

Identification of Histological Changes in Liver and Evaluation of the Effect of Some Probiotics and Amino Acids on the Invasion of *C. jejuni*

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Abstract

Poultry meat represents a worldwide food and can be a source of pathogen agents (*Salmonella enterica* spp., *Escherichia coli*, and *Campylobacter jejuni*). Therefore, identification of the best methods to reduce infections of these pathogens from poultry meat represents a critical aspect for producers. The purpose of this study was to identify the histological changes in liver and to evaluate the influence of some probiotic strains and of some amino acids in the invasion of *Campylobacter jejuni*. The biological material was represented by poultry broilers, the hybrid ROSS 308, which were randomly allocated in seven experimental groups, of 10 individuals/group that were treated as follows: G0 (control); G1-*L. paracasei* CMGB 18 and *L. rhamnosus* CMGB 34 (0-42 days); G2-*L. paracasei* CMGB 18, *L. rhamnosus* CMGB 34, *L. lactis* CMGB 31 and *L. lactis* CMGB 32 (0-42 days); G3-*L. paracasei* CMGB 18 and *L. rhamnosus* CMGB 34 (35-42 days); G4-*L. paracasei* CMGB 18, *L. rhamnosus* CMGB 34, *L. lactis* CMGB 31 and *L. lactis* CMGB 32 (35-42 days); G5-*L. paracasei* CMGB 18, *L. rhamnosus* CMGB 34, L Treonine and DL Methionine (0-42 days); G6-*L. paracasei* CMGB 18, *L. rhamnosus* CMGB 34, *L. lactis* CMGB 31, *L. lactis* CMGB 32, L Treonine and DL Methionine (35-42 days). The microscopic aspects pointed out by us in this study suggest the pathogenicity of *C. jejuni*, marked by the appearance of inflammatory areas, with the presence of perivascular and diffuse leukocytes infiltration and, also, presence of pyknotic nuclei. Moreover, the results show the beneficial effect of probiotic strains *L. paracasei* CMGB 18, *L. rhamnosus* CMGB 34, *L. lactis* CMGB 31 and *L. lactis* CMGB 32, with reduction of the changes caused by *C. jejuni* and the production of toxins, thus preventing the damage of hepatic cells. The most powerful effect was noticed when all the four probiotic strains were administered, during the experiment of 0-42 days.

Keywords: *C. jejuni*, chicken broilers, liver, probiotic, histological changes

1. Introduction

Nowadays, food safety is a major problem for food industry and consumer. Many researches now aim to improve the quality of animal products, in conditions of security and safety for human consumption [1]. However, a large number of food-borne diseases occur as a consequence of

the development of pathogenic microorganisms in food, transmitted directly from animals to humans or through the food chain. In the European Union, most food-borne diseases are associated with *Campylobacter* and *Salmonella* [2, 3].

C. jejuni is a Gram-negative bacterium, microaerophilic and spirillate and *C. jejuni* infection is one of the most common causes of the gastroenteritis worldwide [4-6]. The chicken represents a significant reserve for *C. jejuni* because the chickens are most effectively colonized without causing symptoms. They can cause a number of infections [7] in humans by

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ingestion of undercooked meat [8, 3]. The important source of campylobacteriosis in humans is the chicken meat [9, 10, 11]. In addition, infection with *C. jejuni* leads to complications such as Guillan - Barre syndrome (GBS), an acute inflammatory peripheral polyneuropathy [12], reactive arthritis [13], inflammation of the bowel and irritable bowel syndrome [14, 15]. Although *C. jejuni* colonization of the small intestine and colon mucus, it can be found in other organs such as the liver and spleen.

Therefore, to alleviate infections with these pathogens, it becomes necessary to identify strategies for reducing pathogens in farms [9, 16, 17], but without resorting to antibiotics. An area of interest to biomedical research in prevention and treatment of bacterial intestinal infections is the use of probiotics [18] that have beneficial effects on animal health, increase of growth performance and decreasing of pathogens in the food chain [1].

