

## *In-vivo* Effect of Probiotics on *Escherichia coli* O157:H7 Isolated from Salad Vegetables

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### Abstract

This study was undertaken to isolate *Escherichia coli* O157:H7 from salad vegetables, determine its pathogenicity and effect on the gastrointestinal tract of mice and sensitivity to probiotics; *Lactobacillus lactis* ATCC12315 and *Lactobacillus acidophilus* ATCC4356 in-vivo. Twenty seven samples made up of eight different types of salad vegetables which includes carrot, cabbage, cucumber, lettuce, peas, green pepper, green beans and spring onions were collected from different locations; markets, farm and street vendors in Lagos, Nigeria. Sampling was done between 13<sup>th</sup> October 2009 and 17<sup>th</sup> March 2010. In all experiments with mice the protocol for care of animal was carried out according to National Institute of Health (NIH). The mice given only *E. coli* O157:H7 developed diarrhea which led to loss of body weight and death of majority of them. On the other hand mice that were not given any microbial suspension (control), those given only probiotics, combination of *E. coli* O157:H7 and probiotics showed no symptom of diarrhea and there was increase in their body weight. The histopathology of the intestines of this group of mice showed that their intestines were not damaged, while those given only *E. coli* O157:H7 showed lymphoid hyperplasia, mucosal sloughing and inflammation (enteritis). The maintenance of good health by probiotics has again been re-emphasized.

**Keywords:** *E. coli* O157:H7, probiotics, salad vegetables, gastrointestinal tract

### 1. Introduction

Probiotics refer to a group of non-pathogenic microorganisms which when consumed in certain amount exert beneficial effects on health (Reid et al., 2003). Huang et al. (2002) demonstrated the use of a meta-analysis of randomized controlled trials to show evidence of the efficacy of lactic acid producing bacteria for both prevention and treatment of acute diarrhea in infants and young children. Beneficial activities of probiotics are usually obtained from the complex interactions of the microorganisms with the intestinal microflora and the gut epithelium of the individual (Marteau et al., 2001). The use of probiotics in animal models of inflammatory bowel disease and in diarrhea of premature infants, severe burn patients and acute and chronic colitis (Filho-Lima et al., 2000) has shown potential beneficial effects of probiotic strains. Some *Lactobacillus* species are used industrially for the production of yoghurt, cheese, sauerkraut, pickles; wine and other fermented foods, as well as animal feeds such as silage. The health benefits of *Lactobacillus* include enhancement of immune system, antimicrobial effects inhibiting intestinal and food poisoning pathogens, improvement of gut function by normalizing microflora balance and treatment of diarrhea including infantile, traveler's and antibiotic induced diarrhea (Reid et al., 2003).

Haemorrhagic colitis that characterised the first registered outbreak of *E. coli* O157:H7 in Oregon was described with severe abdominal cramps, little or no fever, severe bloody diarrhea and colonic mucosal oedema (Riley, 1987). This first recognized outbreak was linked to contaminated ground beef, but it is now claimed that numerous foods such as raw milk, yoghurt, lettuce, unpasteurized apple cider juice, potatoes have been implicated. Traditionally, the O157:H7 sero-type has been linked to foodborne illness outbreaks in which undercooked meats, especially ground beef, have been consumed. The CDC estimates 73,000 cases of infection with *E. coli* O157:H7 and 61 deaths on average occur in the United States every year (Seto et al., 2007). The largest outbreak to date occurred in Japan in 1996, affecting over 9000 people, with contaminated radish sprouts as the possible source of infection (Michino et al., 1998). Reports of person-to-person and waterborne

