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Effect of bovine lactoferrin in *Salmonella* ser. Typhimurium infection in mice

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Abstract Lactoferrin (LF) has in vitro antimicrobial activity against Gram-negative bacteria. *Salmonella enterica* subsp. *enterica* serovar Typhimurium causes systemic infection and acute diarrhea in humans, mainly in children younger than 2 years of age. The aim of the study was to determine the in vivo effect of bovine LF in *Salmonella* ser. Typhimurium infection in mice. 58 BALB/c mice were employed. Two hours before the infection with 300 µl of 10^7 CFU of *Salmonella* ser. Typhimurium, 29 mice received LF (2 mg) and 29 placebo (buffer). After the infection, the mice received LF (10 mg/ml) ad libitum or buffer, respectively, for 7 days. Mortality, weight and clinical signs (piloerection, hunched position and reduced movement) were monitored daily. The degree of inflammation and necrosis in the intestine, liver, spleen and brain were studied with a blinded observer. The mortality in the control group (8/29) was higher than in the LF group (1/29) (Kapplan Meier $P < 0.05$). From the third day post-infection the control group were significantly more symptomatic ($P < 0.05$). The blood culture for *Salmonella* spp. was positive for all mice studied in the control group (17/17), but positive in the LF group in only 6/17 animals ($P < 0.05$). In the LF group, the pathologic studies show less inflammation and focal necrosis in the four organs studied, with the greatest difference found in the intestine. Bovine LF protects against *Salmonella* ser. Typhimurium infection in mice, reducing the severity, mortality and the degree of inflammation of this infection.

Keywords Lactoferrin · Salmonella · Mice · Type three secretory system · Inflammation

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Introduction

In recent years it has been suggested that lactoferrin (LF) is protective against various enteropathogens including *Shigella flexneri*, enteropathogenic *Escherichia coli* (EPEC) and enteroaggregative *E. coli* (EAEC) (Gomez et al. 2002; Ochoa et al. 2006; Ochoa et al. 2003).

Human LF interacts with surface bacterial proteins of *Salmonella enterica* subsp. enterica serovar

Typhimurium and reduces their ability to adhere to host cells in an in vitro model (Bessler et al. 2006). In vivo, the LF effect in a Sa*lmonella* ser. Typhimurium infection has not been studied. In mice, this bacterium causes a systemic disease similar to typhoid fever in humans, characterized by enterocolitis, invasion of the liver, the spleen and finally the brain (Hsu 1989; Wickham et al. 2007). Because these findings are striking in this model, it is possible to use it to study the effect of LF in *Salmonella* ser. Typhimurium infection.

Materials and methods

Bacterial strain

Salmonella ser. Typhimurium strain C52, a well characterized virulent strain was used throughout this study. Its virulence is due to the presence of a plasmid (pIP1352) (Kusters et al.1993; Norel et al. 1989; Pardon et al. 1986). Bacteria were cultivated overnight in Trypticase Soy Broth (TSB) and reactivated in the same media. When the culture reached the required optical density (OD), the bacteria were washed by centrifugation and transferred to buffer saline phosphate for animal infections. Three dilutions of the bacterial culture were plated on agar to determine the number of colonization forming units (CFU) to confirm that it corresponded to the bacterial concentration expected from the OD measurement.

Lactoferrin

Bovine LF was obtained from Tatua Biologics (Tatua, New Zealand). The purity of the LF was 90% with an iron saturation of 15%. All experiments were done with a LF concentration of 10 mg/ml, which is approximately the human LF concentration present in colostrum.

Experimental animals

Experimental animals were female mice Balb/c strain between 6 and 8 weeks of age with a weight between 20 and 24 g (Wickham et al. 2007). Animals were healthy prior to inoculation. The use of these mice and the procedures performed, were approved by the Animal Ethics Research Institutional Committee at Universidad Peruana Cayetano Heredia.

Animal infections and tissue samples

Two hours before the infection, the LF group (29 mice) received 200μL of LF (10 mg/ml) and the control group received 200 μl placebo: phosphate buffer saline (PBS). Animal infections were carried out with 300 μl of 10^7 CFU of *Salmonella* ser. Typhimurium. For infection and pre-treatment inoculations, a gavage needle was used. After the infection the mice received LF (10 mg/ml) or PBS ad libitum, respectively, for 7 days. The mortality, weight and clinical signs (piloerection, hunched position and reduced movement) were daily monitored in all mice for 7 days after infection. The incidence of clinical signs was determined by comparing the behavior of each infected mouse with a healthy mouse for 15 min. At day 7 post-infection (p.i.), animals were sacrificed and cardiac puncture was performed for blood cultures. For histopathologic analysis, organs (intestine, liver, spleen and brain) were removed.

