

**ANTI-GIARDIASIS EFFECT OF ZINGIBER OFFICINALE ON GIARDIA LAMBLIA
INFECTION AND THE IMMUNOLOGICAL RESPONSE IN EXPERIMENTALLY
INFECTED IMMUNOCOMPETENT MICE**

By

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Abstract

Giardia lamblia is a common protozoan parasite infects humans and is the most common cause of waterborne outbreaks of diarrhoeal diseases. The present study examined the anti-giardiasis effectiveness of ginger as a natural product in the prevention and treatment of *G. lamblia* in infected mice. Male albino mice were divided into the following four groups: 1. Control uninfected untreated, 2. Infected with *Giardia* cysts and untreated, 3. Prophylactic with ginger before infection, and 4. Treated with ginger after infection. The results showed that Ginger resulted in a complete eradication of *Giardia* cysts in the faeces after infection by day 12 in the protected group and day 10 in the treated group. IFN- γ levels were significantly decreased ($p < 0.05$) in the ginger-protected and ginger-treated groups, and IL-5 levels were elevated in both ginger groups. Histopathological observations of the ileum support the immunological findings

Key words: Saudi Arabia, Anti-giardiasis *lamblia*, Ginger, *Zingiber officinale*, IFN- γ , IL-5.

Introduction

Giardia lamblia infections are considered to be a major public health problem, globally; however, the interactions of this parasite with the host are not fully understood, and effective vaccination strategies against this parasite do not exist for humans. *G. lamblia* is a flagellated unicellular eukaryotic enteric protozoan parasite that is responsible for one of the most common infections of the human intestine; this parasite is a leading cause of diarrhoeal disease worldwide (Jakubowski and Craun, 2002; Tian *et al*, 2010). This parasite is endemic in both high- and low-income countries (Adam, 2001). In developed countries, *Giardia* is the most common cause of waterborne outbreaks of diarrhoeal disease (Slifko *et al*, 2000). Globally, approximately 1.5 million deaths occur each year from diarrhoeal diseases, and these deaths occur primarily in low-income settings and in children under 5 years old (WHO, 2015). A total of 2.0×10^8 people are estimated to have giardiasis, worldwide, each year, and children under the age of five are specifically at risk (Lane and Lloyd, 2002; Thompson, 2000). Associated with substantial economic losses, *Giardia* infection is a common cause of diarrhoeal disease in domestic animals (e.g., dairy calves, dogs,

and cats) (Thompson, 2000). A fraction (20 to 80%) of all stool-positive *Giardia* infections in humans are symptomatic infections (Flanagan, 1992; Nash *et al*, 1987), characterized by diarrhoea, epigastric pain, nausea, vomiting, and weight loss (Stifko *et al*, 2000). Symptoms typically appear 6 to 15 days after infection and last for 2 to 4 days. In more than 85% of cases, Giardiasis is self-limiting, which indicates that efficient host defences exist (Flanagan, 1992). CDC (2013) stated that nearly 2% of adults and 6 to 8% of children are currently infected with *Giardia* in developed countries, whereas a third of the people in the developing world have previously had giardiasis. *Giardia* cysts can survive for months in the soil and on the water surface. A detectable outbreak can occur even with very small concentrations of virulent and infectious cysts (Plutzer *et al*, 2010). These factors can be influenced by the higher *Giardia* prevalence in humans, estimated to be 10%, compared to the 3 to 5% of *Cryptosporidium* (Huang and White, 2006). A better understanding of the immune response to the *Giardia* parasite may aid in the development of an appropriate therapeutic agent since there is currently no effective therapeutic agent with anti-giardial activity. The initial innate responses limit

the parasite numbers; however, the clearance of the infection ultimately necessitates a cell-mediated immune response (Ungar *et al*, 1991; McDonald *et al*, 2004). Cytokines are proteins that play an important role in the modulation of innate and adaptive immune responses. In mice, studies have established that IFN- γ is a primary player in cell-mediated immunity as well as in the early innate immune responses (Theodos, 1998; Riggs, 2002; Lean *et al*, 2002). The majority of studies have reported that IFN- γ activity is the most effective adaptive immune response to *Giardia* infection (Urban *et al*, 1996; Kapel *et al*, 1996). IL-5 is a cytokine that contributes to the humoral response and is produced by Th2 lymphocytes and takes part in the differentiation and growth induction of B- & T-cells. IL-5 also stimulates the proliferation and differentiation of eosinophil precursors as well as their degranulation and production of reactive oxygen compounds. IL-5 induces eosinophilia by exerting a chemotactic effect on eosinophils (Faccioli *et al*, 1997; Weltman, 2000).