Probiotics are defined as “Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [FAO/WHO-19]. They have a great ability to adhere at the epithelial cells and by colonization of the intestinal mucosa, they form a barrier which prevents the infections produced by enteropathogens, especially *Campylobacter* [1, 20].

The aim of this study was to identify the histological changes in the liver and to evaluate the influence of certain probiotics and amino acids in invasion of *C. jejuni*.

2. Materials and methods

The biologic material used was represented by 280 poultry broilers belonging to ROSS308 breed, which were randomly divided in 7 experimental variants and used in difference investigations, including histological evaluation.

Table 1. Experimental scheme used for determining the effect of probiotic administration in policulture at a concentration of 10⁹CFU/kg feed

Group L0	Group L1	Group L 2	Group L 3	Group L4	Group L5	Group L6
The moments of probiotics administration in feed						
Without probiotics (control)	0-42 days	0-42 days	35-42 days	35-42 days	0-42 days	35-42 days
Probiotic strains administered						
-	<i>L. paracasei</i> CMGB 18	<i>L. paracasei</i> CMGB 18	<i>L. paracasei</i> CMGB 18	<i>L. paracasei</i> CMGB 18	<i>L. paracasei</i> CMGB 18	<i>L. paracasei</i> CMGB 18
	<i>L. rhamnosus</i> CMGB 34	<i>L. rhamnosus</i> CMGB 34	<i>L. rhamnosus</i> CMGB 34	<i>L. rhamnosus</i> CMGB 34	<i>L. rhamnosus</i> CMGB 34	<i>L. rhamnosus</i> CMGB 34
		<i>L. lactis</i> CMGB 31		<i>L. lactis</i> CMGB 31	CMGB 34	<i>L. lactis</i> CMGB 31
		<i>L. lactis</i> CMGB 32		<i>L. lactis</i> CMGB 32		<i>L. lactis</i> CMGB 32
Synthetic amino acids added in feed						
-	-	-	-	-	L Treonine DL Methionine	L Treonine DL Methionine

In the structure of compound feed, coccidiostats were not introduced. Chickens were fed with three structures of compound feed according to the recommendations in the growth boom for Ross 308 hybrid. The light regime was 23 hours light and 1 hour darkness. The temperature in the hall was 32⁰C at the beginning of the experiment and was gradually reduced to 21⁰C at 21 days.

At the end of experiment, three individuals were slaughtered from each experimental variant for taking samples of liver. A preliminary stage

consisted in identification the *C. jejuni* presence at individuals of those seven experimental groups, by realization of bacterial cultures isolated from cloaca swabs, cecal content and cecal mucus and identification of species of *C. jejuni* by API Campy assay. This assay was performed according to the manufacturer's instructions. Identification and confirmation of *C. jejuni* strains was performed by PCR and Western blotting.

For the histological examination, the liver samples were fixed in neutral formalin 10% and embedded

in paraffin, before previously being dehydrated in three baths of ethyl alcohol solutions (100°) and clarified in two baths of acetone and two baths of benzene. The paraffin blocks was sectioned to a thickness of 4 µ. Histological sections were stained with Mallory trichrome method [21] and were examined with research microscope CX41 Olympus equipped with digital camera and image analysis software.

3. Results and discussion

In individuals from the Group L0 (control), histological sections performed by liver reveals the classical architecture of hepatic parenchyma, consisting of liver lobules, delimited between them by perilobular connective tissue.

In the Kiernan interlobular spaces are present mild hypertrophic vascular processes. In the structure of hepatic lobules, hepatocytes are arranged in cords oriented toward centrilobular vein. Hepatocytes are hypertrophic, with vacuolar cytoplasm, with a few and finely granules, having clear appearance (Figures 1b, 1c). Among hepatocytes cords are sinusoidal capillaries, slightly hypertrophic. On some areas, it signals histological changes which are characterized by mild congestive aspects (Figure 1b) and the presence of leukocyte cells with diffuse spreading (Figure 1a). In these territories, the hepatocytes have a more intensely colored cytoplasm and their nuclei are pyknotic, aspect that suggests the manifestation of cell death process by apoptosis and involvement of macrophages in the phagocytose of apoptotic bodies.

