Lactobacillus acidophilus Protected Organs in Experimental Arthritis by Regulating the Pro-inflammatory Cytokines

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Abstract Arthritis is an inflammatory disease of joints. Exact etiology of the disease is not understood yet; but histopathological examination of vital organs like liver, kidney, ovary and knee joint can anticipate immune mediated damage. In this study, *Lactobacillus acidophilus* was administered orally by both prophylactic and curative protocol in freund's complete adjuvant induced arthritic rats. Indomethacin was used as standard anti-arthritic drug. Histopathology of liver, kidney, ovary and right hind knee joint were done. Cytokine concentrations were determined by using ELISA. Effects shown by *L. acidophilus* were comparable with indomethacin. Histopathological analysis of liver, kidney, ovaries and knee joints of *L. acidophilus* fed groups revealed significantly less damage as compared with other counterparts. *Lactobacillus* treatment has down-regulated pro-inflammatory level and up-regulated anti-inflammatory cytokines level in serum samples. *L.*

acidophilus managed organs damage associated with arthritis. It has significantly down regulated the pro-inflammatory cytokines.

Keywords Freund's complete adjuvant · Probiotics · Neutrophil infiltrations · Bone damage

Abbreviations

FCA	Freund's complete adjuvant
CIA	Collagen induced arthritis
RA	Rheumatoid arthritis
COX	Coxygenase
CPSCEA	Committee for the purpose of control supervision on experiments animals
IL-6	Interleukin-6
IL-10	Interleukin-10
TNF α	Tumor necrosis factor- α

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Introduction

Inflammation is an important protective process maintaining the integrity of organisms against physical, chemical and infective losses. However, autoimmunity implies that the inflammatory response to these conditions leads to the damage of normal tissues also. Rheumatoid arthritis (RA) is an autoimmune disorder of inflammatory joints and other musculoskeletal regions. Progression of disease results in joint destruction, deformity and significant disability. It involves multiple tissues, which includes brain, liver, kidney, reproductive organs and cardiovascular systems etc [1].

Histopathological examinations in RA are reliable evidences for primary manifestations in understanding

immune mediated damage in arthritic foci. Synovial lesions in rheumatoid arthritis show complex histopathological manifestations. These involve several diagnostic hallmarks such as many layered synovial lining tissues associated with a palisading structure of the innermost lining of cells and presence of non foreign body type giant cells, formation of lymphoid follicles and massive accumulation of plasma cells and macrophages. Essential alterations in joint components are traumatic in origin and superficial lesions with loss of elasticity. Degeneration, proliferation and erosion are most evidenced. In more advance stages, the loss of cartilage elasticity is apparent. First alteration that can be appeared is the fraying of cartilage surface layer and lost in its uniformity. In cross section, there is irregularity in surface outline. Mesenchymoid transformation and fibrinoid degeneration are the definite histopathological features of RA. These lesions are specific to synovium in progression stage of RA and their developmental process remains unclear [2, 3]. Many studies have well documented, in spite of knee region other organs are also damaged as consequence of arthritis. These include liver, kidney, spleen, lymph node, bone marrow, reproductive organs and colon [4, 5].

Hyperplasia, increased vascularity, inflammatory cell infiltration, CD4⁺ T cell, synovial cells, fibroblast and release of large number of cytokines with synergistic and antagonistic effects are prominent symptoms of rheumatoid arthritis. Cytokines are the key mediators responsible for bone damage. These include pro-inflammatory cytokines; mainly 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 as anti-inflammatory cytokines that down regulate inflammation [6]. Some cytokines have dual nature like IL-6, IFN- γ and TGF- β i.e. showing both actions [6]. Imbalances between pro-inflammatory and anti-inflammatory cytokines play an important role in initiation of arthritis. Since cytokines were expressed in tissues derived from autoimmune diseases, they were likely to be of fundamental importance in disease pathogenesis and developing a new class of biological therapeutics [7]. It has been found that TNF- α plays a pivotal role in progression of arthritis [8, 9].

