

Effects of Synbiotics on the Pathological Features of Experimental Septic Peritonitis in Rats

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Abstract: The aim of this study was to investigate the protective activity of the synbiotics Biomin®Imbo and yoghurt against septic peritonitis in rats. Cecal Ligation and Tip Resection (CLTR) model was followed to induce fecal peritonitis. Effects of the synbiotics on survival after CLTR, acute phase proteins, pathological lesions, the Total and Differential Leukocytic Count (TLC and DLC) of the peritoneal fluid, the TLC and DLC of the peripheral blood and mean bacterial growth in the peritoneal cavity were studied. It was found that neither Biomin®Imbo nor yoghurt in the dose schedule used in this study prevented lethality at the end of the first and third day post induction (p.i.) of CLTR. No significant change was found in the values of the serum c-reactive protein in the various groups. Fibrinogen was elevated significantly in the groups in which septic peritonitis was induced. Gross and microscopic lesions that were observed in groups subjected to CLTR were comparable with those of bacteremia. There were generalized sepsis and fibrinous adhesions that were converted to fibrous adhesions after the 7th p.i. day. No significant change was noted in the cytology of the peritoneal fluid in the various groups at the end of the first p.i. day. However, the TLC of the peripheral blood was significantly higher in rats of the experimental groups (with septic peritonitis) than in the control group. Yoghurt and to a lesser extent Biomin®Imbo were found to lower insignificantly the bacterial growth in the peritoneal cavity at the end of the first p.i. day.

Key words: Effects, synbiotics, pathology, experimental sepsis, peritonitis, Biomin®Imbo, yoghurt

INTRODUCTION

A prebiotic has been defined as a non-digestible food that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Shah, 2004, 2006). Probiotics were defined as living microorganisms which upon ingestion in certain number exert health effects beyond inherent basic nutrition (Guarner and Schaafsma, 1998). A number of genera of bacteria (and yeast) are used as probiotics, including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium* and *Enterococcus* but the main species believed to have probiotic characteristics are *L.acidophilus*, *Bifidobacterium* sp. and *L.casei* (Shah, 2007). Members of the genera *Lactobacillus* and *Bifidobacterium* have a long and safe history in the manufacture of dairy products and are also found as a part of gastrointestinal microflora. Probiotic bacteria with desirable properties and well-documented clinical effects include *L. johnsoni* La 1, *L. rhamnosus* GG (ATCC 53103), *L. casei* Shirota,

L. acidophilus NCFB 1478, *B. animalis* Bb12 and *L. reuteri* (Shah, 2004). A number of health benefits have been claimed to products containing probiotic organisms including antimicrobial activity and gastrointestinal infections, improvement in lactose metabolism, antimutagenic properties, anticarcinogenic properties, reduction in serum cholesterol, antidiarrhoeal properties, immune system stimulation, improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection (Guarner and Schaafsma, 1998; Shah, 2004, 2006). When products contain both prebiotics and probiotics they are referred to as synbiotics (Shah, 2004). Synbiotics have the effects of both probiotics and prebiotics to produce health enhancing functional food ingredients (Shah, 2004).

Peritonitis, inflammation of the peritoneum is a very common condition in the large domestic animals and less common in dogs and cats (Sellon and Long, 2007). It could be classified as primary or secondary acute or chronic, local or diffuse, septic or non-septic and according to type of exudate into serofibrinous,

fibrinopurulent, purulent, hemorrhagic or granulomatous (Sellon and Long, 2007). Diffuse peritonitis is usually acute and fatal in horses and is associated in part with the small omentum and a poor capacity to wall off contaminated areas. Clinical peritonitis is caused by rupture or perforation of the stomach or intestine with spillage of contents (Sellon and Long, 2007; Brown *et al.*, 2007). In cattle, acute diffuse fibrinopurulent peritonitis is common and is usually the result of perforation of a viscous, especially the reticulum or uterus. Traumatic reticuloperitonitis (hardware disease) is virtually always caused by sharp foreign body usually a wire or nail penetrating the reticular wall (Sellon and Long, 2007). In dogs fibrinohemorrhagic peritonitis is usually mild and easily overlooked and is common in infectious canine hepatitis and toxoplasmosis (Sellon and Long, 2007; Fossum *et al.*, 2007). Feline infectious peritonitis is the most common form of peritonitis in cats (Sellon and Long, 2007).