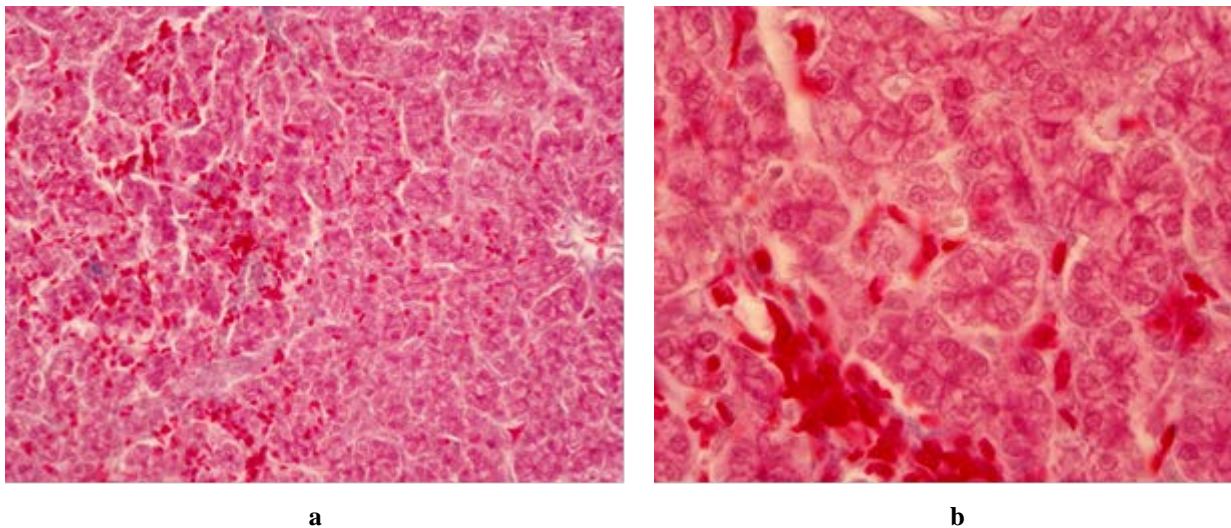


Figure 1. Liver Group L0.

- a. Diffusely infiltrated leukocyte, hepatocyte cells with cytoplasm intensely coloured and pyknotic nuclei (400x);
- b. Mild congestion and hypertrophic hepatocytes with clear cytoplasm (1000x)

The pathogenic microorganisms use a variety of molecular strategies to destroy the machinery of host cells, strategies that allow these pathogens to invade host cells.

Identification and characterization of virulence factors is a major activity of microbiological research. Knowledge of nature, regulation and the mechanism of action of virulence factors are indispensable for prevention and treatment of infectious diseases. Currently are recognized four important properties of bacterial virulence: motility, adhesion, invasion, and production of toxins [22]. A large number of pathogenic Gram Negative bacteria (*Campylobacter spp.*,

