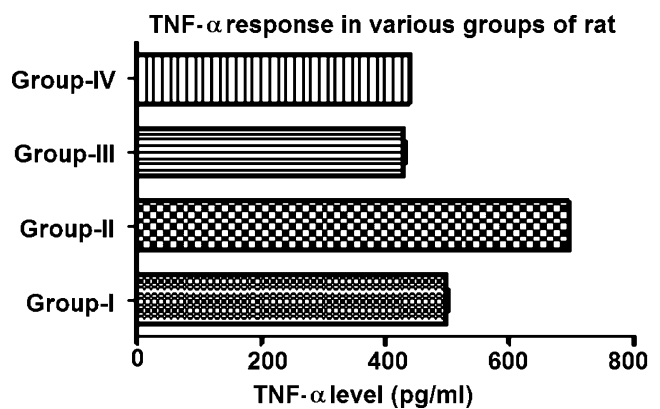## IL10 response in various groups of rat

Fig. 3 The anti-inflammatory cytokines Il-10 (picogram per milliliter) for all the groups. Group III animals show significantly higher value of IL-10, while there is a significantly decreased value for Groups I, II, and IV animals *P<0.0001*. Data are expressed as mean ± standard error of five rats per group

IL-10 concentration of 24.50±0.220 pg/ml, which was not significant at *p<0.0001* (Figs. 2, 3, 4).

Histopathology

The inflammation induced by CIA was associated with cellular infiltration, edema, granuloma formation, and bone destruction on day 29. Group II animals showed significantly increased in inflammatory infiltrate than the Group I animals. The intensity of infiltrate was significantly lower in Groups II and IV animals when compared with their other counterparts. Necrotic and degenerative changes was found in Group II animals while histopthological analysis of Groups III and IV animals showed moderate destruction and less degenerative changes (Fig. 5).

IL6 responce in various groups of rat

Fig. 2 The pro-inflammatory cytokines Il-6 (picogram per milliliter) for all the groups. Group II animals show significantly higher value of IL-6, while there is a significantly decreased value for Group III animals *P<0.0001*. Data are expressed as mean ± standard error of five rats per group

TNF-α response in various groups of rat

Fig. 4 The pro-inflammatory cytokines TNF-α (picogram per milliliter) for all the groups. Group II animals show significantly higher value of IL-6, while there is a significantly decreased value for Group III animals *P<0.0001*. Data are expressed as mean ± standard error of five rats per group

Fig. 5 Histopathological analysis of joint morphology. Photomicrograph (×250) of right hind knee joint after hematoxylin and eosin staining. a Negative control (Group I); b Positive control (Group II); c Group III treated with *L. casei*; d Group IV treated with standard drug Indomethacin. Pictures are representative of distinct five rats per group
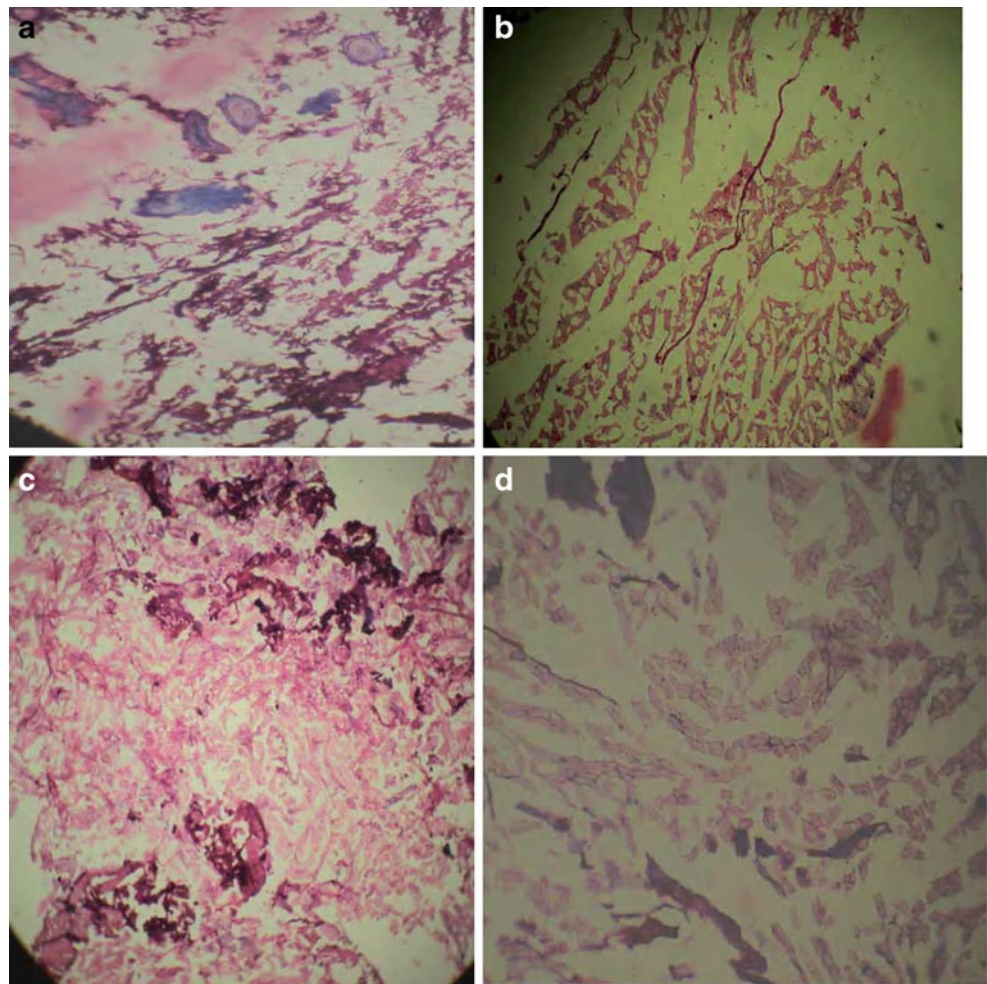
Discussion

