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The effect of *Lacticaseibacillus casei* on inflammatory cytokine (IL-8) gene expression induced by exposure to *Shigella sonnei* in Zebrafish (*Danio rerio*)

[*O efeito do Lacticaseibacillus casei na citoquina inflamatória (IL-8) expressão genética induzida pela exposição a Shigella sonnei em Zebrafish (Danio rerio)*]

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ABSTRACT

This study aimed to evaluate the protective function of probiotics against *Shigella sonnei* pathogenicity. For this purpose, 400 zebrafish were divided into four groups with two replications: (T1): receiving *Lacticaseibacillus casei* for 27 days, (T2): receiving *L. casei* for 27 days followed by 72 hr exposure to *S. sonnei*, (T3): receiving basal diet for 27 days followed by 72 hr exposure to *S. sonnei*, and control group (C): receiving basal diet without exposure to the pathogen. According to the results, feeding with *L. casei* for 27 days reduced the interleukin-8 (IL-8) expression significantly (P<0.05). The results showed a decrease in IL-8 expression in the group exposed to the pathogen and fed with the probiotic compared to the group only fed with the basal diet (P<0.05). Considering the role of IL-8 as a pro-inflammatory cytokine, our results indicated that feeding with *L. casei* could modulate inflammatory responses.

Keywords: *Lacticaseibacillus casei*, *Shigella sonnei*, Interleukin-8, Zebrafish

RESUMO

Este estudo teve como objetivo avaliar a função protetora dos probióticos contra a patogenicidade Shigella sonnei. Para este fim, 400 zebrafish foram divididos em quatro grupos com duas réplicas: (T1): recebendo Lacticaseibacillus casei por 27 dias, (T2): recebendo L. casei por 27 dias seguido por 72 horas de exposição a S. sonnei, (T3): recebendo dieta basal por 27 dias seguido por 72 horas de exposição a S. sonnei, e grupo controle (C): recebendo dieta basal sem exposição ao patógeno. De acordo com os resultados, a alimentação com L. casei por 27 dias reduziu significativamente a expressão da interleucina-8 (IL-8) (P<0,05). Os resultados mostraram uma diminuição na expressão de IL-8 no grupo exposto ao patógeno e alimentado com o probiótico em comparação com o grupo alimentado apenas com a dieta basal (P<0,05). Considerando o papel da IL-8 como uma citocina pró-inflamatória, nossos resultados indicaram que a alimentação com L. casei poderia modular as respostas inflamatórias.

Palavras-chave: Lacticaseibacillus casei, Shigella sonnei, Interleukin-8, Zebrafish

INTRODUCTION

Shigella sp. is one of the most common causes of gastrointestinal diseases and the most important cause of dysentery worldwide (Zhang *et al.*, 2012; Mattock and Blocker, 2017). This Bacterium is responsible for about 165 million shigellosis (Duggan and Mostowy, 2018) and over half a million deaths annually (Lima *et al.*, 2015, Kotloff *et al.*, 2018). Shigella pathogenicity is triggered by penetrating the intestinal epithelium, proliferating within the epithelial cells, and spreading to the adjacent cells (Bolla *et al.*, 2016). This causes an acute inflammatory response known as "shigellosis" Schroeder and Hilbi (2008), which is a common form of bacillary dysentery resulted from infections with four species of Shigella including *S. flexneri*, *S. dysentery*, *S. buidi*, and *S. sonnei* (Moorthy *et al.*, 2010).

The infection with *S. sonnei* is observed in developed and developing countries (Levine *et al.*, 2007; Kotloff *et al.*, 2018). However, the

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pathogenicity of this infection is still not fully understood (Duggan and Mostowy, 2018). Shigella infection is generally known to modulate the inflammatory and innate immune responses by inducing specific mediators, limiting the adaptive immune response, and aiding the infection spread (Mattock and Blocker, 2017). Deaths from shigellosis are commonly associated with complications such as uremic hemolytic syndrome, systemic symptoms, hypoglycemia, and pneumonia (Caboni *et al.*, 2015).