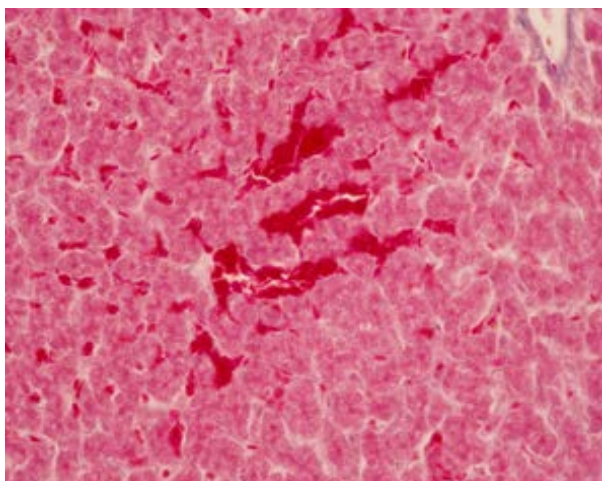
Escherichia coli, *Haemophilus ducreyi*, *Helicobacter spp.*, *Salmonella enterica* serotype *Typhi* and *Shigella spp.*) produce cytolethal distending toxin (CDT) [23]. A number of data indicate that this toxin contributes to the pathogenicity of the bacteria *in vivo*. CDT secreted by *Helicobacter hepaticus* and *Campylobacter jejuni* are essential for persistent infection of the gastrointestinal tract and increases the severity of mucosal inflammation and liver disease in mice [24].

Bacterial CDT belongs of the type A-B2 toxin usually composed of three subunits: CDTA, CdtB and CdtC [25]. CdtB subunit has catalytic activity

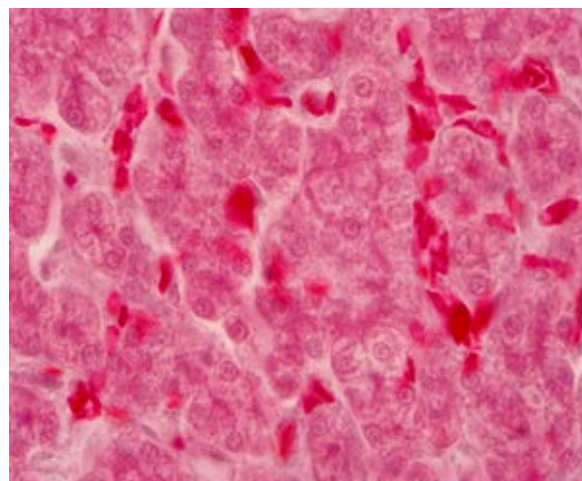
similar DNA-ase I, and the CdtA and CdtC subunits are required for binding holotoxin to the specific receptors from plasma membrane of target cells allowing internalization active CdtB, by dynamin dependent endocytosis [26]. CdtB can translocate into the nucleus [23, 27] where it has a toxic activity, process mediated by a nuclear localization signal NLS [28]. Consequently, CdtB induce host DNA damage, which leads to arrest of the target cells in phases G1 and/or G2 of the cell cycle [29, 30] and the activation of DNA repair mechanisms [24]. These processes are coordinated by ATM-Chk2 and ATR-Chk1 kinase [23].

The arrest of the cell cycle induced by CDT determines the expression of the p53 tumor suppressor protein that induces transcription of the gene p21, increasing of the p21 protein synthesis, a multipotent inhibitor of cyclin dependent kinase. The final consequence is inhibition of Rb protein

phosphorylation and, thus, the arresting cell cycle progression. The cell cannot pass in the S phase of cell cycle and cannot divide until the lesions present into its genome are not repaired. The cells that do not repair the damage will undergo apoptosis [29-31]. The microscopic analysis of liver sections reveals, at individuals from experimental Group L1, in which were administered *L. paracasei* CMGB 18 and *L. rhamnosus* CMGB 34 (0-42 days), the hypertrophic aspect of interlobular blood vessels, centrilobular vein and of sinusoidal capillaries (Figure 2a). On certain territories there were signals of congestive aspects. The liver parenchyma consists of hepatocytes slightly hypertrophic, with finely granular cytoplasm, and among them there are cells with vacuolar cytoplasm and with clear appearance (Figure 2b). On small areas appear hepatocytes with pyknotic nuclei and apoptotic bodies (Figure 2a).



a



b

Figure 2. Liver Group L1.

a.Hypertrophy of sinusoidal capillaries, pyknosis and apoptotic bodies (400x)

b.Hypertrophic hepatocytes (1000x)

Regarding individuals in experimental group L2, in which were administered four probiotics, respectively *L. paracasei* CMGB 18, *L. rhamnosus* CMGB 34, *L. lactis* CMGB 31 and *L. lactis* CMGB 32 during all experimental period (0-42 days), the microscopic analysis do not revealed the histological changes which have been found in individuals from control group.