Based on the immunomodulatory properties of *L. casei*, it was considered that it might have antiarthritic property. The aim of present study was to assess the therapeutic efficacy of *L. casei* in CIA model of arthritis. Oral administration of *L. casei* in Wistar female rats results in protection against arthritis symptoms, hence providing a therapeutic effect. When compared with other arthritis model, collagen-induced arthritis is a promising model for validation of effectiveness of antiarthritic drugs and various other agents for treatment strategy [27].

Most important parameter for the assessment of arthritis is measuring footpad swelling and visual inspection of symptoms, i.e., arthritis scores [28]. The macroscopic signs of arthritis are redness, swelling, and inability to movement [6]. Oral administration of *L. casei* significantly decreased the arthritis score. Arthritis scores of group I animals were 0 until the fourth week of experiment while Group II animals showed a significant increment in arthritis score from 1st to 28th day. In case of Groups III and IV animals, arthritis score was significantly decreased in comparison to Group I animals. This decreased in the arthritis score of *L. casei*-fed group suggests that its immunosuprressive effect from its anti-inflammatory effect. Some factor or protein is responsible for this property of *L. casei* [23, 29].

In case of autoimmune diseases, pro-inflammatory cytokines plays a major role in the disease progression. An imbalance between pro-inflammatory and anti-inflammatory cytokines activities favors the induction of autoimmunity, chronic inflammation, and thereby joint damage [30].

Though various drugs are being available in the market which manages the suppression of pro-inflammatory cytokines and thereby disease severity. Drugs which block pro-inflammatory cytokines would manage the rheumatoid arthritis up to some extent [31, 32]. These drugs have shown better effects, but these are expensive [33]. Some natural remedy, which can manage the disease, would be a better option. Various studies have being performed on *Lactobacillus* species for their potent immunomodulatory activity [17, 29, 34–37].

In this study, we have estimated pro-inflammatory and anti-inflammatory cytokines. Our results are consistent with the results of other groups [29, 38–40]. *L. casei* significantly decreased the secretion of Pro-inflammatory cyto-

kines (TNF-α and IL-6). *L. casei* may bind to surface molecules thereby suppressing the signaling pathway for IL-6 and TNF-α production [41]. Prostaglandins are the majority of molecules responsible for pain and inflammation in case of arthritis. Cyclooxygenase (COX) is rate limiting enzyme in prostaglandins synthesis. This exists in two forms COX-1 and COX-2. COX-1 is responsible for prostaglandins synthesis while COX-2 is induced by lipopolysaccharides or TNF-α.

