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Antiinflammatory, Antimicrobial And Immunomodulatory Properties Of Lactobacillus Casei Against Enteropathogenic Escherichia Coli

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No interest

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Abstract

Purpose: Immunomodulatory as well as *in vivo* antimicrobial properties of *Lactobacillus casei* were investigated. *L. casei* was administered orally in Wistar rats.

Methods: Enteropathogenic *Escherichia coli* were used. Both live and dead culture of *L. casei* were used. $2x10^{8}$ CFU/ml of each culture was given to respective group daily from day 1^{st} upto 28^{th} days.

Results: Beneficial effects such as increased adhesive property shown by *L. casei*, increased hemoglobin content and RBC and decreased WBC counts were observed in LL (live *L. casei*) and LD (dead *L. casei*) group when compared with EC group (Administerd *E. coli*). Serum biomarkers like Serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacteic transaminase (SGOT) and total proteins (TP) were also decreased in LL and LD group. Moreover, *L. casei* in both live and dead form has decreased the production of pro-inflammatory cytokine (IL-6 and α TNF) while it has increased anti-inflammatory cytokine (IL-10). Histopathological analysis also confirmed the protective effect of *L. casei* against enteropathogenic *E. coli.*

Conclusion: Present study revealed that *L. casei* consumption may provide gastrointestinal tract immunity against enteropathogenic *E. coli*

Key words: *Lactobacillus casei***,** Enteropathogenic *Escherichia coli,* IL-6, IL-10, TNF α

Running Title: *Lactobacillus casei* modulate the expression of anti-inflammatory cytokines in gastrointestinal tract.

Introduction

Enteropathogens are the sole source of agents responsible for diarrhoeal sickness. These are the common cause of morbidity and even mortality. An increasing wide variety of pathogens are recognized as cause of serious illness, such as *Escherichia coli*, *Campylobacter jejuni*, *Clostridium difficile, Salmonella, Shigella* and *Giardia*. *E. coli* are responsible for 200 million cases of diarrhoea each year, and death of 380,000 people mainly children in developing countries **[1].** *E. coli* is the normal microflora of human gastrointestinal tract **[2].** Establishment of normal bacterial flora is important to prevent growth of potential pathogens as well as, contribute to the adequate maturation of the immune system, since imbalances in the normal microflora composition have been consistently associated with several disease consequences. Intestinal mucosa also provides protective host defense against the food antigens and microorganisms in the gut lumen **[3].** Intestinal mucosa also provides protective host defense against the constant presence of food antigens and microorganisms in the gut lumen. Various therapeutic treatments have been used through out of the world. Administration of probiotics, prebiotics or their association (symbiotics) has as a new and interesting strategy to modulate intestinal microbiota **[4].** Consumption of *Lactobacillus, Bifidobacterium* and *Enterococcus* etc improves intestinal health **[5-7].**

Various studies have been performed for the immunomodulating properties of *Lactobacillus* against various pathogens like *E. coli, Salmonella, Listeria* **[8 -15].** *Lactobacillus* species has been used previously for its antagonistic activity in *in vivo* and *in vitro*.

The present study aimed at understanding the protective properties *L. casei* both in live and dead form against enteropathogenic *E. coli* used to infect Wistar rats.

Materials and Methods

Reagents

All the chemicals used in this study were of analytical grade. These were purchased from Peekay Chemicals & Glasswares and Scientific Systems & Chemicals, Bhopal, Madhya Pradesh. Cytokines assay kits were purchased from Ray Biotech, Norcross G A and DNA bio, Hyderabad, Andhra Pradesh India. Kits of Liver functional test were purchased from Acurex Private

Ltd. Thane; India and Aggappe Diagnostics, Kerela; India.

Cultures:

Lyophilized culture *L. casei* ATCC 334 was obtained from Hi Media (Navi Mumbai, India). Culture of Enteropathogenic *E coli* was obtained culture collection from Department of Microbiology, Barkatullah University, Bhopal. Culture of *E. coli* was streaked on Nutrient Agar and *L. casei* on MRS agar.