Tremendous work in the field of synthetic drugs during recent years has been done; but the side effects of these synthetic drugs could not be avoided. Therefore the efficacy of various plants extracts and other natural compounds have been exploited for their anti-arthritic activity. Previously, we have studied anti-arthritic property of *L. casei* [10, 11]. *L. casei* has protected joint damaged associated with collagen induced arthritis. Inflammatory condition may lead to systemic manifestations and along with joints; other important organs may get damaged due to arthritis.

Taking these points into consideration, present study was planned to evaluate *L. acidophilus* in protecting liver, kidney, ovaries along with knee joints from inflammatory reaction in adjuvant induced arthritis.

Materials and Methods

Drugs and Chemicals

Freund's complete adjuvant (FCA) was purchased from Genei, Bangalore, India. Standard anti-arthritic drug indomethacin was purchased from Recon, Bangalore, India. Cytokine assay kits were purchased from Ray Biotech, Norcross GA and DNA bio, Hyderabad, Andhra Pradesh. All other chemicals used for histopathology were of analytical grade and purchased locally.

Bacterial Culture

Lactobacillus acidophilus (ATCC 314) was purchased from Hi Media, Navi Mumbai, India. Lyophilized culture was streaked over De Mann Rogosa Agar (MRS) at 37 °C in anaerobic condition.

Animals

Fourty two female Wistar rats, 200 g weight were included in this study. Animals were randomly divided into six groups ($n = 7$). The institutional ethical committee guidelines were followed. Animals without any symptoms of wound/lesion in the skin or legs were selected for the study. These were purchased from rat colony at Indian Institute of Toxicology Research (IITR), Lucknow, India and maintained in the animal house at Institute of Biomedical Sciences, Bundelkhand University, Kanpur Road, Jhansi, Uttar Pradesh, India. Animals were housed in large spacious cages. Pellet feed and water were provided ad libitum to all rats for 10 days (acclimatization period). These animals were maintained under laboratory conditions (Temperature 22 ± 2 °C, Relative humidity 60–70 % and 12–12 h light–dark cycle).

Induction of Arthritis

Freund's complete adjuvant arthritis was induced according to the method described by Pearson, 1956 [12]. 100 μ l of Freund's complete adjuvant (FCA) containing 10 mg/ml of heat killed *Mycobacterium tuberculosis* was injected intradermally into the right hind paw of each rat using sterile needle (Romo JetTM) needle.

Effects of *L. acidophilus* Species on Freund's Complete Adjuvant (FCA) Induced Arthritis

The down regulation or up-regulation of various cytokines in the employed animals could be affected due to the inherent properties of cytokines themselves. Therefore to justify the effect of cytokines in animals due to *L. acidophilus* induced response, all the animals were acclimatized in same environment and were grouped ($n = 7$) randomly to receive various treatments as follows:

- Group (I) Negative control i.e. No FCA and no treatment
- Group (II) Positive control i.e. FCA and 0.5 ml of distilled water
- Group (III) Prophylactic *L. acidophilus* group i.e. 2×10^8 CFU/ml of *L. acidophilus* suspended in 0.5 ml of distilled water before the injection of FCA
- Group (IV) Prophylactic standard drug group i.e. indomethacin before the injection of FCA @ 10 mg/kg of body weight
- Group (V) Curative *L. acidophilus* group i.e. 2×10^8 CFU/ml of *L. acidophilus* suspended in 0.5 ml of distilled water after the induction of arthritis
- Group (VI) Curative standard drug group i.e. indomethacin after the induction of arthritis @ 10 mg/kg of body weight

In curative groups, oral treatments were started after the induction of arthritis i.e. on day 8th after the induction of arthritis symptoms, while in prophylactic groups; oral treatment were started before the induction of arthritis i.e. on day 1st, just before the injection of Freund's complete adjuvant (FCA). Oral administrations of *L. acidophilus* and standard drug were started on day 1st and 8th and continued up to 21st day for prophylactic and curative groups respectively. Dose of *L. acidophilus* and standard drug were selected according to previous literature and preliminary studies performed in our laboratory [10, 11, 13]. Dose of *L. acidophilus* was prepared by using serial dilution method. Simply, 0.1 ml from 10^{-7} dilution was used for spreading over MRS. Suspension obtained by mixing 2 colonies from the plate of 10^{-7} th dilution in 0.5 ml will be equivalent to 2×10^8 CFU/ml. Both prophylactic and curative protocols were performed. On 22nd day, all the animals were sacrificed by following the guidelines of institutional ethical committee. Blood was collected for serum isolation to perform cytokines ELISA assay. Liver, kidney, ovary and right hind knee joints were amputated from all the animals. These were dissected and kept in 10 %