Nowadays, bioactive plants are anti-parasitic treatment due to significant increase in resistance to chemical drugs (Kelley *et al*, 2016). *Zingiber officinale* Roscoe (ginger, Zingiberaceae), which is known for its flavour and pungency, is one of the most widely used spices in various foods and beverages. The rhizome of this plant is the primary part that is used for Chinese cuisine, medicinal herbs and condiments. It has been reported that *Z. officinale* exerts numerous pharmacological effects, such as antiulcer (Yoshikawa *et al*, 1994), antioxidant (Sadhana and Gupta, 2013), antibacterial (Mahady *et al*, 2003), antifungal (Ficker *et al*, 2003), anti-inflammatory (Jeena *et al*, 2013), antidiarrheal (Abouel-Nour *et al*, 2016), anthelmintic (Iqbal *et al*, 2001; El-Sayed *et al*, 2015), and anticancer effects (Shukla and Singh, 2006). Extracts of *Z. officinale* were studied extensively for a wide range of biological activities, including anticonvulsant,

analgesic, gastric anti-secretory, antispasmodic, and antiallergenic activities, as well as increasing digestive fluids and absorbing and neutralizing toxins and stomach acid. *Z. officinale* increased bile secretion and to increase the action and tone of the bowels (Bradley, 1992). Al-Masoudi (2011) reported the anti-giardial activity of *Zingiber officinale*. The watery extract of this plant was shown to reduce the number of *G. lamblia* trophozoites.

This study aimed to investigate the anti-giardiasis effectiveness of ginger as a natural medicinal plant to prevent and treat *Giardia* infections in immunocompetent mice that were experimentally infected. Though the role of cytokines in parasitic infections has been extensively studied in animal models, there is insufficient literature and clinical trials on the significance of the Th1 cytokine, IFN-gamma (IFN- γ), and the Th2 cytokine, interleukin-5 (IL-5), in human giardiasis. Therefore, this work was performed to investigate the levels of these cytokines in the sera of mice infected with *G. lamblia*. Hence, this study aimed to assess the protective and therapeutic capacity of ginger against giardial infection.

Materials and Methods:

Plant material: Rhizomes of *Z. officinale* were obtained from a local market, and the roots were cut, washed with distilled water and dried in an oven at 50 °C for 5 to 7 days until they were completely dried. A grinder was used to grind the dried roots into a powder, which was stored at 4 °C. The dose of ginger used for treatment was 50 mg/kg body weight/day, which was administered orally using a gastric tube 1 hour before breakfast.

Animals: A total of 20 male Swiss albino mice, three to five weeks old and weighing 25 to 30 grams, were used. The mice were housed in well-ventilated cages and provided with standard pellet food and water. All experimental procedures were approved by the Princess Nourah bint Abdulrahman University research ethics committee, IRB

No. 18-0177. The mice were permitted to adjust to the laboratory environment for one week before the experiment (El-Fakhry *et al*, 1998), and they were free from any infection.

Mice infection: *Giardia lamblia* cysts were purchased from waterborne™, Inc. (New Orleans, Louisiana, USA) and stored in a shipping medium (phosphate-buffered saline with penicillin, streptomycin, gentamycin, amphotericin B & 0.01% Tween 20) at 4°C until use. Each mouse of the infected, experimentally protected, and experimentally treated groups was orally inoculated with the isolated *Giardia* cysts in a dose of 125×10^3 cysts/mouse (Gaafar, 2007). A 23-gauge needle tipped with plastic tubing was used for ingestion by gastric gavage (Riad *et al*, 2009).

Experimental design: Male Swiss Albino mice were divided into four equal groups of 5 mice each as follows: 1- Negative control group (uninfected, untreated) did not receive any treatment. 2- Infected group (positive control, infected, untreated); mice were inoculated orally with *G. lamblia* cysts in a dose of 125×10^3 cysts/mouse (Gaafar, 2007). 3- Protected group; the mice received ginger (50mg/b.w./day) two days before infection, and continued to receive ginger daily for 12 days post-infection. 4- Experimental treated group; mice received ginger (50mg/ b.w./day) one day after infection, and continued to receive ginger daily for 2 weeks.