Animals

60 female Wistar rats, two months old (190-200 gm) were purchased from Institute of Industrial Toxicological Research Centre, Luckhnow, India. Animals were housed in Institute of Biomedical Sciences, Bundelkhand University, Jhansi, Uttar Pradesh. Stainless steel cages (34 x 47 x 18 cm) with soft wood shavings were used as bedding. Wistar rats were fed with normal commercial pellet diet (Amrut feed Ltd. India) and water *ad libitum* and maintained under laboratory conditions (Temperature 20-22°C, Relative humidity 60-70%, and 12 hrs light-dark cycles). All procedures and techniques used in this study were in accordance CPCSEA guidelines and were approved by the Departmental Ethics committee. Animals were randomly divided into five groups (n=12). Groups were designated as:

1. **LL group** : Live culture of *L. casei* in 1 ml distilled water
2. **LD group** : Dead culture *L. casei* in 1 ml distilled water
3. **EC group** : Live culture of *E. coli* in 1 ml distilled wa
4. **LE group** : Live culture of both *L. cei* and *E. coli* in 1 ml distilled water
5. **CON group** : Control group fed on 1ml distilled water

Experimental schedule

Dose $2x10^8$ CFU /ml were suspended in 1ml distilled water and orally administered with syringe cannula to the prescribed group according to the protocol from day 1st to 28th day. For dead culture of *L. casei*, the method given by Bhatia K and Rani U **[16]** was used with some modification. $2x\ 10^8$ CFU/ml was taken and suspended in 1ml distilled water and kept in water bath for 60 min at 90 °C. Control group was administered with 1 ml distilled water. These treatments were given up to day 28 th. On 29 th day animals were euthanized with the help of Di ethyl ether.

Sample collection and processing

Blood samples were drawn. Blood was mixed with EDTA for the hematological parameters. Serum was obtained by centrifuging the blood sample at 3500 RPM for 30 minutes at 4°C. Serum were isolated from blood and stored at -80°C for further investigations. Spleen and thymus were also taken. Piece of small intestine (2 cm long) were taken for histopathological analysis.

Body weight

Weight of all the animals were checked on day 0, 7th, 14 th, 21 th and 28th day. Spleen and Thymus of all the wistar rats were isolated and weighed on an electronic balance.

Hematological parameters

At 29 **th blood sample was collected. Hematological parameters were analyzed. Red blood cell count (RBC count), White blood cell count (WBC Count) and Hemoglobin content (Hb %) were observed.**

Cytokines assay

For the estimation of cytokines, serum was separated from blood. Estimation of IL-6 and TNF-α (Pro-inflammatory cytokines) and IL-10 (Anti-inflammatory cytokines) was done with the ELISA Reader (Lisa Plus). IL-6 and TNF-α (Ray Bio ®) and IL-10 (DNA BIO) ELISA kits were used. Assays were performed according to the instructions of manufacturer.

Bacterial count in feces of Wistar rats

Freshly voided fecal materials were collected at day 0 and 4 from each rat (1 gm/rat) of LE group. This was done to check the growth of *L. casei* in presence of *E. coli*. Faeces were homogenized in normal saline and serially diluted. 0.1 ml fecal sample was spread on MRS agar for the enumeration of *L. casei* and on MacConkey agar for the enumeration of *E. coli*. Plates were incubated at 37 ? C for 24 hours and colony forming units were on recorded **[15]**.

Biochemical test

Some of major serum biochemical markers were assayed. Enzyme assays for liver functioning *i,e* Serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacteic transaminase (SGOT) and Total protein (TP) were performed with the help of Spectrophotometer (UV-Vi Shimadzu, Japan, 1601). Enzyme assays were done according to the instruction of manufacturer.

Histopatological analysis

Small intestines of wistar rats were removed. It was preserved in 10% formalin, dehydrated in increasing percentages of ethanol. These tissues were than Cleared in xylene for 2 hours for embedding. The embedded organs were sectioned using microtome and stained with Haematoxylin-eosin **[17].**

Statistical analysis

The results were presented as a Mean ± SEM of twelve rats per group. The significance of the difference was evaluated by one-way ANOVA followed by Dunnett's multiple comparisons test. Data were considered statistically significant if P<0.0001.

Observation and Results

Effect of cultures on the diarrhoeal symptoms

Rats of CON, LL and LD group were not showing any symptoms of diaarhoea till last day of experiment. Symptoms of diarrhoea were prominent in EC group. These animals were weak and lean in comparison to other treated animal group. LE group was showing mild symptoms of diarrhea but severities of symptoms were mild in comparison to the EC group animals. Animals of CON, LL and LD groups were healthy till last date of experiment.