The aims of the study were to test the efficacy of two types of synbiotics (Biomim@Imbo and yoghurt) in preventing or alleviating the pathological features of experimental septic peritonitis to evaluate the possibility of using the concentrations of acute phase proteins as biomarkers of septic peritonitis and to study the effects of synbiotics on bacterial growth in the peritoneal cavity in case of septic peritonitis.

MATERIALS AND METHODS

Experimental animals: Eighty apparently healthy adult white rats (250-300 g) of both sexes were used in this study. They were obtained from the animal house of the College of Veterinary medicine, University of Mosul and housed in plastic cages in a temperature-controlled room maintained in 12 h light/dark cycles.

The standard laboratory food and water were freely available *ad libitum* except for overnight fasting before inducing experimental sepsis. Male rats were separated from the females to prevent mating and the animals were left for 1 week for acclimatization before being used in the experimentation.

Biomim@Imbo: This synbiotic was obtained from Biomim Gesunde Tierernährung International GmbH, Austria and was stored in the refrigerator till being used. It contains a stable strain of probiotic bacteria (*Enterococcus faecium* strain IMB52 in a concentration of 5×10^{11} CFU kg^{-1} of the preparation) a prebiotic (fructooligosaccharide) and a mixture of algae and bacterial cell wall. The synbiotic was added to the food at a rate of 1.5 g kg^{-1} food and was given to the rats for 42 days.

The yoghurt: The yoghurt was prepared in the laboratory following a unified fermentation procedure using pasteurized cow milk. Following fermentation the bacterial count was determined using the formula (Benson, 2002) Number of bacteria in 1 mL of the original sample = the total number of colonies in the dish $\times 10 \times$ the inverse of the dilution. The yoghurt sample was also checked for the presence of *Lactobacillus acidophilus* and *Streptococcus thermophilus* in a count of 6×10^9 CFU mL^{-1} .

Induction of septic peritonitis: The model used was a modification of the Cecal Ligation and Puncture (CLP) model of fecal peritonitis developed by Wichterman *et al.* (1980) and adapted by Tsunoda *et al.* (2002). The rats were anesthetized with ketamin and xylazin and shaved over the anterior abdominal wall.

A midline incision was made approximately 2 cm long sufficient to expose the cecum. When the tip of the cecum was vacant feces in the proximal cecum were moved into the tip of the cecum by manipulation. The cecum was then tightly ligated with a 3-0 silk suture about 6-12 mm from the tip.

The tip was resected with scissors to leave the small amount of opened bowel containing feces. The length of the opened bowel was defined using a ruler and the tip of the cecum was resected as its length was made as short as possible, usually 1-2 mm long. The treated cecum was then returned to the peritoneal cavity. The abdomen was closed with 5-0 catgut suture following a simple continuous pattern. The peritoneum was sutured with the muscles. Suturing of the skin incision was done using 4-0 silk suture following a simple interrupted pattern. The rats were returned to their cages and given access to water and food *ad libitum*.

Surgical intervention after CLTR (CLTRI) was performed at 6 h after CLTR. The abdomen was reopened by a 2 cm midline laparotomy. Excision of the opened bowel and an intensive peritoneal lavage using 5 mL of sterile saline were performed. The abdomen was closed with 5-0 catgut suture.