Stool analysis: Samples were collected from all mice in separate plastic containers. Stools were inspected by direct wet saline smear, iodine and Sheather's sugar flotation method (Garcia and Brucker 1997). The experiment was conducted as follows: using an applicator stick, a small amount of faeces (approximately 2mg) was placed in the centre of the slide with a drop of saline. The sample was lightly stirred until thoroughly mixed with saline, and then a 22x22mm coverslip was placed on top of the mixture.

The mixture was examined using bright-field microscopy with low and high power objectives.

Immunological analysis: On day 17 post-infection, mice were anaesthetized (24 hrs after last dose in all groups). Each animal was kept fast for 12hrs before anaesthetization ether. Blood samples were withdrawn directly from heart. Blood was centrifuged at 3,000rpm to separate sera, which was stored at -20°C until the concentration of IFN- γ was determined using the Quantikine-MIF00 mouse IFN- γ ELISA immunoassay kit (R&D, Minneapolis, MN, USA). IL-5 concentration was analysed using mouse IL-5 ELISA Kit from RayBio® Company, according to the manufacturer's instructions.

Histological examination: After anaesthetized, the mice were sacrificed 17 days post-infection for the control, infected, and treated (using ginger) groups, while the mice of the ginger-protected group were sacrificed 15 days post-infection. Autopsy samples were obtained from the mice intestines of all groups and fixed in 10% neutral formalin for 24hrs, washed with tap water, and then serial dilutions of ethanol (absolute) to dehydrate the samples. Specimens were cleared in xylene and embedded in paraffin at 56 °C in a hot air oven for 24hrs. Paraffin beeswax tissue blocks were sectioned at a thickness of 4 μ m by sledge microtome. The tissue sections were collected on glass slides, deparaffinized, and stained with haematoxylin & eosin for routine examination by a research light microscope (Banchroft *et al*, 1996).

Statistical analysis: Analysis was conducted among groups. Results were expressed as mean \pm S.E, and significance was analysed using the t- test.

Results

Stool showed cysts in infected group; in protected and treated groups. Cysts were absent in 12 days post-infection in protected group and 10 days in the treated group.

Table 1: Stool analysis for all groups.

Data collection	Negative Control	Positive Control	Protected	Treatment
1 week before infection	- ve	- ve	- ve	- ve
2 days before infection	- ve	- ve	- ve	- ve
2 days post infection (PI)	- ve	+ ve	- ve	- ve
4 days PI	- ve	+ ve	- ve	- ve
6 days PI	- ve	+ ve	+ ve	+ ve
8 days PI	- ve	+ ve	+ ve	+ ve
10 days PI	- ve	+ ve	+ ve	- ve
12 days PI	- ve	+ ve	- ve	- ve
14 days PI	- ve	+ ve	- ve	- ve
16 days PI	- ve	+ ve	- ve	- ve

- ve =no *Giardia* cysts, + ve = *Giardia* cysts.

Effect of *G. lamblia* infection and ginger protection and treatment on IFN- γ levels in serum: Infection with *Giardia* cysts caused a significant increase ($P < 0.05$) in the levels of IFN- γ in the serum of the mice in the infected, untreated group compared with the uninfected and untreated group of mice. In

the ginger-protected and -treated groups, there was a significant decrease ($P < 0.05$) in IFN- γ levels in serum compared to infected group. Moreover, there was a significant increase in IFN- γ levels in serum of the ginger-protected and -treated groups compared to uninfected control group.

Table 2: Concentration of IFN- γ & IL-5 in serum of mice in each group.

Treatment	Negative Control	Positive Control	Protected	Treatment
IFN- γ (pg/mL)	126.7 \pm 0.6 ^b	800.8 \pm 1.5 ^a	207.8 \pm 0.8 ^{ab}	168.8 \pm 0.7 ^{ab}
IL-5 (pg/mL)	182 \pm 6 ^b	95.3 \pm 0.9 ^a	143 \pm 4 ^{ab}	103 \pm 0.8 ^a

^a significant compared to control ($P < 0.05$). ^b significant compared to infected group ($P < 0.05$).