Pathology scoring

Tissues from the four organs removed from the LF ($n = 13$) and control group ($n = 13$), were fixed in 1% buffered formaldehyde, embedded in paraffin and stained with hematoxylin and eosin (Coombes et al. 2005). The degree of inflammation and necrosis in the organs was studied with a pathologist blinded to group assignment in order to prevent bias. The degree of inflammation was scored on a 0 to 3+ scale (1+, 2+ mild inflammation and 3+ moderate inflammation).

Statistical analysis

Survival was analyzed with Kaplan–Meier (log rank). The changes in weight were evaluated for significance using the Student's t test (mean ± SEM). For categorical variables (clinical signs, blood cultures and histopathology scores), χ^2 test was performed. Significance was defined as a $P < 0.05$ for all analyses. SPSS v12 was used for the analyses.

Results

Lactoferrin group suffered lower mortality

Fifty-eight mice were challenged with 10^7 *Salmonella* ser. Typhimurium, (29 in the LF group, 29 in the placebo), and monitored daily for survival over the subsequent week. A total of nine deaths occurred, eight in the control group from day 3 p.i to day 7 p.i., and only 1 death in the LF group at second day p.i. Kapplan Meier (log rank, $P < 0.05$) (Fig. 1).

Lactoferrrin group did not lose weight

Weight loss was daily monitored from all studied mice ($n = 58$) at the same time of day for a week. The control group ($n = 29$), had weight loss at the third day post infection compared with day 1 ($P < 0.05$). In both groups (LF and control), the mice gained weight at days 6 and 7 p.i. compared to day 4 in the LF group and compared to day 5 in the control group ($P < 0.05$) (Fig. 2).

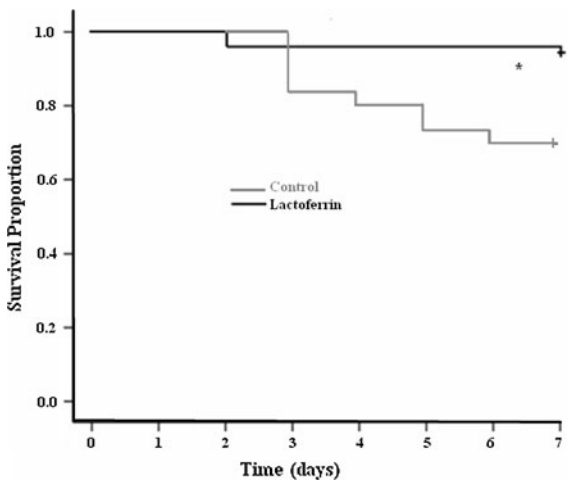

Fig. 1 Survival rates of female Balb/c mice orally challenged with 10^7 *Salmonella* ser. Typhimurium. Two hours before the infection the animals received either 2 mg of LF (LF group) or 200 µl PBS (control group) and subsequently they received LF (10 mg/ml) or PBS ad libitum for a week. The Kaplan–Meier survival analysis showed less deaths in the LF group than in control group (* $P < 0.05$; long rank). Number of mice in the LF group $n = 29$ and in the control group $n = 29$

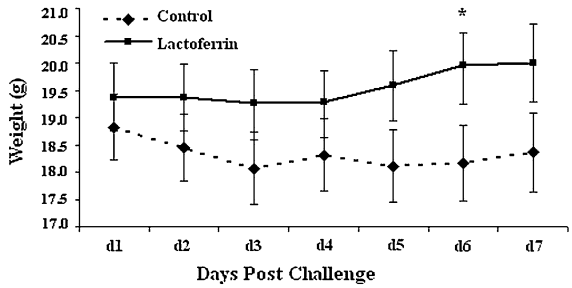

Fig. 2 Weight changes in LF (*filled square*) and control group (*filled triangle*) of mice challenged with 10^7 *Salmonella* ser. Typhimurium. There was a tendency for differences in weight between LF and control group for day 6 p.i. (* $P = 0.06$)

Lactoferrin group had less clinical signs of infection

Three clinical signs (piloerection, hunched position and reduced movement) typical of acute *Salmonella* ser. Typhimurium infection were studied in both groups. More mice from the control group developed significantly more clinical signs compared to mice from the LF group (Fig. 3).