Effect of Cultures on Body weight

In the first two weeks, the increment in body weight was almost similar in all the rats of all groups. However after two weeks, weight was gained by LL (211 ± 3.91 gm) and LD (203 ± 9.20 gm). It was significantly higher in LD in comparison to control group at P<0.024. It was found that a loss in body weight was observed in EC (200 ± 4.08 gm to 183 ± 4.76 gm) Group as compared to CON (205 ± 4.08 gm to 206.75 ± 4.57 gm) at the end of second week. There was a decreased in weight of LE group from 1st week (208.75 ± 2.16 gm) upto to end of 2nd week (201.75 ± 4.99 gm). There was a significant increased in the body weight of LD groups as compared to the control group. At the end of 4th week body weight of LD group animals was (216 ±8.42 gm) which was statistically significant at the level of P< 0.0024**. [Fig 1]**

Bacterial count in feces of Lacto-Ecoli (LE) group

The ability of the isolates to protect the Gastrointestinal tract against pathogens can be confirmed by monitoring the count of enterobacteria, especially *E. coli* and beneficial bacteria *L. casei* in rat faeces. On day zero the colony count of Enterobacteria was (42 ± 2.54 x 10^7 CFU/gm) and on day 4 it was (28 ± 2.86 x 10^7 CFU/gm), which is 66.66% decreased. However in case of *L. casei* colony count was increased from (37 ± 3.67 x 10^7 CFU/gm) to (51 ± 4.87 x 10^7 CFU/gm). It has increased to 72.11%.

Weight of spleen and thymus

Spleen and thymus were collected from all the animals on 29th day. Weights of spleen were significantly reduced in LL (0.821 ± 0.008 gm) and LD (0.831 ± 0.015 gm) group as compared to EC (1.015± 0.015 gm) group which was significantly higher. However, CON group was showing a small increased weight of spleen (0.921 ± 0.015 gm). Weight of spleen was highest in comparison to other counterparts. Similar results were also obtained in case of thymus. Weight was found to be highest in EC (0.676 ± 0.018 gm) group and lowest in CON (0.311± 0.008 gm) group. While LL and LD have (0.335 ± 0.0057 gm) and (0.373 ± 0.005gm) respectively. LE group has shown a medium value of spleen and thymus (1.013 ± 0.00721 gm) and (0.550 ± 0.019gm) respectively **[Fig 1]**

Hematological parameters

There was a significant increase in WBC count which was administered with *E. coli* orally (10.45 ± 0.24) x10^3 /mm^3. In LL (7.63 ± 0.053) x10^3/mm^3 and LD (8.25 ± 0.127) x10^3/mm^3, WBC count was significantly lower than CON (9.73 ± 0.066) x10^3/mm^3 group. WBC count of LE group was (7.56 ± 0.175) x10^3/mm^3 and it is nonsignificant as compared to CON. RBC count was significantly higher in LL (8.39 ± 0.069) x10^6/mm^3 and LD (8.50 ± 0.082) x10^6/mm^3 .However, RBC count of LE was (7.56 ± 0.335) x10^6/mm^3 but it was non significant. RBC count of EC group significantly lowest and it was (5.84 ± 0.061) x10^6/mm^3. Similarly hemoglobin content was significantly higher in LL (15.23 ± 0.89 gm/dl), LD (14.77 ± 0.49 gm/dl) and LE (10.36 ± 0.19 gm/dl) group and significantly lower in EC (9.38 ± 4.62 gm/dl) group as compared to CON group. **[Fig 1]**

Biochemical parameters

Serum Glutamic Pyruvic Transaminase (SGPT or ALT) and **Serum Glutamic Oxaloacetic Transaminase (SGOT or AST)** levels were assayed. These are enzymes of the liver and their presence in the blood stream indicates that the walls of the liver have been compromised and that these enzymes are leaking into the blood stream. Value of SGPT were significantly highest in case of EC (143 ± 1.09 IU/L) group animals. SGOT levels were significantly different in case of LD and LE group and these were found to be (103.28 ± 1.06 IU/L) and (123.94 ± 0.90 IU/L) respectively. SGPT level was significantly different in case of LL group and it was (28.25 ± 0.84 IU/L) as compared to CON group. Concentration of Total Protein was significantly different in case of EC group (4.19 ± 2.06 gm %) and LL (5.55 ± 0.06 gm %) at the level of P< 0.0001. **[Fig 2]**

