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Full Length Research Paper

The healing effect of *Lactobacillus plantarum* isolated from Iranian traditional cheese on gastric ulcer in rats

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Probiotics are living organisms which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition. The lactic acid bacteria are one of the most significant groups of probiotic organisms. These bacteria are commonly used in fermented dairy products and have been advocated for the treatment of gastrointestinal disorders. The aim of the present study is to investigate the effect of a probiotic strain *Lactobacillus plantarum* isolated from Iranian traditional cheese on gastric ulcer. Gastric ulcers were induced by luminal application of acetic acid (60% v/v) in rats. *L. plantarum* was given intragastrically at 10^{10} CFU/day for three, five, seven and fourteen consecutive days after ulcer induction. *L. plantarum* significantly reduced gastric ulcer area as compared to control and negative control groups. Neutrophils were strongly increased on day three and decreased on days five, seven and fourteenth after ulcer induction in treatment groups. Macrophages and fibroblasts significantly increased on three and five days after ulcer induction and decreased on days seven and fourteenth. These finding suggested that *L. plantarum* isolated from Iranian traditional cheese enhanced gastric ulcer healing via stimulating immune system and fibroblast increasing.

Key words: *Lactobacillus plantarum*, probiotic, gastric ulcer, healing.

INTRODUCTION

The insight of diversity and function of the human intestinal microbiota has been obtained by clinical studies with bacteria exhibiting specific functions and marketed as probiotics to positively affect our health. Initial attempts focused on establishing sound scientific support for the efficacy of these probiotic bacteria, which mainly include *Lactobacillus* and *Bifidobacterium* species (Saxelin et al., 2005). The action of lactic acid bacteria depends on species, strain and sufficient numbers of bacteria being available in the intestines. The difficulties of identifying and classifying strains, has complicated research, since benefits may only belong to particular strains (Herich and Levkut, 2002). The main product of lactic acid bacteria is lactic acid (Korakli and Vogel, 2006). These bacteria are widespread in nature, in soil, meat, vegetables, milk and the human body. They are used in fermented dairy products (Olusegun and Iniobong, 2011). The probiotic effects attributed to lactic acid bacteria (LAB) and their fermented dairy products arise not only from the whole microorganisms and cell wall components, but also from the peptides and extra-cellular polysaccharides (exopolysaccharides) produced during the fermentation of milk (Vinderola et al., 2006). They are believed to intervene the adhesion of the microorganisms to the gut wall (Schiraldi et al., 2006). It is shown that exopolysaccharides (EPS) have health stimulating properties, such as immunity stimulation (Chabot et al., 2001; Oda et al., 1983), anti-ulcer activity (Nagaoka et al., 1994) and cholesterol reduction (Nakajima et al., 1992). They have been shown to improve the symptoms of various gastrointestinal diseases (Lam et al., 2005). Environmental parameters of the fermentation process, such as pH, temperature and availability of oxygen are important in EPS production, thus composition of the media plays a vital role. EPS-producing LAB isolated from fermented dairy products attracts interest (DeVuyst and Degeest, 1999; Levander and Rådström, 2001; Svensson et al., 2005), due to their generally regarded as safe (GRAS) status and wide application in traditional and industrial fermentation processes. So, it is interesting to determine for each

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probiotic strain whether they actually have the ability to produce EPSs (Desai et al., 2006) and to find newer strains possessing a high EPS productivity (Gotteland et al., 2006).

Since the probiotic strains with high EPS productions are capable to be beneficial for health especially gastro-intestinal tract. The objective of this study is to screen high EPS-production lactic acid bacteria from Iranian traditional cheese and investigate its role on gastric ulcer healing.

MATERIALS AND METHODS

Experimental animals

The use of animals in this study was approved by the committee on the use of live animals in teaching and research of Islamic Azad University of Parand. Male Wistar rats (260 to 290 g) were reared on a standard laboratory diet and given tap water. They were kept in a room where humidity (65 to 70%), temperature (22 ± 2°C) and day/night cycle (12:12 light/dark) were controlled. Rats were divided into 3 groups; experimental, control and negative control (n = 7).

Bacteria

Lactobacilli strain originally isolated from Iranian traditional cheese was used which had been previously approved for the ability of survival and growth in digestive system and having high EPS production. The lactobacilli strain was identified by 16S rRNA gene sequencing and showed 98% similarities to *Lactobacillus plantarum* (Gen Bank accession no GQ423760) (Ebrahimi et al., 2011).