transmission have been increasing (Meng et al., 2001). *E. coli* O157:H7 serotypes are closely related, descended from a common ancestor, divergent in plasmid content more than chromosomal content, and are no more related to other shiga-toxin producing strains than any other randomly chosen *E. coli* serotype. *E. coli* O55:H7 and *E. coli* O157:H7 are most closely related and diverged from a common pathogenic ancestor that possessed the ability to form attaching and effacing lesions. *E. coli* O157:H7 can naturally be found in cow intestines. Therefore, any object that encounters cow feces could potentially be contaminated with *E. coli* O157:H7 (Whittam et al., 1988). Naturally, cows eat grass and hay, but in these massive feed lots they are fed corn as this diet is cheaper for the farmer and can mature the size of the cow much quicker than in nature. Rain water run-off from these feed lots has also made its way into our streams and lakes. Stream and lake water is regularly used to hydrate vegetable gardens which can then contaminate these vegetables (leafy greens) with *E. coli* (Rodrigue, 1995). The toxin requires highly specific receptors on the cells' surface in order to attach and enter the cell; species such as cattle, swine, and deer which do not carry these receptors may harbor toxigenic bacteria without any ill effect, shedding them in their feces, from which they may be spread to humans. Flesh can become contaminated during slaughter and butchering, and organisms can be thoroughly mixed into beef when it is ground into hamburger. Bacteria present on the cow's udders or on equipment may get into raw milk. Although the number of organisms required to cause disease is not known, it is suspected to be very small (Rodrigue, 1995). The consumption of contaminated meat (especially ground meat), vegetables or produce that has not been cooked sufficiently to kill *E. coli* O157:H7 can cause infection.

The aim and objectives of this research therefore are to:

- Isolate, characterize and identify *E. coli* O157:H7 from salad vegetables.
- Determine the pathogenicity of *E. coli* O157:H7 in the gastrointestinal tract.
- Determine the effect of probiotic organisms in the gastrointestinal tract.
- Determine the effect of the presence of probiotics associated with *E. coli* O157:H7 in the gastrointestinal tract.

## 2. Materials and Methods

### 2.1 Collection of Salad Vegetables

Twenty seven samples made up of eight different types of salad vegetables which includes carrot, cabbage, cucumber, lettuce, peas, green pepper, green beans and spring onions were collected from different locations; markets, farm and street vendors in Lagos, Nigeria. They were collected between 7-8 am on each sampling day. Sampling was done between 13<sup>th</sup> October 2009 and 17<sup>th</sup> March 2010, that is, for a period of 5 months.

### 2.2 Isolation of Microorganisms

From each salad vegetable 25g was weighed into 225 ml of sterile distilled water in a sterilized blender and homogenized at high speed for 3min. Double strength MacConkey broth medium were inoculated with each of the homogenized salad vegetables and incubated at 37°C for 48hrs for enrichment. Broth cultures were serially diluted and 0.1ml aliquots from 10<sup>-2</sup>, 10<sup>-5</sup> and 10<sup>-8</sup> dilutions were spread on a set of MacConkey agar and Eosin Methylene Blue agar. The plates were incubated at 37°C for 24hrs. After incubation the isolates were counted and observed for colonies resembling *E. coli* (rose pink colonies and metallic green sheen colonies) on MacConkey agar and Eosin Methylene Blue agar plates respectively. Pure cultures were obtained by sub-culturing.

### 2.3 Identification of Isolates

The cultural characteristics of the colonies on the plates were observed and recorded. The colonies were Gram-stained to determine their Gram reaction and biochemical tests were also done. The colonies suspected to be *E. coli* were sub-cultured onto sorbitol MacConkey agar and incubated at 37°C for 24 hrs. After incubation, the sorbitol fermenters appeared pinkish while the non-sorbitol fermenters appeared colourless on the plates.

### 2.4 Antigenic Characteristics (Serotyping)

The colonies that did not ferment sorbitol were then serotyped using Oxoid diagnostic kit for the identification of *E. coli* O157:H7. The test was carried out by picking the isolates with an inoculation loop and mixing it with the antisera on the tile. The mixture was swirled for some seconds and presence of agglutination confirmed the isolates to be *E. coli* O157:H7.

### 2.5 Experimental Animals

The animals used for the experiment were mice. A total of 31 mice were obtained from Nigerian Institute of

Medical Research (NIMR), Yaba, and Lagos. Their stool samples were collected and tested for the presence of the pathogen *E.coli* O157:H7. On getting a negative result, the animals were certified as free from an already existence of the pathogen and were therefore fit for the experiment.