In these individuals, the hepatic parenchyma has a normal aspect, which is formed from polygonal

hepatocytes, with their cytoplasm, predominantly, finely granular (Figures 3a, b) and only on some territories, the hepatocytes are slightly hypertrophic, with vacuolar cytoplasm. Among the hepatocyte cords there are sinusoidal capillaries which have not hypertrophic appearance. Also, the venules and arterioles from the portal triads have normal appearance. Was not signaled the presence of infiltrate leukocyte cells (Figures 3a, b).

These microscopic aspects suggest the beneficial effect of probiotics administered from the first day post-hatched, by attenuating the growth of *C.*

Jejuni and reducing the toxins production and thus preventing cell damage.

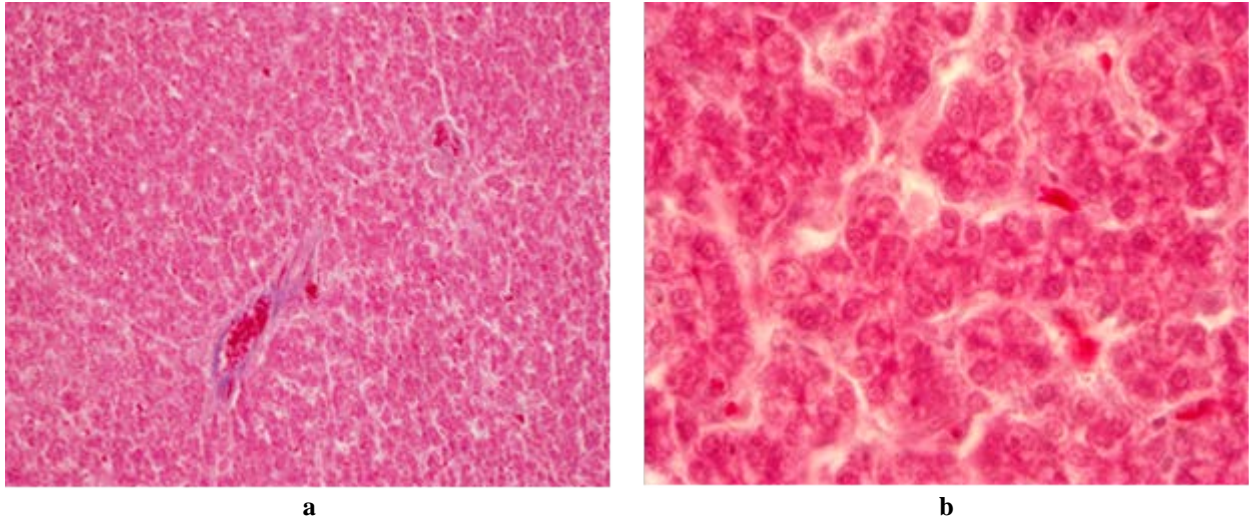


Figure 3. Liver Group L2.
a. Overview of liver parenchyma (100x);
b. Hepatocytes with finely granular cytoplasm and sinusoidal capillaries (1000x)

The bacterial mixtures are used to intensify the beneficial effects of probiotics [32].

Numerous studies have shown that probiotics reduce harmful microorganism in the intestinal tract and their toxin production by stimulating the immune response and producing antimicrobial factors [33-35]. Probiotics such as *Lactobacillus* strains can use different mechanisms of protection [9, 1, 36] as co-aggregation with the pathogen, competition with pathogens for attachment at the membrane sites of mucosal surface [37], production of the antimicrobial compound, lactic acid and hydrogen peroxide [Campana 2012, Neal- McRinnery et a., 2012, Zhao et al., 2006, cited by 9]. Chaveerach et al., 2004 [38] have shown that probiotics such as *Lactobacilli* and *Bifidobacteria* inhibited the growth of different *C. jejuni* and *C. coli* strains, aspect correlated with the flagellar factor *flaA* [39].