L. plantarum was grown in MRS agar (Fluca, catalogue no. 69966) for 48 h at 37°C to achieve the stationary phase. Bacteria were suspended in sterilized water in order to determine the optical density (OD) by spectrophotometer to procure extent bacteria. Then the bacteria were harvested by centrifugation (4000 rpm, 20 min) and re-suspended in ultra-high-temperature (UHT) milk. Inoculums of *L. plantarum* containing 10^{10} CFU/ml were prepared for experimental groups.

Groups

Experimental groups

Experimental groups 1, 2, 3 and 4 were fed with sterilized milk containing 10^{10} CFU/ml *L. plantarum* after ulcer induction.

Control groups

Control groups 1, 2, 3 and 4 were fed with sterilized milk after ulcer induction.

Negative control groups

Negative control groups 1, 2, 3 and 4 were fed with normal saline after ulcer induction.

Ulcer induction

Rats were deprived of food but not water for 18 h and gastric ulcers were induced by luminal application of acetic acid solution (Ma et al., 1999). Briefly, rats were anesthetized by ketamine and xylazine, a midline laparotomy was performed and the stomach was gently exteriorized. The barrel of a 1 ml syringe was placed on the serosal surface of the stomach in the body region. Acetic acid (0.12 ml of 60% v/v) was instilled into the barrel of the syringe and allowed to remain in contact with the stomach for 45 s, after which the time it was aspirated and the area was rinsed with normal saline. After the different treatment regimens, rats were anesthetized. The stomachs were removed and washed with normal saline and the ulcer area was determined in each stomach.

L .plantarum treatment and measurement of gastric ulcer area

One day after ulcer induction, rats were gavaged intragastrically (i.g.) with normal saline, sterilized milk or sterilized milk with *L. plantarum* at a concentration of 1×10^{10} CFU/ml through oral gavage via a gavage needle for some consecutive days. Rats were sacrificed on days three, five, seven and fourteen after ulcer induction and the ulcer sizes (mm^2) in the anterior and posterior walls were determined and summed in each stomach as previously described (Ma et al., 1999). Gastric tissues were excised and fixed in 10% buffered formalin for histological analysis. After sectioning and staining with hematoxylin and eosin (H & E), samples were surveyed for neutrophil, macrophage and fibroblast in the surface unit by a light microscope.

Ulcer healing assay method

Ulcer healing were estimated by ulcer size and healing percent. Ulcer sizes were determined on days 3, 5, 7 and 14 after ulcer induction.

$$\text{Ulcer healing percent} = \frac{\text{Ulcer size on day 1 } (mm^2) - \text{ulcer size on day} \times (mm^2)}{\text{Ulcer size on day 1 } (mm^2)} \times 100$$

Statistical analysis

The data were expressed as mean ± S.E.M. Statistical analysis was performed with ANOVA, followed by Post-Hoc Tukey multiple range tests using the statistical package for the social sciences (SPSS) for Windows. $P < 0.05$ was considered statistically significant.

RESULTS

L. plantarum decreased ulcer area and enhanced gastric ulcer healing

L. plantarum could successfully reduce ulcer area and enhance gastric ulcer healing as compared to control and negative control groups within the 4 days of experiments. The most significant reduction of ulcer area in experimental group was observed on day 7 after ulcer induction. Also, control groups showed significant in-crease in healing percent on Days 3 and 7 in comparison with negative control groups (Figure 1).