Cytokines assay

Serum was used for the assay of Cytokines IL-6, IL-10 and TNF α for all respective groups. It was found that for LL, LD, EC group, IL-6 values and TNF α were significantly different as compared to CON group. However in case of IL-10, only LL group was showing significantly different values as compared with the CON group. For all other groups concentration of IL-10 was not significantly different at the level of P<0.0001 **[Fig 2]**

Histopathogical analysis of small intestine

Histopathology of small intestine was performed. Villus pattern of intestine was intact in CON group. In case of LL and LE group villus pattern was normal in comparison to EC group. There was necrosis found in small intestine of EC group when compared with LE group. Villus pattern were well preserved in case of CON, LL and LE group. Vacuolated cell were found in case of EC group while in case of LE group very less vacuolated cell were observed. **[Fig 3]**

Discussion and conclusion

Present study was undertaken to evaluate *L. casei* as an immunomodulator and a protective agent against enteropathogenic *E. coli*. *In vitro* and *in vivo* studies have shown that *Lactobacillus* sp. protect against both animal and human model **[18].** There are 300-500 different species of bacteria in the intestinal tract, and their location remains unclear at present. Since probiotics isolated from intestinal tract have an excellent permanent planting ability **[19].** In this study it was found that Colony forming unit of *L. casei* were higher than enterobacteria. This suggests that *L. casei* has a good planting activity **[20].** Mechanism of protection is unclear however several factors are responsible for the protections against enteropathogens. Enzymes (α Glucosidase, β Glucosidase, Nitroreductase), Acids (Lactic acid, Hydrogen peroxide, Acetoin, Formic acid), Adhesion property (α Enolase) and Bacteriocins as being responsible for their ability to inhibit other bacteria **[21].** Studies suggested that it is a vaccine candidate against many pathogens **[22]**.

We have used dead culture of*L. casei*. It has also protected the rats. Feeding rat with inactive dead cells of *L. casei* prevented rats by ameliorating the production of IL-6 and α-TNF (Both are Pro-inflammatory cytokines) while increasing the serum level of IL-10 (Anti inflammatory cytokine). Various similar studies have been performed for the production of both pro-inflammatory and anti-inflammatory cytokines by live *Lactobacillus* species **[23]**. This is suggestive of the fact that immunomodulatory and protective property does not depend on the viability. Thus, we assume that a heat stable bacteria cell wall constituent, cell membrane component or intercellular component is responsible for this effect. This may also possible that some factor secreted by *L. casei* is responsible for this immunomodulation and protection.

We have performed hematology to access the effect of *L. casei* on different parameters like RBC count, WBC count and Hemoglobin concentration. It was found that RBC count and Hemoglobin concentration has increased in case of LL and LE groups however, these groups were having lowest WBC count when compared with CON group **[24].** Ability of *L. casei* to protect GIT against pathogen can be confirmed by monitoring the total *L. casei* and enterobacteria count. It was found that *L. casei* count was higher in LE group on 4th day when compared the fecal count of LE group on day 0. Our results were consistent with the results reported by **Mitsuoka [25] and Oyetayo V O et al [15].**

Serum biochemical markers of liver functions i.e SGOT (AST), SGPT (ALT) and Total protein (TP) and Alanine aminotransferase (ALT) is mainly found in the liver and is regarded as more specific enzyme for detecting liver cell damage. Highest values of ALT found in EC group. This shows the toxicological damage to liver in EC group animals challenged with *E. coli.* The lower ALT values in LL, LD and LE groups compared to the control indicate liver function improvement which is brought about by the *L. casei*. Hepatocytes play a major role in absorbing and metabolising many toxic chemicals **[26].** Serum AST level increases in bacterial infections, malaria, pneumonia, pulmonary infarcts and heart and muscle tumors. In case of EC group concentrations of AST were highest in comparison to LL, LD and EC groups. This may due to tissue injury which is responsible for the higher value of serum AST in case of EC groups. Administration of *L. casei* in both live and dead form brought a downfall in serum SGPT and SGOT level.