Unlike the individuals from experimental groups L2 and L1, in case of individuals in the **groups L3 and L4** in which probiotics were administered only in the last week of experiment, in the range of 35-42 days, respectively, although the liver architecture is normal and the hepatocytes have finely granular cytoplasm (Figures 4a, 4b, 5b), on

small areas it was signaled the presence of pyknotic nuclear and perivascular leukocytes (Figures 5a, b). Sinusoidal capillaries, centrilobular veins and interportal vessels are slightly hypertrophic.

The human clinical studies show systemic spread of *C. jejuni*, at the hepatic level, through the bloodstream, during early stages of infection. This is experimental supported by the fact that *C. jejuni* can be recovered from blood and liver of infected mice, 60 minutes after oral inoculation [40, 41]. The *C. jejuni* ability to persist in hepatobiliary tract varies depending on strain [41].

In the individuals from the experimental group L5, in which were administered two probiotics (*L. paracasei* CMGB 18 and *L. rhamnosus* CMGB 34) and two amino acids (L threonine and DL methionine), throughout the experiment (0-42 days), microscopic examination reveals a slightly hypertrophic process of the blood vessels (Figures 6a, b). The hepatocytes are arranged in uniform cords oriented to centrilobular vein and their cytoplasm is, generally, finely granular (Figure 6b). Perivascular, in some areas, it noticed the presence of hepatocytes with intense pyknotic nucleus and apoptotic bodies (Figure 6b).

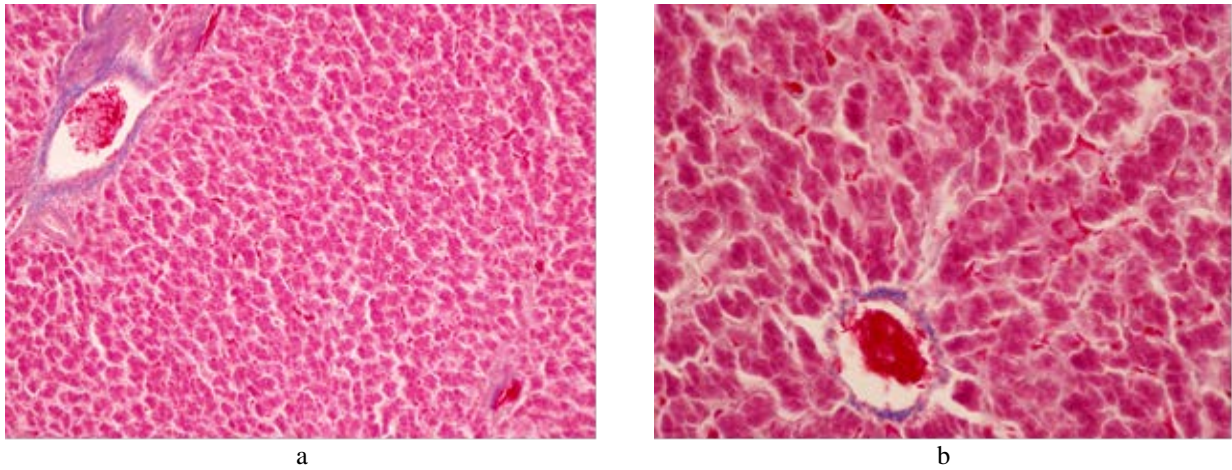


Figure 4. Liver Group L3. a. Overview of liver parenchyma (100x);
b. Slightly hypertrophic vascular processes (400x)