## 2.6 Probiotic Cultures and Inoculation of Mice

Pure cultures of probiotics were obtained from Federal Institute of Industrial Research (FIIRO), Oshodi, Lagos. The cultures are *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus lactis* ATCC 12315. *L. acidophilus* and *L. lactis* were inoculated into MRS broth and incubated at 37°C for 48hrs using anaerobic jar. Then they were serially diluted up to 10<sup>8</sup>-fold with sterile saline. An aliquot of 200µl of the suspension was given orally to mice using catheter tube.

## 2.7 Experimental Design

In all experiments with mice the protocol for care of animals was carried out according to National Institute of Health (NIH). Animals were divided into six groups:

- Group A was made up of 2 mice and were not given any microbial suspension (control).
- Group B was made up of 4 mice and were given *L. lactis* at a concentration of 10<sup>8</sup> cfu/ml on days 1, 2, 3, 4, 5 and 6.
- Group C was made up of 4 mice and were given *L. acidophilus* at a concentration of 10<sup>8</sup> cfu/ml on days 1, 2, 3, 4, 5 and 6.
- Group D was made up of 7 mice and were given *E. coli* O157:H7 at a concentration of 10<sup>3</sup> cfu/ml on days 4, 5 and 6
- Group E was made up of 7 mice and were given *L. lactis* at a concentration of 10<sup>8</sup> cfu/ml on days 1, 2, 3, and *E. coli* O157:H7 at a concentration of 10<sup>3</sup> cfu/ml on days 4, 5 and 6.
- Group F was made up of 7 mice and were given *L. acidophilus* at a concentration of 10<sup>8</sup> cfu/ml on days 1, 2, 3, and *E. coli* O157:H7 at a concentration of 10<sup>3</sup> cfu/ml on days 4, 5 and 6.

Mice were observed for presence of diarrhea and their body weight taken each day. On day 4, 1 mouse each from groups A, B and C were sacrificed under anesthesia with formalin. Their intestines were homogenized, centrifuged at 4000 rpm for 5 mins and the supernatant was decanted, serially diluted and plated on MRS agar plates and incubated anaerobically at 37°C for 48 hrs. Developed colonies were counted. The intestines were also fixed with formalin for histopathology examination. On day 7, the remaining mice from groups D, E, and F were sacrificed under anesthesia with formalin. Their intestines were homogenized, centrifuged at 4000 rpm for 5 mins and the supernatant was decanted, serially diluted, plated on MRS agar plates and sorbitol MacConkey agar plates. MRS plates were incubated anaerobically at 37°C for 48hrs while sorbitol MacConkey agar plates were incubated aerobically at 37°C for 24 hrs. Developed colonies were counted. The intestines were also fixed with formalin for histopathology examination.

## 2.8 Gastrointestinal pH

Supernatants from homogenized intestines were filtered with 0.8 µm pore sized filter. pH of filtrates were taken with a pH meter.

## 2.9 Histopathology

The intestines were fixed in 10% neutral buffered formalin for 6hrs. The tissues were grossed by placing them on a board and looking for areas of interest. They were processed by cutting off a length of 5mm, put on already labelled cassette and transferred into an automatic tissue processor which consists of 12 beakers. From the beaker containing 10% formalin it was fixed in 70% alcohol for 2 hrs. In 3 beakers containing 95% alcohol, it was fixed in the first one for 1 hr, transferred into the second one for 1hr and transferred into the third one for 1hr. In 3 beakers containing absolute alcohol (100% alcohol), it was fixed in the first one for 1hr, transferred into the second one for 1hr and into the third one for 1hr. In 2 beakers containing xylene, it was fixed in the first one for 2hrs and in the second one for 1hr. In 3 beakers containing wax, it was fixed in the first one for 1hr, transferred into the second one for 2hrs and into the third one for 1hr.

Then the intestines were brought out and embedded in a mixture of molten paraffin wax and parablast, allowed to solidify on ice, microtomed at 3-5µ on albuminized glass slides, floated on hot water bath at 56°C, drained and dried on a hot plate, then stained using haematoxylin and eosin staining technique. Slides were viewed under the microscope.