We have analyzed the anti- and pro inflammatory responses by feeding *L. casei* and *E. coli* alone and in combination. Bacterial pathogens and their products trigger the inflammatory response by transcriptional activation of inflammatory genes, leading to an excessive and uncontrolled release of a large number of inflammatory mediators, including cytokines, chemokines, adhesion molecules, reactive oxygen and nitrogen species **[4].** Several biochemical and immunological alterations are responsible in state of infection. TNF-α and some other cytokines like IFN-g, IL-2, IL-4, IL-5 and IL-6 is also considered one of the most important mediators in the pathogenesis of

sepsis and septic shock. This leads to vasodialation, impaired coagulation and fibrinolysis. Oral administartion of *L. casei* in both live and dead form showed a significant decrease in IL-6 and TNF α when compared with untreated control rats **(Table 5)**. As expected, LPS treated rat i.e. EC group and LE group showed an alteration of the cytokine profile production including increased levels of TNF- α, IL-2, IL-5 and IL-6 and a reduced expression of the regulatory cytokine IL-10. This increase leads to multiple organ dysfunctions. Increase in spleen size and Liver functional tests **(Table 3 and Table 5)** were also evidenced of organ damage. *L. casei* restored the regulatory cytokine IL-10. Administration of *L. casei* downregulate pro inflammatory cytokine and induce the production of anti inflammatory cytokines. Similar results were observed by **Rochat T et al [27].** Results of histopathological analysis also revealed that the protective property of *L. casei* alone as well as in presence of *E. coli*. The protection and normal villus pattern of gastrointestinal tract was observed in rats treated with *L. casei* in live and dead form. There was partial protection in case of rats treated with *L. casei* and *E. coli.* While the group treated with *E coli* only was showing destruction in villus pattern. Similar results were also reported by **Oyetayo V O et al [4] and Chen LL et al [28].**

In conclusion, our result showed an antiinflammatory, Immunomodulatory and protective property of *L. casei* against enteropathogenic *E. coli*. Lower serum AST and ALT suggests better liver functions. The ability of *L. casei* to antagonize the enterobacteria, protection of gastrointestinal tract and as an immune enhancer makes it a potential strain against enteropathogen clinical diagnostics.

Conflict of interest

There is no conflict of interest to declare by any of the author's

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Author's contribution

VS, RS and PS conceived the design of this study and coordinated all phases of the preparation of the manuscript. SA, DKG and LKD performed experiment. AK participated in statistical analysis. All authors read and approved the final Manuscript

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Illustrations

Illustration 1

Fig-1

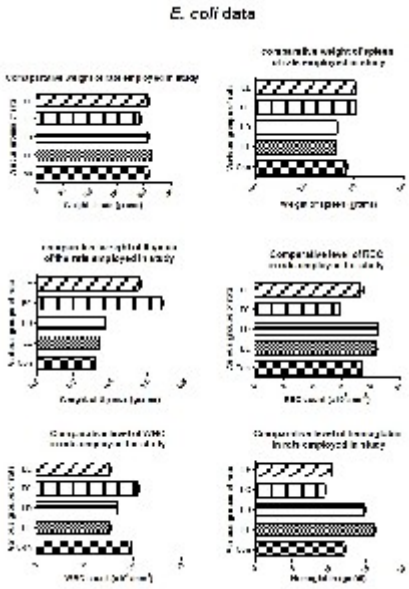


Illustration 2

Fig-2

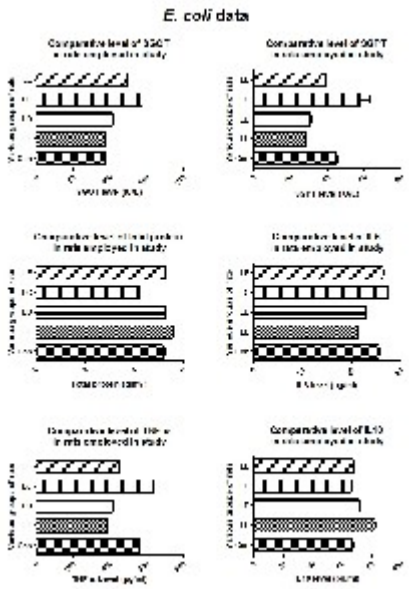
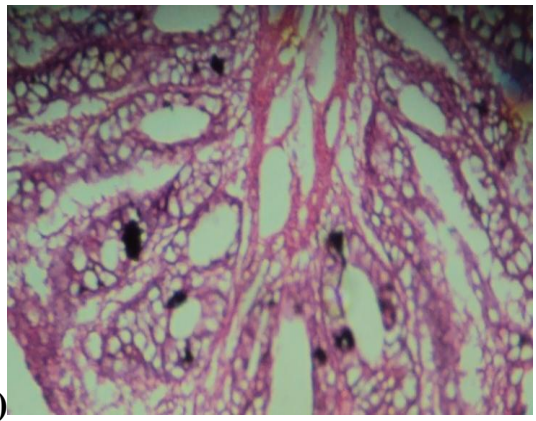