Nowadays, the emergence of antibiotic-resistant bacteria is a significant threat, and Shigella is one of the bacteria associated with the resistance to antibiotics such as fluoroquinolones, cephalosporins, and azithromycin (Puzari *et al.*, 2018). The resistance of Shigella species to antibiotics, like other Gram-negative bacteria, is increasing (Harrington, 2015), and the World Health Organization (WHO) has placed the shigellosis among the 12 major diseases (Tacconelli *et al.*, 2018). Considering the limited applicability of the current antimicrobial drugs for treating shigellosis (Moorthy *et al.*, 2010), it is important to seek alternative therapeutic agents.

One of the critical issues in the pathogenesis of *S. sonnei* is to understand how the bacterium interacts with the intestinal epithelial cells, which ultimately leads to the inflammation. Due to their protective roles as barriers against penetrating pathogens and modulators of inflammatory processes in the mucosa, the intestinal epithelial cells are particularly important in *S. sonnei* pathogenesis (Moorthy *et al.*, 2010). Besides, many intestinal functions such as nutrient uptake, creating a mucosal barrier, and angiogenesis are, in part, promoted by the bacteria, which are a part of the intestinal flora (Hooper *et al.*, 2001; Stappenbeck *et al.*, 2002). In this regard, it has been shown that some bacteria of the intestinal flora including Lactobacilli can protect the host against pathogens, including those that cause diarrhea (Moorthy *et al.*, 2010). Lactobacilli, which belong to the natural bacterial flora of intestine, are among the most common probiotic groups (Cui *et al.*, 2004; Zhang *et al.*, 2012).

The probiotics include microorganisms with numerous health benefits for the host FAO/WHO (Guidelines..., 2002). Several studies have reported the Lactobacilli ability to protect the host against infectious diseases caused by intestinal pathogens (Gupta and Garg, 2009). Lactic acid bacteria (LABs) are used in the processed food industries and the products containing Lactobacillus selectively modulate gut microbiota composition by producing antimicrobial compounds such as bacteriocin, organic acids, and hydrogen peroxide, which inhibit the growth of pathogens and improve the intestinal barrier function (Eor *et al.*, 2020). *Lacticaseibacillus casei* (*L. casei*) is one of these probiotics and a component of the natural intestinal flora, which is used either alone or in an enriched form (García-Peñalvo, 2019). It has been shown to modulate mucosal innate immune response and inflammatory reactions (Tien *et al.*, 2006; Borruel *et al.*, 2002). Probiotics apply their effects by neutralizing the pathogens, augmenting the intestinal barrier, and modulating the immune response (Plaza-Diaz *et al.*, 2014). In general, probiotics beneficial effects may be derived by regulating the innate and adaptive immune responses. For example, Lactobacilli have been shown to reduce the inflammatory response induced by the Shigella strains invading the intestinal epithelial cells (Tien *et al.*, 2006). The mechanisms of action of *L. casei* include improvement in the antioxidant activity of blood, increase in the concentration of bioactive peptides caused by hyperglycemia, immune system regulation, improvement in inflammation, and blood cholesterol lowering (Grom *et al.*, 2020).

Cytokines are the key inflammatory mediators in inflammatory bowel disease (IBD) (Gopal *et al.*, 2017). Investigation of the cytokine gene expression in the intestine of the patients with acute shigellosis revealed the elevated levels of pro-inflammatory interleukins (ILs) such as IL-8 (Phalipon and Sansonetti, 2007). The IL-8 is a potent pro-inflammatory chemokine produced by the epithelial cells and basolateral epithelial membrane in response to Shigella (Köhler *et al.*, 2002).

Zebrafish is an important laboratory model because of the numerous features such as rapid growth, high genetic similarity to humans, body transparency, and the lack of adaptive immunity at early stages of embryogenesis, which allows the specific studies on the innate immunity (Lieschke and Currie, 2007; Sullivan *et al.*, 2017;

Lieschke, 2009. This fish is also a valuable model to study Shigella pathogenesis as many of the disease symptoms observed in humans have also been identified in zebrafish (Duggan and Mostowy, 2018).