Measurement of acute phase proteins: The c-reactive protein and fibrinogen were selected as representatives of the acute phase proteins in rats. Measurement of level of c-reactive protein in the serum was done using a commercial kit (CRP Latex Test, Plasmatec Laboratory Products Ltd, Unit29, Dreadnought Trading Estate, Birdport, Dorest DT6 SBU, UK). The plasma level of fibrinogen was measured using BIO-FIBRI kit produced by BIOLABO (BIOLABO SA.02160, Maizy, France).

Histopathological examination: Tissue specimens were collected from the peritoneum, intestine, liver, spleen, kidneys and mesenteric lymph nodes. They were fixed in 10% formalin solution for 48 h, trimmed to suitable sizes washed, dehydrated, cleared in xylol, embedded in paraffin wax, sectioned at 5-6 μm thickness stained with hematoxylin and eosin and examined with a light microscope (Kiernan, 1999).

Examination of peritoneal fluid: After aseptic opening of the abdomen, a 2.5 mL of the peritoneal fluid was withdrawn using a sterile glass pipette. The fluid was subjected to Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC) (Coles, 1986).

Hematological examinations: Blood samples (2.5 mL each) were collected from the eye vein using capillary tubes containing Ethylene Diamine Tetraacetic Acid (EDTA). TLC and DLC were done within 24 h after collection of the samples (Coles, 1986).

Calculation of average bacterial growth: Within 6 h after induction of the CLTR each rat was injected with 6 mL sterile physiological saline solution intraperitoneally. The abdomen was slightly massaged for 30 sec to assure homogenization of the injected fluid. Immediately after that a 5 mL of the peritoneal fluid was collected using EDTA containing tubes. The sample was diluted decimally and vital bacterial count was accomplished and the sample was incubated at 37 $^{\circ}\text{C}$ for 16-24 h and cultured on nutrient agar medium. The number of colonies was calculated according to the formula (Benson, 2002).

$\text{No. of bacteria mL}^{-1} (\text{CFU mL}^{-1}) = \text{total number of colonies in the plate} \times 10 \times \text{inversion of the dilution.}$

Statistical analysis: Data concerning the different animal groups were analyzed using the one way analysis of variance and the results were evaluated through the least significance difference test (Petri and Watson, 1999).

Experimental design: The rats were divided into four groups, group one (15 rats) served as negative control (no any treatment), the second group (15 rats) served as positive control in which septic peritonitis was induced the third group (25 rats) was given Biomin®Imbo with food for 6 weeks and then exposed to induction of CLT and then CLTR, the fourth group (25 rats) was given 2 mL yoghurt twice daily orally for 6 weeks and then exposed to CLT and CLTR. Parameters that were studied included mortality following induction of the septic peritonitis, estimation of the serum level of c-reactive protein and

fibrinogen at the end of the first day following the induction of septic peritonitis, gross and microscopic lesions that occurred in the peritoneum and various internal organs at 1, 3, 7, 15 and 21 days periods following induction of septic peritonitis, estimation of the TLC and DLC of the peritoneal fluid at 24 h following the induction of septic peritonitis, estimation of the TLC and DLC of the blood at the end of the first day following induction of septic peritonitis and estimation of the bacterial growth in the peritoneal fluid at the end of the first day after induction of septic peritonitis.

RESULTS

Mortality rates that occurred in the various groups of rats at the end of the first, 3rd and 7th days following induction of septic peritonitis are shown in Fig. 1-3. No mortality was seen in the various groups at 15-21 days following induction of septic peritonitis. Mortality was zero in rats of the first group (negative control) during the whole experimental period. In group two (positive control) the mortality percentage was 33.3% at the end of the 24 h following induction of septic peritonitis. Mortality was zero in this same group at the end of the 3rd, 7th, 15th and 21st day after induction of septic peritonitis. In group three (given Biomin®Imbo) the mortality rate was 64% at the end of the first 24 h after induction of septic peritonitis. At the 3rd day in the same group, the mortality rate was 12%. In this same group, no mortality occurred during the 7, 15 and 21 days following induction of septic peritonitis. In the 4th group (given yoghurt) mortality was 72% at the end of the first day following induction of septic peritonitis. The mortality was 12% at the 3rd day and 4% at the 7th day following induction of septic peritonitis. Death was not encountered among rats of this group at the 15 and 21 days periods.