### 3. Results

*Escherichia coli* O157:H7 was isolated from all salad vegetables analyzed in this study. *E. coli* O157:H7 a non-sorbitol fermenting organism appeared colourless on sorbitol MacConkey agar.

#### 3.1 Serotyping of *E. coli* O157:H7 Isolates

The result of the reaction when non-sorbitol fermenters were serotyped using Oxoid diagnostic reagent for the identification of *E. coli* O157:H7 indicated that agglutination was produced by them. The result of this test confirmed *E. coli* O157:H7.

#### 3.2 Changes in Weight of Mice

The weight of mice in all the groups changed daily. The weight of mice in groups B, C, E, and F increased from day 1 to 7, while those in group D increased up to day 3, then decreased afterwards (Figure 1).

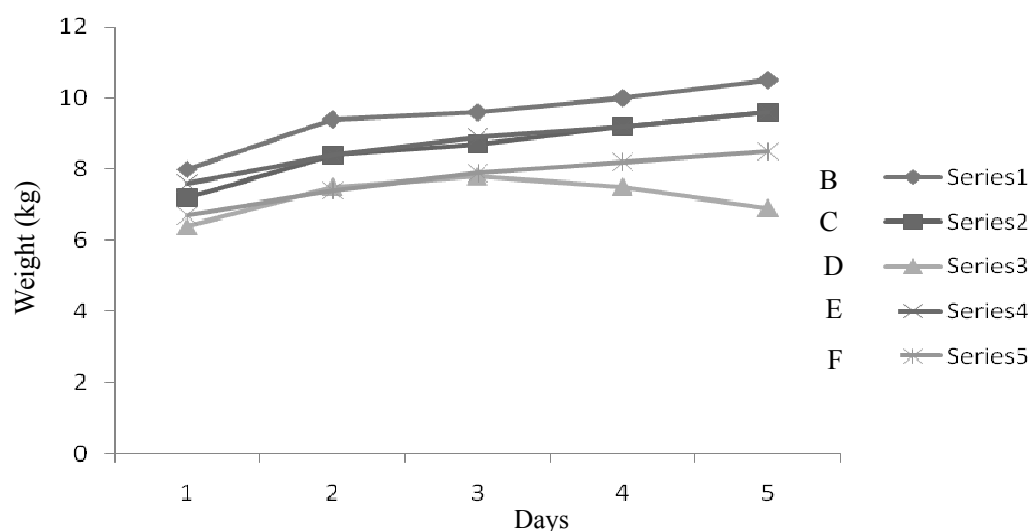


Figure 1. Change in weight of mice given *Lactobacillus lactis* (B), *Lactobacillus acidophilus* (C), *Escherichia coli* O157:H7 (D), *L. lactis* + *E. coli* O157:H7 (E), *L. acidophilus* + *E. coli* O157:H7 (F)

#### 3.3 Clinical Symptoms

Some of the infected mice showed no signs of diarrhea while some did. On day 5, decrease in weight and softening of stool was observed on 5 mice (71.4%) in group D. On day 6, the stooling observed on the 5 mice worsened and they became less active, while the remaining 2 mice (28.6%) in the same group developed slight diarrhea and loss of weight. On day 7, 5 of the mice in group D died (severe diarrhea) but no bloody diarrhea was observed. The mice in groups E and F did not show sign of diarrhea, they remained active till day 7 (Table 1). Viable microbial counts in the gastrointestinal tracts of mice increased dramatically. *Lactobacillus* strains in groups B and C increased more than in groups E and F. *E. coli* O157:H7 in group D also increased (Figures 2-4).