Effect of giardiasis, ginger protection and treatment on serum IL-5 Levels: Infection with *Giardia* cysts resulted in a significant decrease ($P < 0.05$) in IL-5 levels in the serum of the infected untreated group of mice compared with the uninfected control group. In contrast, there was a significant increase in IL-5 levels in the ginger-protected group compared to the infected group. In addition, there was a significant decrease ($P < 0.05$) in the ginger-protected and ginger-treated groups compared with the uninfected group. Histopathological study: 1- Control group with their ileum sections of the control mice exhibited normal architecture of the villi. 2- Infected group but untreated, their ileum sections exhibited histopathological alterations. 3- Experimental prophylactic group their ileum sections prepared from mice treated with ginger exhibited histopathological alterations compared to controls. 4- Experimental therapeutic group their ileum sections prepared from mice treated with

ginger exhibited histopathological alterations compared to controls

Discussion

In the present study, mice that were used were free from any type of infection. Additionally, the infectivity of the mice was successful, and the protected and treated groups were all infection-free by day 12 and 10 post-infection (PI), respectively. This finding indicates that ginger effectively removed *Giardia Lamblia* from the body and, when compared to the control infected group, which was still showing positive results of infection by passing cysts in the stool until the end of the experiment at day 16 PI, there were no signs of infection in the stools of the ginger-protected and ginger-treated groups.

Giardia is primarily an intestinal parasite, which induces changes in the immune system to overcome the infection; these changes may be reflected in the production of immune mediators. The variations in cytokines

and immunoglobulin levels may allow for the establishment of a distinctive profile related to infection by *Giardia*, although these cytokines are produced in small quantities.

In this study, an increase in IFN- γ cytokine secretion was detected in the infected mice compared to the non-infected control mice, which indicates that the infected mice exhibited an immune response, which attempted to overcome the infection. The increased expression of the immune mediator IFN- γ was observed during the infection, indicating that the immune response seems to be mostly a Th1-mediated response. This finding is in line with (McDonald, 2000). Multiple studies have demonstrated the critical role of IFN- γ in the host resistance to infection with another parasite, *Trypanosoma cruzi* (Aliberti *et al*, 1996; Cardillo *et al*, 1996). After infection, a primary response against *Giardia* was detected by the significant increase in the level of IFN- γ . The balance between Th1 (to control parasite growth) and Th2 cytokines (to limit pathology) is delicate, and this finding is in line with (Lean *et al*, 2002; Tessema *et al*, 2009).

The administration of *Giardia* cysts caused a significant decrease ($P < 0.05$) in the IL-5 levels in the serum of the infected untreated group of mice compared with the control group. On the other hand, there was a significant increase in IL-5 levels in the ginger-protected group compared to the infected group. Comparing ginger-protected group with the uninfected group, there was a significant decrease in IL-5 levels. Nevertheless, in the ginger-treated groups, there was a significant decrease in IL-5 level in comparison to the uninfected group. Several studies have indicated the variations in the levels of immunological indices during parasitic invasions, e.g., the observed increase in IL-5 that is typical of parasitosis (Faccioli *et al*, 1997). Matowicka-Karna *et al*. (2009) performed research on patients infected with *G. lamblia* and confirmed a statistically significant increase in IL-5 levels. In the acute form of *Schistosoma mansoni* infection

course, the levels of IL-5 & IFN- γ exhibited a marked increase (de Jesus *et al*, 2002). In response to the administration of the extract of a soluble antigen of *Onchocerca volvulus*, an increase in IL-5 & IL-13 was observed (Brattig *et al*, 2002). Cooper *et al*. (2001) described the early stage of *Onchocerca volvulus* infection, which caused an increase in the production of IL-5 & IFN- γ . Ajdary *et al*. (2009) detected a significant increase in the levels of IL-5, IFN- γ & IL-13 in patients who were chronically infected with *Leishmania* (but untreated), produced by peripheral blood mononuclear cells (PBMC) *in vitro*. These findings suggest the occurrence of a mixed Th1/Th2 response; nonetheless, patients with acute disease displayed a Th1 response, which was confirmed by the increase in the levels of IFN- γ and the low levels of IL-5 & IL-13. Ishikawa *et al*. (1998) reported that experimental mice infected with *Trichinella spiralis* and *Nippostrongylus brasiliensis* exhibited the release of IL-5 and IFN- γ from Th1 and Th2 cells. This finding is in accordance with the present study. The synthesis and release of IgE are suppressed by IFN- γ , which is produced by activated Th1 cells. IFN- γ stimulates the elimination of intracellular parasites by macrophages by inducing the production of reactive oxygen and the secretion of hydrogen peroxide (Lucey *et al*, 1996). Touil-Boukoffa *et al*. (1997) demonstrated that the defence mechanisms that are activated in the course of echinococcosis also involve IL-6 and IFN- γ . Ajami and Rafiei (2007) on *Hymenolepis nana*-infected patients found an increase in the levels of IL-5, IL-12, IL-13 and IFN- γ .