Lactoferrin group had less bacteremia

At day 7 p.i cardiac puncture was performed in order to do blood cultures. The bacteria obtained from blood cultures were biochemically and serologically identified. The blood culture for *Salmonella* spp. was positive for all mice studied in the control group (17/17), but positive in only 6/17 (35%) in the LF group ($P < 0.05$).

Lactoferrin group had less histopathologic abnormalities

At day 7 p.i all animals were sacrificed and 4 organs (intestine, liver, spleen and brain) were removed for pathologic studies. Histopathology analyses were performed for 26 mice, 13 from each group (LF and control). In the intestine, the control group had necrotizing inflammation and damage in the intestinal mucosa in 85% compared with the 15% present in the LF group ($P < 0.001$) (Fig. 4a, b; Table 1). In the liver, granulomas and parenchymal necrosis were present in some animals in both groups; the control group had inflammation in all animals compared with 69% with inflammation in the LF group ($P < 0.05$).

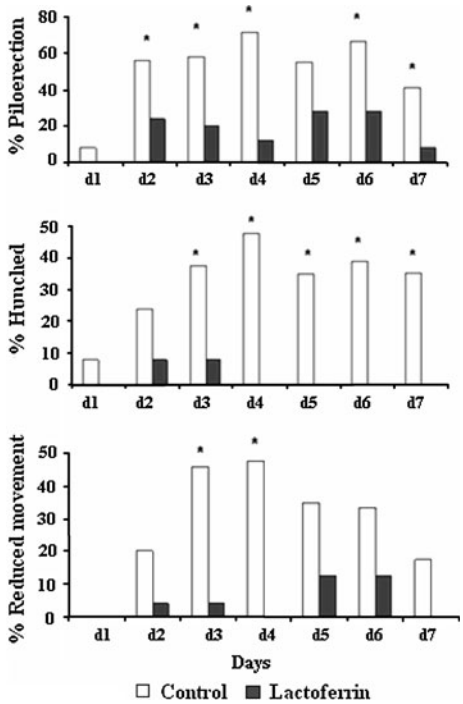

Fig. 3 A total of 58 mice, 29 from each group (LF and control) were monitored daily over a week for clinical sings of acute infection with *Salmonella* ser. Typhimurium to determine the percentage of animals in each group with the presence of specific clinical signs. * $P < 0.05$ for the comparison between LF (*filled square*) and control (*open square*) groups

Kuppfer cell hyperplasia, a liver inflammation marker was not different between the groups (Fig. 4c, d; Table 1). In the case of spleen, focal necrosis and granulomas were present in both groups with identical frequencies (92% in each group) (Fig 4e, f; Table 1). In the brain, gliosis, glia cell proliferation, (92 vs. 100%) and cerebral edema (100 vs. 77%) were observed in LF and control group, respectively; these differences were not significant (Fig. 4g, h; Table 1).

Discussion

In this study there were three main results: the mice infected with *Salmonella* and treated with LF had lower mortality, less clinical signs of the infection and less damage in the epithelial integrity of the gut. In susceptible strains like BALB/c, a concentration of 10^8 CFU is considered a lethal dose, which causes death in 4 or 5 days post-infection (Sebastiani et al. 2002); therefore, the dosis that we used (10^7 CFU) was sub-lethal but high enough to cause a systemic infection that sometimes led to death. We found a significant difference in mortality between the LF and control groups supporting the protective effect of LF in this bacterial infection. The second significant result was the change in weight and clinical signs of acute infection (piloerection, hunched position and reduced movement) due to enterocolitis in the LF group in concordance with previous reports (Bessler et al. 2006; Ochoa and Cleary, 2009). The third significant result found was the difference in the histopathology of the four organs studied between LF and control group. The gut, which is the first organ affected by *Salmonella* ser. Typhimurium, had a significantly less epithelial damage in the LF treated group (15 vs. 85%), which could be explained by a lower bacterial load and less adherence of the bacteria to the gut ephitelium.