formalin for 24 h for histopathology. Tissues were washed over night in running water to remove fixative. Dehydration was carried out to remove water content present in tissues by transferring them to a series of gradually increasing percentages of alcohol. The knee joint regions were decalcified by nitric acid method and fixed in 70 % alcohol. These materials were then cleared in xylol and embedded in wax (melting point 52 °C). Sections were cut at 5 μ m thicknesses and stained in haematoxylin and eosin [14].

Cytokines Assay for Pro-inflammatory (IL-6 and TNF- α) and Anti-inflammatory (IL-10) Cytokines

IL-6, TNF- α (pro-inflammatory cytokines) and IL-10 (anti-inflammatory cytokines) in picogram per millilitre (pg/ml) were estimated with the help of ELISA Reader (Lisa Plus, Germany). Serum samples were used for estimation of IL-6, TNF- α and IL-10. Assays were performed according to the manufacturer's recommendations.

Statistical Analysis

The results of cytokines are expressed as a Mean $\pm$ SEM of seven 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. acidophilus has shown protective effects against the FCA induced arthritis in female Wistar rats

Down-Regulation of Pro-inflammatory (IL-6 and TNF- α) and Up-regulation of Anti-inflammatory (IL-10) Cytokines

Figure 1 is showing the concentration of cytokines in serum. Serum level of IL-6 (60.085 ± 0.04 pg/ml) and TNF- α (669.46 ± 0.18 pg/ml) were highest and IL-10 (15.9 ± 0.13 pg/ml) was lowest in case of group II animals at $P < 0.0001$ when compared with group I, III, IV and V animals. On the contrary, group I animals were showing lowest values of IL-6 (48 ± 0.03 pg/ml) and TNF- α (497.12 ± 0.05 pg/ml) and highest value of IL-10 (21.99 ± 0.04 pg/ml) at $P < 0.0001$ when compared to all other groups. On the other way, TNF- α was lowest (444.99 ± 0.04 pg/ml) in case of group III animal while group IV, V and VI were showing significantly lower values of TNF- α i.e. 453.53 ± 2.18 , 463.69 ± 0.23 and 469.25 ± 0.47 pg/ml respectively. IL-6 concentration for group IV, group V and group VI animals were 48.20 ± 0.20 , 52.00 ± 0.09 and 53.97 ± 0.05 pg/ml respectively. *L.*

Fig. 1 Figure showing the concentration of cytokines IL-6, TNF- α and IL-10 (pg/ml) in serum of FCA induced arthritic Wistar rats. Results are expressed as the mean of seven rats per group. $P < 0.0001$: group II versus all other groups (normal and treated groups). **a** Negative control (group I); **b** Positive control (group II); **c** Prophylactic *L. acidophilus* (group III); **d** Prophylactic drug (group IV); **e** Curative *L. acidophilus* (group V); **f** Curative Standard drug (group VI)

acidophilus treatment significantly decreased the IL-6 and TNF- α concentration while it has increased the IL-10 concentration at $P < 0.0001$. Standard drug in both protocols was showing comparable results (Fig. 1).

Histopathology of Various Organs

Knee Joint

Histopathological study revealed that along with the knee joint, various other important organs were also damaged with FCA arthritis. Knee joint of group II animal was showing pannus formation along with the infiltration of inflammatory cells, while in other groups, damaged was less as compared to group II. Group I was showing normal histopathology having no damage at all. Results of prophylactic treatment of group III were comparable with that of group IV animals (Fig. 2).

Liver

Normal distribution of hepatocytes was observed in all the groups except group II animals. Necrosis was observed in group II animals. Tissue macrophages were also less in group III and V animals. Vacuoles like spaces which were empty was tremendous in case of group II animals; while these were less observed in III, IV, V and VI groups animals. Mononuclear infiltrates were also nominal in case on group III and V animals when compared with all other groups (Fig. 3).