Histological examination

Ulcer healing involves complex processes including

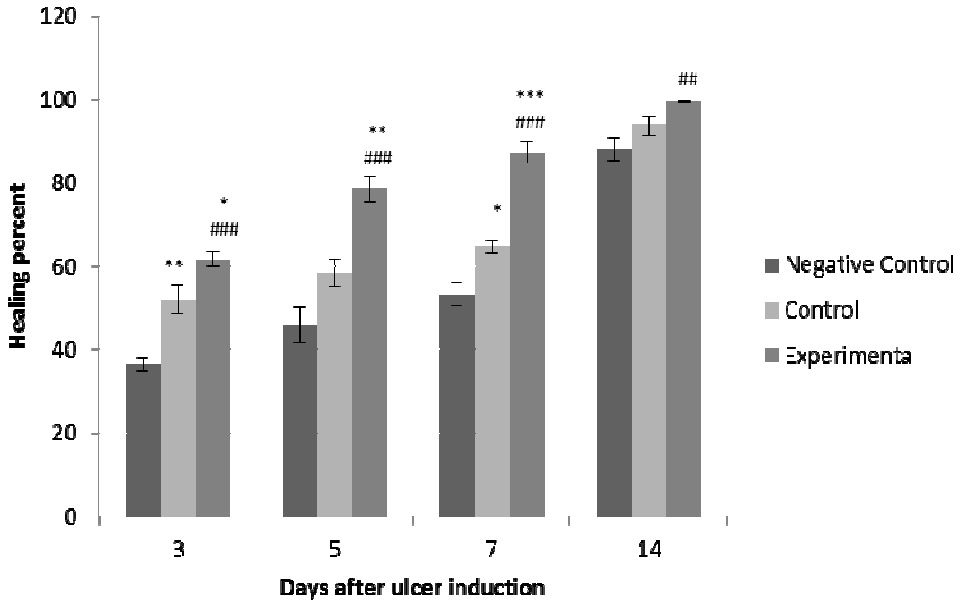

Figure 1. *L. plantarum* supplementation reduced gastric ulcer area. One day after ulcer induction, rats were intragastrically gavaged with normal saline (negative control groups), sterilized milk (control groups) or 10^{10} CFU/day of *L. plantarum* suspended in sterilized milk daily for three, five, seven and fourteen days. The healing percent was determined and summed in each stomach. Results are presented as mean ± S.E.M. Data are representative of four individual experiments. *P < 0.05, **P < 0.01, ***P < 0.001 for experimental and control groups as compared to the negative control groups, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 for experimental groups as compared to the negative control groups (one-way ANOVA).

inflammation and proliferation (Diegelmann and Evans, 2004). Therefore, it is investigated whether *L. plantarum* promoted ulcer healing through the induction of cell proliferation and effect on inflammation. In this study, we showed that 10^{10} CFU/day *L. plantarum* significantly increases neutrophils on day three and decreases them on days five, seven and fourteen after ulcer induction (Figure 2). This probiotic remarkably moved up macrophages on days three and five and decreased on days seven and fourteen after ulcer induction when compared with control and negative control groups (Figure 3). This represents that *L. plantarum* could significantly affect the inflammation phase of healing. It has significantly stimulated influx of neutrophils and macrophages on early phase and reduced them on late phase of healing process.

Also, control group had a prominent increase in macrophages and in neutrophils and macrophages on days three and five after ulcer induction when compared with negative control groups.

L. plantarum had a significant effect on both fibroblasts and the proliferation phase of healing (Figure 4). It was illustrated that fibroblasts significantly increased on days three and five and decreased on days seven and fourteen after ulcer induction, which means collagen deposition phase initiates within a shorter time than control and negative control groups. On day seven after ulcer induction, a significant increase in fibroblasts was observed in control group as compared to negative control group.

DISCUSSION

Previous studies have documented the ability of probiotic strains to accelerate the healing of experimental gastric ulcers (Uchida and Kurakazu, 2004; Lam et al., 2007; Bing et al., 1998). Moreover, high EPS-producing strains have more potential to colonize and affect the gastrointestinal tract (Bauer et al., 2009). *L. plantarum* isolated from Iranian traditional cheese was chosen from some strains for the study since the strain has a high tolerance to acidic conditions of the stomach and is effective to colonize in the gastrointestinal tract (Ebrahimi et al., 2011). Also, this strain is one of the most widely studied probiotic bacterium. To study the effect of this probiotic strain on gastric ulcer healing, *L. plantarum* was orally administrated on day one after ulcer induction for some consecutive days. *L. plantarum* supplementation at the dose of 10^{10} CFU/day for three, five, seven and fourteen days reduced ulcer size by 61.63, 78.46, 87.30 and 99.56%, respectively. These findings suggest that the effect of *L. plantarum* is not limited to the early phase, but may be continued to the later stage of the whole healing process.