Significant differences ($p < 0.05$) was not encountered in the values of c-reactive protein in rats of the four groups at the end of the first day after induction of septic peritonitis. The values of fibrinogen in the various groups were 220 ± 14.14 (negative control), 342 ± 17.14 (positive control), 283 ± 3.19 (Biomin®Imbo) and $325 \pm 10.20 \text{ mg dL}^{-1}$ (yoghurt group). The level of fibrinogen was higher significantly ($p < 0.05$) in the positive control than in negative control rats. Similarly, the values of fibrinogen in the 3rd and 4th groups were significantly higher ($p < 0.05$) than in the negative control group.

Pathological lesions were not encountered in the peritoneum and various abdominal organs of any of the rats of the negative control group for the whole experimental period. In the experimental groups in which

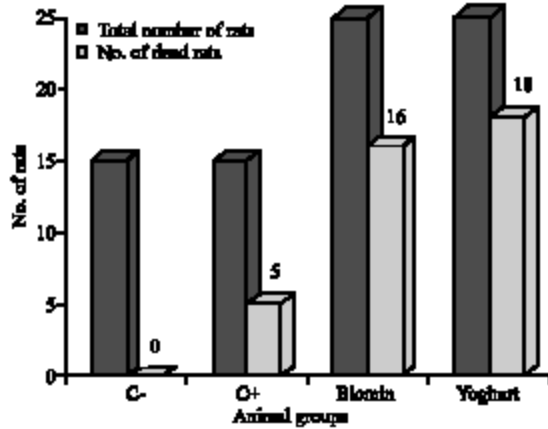


Fig. 1: Mortality of rats at 24 h following induction of septic peritonitis in the experimental group

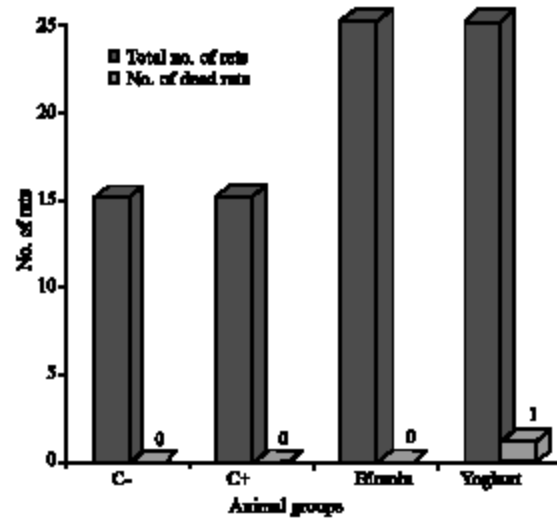


Fig. 3: Mortality of rats at the end of the 7th day after induction of septic peritonitis in the experimental groups as well as in the negative control group

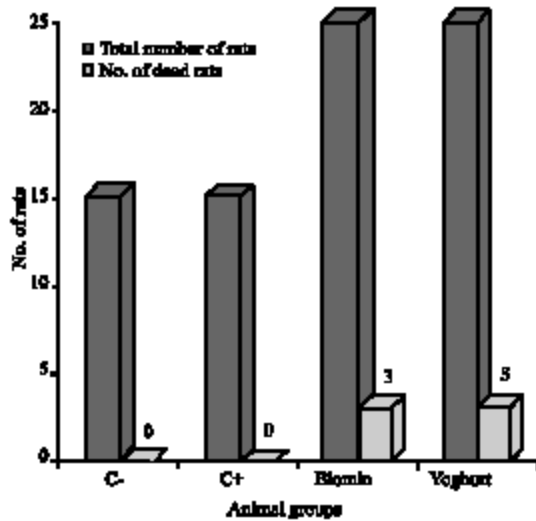


Fig. 2 : Mortality of rats at the end of the 3rd day after induction of septic peritonitis in the experimental groups as well as in the negative control group