The effect of *L. casei* in reducing Shigella pathogenicity has been proven (Tien *et al.*, 2006); however, the exact mechanisms involved in this effect have not yet been investigated thoroughly. In this study, the probiotic *L. casei* effects were investigated on the intestinal epithelium structure and inflammatory response in zebrafish exposed to *S. sonnei*.

MATERİALS AND METHODS

The clinical strain of *S. sonnei* (ATCC 9290) was prepared from the Microbial Bank of Iranian Biological Resources (Tehran, Iran) and grown in Shigella-Salmonella (SS) agar (Merck, Germany) for 18 hr at 37°C (Gopal *et al.*, 2017). After ensuring the appropriate growth of the bacteria, they were transferred to MacConkey culture medium and incubated for 20 hr at 37°C (Andrews and Jacobson, 2019). Finally, 0.5 McFarland (1.5×10^8 CFU/mL) concentration was determined based on the method of Eduardo *et al.*, (2018) by preparing bacterial suspension in Muller Hinton broth and measuring turbidity using a spectrophotometer at 625nm wavelength of (Eduardo *et al.*, 2018).

The lyophilized *L. casei* was provided from the Microbial Bank of Iranian Biological Resources (Tehran, Iran) and grown in MRS broth culture medium (Merck, Germany) for 18-20 hr at 37°C (Moorthy *et al.*, 2010). Afterward, the grown bacteria were centrifuged (2500 rpm) for 25 min at 4°C, and then washed three times with phosphate-buffered saline (PBS). Finally, a suspension with a concentration of 1.5×10^8 CFU was prepared (Zang *et al.*, 2019). The suspension was kept at -20°C with 20% glycerol until use (Barbour and Priest, 1986).

The commercial feed used in this study was BioMar (France). The probiotic diet was prepared by combining probiotic *L. casei* suspension (1.5×10^8 CFU/mL) (2mL) with commercial feed based on 2% fish body weight. The diet was prepared daily. Briefly, probiotic was mixed with commercial feed, then; the mixture was incubated on ice for 15 minutes to allow bacteria to absorb and finally, combined with 1% skim milk as a preservative. The basic diet was prepared by combining the commercial feed with 2mL sterile PBS (Wang *et al*, 2016). Feeding was done twice a day at 9 a.m. and 16 p.m.

Zebrafish were purchased from an ornamental fish breeding center in Tehran, Iran, and transferred to the "Razef" Research Center of the Science and Research University (Shahriar, Tehran, Iran). The fish were kept at 26±1°C and dark/light cycles of 8:14 for 2 weeks to adapt to the condition. They were fed twice a day with commercial feed. During this time, the physicochemical factors were examined to ensure an ideal maintenance condition.

Fish were divided into four groups with two replications in the form of T1; Feeding with probiotic *L. casei* (1.5×10^8 CFU/mL) for 27 days, T2; Feeding with probiotic *L. casei* for 27 days and then 72 hr exposure to *S. sonnei* (1.5×10^8 CFU/ml), T3; Feeding with the basal diet for 27 days and then 72 hr exposure to *S. sonnei*, and control group (C); Feeding with the basal diet for 27 days and no exposure to *S. sonnei*.

Sampling was performed on days 1, 14, 27, and 30. To examine the IL-8 gene expression, three fish were randomly collected from each tank, anesthetized by clove powder, and euthanized (Wong *et al.*, 2014). The prepared samples were placed in RNAse-free microtubes and kept at -80°C for the gene expression analysis.

Real-time PCR was performed to evaluate the expression level of IL-8 gene. First, total RNA was extracted from fish intestine using TRIzol reagent according to the manufacturer's protocol. Three replications were performed for each test. Extracted RNA was quantified using Nanodrop Spectrophotometer (Thermo Scientific™). CDNA synthesis was performed using the Revert Aid^{TM} First Strand cDNA Synthesis Kit (Thermo Scientific™ Fermentas, USA), and real-time PCR was performed by Real-Time PCR Detection Systems (Bio-Rad). The primers were designed using GeneRunner software (Table 1). The gene expression was analyzed using the $2^{-\triangle\triangle^{ct}}$ method, and β-actin was considered as the endogenous reference (Yang *et al.*, 2019).