Multiple mechanisms have been proposed to explain the LF effect on enteropathogens: it can chelate iron and is therefore bacteriostatic; LF can also adhere to lipid A of bacterial lipopolysaccharide (LPS) changing its rigidity and desestabilizating it (Appelmelk et al. 1994; Brandenburg et al. 2001; Ellison et al. 1988). It has been determined that LF in EPEC and *S. flexneri* blocks actin polymerization in epithelial cells by degradating proteins of the type three secretory system (TTSS), the needle complex mechanism by which the bacteria can attach to or invade host's tissues (Gomez et al. 2002; Ochoa et al. 2003). In vivo studies have shown that LF protects mice from lethal intravenous doses of *E. coli* (Zagulski et al. 1989) and rabbits from enteritis induced by *S. flexneri* (Gomez et al. 2002). In *Salmonella* ser. Typhimurium infection, LF reduces bacterial ability to adhere to host cells in an in vitro model (Bessler et al. 2006). Independent of iron, LF interacts with this bacteria and produces the decrease of the bacterial metabolic rate, increasing the bacteriostasis. It has been postulated that LF damages the membrane of *Salmonella* at LPS, causing permeability problems and destabilizing the bacterial membrane (Naidu et al. 1993). We speculate that LF protective effect in Salmonella infection in mice, could be explained by the interaction of LF with the TTSS, similar to its effect on EPEC and *Shigella*.

There are over 2,460 *Salmonella* serotypes. *Salmonella* ser. Typhimurium and *Salmonella* ser.