It is demonstrated that live probiotic strains induce the

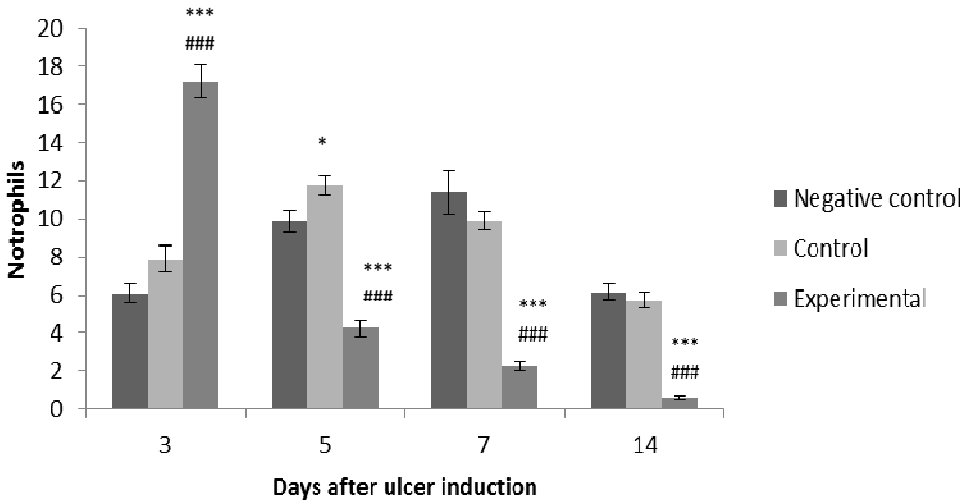

Figure 2. *L. plantarum* significantly affected a number of neutrophils. *L. plantarum* significantly increased neutrophils on day three and decreased on days five, seven and fourteen after ulcer induction. Neutrophils in control group had a significant increases as compared to negative control group on day 5 after ulcer induction. Data are representative of four individual experiments. *P < 0.05, **P < 0.01. ***P < 0.001 for experimental and control groups as compared to the negative control groups, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 for experimental groups as compared to the negative control groups (one-way ANOVA).

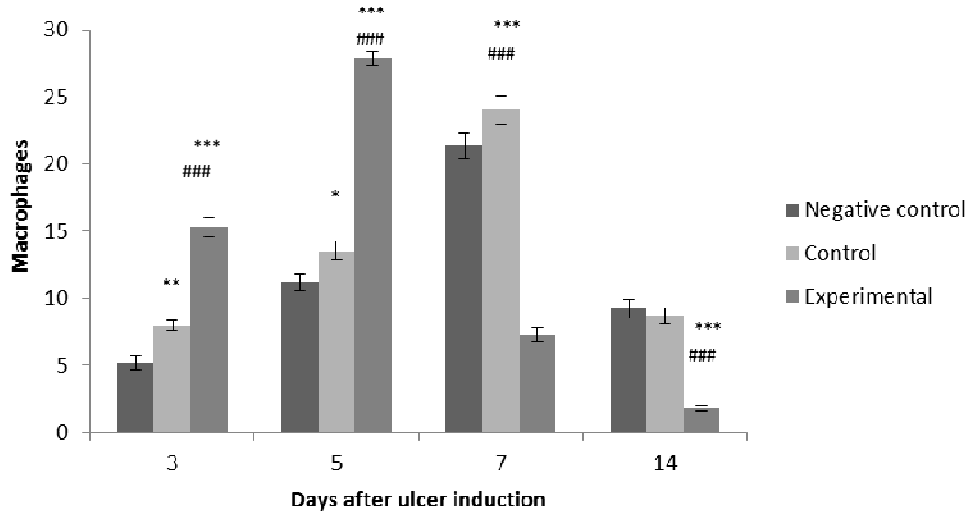

Figure 3. *L. plantarum* significantly affected a number of macrophages. *L. plantarum* significantly increased macrophages on days three and five and decreased on days seven and fourteen after ulcer induction. Macrophages in control group had a significant increase as compared to negative control group on days three and five after ulcer induction. Data are representative of four individual experiments. *P < 0.05, **P < 0.01, ***P < 0.001 for experimental and control groups as compared to the negative control groups. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 for experimental groups as compared to the negative control groups (one-way ANOVA).

production of protective cytokines that enhances the epithelial cell regeneration and inhibit epithelial cell apoptosis (Eswara Murali et al., 2010). In a result of a study cytokine-induced apoptosis was prevented in intestinal epithelial cells in the presence of *Lactobacillus rhamnosus* GG (Yan and Polk, 2002). Probiotic bacteria in a culture of mouse or human colon cells activated anti apoptotic Akt/protein kinase B and inhibited activation of the proapoptotic p38/mitogen activated protein kinase by tumor necrosis factor-α (TNFα), IL-1α, or interferonγ