Table 1. Primer specifications used for IL-8 and β-actin genes

Primers	Primer sequence	Length of product
Forward primer IL-8	CGTGAAGTGAACGTGGTGGA	160bp
Reverse primer IL-8	CTTTCAAGTCGCTGCTTCCG	
Forward primer Beta Actin	CCTTCTTGGGTATGGAATCT	194bp
Reverse primer Beta Actin	GATCTTGATCTTCATTGTGCTA	

The statistical data analysis was performed by SPSS 16 software (SPSS Inc., Chicago, IL., USA) using the one-way analysis of variance (One-way ANOVA). Tukey's HSD *post-hoc* test was used to investigate the differences in the expression of the desired genes between the control and experimental groups. Finally, the data was presented as mean ± standard deviation (SD). The P<0.05 was considered as statistically significant.

To determine the effect of *L. casei* on the cellular and molecular immune responses of zebrafish against *S. sonnei* invasion, the IL-8 gene expression was assessed using real-time PCR molecular technique.

The expression levels of IL-8 (mean ± SD) in various groups are summarized in Table 2. Based on these results, the IL-8 pro-inflammatory cytokine expression decreased in zebrafish intestine in the groups fed with the probiotic diet on days 14 (P>0.05) and 28 (P<0.05), and this trend continued in the T1 group until day 30 (Figure 1). This observation indicated the immunomodulatory effects of probiotic *L. casei* diet. On the other hand, the expression of this cytokine gene was down-regulated significantly after exposure to *S. sonnei* in the T2 group compared to the T3 group (Figure 1, P<0.05), reflecting the inflammatory damaged induced by *S. sonnei* in zebrafish intestine and modulation of the cellular and molecular immune responses in the group fed with the probiotic regimen.

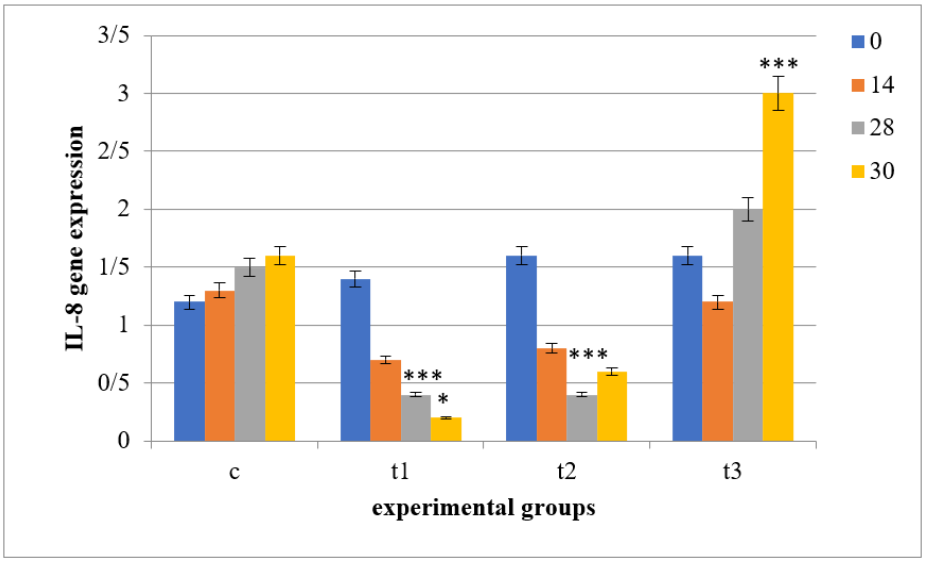

Figure 1. The expression of IL-8 gene in different groups.