Kidney

Normal structure of the cortex and medulla was observed in the kidney of group I animals while degenerative changes were observed in group II animals. Group II animals were showing empty nucleated cells these were less in group III, IV, V and VI animals. Here in this case prophylactic treated groups i.e. group III and group IV were less damaged in comparison with group V and group VI (i.e. curative groups) (Fig. 4).

Ovaries

Histological evidence suggest that there were major changes occurred in ovaries of group II. Lysed follicular cells were observed in group II animals and these were very less as compared to *L. acidophilus* treated groups. Drug also disrupted the normal histology of ovary in group III and VI animals. Lysed ova were apparent in group II, III and VI ovum. Group I was showing normal histopathology of ovary (Fig. 5).

Discussion

This study clearly suggests that, along with knee joints, some other important organs are also affected by arthritis as evaluated in adjuvant induced arthritis experimental

Fig. 2 Histopathological analysis of knee joints of FCA induced arthritis Wistar rats. Paraffin sections of knee joints were stained with hematoxylin and eosin (H&E). Pictures are representative of seven distinct rats per group. **a** Negative control (group I); **b** Positive control (group II); **c** Prophylactic *L. acidophilus* (group III); **d** Prophylactic drug (group IV); **e** Curative *L. acidophilus* (group V); **f** Curative standard drug (group VI). Arrow showing the damaged portions which are less in *Lactobacillus* treated group in comparison to Group II (**b**)

Fig. 3 Histopathological analysis of liver of FCA induced arthritis Wistar rats. Paraffin sections of rat liver were stained with hematoxylin and eosin (H&E). Pictures are representative of seven distinct rats per group. **a** Negative control (group I); **b** Positive control (group II); **c** Prophylactic *L. acidophilus* (group III); **d** Prophylactic drug (group IV); **e** Curative *L. acidophilus* (group V); **f** Curative standard drug (group VI). Arrows are showing the necrotic areas and lacunae; in group II (**b**) and group IV (**d**) are prominent when compared to other counterparts

model. *L. acidophilus* prevents joint damaged as well as protected some important organs like liver, kidney and ovaries as suggested by histopathological analysis.

Cytokines are the main culprit responsible for the inflammatory damage caused due to arthritis. TNF- α and IL-1 β play a significant role in the pathogenesis of adjuvant arthritis, but other mediators such as IL-6, IL-15, IL-18 and leukotriene B4 are also implicated in this process, with

some of them playing a key role in neutrophil recruitment during immune inflammation. TNF- α , which is involved in inflammation, differentiation and proliferation of T and B cells, and bone resorption, is the primary agent in the inflammatory process [15], whereas IL-1 β is responsible for the destruction of cartilages and bones [16]. Blocking TNF- α suppresses the inflammation and ameliorates cartilage destruction.

Fig. 4 Histopathological analysis of kidney of FCA induced arthritis Wistar rats. Paraffin sections of rat kidneys were stained with hematoxylin and eosin (H&E). Pictures are representative of seven distinct rats per group. **a** Negative control (group I); **b** Positive control (group II); **c** Prophylactic *L. acidophilus* (group III); **d** Prophylactic drug (group IV); **e** Curative *L. acidophilus* (group V); **f** Curative Standard drug (group VI). Arrow showing damaged area which is more in non treated arthritic group (**b**) as compared to other counterparts

Fig. 5 Histopathological analysis of ovaries of FCA induced arthritis Wistar rats. Paraffin sections of rat ovaries were stained with hematoxylin and eosin (H&E). Pictures are representative of seven distinct rats per group. **a** Negative control (group I); **b** Positive control (group II); **c** Prophylactic *L. acidophilus* (group III); **d** Prophylactic drug (group IV); **e** Curative *L. acidophilus* (group V); **f** Curative standard drug (group VI). Arrow is showing the damaged section in ovary; ovary is deshaped in **b** (non treated arthritic rats) and **d** (Curative drug group)

IL-6 is also an important cytokines which is responsible for joint destruction. Some studies have established that IL-6 has an important role in leukocyte recruitment, apoptosis and T cell activation [17]. Present study has shown that *L. acidophilus* inhibits TNF- α and IL-6. TNF- α play critical role in both acute and chronic inflammation. TNF- α facilitates inflammatory cell infiltration by promoting adhesion of neutrophils and lymphocytes to endothelial cells.