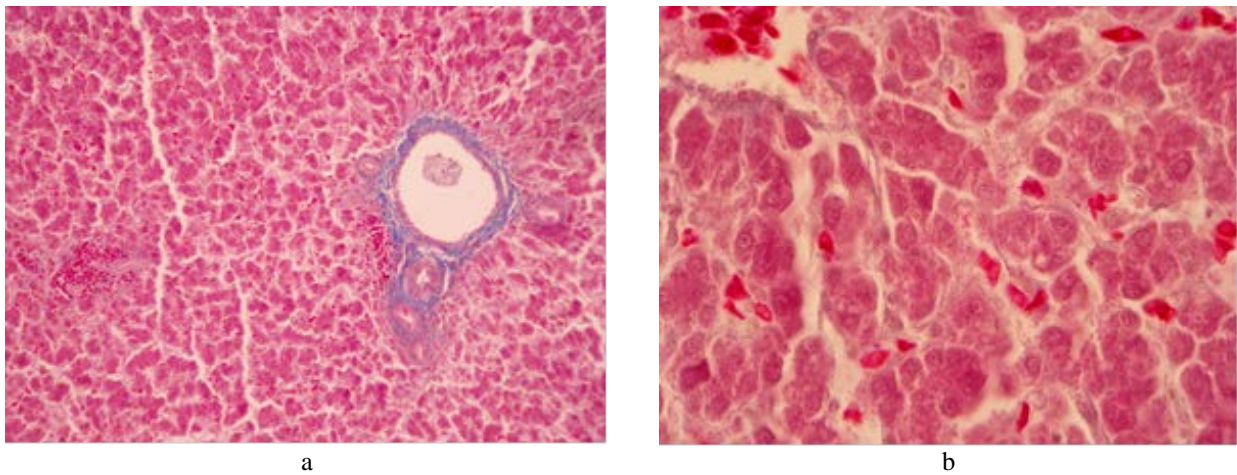


Figure 5. Liver Group L4. a. Perivascular leukocyte (200x); b. Hepatocytes with finely granular cytoplasm between them are the hepatocytes slightly hypertrophic and vacuolar (1000x)

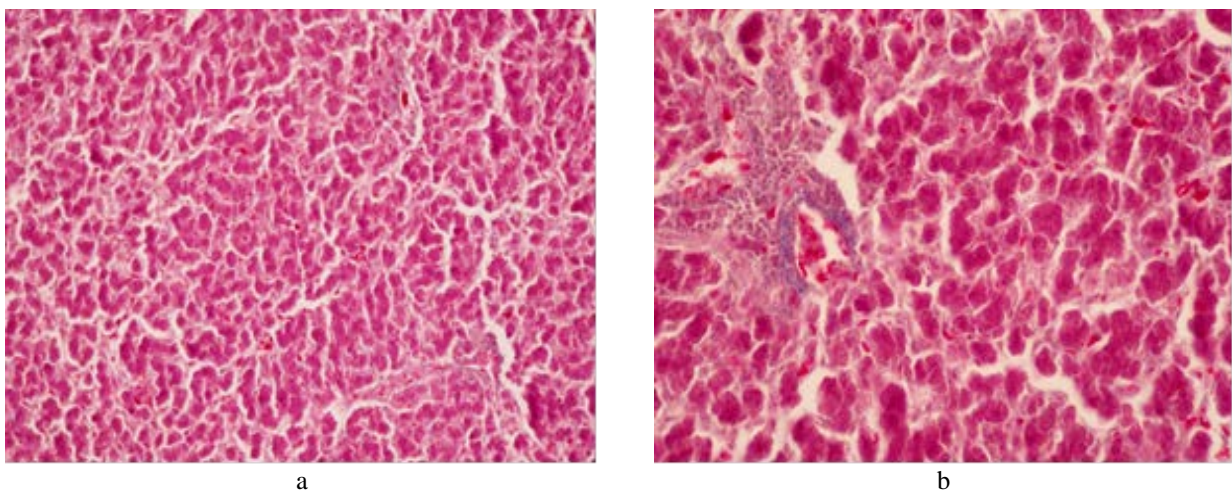


Figure 6. Liver Group L5. a. Overview (200x); b. Perivascular pyknosis (400x)