Table 2. The IL-8 gene expression in different experimental groups in different days

Day/ treatment	T1	T2	T3	C
0	1.38±0.39	1.56±0.19	1.62±0.29	1.23± 0.23
14	0.722±0.63	0.816±0.23	1.29±0.44	1.29±0.82
27	0.409±0.55	0.429±0.82	2.06±0.19	1.52±0.16
30	0.268±0.560	0.602±0.39	3.29±0.38	1.69±0.34

The IL-8 is a pro-inflammatory cytokine produced by the intestinal epithelial cells in response to the invasive agents (Eckmann *et al.*, 2000; Köhler *et al.*, 2002). The production of IL-8 by intestinal epithelial cells may be one of the first signals of the acute mucosal inflammation in bacterial infections (Eckmann *et al.*, 1993).

In this study, we initially examined the effects of feeding with non-pathogenic bacteria, *L. casei*, on the IL-8 gene expression. According to the obtained results, feeding with *L. casei* led to a significant reduction in the gene expression of IL-8 pro-inflammatory cytokine in zebrafish intestine, which was consistent with the results of Wallace *et al*., (2003) who assessed the effects of treating HT-29 human intestinal epithelial cell line (IEC) with *L. rhamnosus*, *L. delbrueckii*, and *L. acidophilus* (Wallace *et al.*, 2003).

Regarding the mechanism of action of *L. casei*, Tien *et al*. (2006) stated that *L. casei* can reduce the expression of some pro-inflammatory cytokines through the ubiquitin-proteasome pathway (Tien *et al.*, 2006). It was also shown that DNA of probiotics reduced the production of IL-8 by the epithelial cells via a mechanism involved in I-κB stabilization (Jijon *et al.*, 2004).

In this regard, several studies have investigated the effects of probiotic bacteria on pro-inflammatory immune responses and IL-8 gene expression (Lazado and Caipang, 2014b; Al-Hisnawi *et al.*, 2019).

Some studies presented opposing results from our study. Lammers *et al*., (2002) and Bolla *et al.* (2016) reported that treating intestinal epithelial cells with Lactobacilli had no effect on the IL-8 gene expression (Bolla *et al.*, 2016; Lammers *et al.*, 2002). Also, in a study by Lazado and Caipang (2014), the host-derived potential probiotics (GP21 and GP12), which were either alive or inactivated by heat, did not modify the IL-8 gene expression significantly in the intestinal epithelial cells (IEPC) of Atlantic cod fish (Lazado and Caipang, 2014a). On the other hand, Pérez-Sánchez *et al.* (2011) found that feeding rainbow trout with probiotics *L. plantarum*, *L. lactis*, and *Leuconostoc mesenteroides* increased the of IL-8 gene expression (Pérez Sánchez *et al.*, 2011). Accordingly, Wallace *et al*. (2003) declared strain-specific differences in the ability of LABs to modulate the intestinal production of cytokines. Therefore, the differences observed among the results of the mentioned studies can be attributed to the variability in the type of probiotics, as well as different test conditions (*in vivo* and *in vitro*), different hosts, and variable treatment periods (Wallace *et al.*, 2003).

The induction of pro-inflammatory cytokines can act like a double-edged sword, causing cellular damage and tissue inflammation in certain conditions (Wallace *et al.*, 2003). In this regard, the results of the present study showed an increase in the IL-8 gene expression in both groups exposed to *S. sonnei*; however, this elevation was lesser in the group treated with *L. casei* compared with the group fed with the basic diet. In accordance with our results, several studies, including those of Bolla *et al.* (2016), Tien *et al.* (2006), and Moorthy *et al.* (2010), have shown the inhibitory effects of probiotics on the IL-8 gene expression after exposure to Shigella bacteria.