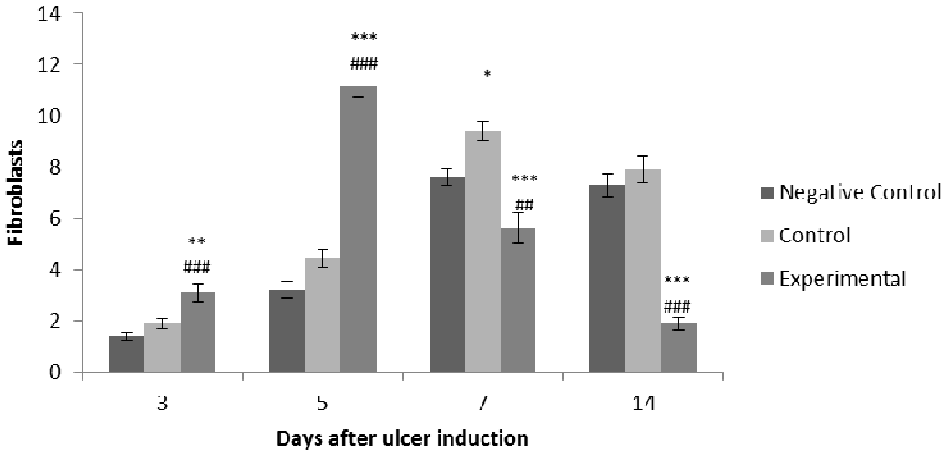

Figure 4. *L. plantarum* significantly increased migration and proliferation of fibroblasts and collagen synthesis. *L. plantarum* increased fibroblasts on days three and five and decreased on days seven and fourteen after ulcer induction significantly. Fibroblasts in control group had a significant increase as compared to negative control group on day seven after ulcer induction. Data are representative of four individual experiments. *P < 0.05, **P < 0.01, ***P < 0.001 for experimental and control groups as compared to the negative control groups, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 foe experimental groups as compared to the negative control groups (one-way ANOVA).

(IFNγ) (Neish et al., 2000). Inhibition of apoptosis enhances survival of intestinal cells and promotes proliferation during recovery from epithelial injury (Hausmann, 2010). Also, probiotic metabolites have been reported to induce angiogenesis, proteoglycans deposition and heal wounds (Halper et al., 2003; Resta-Lenert and Barrett, 2003; Valdez et al., 2005).

Histological studies showed that *L. plantarum* at the dose of 10^{10} CFU/day significantly increased neutrophils, macrophages and fibroblast on day three after ulcer induction. Neutrophils significantly reduced but macrophages and fibroblasts increased in experimental group on day five after ulcer induction. Thus, immune system stimulation, influx and activation of macrophages increase in fibroblasts on days three and five is obvious.

Probiotic strains can stimulate and modulate immune system (Ogueke et al., 2010). *L. plantarum* can increase IL-10 synthesis and secretion in macrophages derived from the inflamed colon (Pathmakanthan et al., 2004). Moreover, they may affect lymphocytes via changes in stimulation induced by alterations in antigen-presenting macrophages (Ng et al., 2009). Migration of fibroblasts into the granulation tissue and their proliferation are triggered by growth factors: TGFβ, PDGF, EGF, FGF and cytokines (Tarnawski, 2005). It is likely that *L. plantarum* could affect one or some of these factors and increase fibroblasts.

Neutrophils, macrophages and fibroblasts significantly reduced on days seven and fourteen after ulcer induction in experimental groups as compared to control and negative control groups. These data indicate that inflammation has been reduced and collagen synthesis increased in experimental groups whereas in control and negative control groups inflammation is kept on. Probiotics induce proinflammatory cytokines release *in vitro*, such as IL6 and TNF-α (Hegazy and El-Bedewy, 2010). In addition, cell wall components of *Lactobacillus casei* have been shown to be anti-inflammatory (Chapat et al., 2004). Conclusively, *L. plantarum* could stimulate immune system in early phase and then reduce inflammation in late phase of healing process. Fibroblasts reduction implies that collagen synthesis has been initiated in a short time than control and negative control groups.

In the present study, *L. plantarum* positively affects gastric ulcer healing. Also, increase in healing percent in control groups has been shown. Healing percent increases in control groups as compared to negative control groups, because of probiotic EPSs, vitamins, proteins and specially calcium in milk that inactivate gastric acid. While *L. plantarum* suspended in sterilized milk, promotes significant increase in healing percent in experimental groups that have occurred. These data suggest that enriching milk with this probiotic strain could be effective in gastric ulcer healing.

Conclusion

In total we demonstrate that *L. plantarum* isolated from Iranian traditional cheese is capable of accelerating gastric ulcer healing. Further studies are required to develop a detailed mechanism of *L. plantarum* during ulcer healing.

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