In this study, there was a decrease in IL-5 levels, whereas IFN- γ levels were elevated in *Giardia*-infected mice. Th1 lymphocytes produce IFN- γ , which contributes to the cellular type response and inhibits the proliferation and action of Th2 cells. Th2 lymphocytes produce IL-4, IL-5, IL-6, IL-10 & IL-13 and stimulate the humoral response. In the present study, the antiparasitic treatment

in mice infected with *Giardia* caused a statistically significant increase in the levels of IL-5 and a significant decrease in the IFN- γ levels of the treated groups, as shown above. This indicated that immune and inflammatory responses in parasitic invasions course wherein IFN- γ , TNF, IL-1b, IL-5 & IL-13 are generated (Ajdary *et al*, 2009).

These results could serve as guidelines for future studies on the immune response to *Giardia* in immunocompetent populations. Significant advances have been made towards understanding the host responses against *Giardia*, along with immune effector mechanisms that control infection, although there are still important gaps in the understanding of the immune mediators that are involved. In this context, this work provides preliminary information, supporting the role of IFN- γ and IL-5 as the key cytokines in both innate and adaptive immunity during primary and secondary *G. Lamblia* infection. The kinetics of IFN- γ expression reflect the pattern of acute infection, which has been observed in neonatal mice, with the levels of IFN- γ increasing as infection progresses to a peak level and falling rapidly as recovery begins (Kapel *et al*, 1996; McDonald *et al*, 2004). Principal functions of IFN- γ and other proinflammatory cytokines include triggering antimicrobial killing mechanisms, such as the generation of toxic nitric oxide derivatives or oxygen radicals, or causing a deficiency of metabolites which are essential for the growth of microorganisms, such as tryptophan or cellular iron (Rottenberg *et al*, 2002). IFN- γ stimulates the production of high levels of nitric oxide and frequently acts in concert with other cytokines, such as TNF- α (Nacy *et al*, 1991). The results of the histopathological examination of the intestinal mucosa of the infected non-treated group are in agreement with multiple studies (Buret, 2005; Abdel-Fattah and Nada, 2007; Abdalla *et al*, 2011; Fathy, 2011; Mahmoud *et al*, 2014; Ismail *et al*, 2017). Numerous host factors, in addition to the interaction between trophozoites and the

intestinal epithelium, were responsible for the microvillus changes and epithelial barrier dysfunction observed in the case of giardiasis (Scott *et al*, 2000; Chin *et al*, 2002). The histopathological examination of the intestinal mucosa of both ginger groups revealed some recovery of the pathological changes of the ileum mucosa and villous architecture. These results were similar to those of other studies (Mahmoud *et al*, 2014; Dyab *et al*, 2016), and this finding suggests the protective and therapeutic capacity of ginger against giardial infection.

Conclusion

It is clear that the immune response was elevated to clear the infection as a result of the treatment strategies employed in this study, which appeared to increase the release of cytokines when compared with the uninfected and infected controls. *Z. officinale* exerts an effective anti-parasitic activity against *G. lamblia* and can be used for the prevention of drug-resistant diseases; however, more evaluation is necessary to isolate the active components and determine their toxicity, side effects and pharmacokinetic properties.

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Explanation of figures

Fig. 1: Cross-section of ileum of uninfected control mice showing normal structures of villi (V), a regular and continuous brush border (BB), absorptive columnar cells (AC) and lamina propria (LP) (H&E, x100).