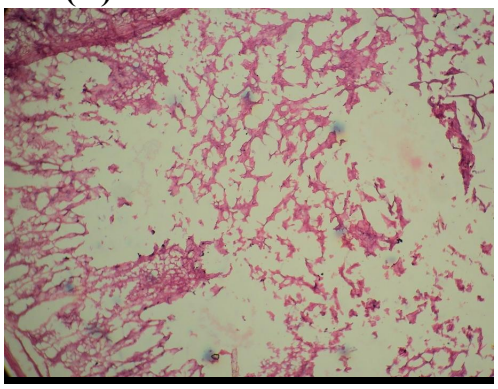
Illustration 3

Fig-3

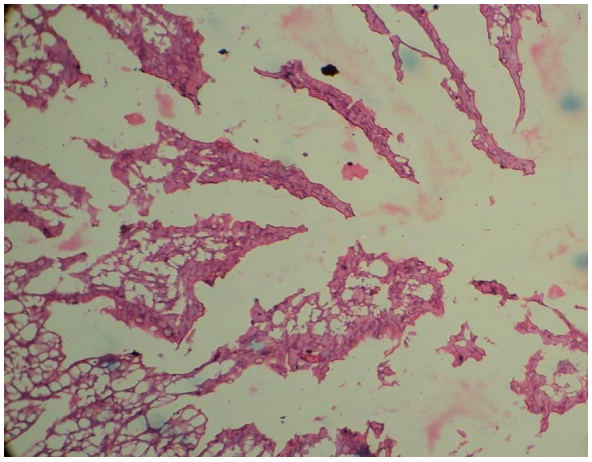
(A)



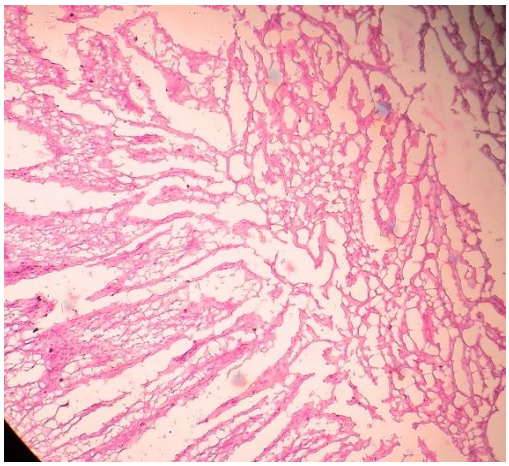
(B)

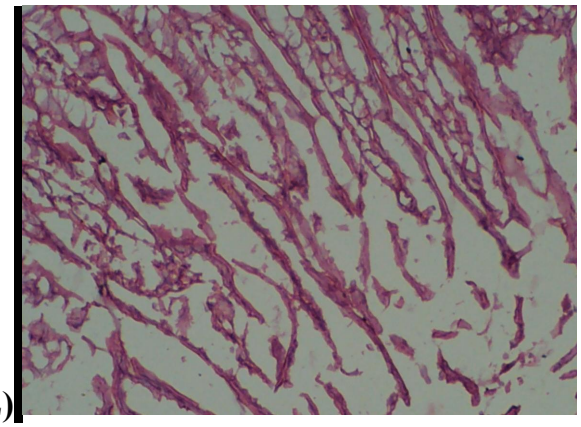


(C)



(D)





(E)

Figure 1: Histopathological section of small intestine dosed with Live *L. casei* (A) *L. casei* and challenge with *E. coli* (B), *E. coli* only (C) Control(D) and Dead *L.casei* (E) (100X)

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