Fig. 4: Abdominal organs of a rat one day after induction of septic peritonitis. White fibrinous exudate covers most of the organs leading to adhesion

septic peritonitis was induced, the gross and microscopic lesions were those of septicemia. The extension and severity of these lesions increased with time (from the first to the 21 p.i. day). The lesions were in the form of fibrin deposition over the visceral peritoneum and fragile adhesions between the peritoneum and adjacent internal organs (Fig. 4 and 5). Foci of necrosis or nodular cellular infiltration were seen on the serosa and extended into the organs or wall of the intestine. Suppurative inflammation (or local abscessation) of the peritoneum, cecum, kidneys, spleen and liver was observed (Fig. 6). Organization of the fibrinous exudate by fibrous tissue occurred with the formation of fibrous adhesions. These adhesions were fully mature at the end of twenty first day p.i (Fig. 7). In

comparison between the various animal groups, the lesions were more severe in rats of the 4th group (positive control group) for all of periods of the study.

Results of examination of the peritoneal fluid indicated that there were no significant differences in the TLC and DLC of rats of the various groups. Examination of the peripheral blood showed no significant differences between the total numbers of the basophils, eosinophils and platelets in rats of the various groups. Similarly, no significant differences were encountered in the neutrophilic count in rats of the 2nd, 3rd and 4th groups. However, the numbers of neutrophils were significantly higher ($p < 0.05$) in rats of the positive control and the 3rd (Biomn@Imbo) groups than in rats of the negative control

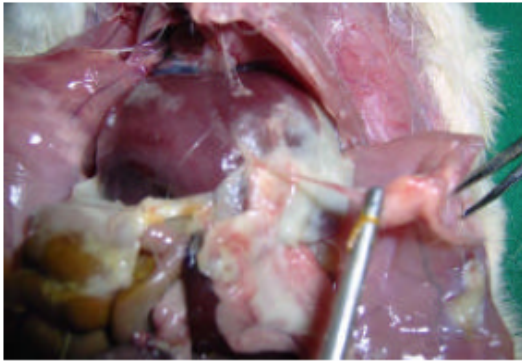


Fig. 5: Fibrous adhesion could be seen between the peritoneum and various internal organs in a rat, 15 days induction of septic peritonitis

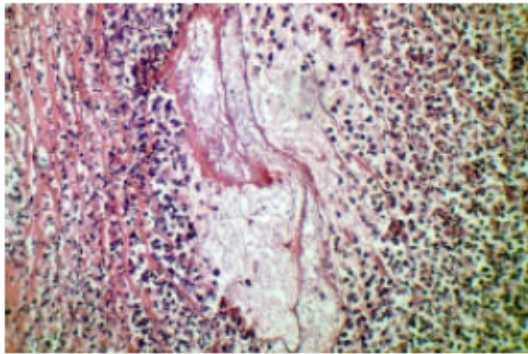


Fig. 6: Purulent inflammation of the cecum of rat 3 days after induction of septic peritonitis (H and E x115)

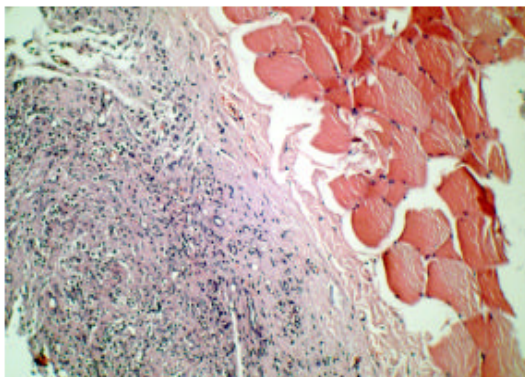


Fig. 7: Mature fibrous tissue replicating the peritoneal exudate and causing adhesion of the peritoneum to the adjacent organs in a rat, 21 days after induction of septic peritonitis (H and E x115)