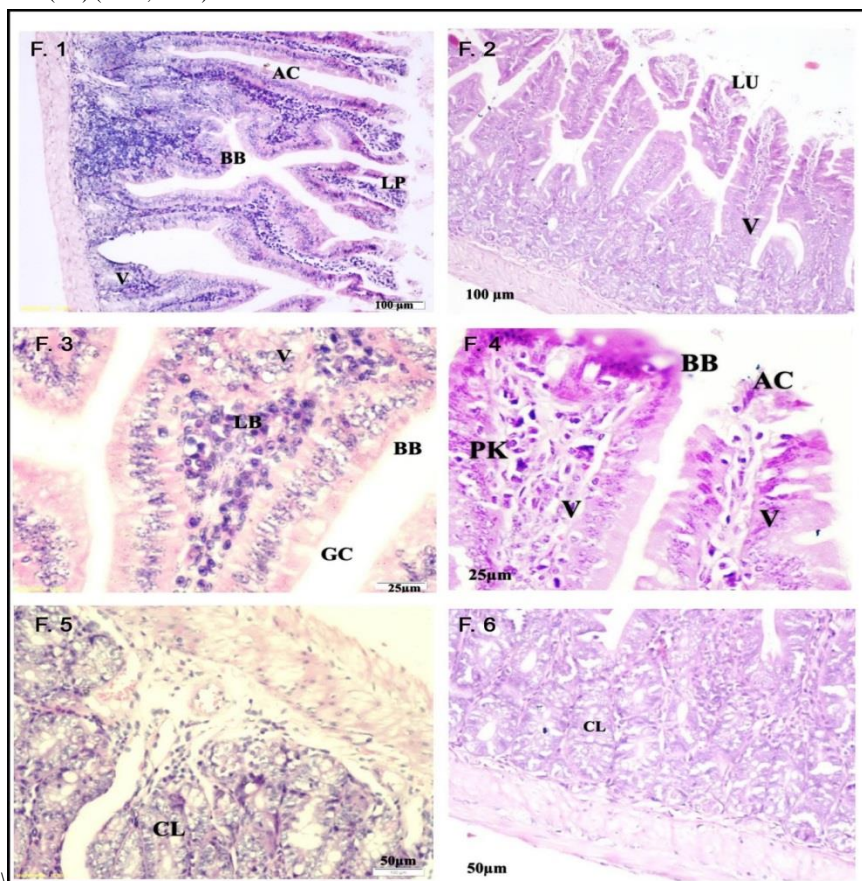
Fig. 2: Cross-section of ileum of control infected mice showing disturbed villous architecture, hyperplasia of the villi (V) with local ulceration (LU) (H&E, x100).

Fig. 3: Villi of uninfected control mice showing normal structure of villi (V), a regular and continuous brush border (BB), presence of goblet cells between enterocytes (GC), and normal connective tissue core of villi (LP) (H&E, x400).

Fig. 4: Villi of control infected mice showing degeneration of columnar epithelium (AC) with many pyknotic nuclei (PK) and a denudeated brush border (BB) (H&E, x400).

Fig. 5: Ileum of uninfected control mice showing normal structures of crypts of Lieberkühn (CL) (H&E, x200).

Fig. 6: Photomicrograph of a representative ileum of control infected mice showing mild oedema with very mild inflammatory infiltrate in the crypts of Lieberkühn (CL) (H&E, x200).



Explanation of figures

Fig. 7: Cross-section of ileum of ginger-protected mice showing nearly normal villi (V), apart from mild atrophy (AT) (H&E, $\times 100$).

Fig. 8: Villi of ginger-protected mice showing very mild degeneration of columnar epithelium (AC) with a preserved brush border (BB) (H&E, $\times 400$).

Fig. 9: Ileum of ginger-protected mice showing normal crypts of Lieberkühn (CL) with no evidence of inflammation (H&E, $\times 200$).

Fig. 10: Cross-section of ileum of ginger-treated mice showing nearly normal villi structure (V), a regular and continuous brush border (BB), normal absorptive columnar cells (AC) and lamina propria (LP) (H&E, $\times 100$).

Fig. 11: Villi of ginger-treated mice showing nearly normal villi (V), normal absorptive columnar cells (AC), a regular and continuous brush border (BB), and normal connective tissue core (LP) (H&E, $\times 400$).

Fig. 12: Cross-section of ileum of ginger-treated mice showing normal crypts of Lieberkühn (CL) with no evidence of inflammation (H&E, $\times 200$).