Additionally, TNF- α stimulates neutrophils to transcribe and release cytokines and chemokines biosynthesis [18]. Inhibition of TNF- α release can reduce the severity of inflammation. TNF- α is shown to accelerate both angiogenesis and matrix degradation by induction of vascular matrix metalloproteinase. Anti-inflammatory cytokines like IL-4 and IL-10 prevent the inflammatory damaged associated with arthritis [19]. *L. acidophilus* induced suppression of IL-

6 and TNF- α and increased the production of IL-10 represents the two of the major mechanism that could afford protection against adjuvant arthritis. Results of the cytokines assay in Fig. 1 clearly suggests that *L. acidophilus* significantly increased the IL-10 not only this it decreased the IL-6 and TNF- α at $P < 0.0001$. In previous study also, *L. casei* was showing the same result in collagen induced arthritis animal model [10, 11]. *L. acidophilus* might be inhibiting the inflammatory pathways leads to the synthesis of pro-inflammatory cytokines i.e. IL-6 and TNF- α . IL-10 is an anti-inflammatory cytokines which inhibits coxygenase-2 [7]. Hence the production of prostaglandins which causes inflammation and pain [6, 10].

Over the centuries, a number of medicinal plants have been exploited for the treatment of the disorders associated with inflammatory conditions or for to control inflammatory aspects of diseases. Present study revealed that *L. acidophilus* minimized the damaged of crucial organs in comparison to standard drug. Along with joints, others organs are also affected by arthritis. The pathological changes were well documented by histopathological analysis of knee joint, liver, kidneys and ovaries. Arthritis and other rheumatic diseases mainly affect joints [20]. Liver damage is common in various diseases along with rheumatic diseases. Inflammation in an organ may affect the overall homeostasis of the body. Studies have suggested that during inflammatory diseases, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) increases [21], [22]. These enzymes assays are good indices of liver damage. Their concentrations were increased in case of group II, III and VI animals (results have not shown here). Due to arthritis, walls of liver become leaky therefore enzyme concentration in arthritic condition increases [21, 22]. Histopathological analysis of liver corroborated these findings. *L. acidophilus* treatment minimized the damage associated with FCA induced arthritis. *L. acidophilus* as curative and protective treatment maintained some other biochemical parameters rheumatoid factor, ceruloplasmin, serum calcium and phosphorous level (unpublished data) and hence protected the experimental animals from the damage induced by freund's complete adjuvant (FCA).

Prophylactic and curative administration of *L. acidophilus* decreased the inflammatory reaction mediated with arthritis and hence damage. Amdekar et al. [10], 2011 have shown anti-inflammatory activity of *L. casei* in case of collagen induced arthritis. Also *L. acidophilus* treatment reduces the damage associated with arthritis. Previous studies suggested that *L. casei* and *Lactobacillus* GG have shown antiarthritic properties in collagen induced arthritis model [10, 11, 13].

In this study, nominal damage were observed in *L. acidophilus* treated groups to kidney as reported by histopathological analysis and it has decreased the paw

thickness significantly (results have not shown here). Kidney is the target of NSAID's and this also inhibits Coxygenase (COX) activity. Renal functions are COX dependent. Also prostaglandins are active in regulating vascular tone, salt and water homeostasis by modulating glomerular hemodynamics and distal nephron functions. Therefore NSAID's if taken may lead to renal failure [23].

L. acidophilus treated groups were showing less infiltration of neutrophils in knee joint. Bone erosion and pannus formation was also significantly reduced in group III and V as compared with group I, II, IV and VI. *L. acidophilus* protected the ovary from the adjuvant induced arthritis. Ovaries of group III and V were near to normal as compared to group I animals.

Thus, present study showed that *L. acidophilus* protects joints as well as important organs damage against FCA induced arthritis. It can be concluded that *L. acidophilus* can be used as supplement and future drug target against arthritis. However further studies are warranted to observe safety, efficacy, potency and long term use of *L. acidophilus* against arthritis.

Conflict of interest There are nonfinancial competing interests (political, personal, religious, ideological, academic, intellectual, commercial, or any other) to declare in relation to this manuscript.

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