Table 1. Severity of diarrhea in mice infected with *E. coli* O157:H7 by day 7

| Diarrhea Severity | Number of mice/total (%) |             |             |            |             |             |
|-------------------|--------------------------|-------------|-------------|------------|-------------|-------------|
|                   | Group A                  | Group B     | Group C     | Group D    | Group E     | Group F     |
| No diarrhea       | 2/2 (100.0)              | 4/4 (100.0) | 4/4 (100.0) | 0/7 (0.0)  | 7/7 (100.0) | 7/7 (100.0) |
| Slight diarrhea   | 0/2 (0.0)                | 0/4 (0.0)   | 0/4 (0.0)   | 2/7 (28.6) | 0/7 (0.0)   | 0/7 (0.0)   |
| Severe diarrhea   | 0/2 (0.0)                | 0/4 (0.0)   | 0/4 (0.0)   | 5/7 (71.4) | 0/7 (0.0)   | 0/7 (0.0)   |

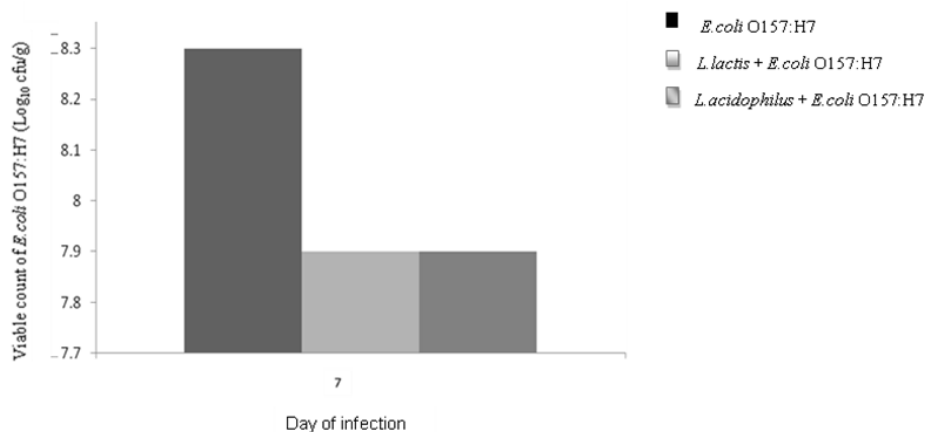


Figure 2. Colonization and distribution of *E. coli* O157:H7, *L. lactis* + *E. coli* O157:H7 and *L. acidophilus* + *E. coli* O157:H7 in the gastrointestinal tract of mice

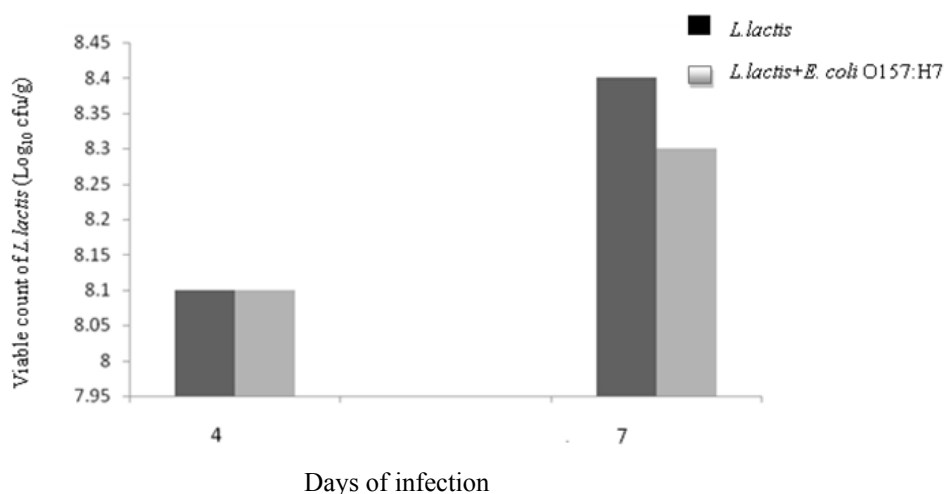


Figure 3. Colonization and distribution of *L. lactis* and *L. lactis* + *E. coli* O157:H7 in the gastrointestinal tract of mice