group. The lymphocytic counts were similar in the 2nd, 3rd and 4th groups. However the lymphocytic count was significantly lower ($p < 0.05$) in rats of the positive control

group than in rats of the negative control group. Significant differences were not encountered in the count of the mononuclear cells in rats of the 2nd, 3rd and 4th groups. However, these counts were significantly lower ($p < 0.05$) than those of rats in the negative control group. The total leukocyte count was significantly higher in rats of the 3rd and 4th groups than in rats of the negative control groups. A similar significant difference was found between the total leukocyte count in rats of the 3rd and 4th groups and rats of the second (positive control) group.

Estimation of the mean viable bacterial growth in the peritoneal fluid at the end of the 1st day following induction of septic peritonitis showed that the highest mean of bacterial growth was in rats of the positive control (1×10^{11}) followed by rats of the 3rd group (89.6×10^6) and then rats of the 4th group (26×10^6). No bacterial growth was seen in the peritoneal fluid of rats of the negative control group.

DISCUSSION

In the study, a CLTR model similar to that described by Tsunoda *et al.* (2002) was used to induce septic peritonitis. This model is a modification of the CLP model and it provides many advantages over the commonly used endotoxemia models since it recapitulates the clinical septic situation in which bowel contents can escape into the peritoneal cavity following trauma or surgical manipulation. The initial CLTR experiments accomplished by Tsunoda *et al.* (2002) showed that mortality varied directly with the length of the opened bowel and it was fixed at 4 mm to obtain sublethal experimental groups. In the study, lethality occurred in the various experimental groups within 24 h after induction of septic peritonitis. The highest lethality (72%) was noted among rats of the 4th group (given yoghurt), then (64%) among rats of the third group (given Biomin@Imbo) and 33.3% in rats of the positive control group. At the 3rd p.i. day, lethality was 12% in rats of the 4th group (given yoghurt). At the 7th p.i. day, lethality was 4% in rats of the 4th group (given yoghurt). Lethality was not seen in rats of the various experimental groups after the 7th p.i. day. Similarly, mortality did not occur in the negative control group at any of the periods following induction of septic peritonitis. From these results it could be concluded that the period 24-72 p.i. h constitutes the critical period in the occurrence of lethality among rats of the various experimental groups. The lethality could be attributed to surgical trauma, manual manipulation of the cecum and the sudden and rapid entrance of large numbers of bacteria into the peritoneal cavity. It could be also concluded that neither Biomin@Imbo nor yoghurt was

efficient in protecting the rats from death particularly during the first 24 h after induction of septic peritonitis. In rats, stimulation of the acute phase proteins with Freund's complete adjuvant showed a 7-fold increase in haptoglobin (Hp) with no change in albumin or CRP following short increase in concentration of interleukin 1 and interleukin 6 (Giffen *et al.*, 2003). The acute phase proteins have been used to assess inflammatory change caused by Phosphodiesterase (PDE) inhibitor based drugs (Eckersall, 2006). In a study of the gastrointestinal toxicologic response to a PDE 4 inhibitor serum Hp concentration was found to mirror histological damage, as did the concentration of interleukin 6 (Dietsch *et al.*, 2006). These findings are in accordance with the results in the present study. Significant difference was not found in the values of CRP in the various groups of rats. In rats the Hp and Acid Glyco Protein (AGP) are moderate APP, while $\alpha 2$ macroglobulin (A2M) has been reported as being the primary major APP to monitor in this species (Giffen *et al.*, 2003). In the study, the levels of fibrinogen were significantly higher ($p < 0.05$) in rats of the experimental groups than in rats of the negative control group. This elevation of the level of fibrinogen is non-specific since it is known to occur in many acute bacterial infections, cancers, coronary artery disease, myocardial infarction, stroke, inflammatory disorders (rheumatoid arthritis and glomerulitis) and trauma (Lab Tests Online, 2008).