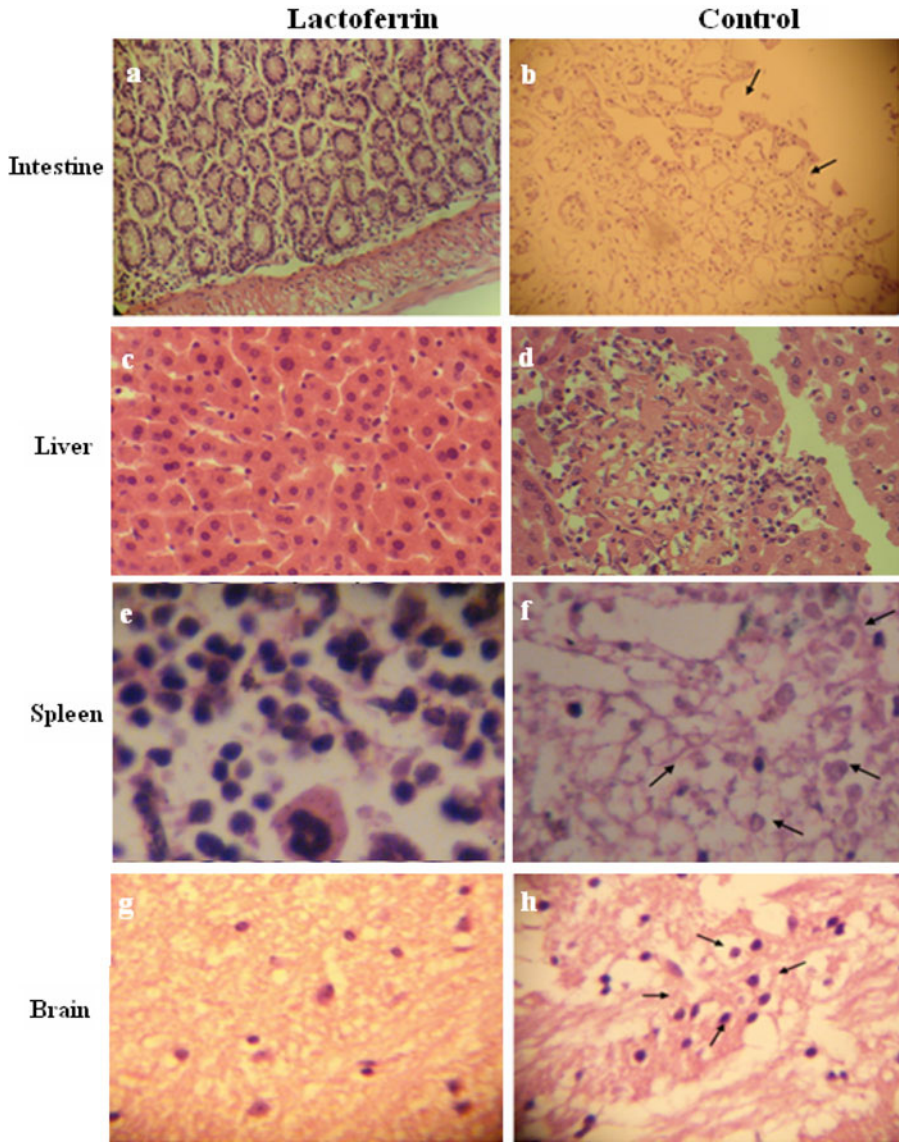

Fig. 4 At day 7 p.i. animals were sacrificed (13 mice from LF and 13 from control group) and the histopathology was evaluated for intestine, liver, spleen and brain. In intestine, the LF group had normal mucosa (**a**); the control group had transmural necrosis indicated with *arrows* (**b**). In the case of liver, the control group had granulomas (**d**), a finding not present in the LF group (**c**). In the spleen, focal necrosis was found (see *arrows*) in the control group (**f**) but not in the LF group (**e**). In the brain, edema and gliosis were present in both groups (**g**, **h**); the *arrows* in (**h**) indicate the gliosis

Enteritidis are the species mainly associated with human infections. *Salmonella* ser. Typhimurium causes systemic infection and acute diarrhea in humans, mainly in children younger than 2 years of age (Foley et al. 2006). *Salmonella* adheres to and enters into the M cells of the Peyer patches, arrives to the basal membrane of the epithelium, where it can be phagocytized by the neutrophils or macrophages which take it to the mesenteric lymph nodes and then to the circulatory system. The bacteria can also be phagocitized in the gut lumen by macrophages CD18+ and then transported to the circulatory system (Sansonetti 2004; Vazquez-Torres et al. 1999). Once in the blood, the bacteria travel intracellularly inside the macrophages and arrive to the liver and spleen where exponential replication may occur (Haraga et al. 2008; Nilsson et al. 2004).

The exponential replication of the bacteria produces a high level of inflammation in the liver and spleen. We found a significant diffference in liver inflammation in the control group versus the LF group, consistent with a LF protective effect (Table 1). The degree of inflammation in the spleen was mild in the LF group and moderate in the control group. *Salmonella* ser Typhimurium infection reaches the brain using macrophages as "Trojan Horses" for

Table 1 Histological analysis of the recovered organs

	Lactoferrin *n/N* (%)	Control *n/N* (%)
Liver		
Inflammation	9/13 (69)	13/13 (100)*
Mild	3/9 (33)	3/13 (23)
Moderate	6/9 (67)	10/13 (77)
Kuppfer cells hyperplasia	13/13 (100)	13/13 (100)
Mild	9/13 (69)	8/13 (62)
Moderate	4/13 (31)	5/13 (38)
Intestine		
Epithelial integrity damage	2/13 (15)	11/13 (85)**
Mild	2/2 (100)	4/11 (36)
Moderate	0	7/11 (64)
Spleen		
Inflammation	12/13 (92)	12/13 (92)
Mild	7/12 (58)	2/12 (17)
Moderate	5/12 (42)	10/12 (83)
Brain		
Cerebral edema	12/13 (92)	13/13 (100)
Mild	7/12 (58)	7/13 (54)
Moderate	5/12 (42)	6/13 (46)
Gliosis	13/13 (100)	10/13 (77)
Mild	13/13 (100)	8/10 (80)
Moderate	0	2/10 (20)

* $P < 0.05$ and ** $P < 0.001$ for the comparison between LF and control groups

crossing the hematoencephalic barrier (Nilsson et al. 2004). In the brain, high concentrations of bacteria cause edema in several mice strains, including Balb/c (Vidal et al. 1995). In this study we observed edema, gliosis, and cerebral macrophages (glia cells) proliferation. We did not find statistically signficant differences in edema but we did find that gliosis was more severe in the LF group. This result could be explained by LF antimicrobial properties, that activate TNFα, IL8 and nitric oxide at equal or higher concentrations than 10 mg/ml (Sorimachi et al. 1997).

One limitation of the study was that the clinical signs evaluations were not performed by a blinded observer, however the observed differences were not subtle. We do not know if LF's protective effect represented prevention or treatment since in this study we used LF before the infection and daily afterwards. The mechanism by which the LF interacts with *Salmonella* ser. Typhimurium to blunt infection remains unclear. We speculate that LF binds to the LPS with disruption of the TTSS, blockade of actin polimerization, and stimulation of the immune system.

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