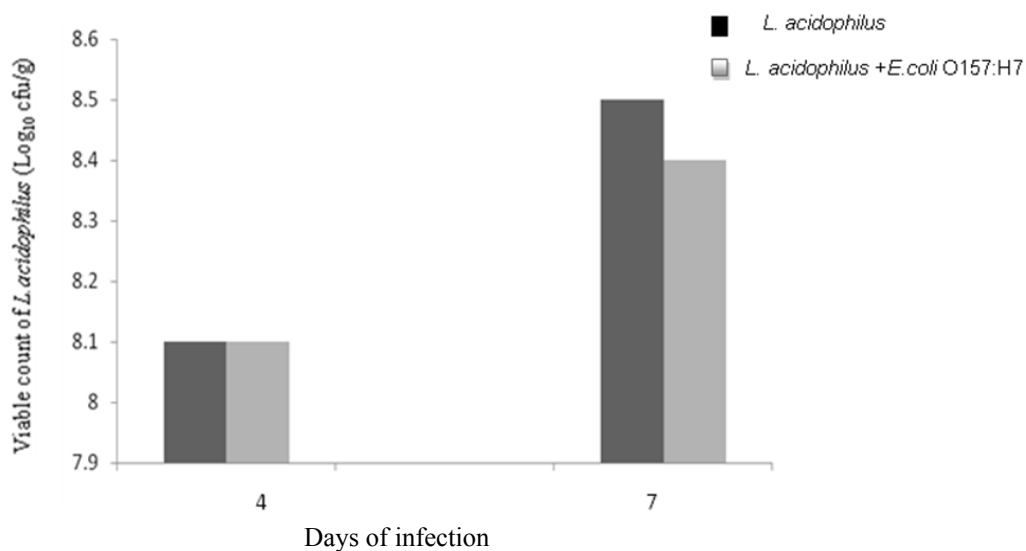


Figure 4. Colonization and distribution of *L. acidophilus* and *L. acidophilus* + *E. coli* O157:H7 in the gastrointestinal tract of mice

### 3.4 pH of Gastrointestinal Tract of Mice

There was no difference in the pH of the gastrointestinal tract of mice in various groups on day 7 even after infection was observed in some of the groups. The pH was approximately 6.6.

### 3.5 Histopathology

From the histopathological results, intestine of mice from group A(control) was normal. Intestines in groups B, given *L. lactis*, C, given *L. acidophilus*, E, given *L. lactis* + *E. coli* O157:H7, F, given *L. acidophilus* + *E. coli* O157:H7 were also normal (Figure 5). That of group D fed with only *E. coli* O157:H7 showed lymphoid hyperplasia, mucosal sloughing and inflammation (enteritis) (Figures 6 and 7).