Gross and microscopic lesions that were observed in the peritoneum and internal organs of rats of septic peritonitis are due to bacteremia that results from the rapid transfer of specific pathogens from the peritoneal cavity in to the general circulation. Gram-negative aerobes/facultative anaerobes such as *E. coli* are by far the most common agents identified in the circulation in man and the laboratory species (Fubini and Ducharme, 2004). The occurrence of fibrinous adhesions is a part of the healing process of the peritoneum in response to damage. Persistence of the fibrin scaffold leads to invasion by fibroblasts which begin at 3-4 days. Collagen deposition by fibroblast and subsequent infiltration of capillaries create the more solid and potentially detrimental. Fibrous adhesion, usually within 7-14 days after injury (Fubini and Ducharme, 2004).

In study, no significant differences were found in the TLC and DLC of the peritoneal fluid of rats in the various experimental groups when compared to those of rats in the negative control group at the end of the first day after induction of septic peritonitis. This finding is in discrepancy with that of others (Tsunoda *et al.*, 2002) who found that peritoneal exudate cell accumulation observed 24 h after intraperitoneal injection of heat-killed *Lactobacillus cases* (LC9018) was significantly enhanced,

suggesting that augmentation of the resistance of mice to CLTR was caused especially by the induction of polymorphonuclear cells. This discrepancy could be explained in the basis of differences in the types of the probiotic used (LC9018 as compared to Biomin®Imbo and yoghurt). However, a significant increase has been reported in the total cell count (particularly neutrophils) in cases of peritonitis in cattle (Lab Tests Online, 2008), dogs (Tontis, 2004; Fossum *et al.*, 2007) and horses (Hendrix, 2002).

Results of hematological examinations accomplished in this study revealed that the TLC was higher in rats of 2nd, 3rd and 4th (experimental) groups than in rats of the negative control group. This increase in TLC of rats in which specific peritonitis was induced was found to be due to increase in the total number of neutrophils. These results are in accordance with those reported in horses (Mendes *et al.*, 2000) and with the statements mentioned by Radostits *et al.* (2007) that the TLC and DLC constitute a useful aid in the diagnosis of peritonitis and in assessing its severity. In acute diffuse peritonitis with toxemia there is usually a leukopenia, neutrophilia and marked increase in immature neutrophils (a degenerative left shift). In less severe forms of acute peritonitis of a few days duration there may be a leukocytosis due to a neutrophilia with the appearance of immature neutrophils (Radostits *et al.*, 2007).

In the study, the counts of bacterial growth in the peritoneal cavity of rats of the experimental groups arranged in order of decreasing frequency were the positive control group, the third group (given Biomin®Imbo) and finally the 4th group (given yoghurt). These findings indicate that the administration of yoghurt and to a lesser degree of Biomin®Imbo exerted a degree of protection to rats against bacterial growth in the peritoneal cavity. Similar results have been reported by Tsunoda *et al.* (2002) using LC9018 intraperitoneally in mice to decrease bacterial growth in the peritoneal cavity. These effects have been attributed to the activation of macrophages since the numbers of the peritoneal macrophages were increased 5 days after i.p. administration of LC9018.

CONCLUSION

It was observed that neither Biomin®Imbo nor yoghurt in the doses used in this study prevented lethality during the first 24-72 h p.i. In addition, the acute phase proteins (c-reactive protein and fibrinogen) could not be used as biomarkers for septic peritonitis. Gross and microscopic lesions that were seen in the present study were comparable to those of sepsis and were followed by healing of the peritoneum with the formation of fibrous

adhesions. TL and DLC of the peritoneal fluid were of no value in judging the presence or absence of peritonitis. Biomin®Imbo and yoghurt may have a lowering effect on the total bacterial growth in the peritoneal cavity at the end of the first 24 h p.i.

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