Prostaglandins have not only play pivotal role in inflammation, but these diverse molecules also regulate some critical physiological responses. These includes blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone, and immune responses [42]. COX-2 has been found responsible for increased production of prostaglandins. Previous studies on the COX-2 suggest that it has key role in inflammation in both cases of osteoarthritis and rheumatoid arthritis [43, 44]. Various studies reported that Prostaglandins synthesis is down regulated by anti-inflammatory cytokines. IL-4, IL-10, and IL-13 are the important candidates which lower down the synthesis of COX-2 and, hence, prostaglandins synthesis. Treatment with *L. casei* increased the serum IL-10 concentration of Group III animals. Similar results were also reported previously that IL-10 has inhibitory effect on COX-2 synthesis and, hence, the further action of prostaglandin which is a potent inflammatory mediator [41]. They have reported that *L. casei* inactivates the NF-κB and hence synthesis of COX-2 inhibited. Also, the induction of TNF-α upregulated the NF-κB, which in turn is responsible for COX-2 synthesis. IL-10 is a cytokine with potent anti-inflammatory activity. IL-10 has been shown to be a potent macrophage deactivator, blocking the induced synthesis of

TNF-α, IL-1, IL-6, IL-8, and GM-CSF by human monocytes [45]. Similar results of COX-2 inhibition were reported by some authors [46, 47] (Fig. 6).
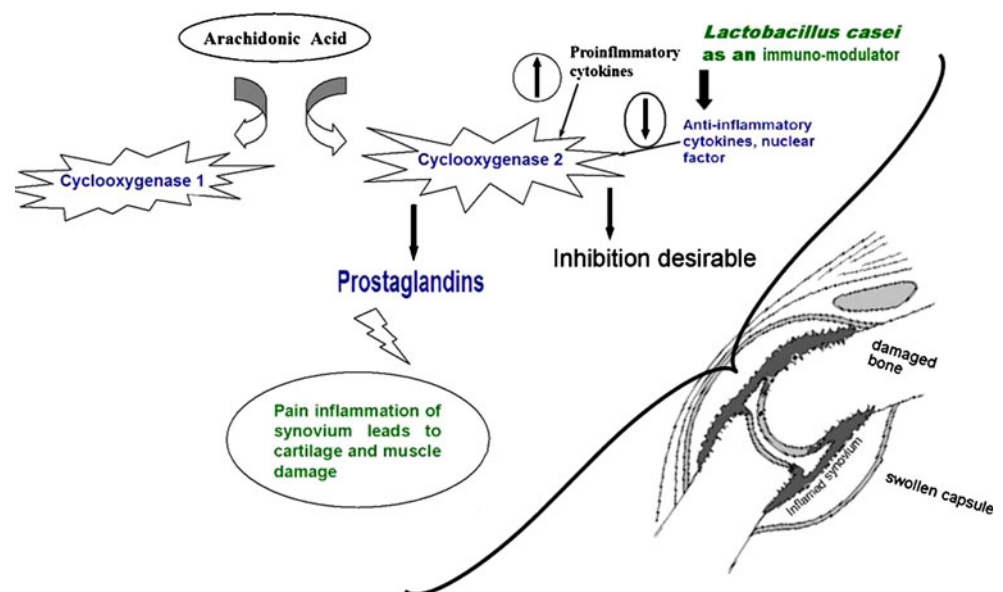
Histopathological analysis corroborated these findings. Showing less infiltration of neutrophils in knee joint are of the group which has orally administered with *L. casei*. Bone erosion and pannus formation are also significantly reduced in bacteria-treated group as compared with standard reference drug and normal and arthritic control. As in case of collagen-induced arthritis, lysosomal enzymes increases, but there may be possibility that *L. casei* treatment inhibited or stabilized the secretion of lysozomal enzymes and hereby destruction due to collagen [48].

In the present study, *L. casei* significantly decreased the inflammation and also the severity of arthritis. The probable mechanism is by prostaglandins inhibition due to anti-inflammatory cytokines raised due to *L. casei* treatment. Prostaglandins plays significant role in different phases of inflammatory reactions.

Conclusion

In summary, this study suggests that *L. casei* exerts an anti-inflammatory effect against CIA model. This event would be the result of COX-2 and NF-κB inactivation which are the potent mediators responsible for inflammatory reactions associated with rheumatoid arthritis. Some more study should be done in this direction for the assessment of cyclooxgenase inhibition by using *Lactobacillus* species either alone or in combination to find out the reason behind the antiarthritic property of *Lactobacillus* species. It is hypothesized that *L. casei* might offer an alternative cartilage and bone protective therapy that is complementary to COX-2 inhibitors.

Fig. 6 Flowchart showing the probable mechanism of action of *L. casei* in blocking the cyclooxygenase-2 which inhibition is desirable for the host. Anti-inflammatory cytokines enhanced due to oral administration of bacteria which in turn downregulate NF-κB and cyclooxygenase-2 and, hence, inflammatory reactions leading bone and muscle damage

Author's contribution VS, RS, PS designed all experiment in this study and drafting of the manuscript. SA and PK handled animals and dosing protocols and other experiments. AK helped in statistical analysis.

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