It has been shown that lipopolysaccharides from Gram-negative bacteria can disrupt the integrity of the intestinal barrier by activating Toll-Like Receptor (TLR) 4 signaling pathway, which leads to the intestinal inflammation (Eor *et al.*, 2020) or. In this regard, the ability of Shigella in inducing pro-inflammatory responses and IL-8 gene expression has been reported in previous studies (Bolla *et al.*, 2016). Also, Pédron *et al*. (2013) showed the prominent effects of *Shigella flexneri* on inducing IL-8 gene expression in the intestinal epithelial cells, suggesting this cytokine as a major regulator of mucosal inflammation in shigellosis (Pédron *et al.*, 2003). Gopal *et al.* (2017) highlighted the role of β-catenin/NF-κB signaling pathway as a mechanism through which Shigella infection could activate the pro-inflammatory response and induce the IL-8 gene expression in the rat model of ileal loop.

The intestinal immune responses are mediated by the receptors expressed on the apical and/or basolateral surfaces of the epithelial cells. Commensal and pathogenic bacteria in the gastrointestinal tract can activate these signaling cascades and therefore trigger pro-inflammatory genes transcription and immune responses (Kelly *et al.*, 2004). Although the presence of virulence factors in the pathogenic bacteria is responsible

for the development of inflammatory responses, commensal bacteria (including Lactobacilli) modulate immune responses via various mechanisms. It is important to understand the molecular and cellular mechanisms through which probiotic bacteria balance the immune homeostasis, which can pave the path for developing new treatments for the inflammatory bowel diseases (Kelly *et al.*, 2004).

The primary target of probiotic bacteria includes intestinal epithelial cells (Kelly *et al.*, 2004), and such interaction blocks the binding of pathogens to the epithelial cells (Zhang *et al.*, 2012). In this regard, according to the report of Moorthy *et al*. (2010), *L. rhamnosus* and *L. acidophilus* reduced *S. sonnei* pathogenicity in the human intestine by inhibiting the pathogen attachment to the epithelial cells and suppressing the release of pro-inflammatory cytokines (Moorthy *et al.*, 2010). Also, according to the results of Bolla *et al.* (2016), the treatment of intestinal epithelial cells with three Lactobacillus strains and two kefir-isolated yeasts reduced pathogen invasion to HT-29 and Caco-2 cells and reduced pathogen entry into HT-29 and Caco-2 cells, suppressing the pro-inflammatory response and IL-8 gene expression upon exposure to *S. flexneri* (Bolla *et al.*, 2016). This was consistent with the findings of the present study. In accordance with our observation, Tien *et al.* (2006) also assessed the anti-inflammatory effects of *L. casei* on the intestinal cells exposed to *S. flexneri* and showed that the probiotic blocked the NF-κB pathway induced by the pathogen, alleviating the pro-inflammatory responses.

In another study, Hersh *et al.* (1998) stated that *Shigella flexneri* induced macrophage apoptosis, resulting in the release of pro-inflammatory cytokines from the dying cells and subsequently the initiation of inflammatory responses and mucosal tissue destruction (Hersh *et al.*, 1998). On the other hand, Kelly *et al*. (2004) described a unique anti-inflammatory mechanism, which was activated by non-pathogenic commensal bacteria that selectively antagonized NF-κB transcription factor. This bacterium attenuated pro-inflammatory cytokines expression by promoting the nuclear export of NF-κB RelA subunit via PPAR-γ-dependent pathway (Kelly *et al.*, 2004). In another report, Eor *et al.* (2020) noted that feeding with *L. casei* reduced osteoporosis and subsequently diminished pro-inflammatory factors and the intestinal inflammation was induced by liposaccharides derived from Gram-negative bacteria (Eor *et al.*, 2020).

Based on our results, probiotic *L. casei* suppressed the excessive production of IL-8 in the intestine of zebrafish exposed to *S. sonnei*, thus, it can play a protective role against pathogenic agents, suggesting a novel therapeutic strategy to treat this infection and other intestinal inflammatory diseases. However, further studies are needed to determine the mechanisms and signaling pathways involved in the IL-8 alternations in the zebrafish infected by *S. sonnei*.

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ETHİCS APPROVAL

Approved by Islamic Azad University Ethical Committee by the code: IR.IAU.TMB.REC.42.440

CONSENT TO PARTİCİPATE

No clinical trials were included in this research.

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