In the experimental group L6, in which were administered four probiotics (*L. paracasei* CMGB 18, *L. rhamnosus* CMGB 34, *L. lactis* CMGB 31, *L. lactis* CMGB 32) and two amino acids (L threonine, DL methionine) but only in the last week of the experiment (35-42 days) it was signaled, in some area, compact groups of cells

with pyknotic nuclei accompanied by infiltrating leukocytes (Figures 7a, b). In the structure of hepatic lobules, hepatocytes have the cytoplasm with finely granulation and one or two nuclei. The sinusoidal capillaries arranged at the vascular pole of hepatocytes have a normal appearance (Figure 7a).

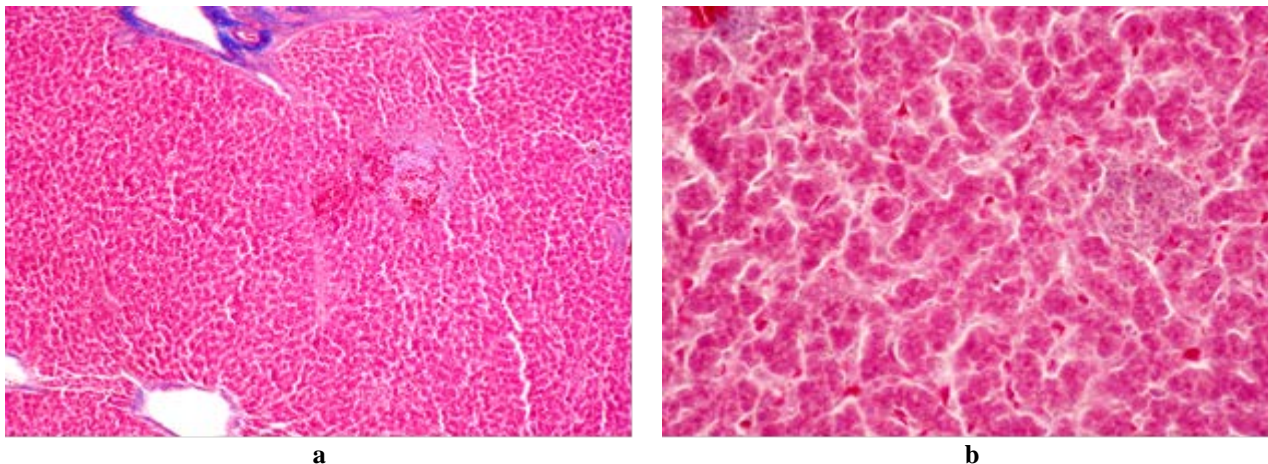


Figure 7. Liver Group L6. a-b. Overview: leukocytes infiltration and pyknosis (100x; 400x)

Analyzing microscopic appearance of the liver in individuals from experimental groups compared with the control group (L0) it was found that histological changes are more intensively in individuals in control group.

Although the beneficial effect of probiotics was reported in individuals from all experimental groups, it was more pronounced in individuals from the experimental group L2, in which was administered a mixture of four probiotics in the feed, immediately post hatched, until the end of the experimental period (42 days). In these individuals there were not reported histological changes in the liver parenchyma.

4. Conclusions

Microscopic aspects reported in this study suggest pathogenicity of the *C. jejuni* in liver, aspects supported by the appearance of inflammatory areas, with the presence of perivascular and diffuse of leukocyte cells and the presence of pyknotic nuclei which suggests the manifestation of process of cell death by apoptosis. Also, the use of probiotics attenuates the pathogenicity of *C. jejuni*, by inhibiting growth and by reducing production of toxins, thus preventing damages of the liver cells.

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