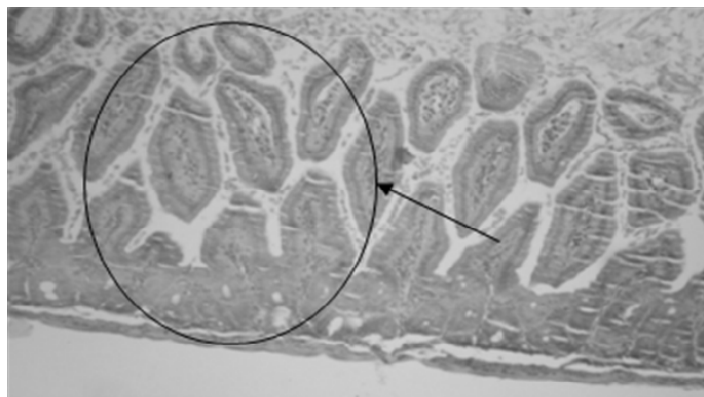


Figure 5. Normal intestine of mice

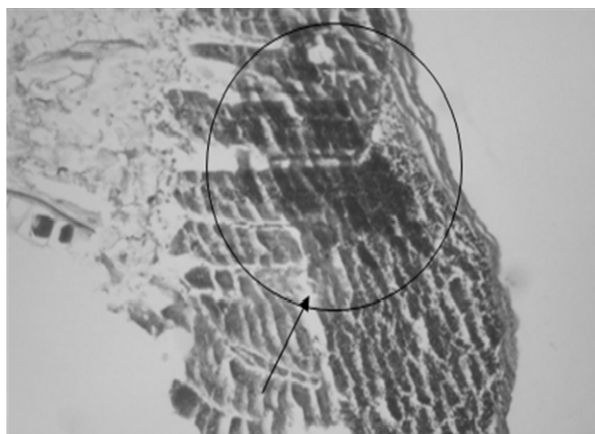


Figure 6. Intestine of mice showing lymphoid hyperplasia

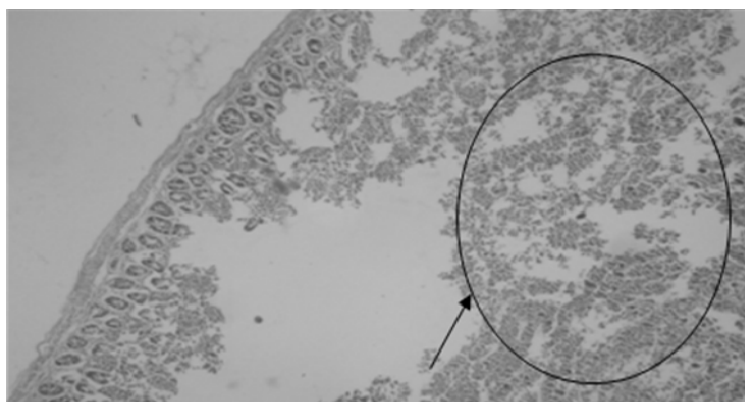


Figure 7. Intestine of mice showing mucosal sloughing, inflammation (enteritis)

#### 4. Discussion

The isolation of *E. coli* O157:H7 from salad vegetables is of public health significance because this organism is a known food pathogen and salad vegetables are consumed with minimal processing and in most cases without heating. Cattle have been recognized as the principal reservoir of the microorganism in water and food borne *E. coli* O157:H7 outbreaks and sporadic infections (Smith et al., 2003). Outbreaks of diseases caused by pathogenic strains of *E. coli* have been described in many parts of the world. Animal faeces can easily be washed by rain water into salad vegetable farms or even into rivers or ground water which may be used to water salad vegetable farms. Some farms may even be located close to a septic tank or broken sewer line. Contamination may also be caused by animals and humans defecating in vegetable farms. The organism can survive in the environment for months, making the risk of infection in contaminated areas even higher (Crump et al., 2002).

The diarrhea observed in mice in group D may have been caused by *E. coli* O157:H7 which was used to infect the mice because there was no diarrhea in the control mice without *E. coli* O157:H7. Diarrhea was also not observed in mice in groups B, C, E and F. This may also be because of the absence of the organism in mice in groups B and C which were given only probiotic organisms. Although *E. coli* O157:H7 was present in mice in groups E and F, they were also given probiotic organisms which may have prevented occurrence of diarrhea in these groups. Probiotic *Lactobacillus* strains have been shown to protect against infection by pathogens including *E. coli* O157:H7 (Nomoto et al., 1992). *Lactobacillus* is a genus of Gram-positive facultative anaerobic or microaerophilic bacteria. They are a major part of the lactic acid bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid (Dicks et al., 2000). The production of lactic acid makes its environment acidic, which inhibits the growth of some harmful bacteria. The probiotic bacterium *Clostridium butyricum* MIYAIRI strain 588 have been reported to have preventive and therapeutic effects on Enterohemorrhagic *Escherichia coli* O157:H7 infection in gnotobiotic mice (Motomichi et al., 2004).

The histopathology of mice also revealed that the intestines of mice in all the groups except group D had normal intestines. The abnormalities (lymphoid hyperplasia, mucosal sloughing and inflammation) observed in mice in group D may have been due to the action of *E. coli* O157:H7. The probiotics may have equally prevented these abnormalities in mice in groups E and F. Thus the probiotic bacteria *Lactobacillus lactis* ATCC 12315 and *Lactobacillus acidophilus* ATCC 4356 helped to maintain good health in mice by their preventive and therapeutic effect on *E. coli* O157:H7 infection in the mice.

#### 5. Conclusion

*Escherichia coli* O157:H7 was isolated from all salad vegetables. The isolate was pathogenic and caused diarrhea in mice. It also resulted in lymphoid hyperplasia, mucosal sloughing and inflammation (enteritis) in the intestines of mice. However, the probiotic organisms exerted therapeutic effect on the pathogen.

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