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Enterococci Isolated from Traditional Cheese Alters Serum Lipid and Cytokine Levels in Adult Male NMRI Mice

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ABSTRACT

Introduction: The present study aimed to isolate *Enterococci* bacteria from Iranian traditional cheese and to evaluate the effects of the isolated bacteria on serum lipid levels and the activation of the immune system in animals. **Methods:** *Enterococci* bacteria were isolated from cheese using particular cultures and identified using analytical profile index (API) kits. NMRI mice were divided into different groups and *Enterococci* was orally administrated at doses of 2 ($6×10^8$ CFU/ml), 3 ($9×10^8$ CFU/ml), and 4 ($12×10^8$ CFU/ml) MacFarland for 2 weeks. Blood samples were collected from a retro-orbital sinus, and serum lipids level was measured using the spectrophotometer method and interleukin levels were measured using ELISAkits. **Results:** Treatment with *E.faecium* serotype decreased serum total cholesterol and LDL and increased serum IL-10 levels, while having no significant effect on serum triglycerides, HDL, IL-2, and IL-6 concentrations ($p < 0.05$). Administration of *E. faecalis* serotype had no significant effect on serum lipid levels. Moreover, results revealed that treatment with the *E. faecalis* serotype increased Il-6 and IL-10 concentrations. None of the mentioned serotypes significantly affected gut pathogen growth ($p < 0.05$). **Conclusions:** *Enterococci* bacteria from Iranian traditional cheese showed no significant inhibitory effects on gut pathogens, but exhibited significant decreases in serum total cholesterol and LDP-chol. More studies are suggested to confirm the potential role of the *E.faecalis* serotype and anti-inflammatory/inflammatory reactions in the body.

Key words: Cheese, enterococci, cheese, lipid level, probiotics, serum

INTRODUCTION

Over the past decade, we have witnessed great changes in the concept of food rules in relation to improvements in human health (Nami *et al.*, 2015). Researchers have changed their direction from considering food as a source of energy and its structural role in the body to analysing the biological metabolism of the food on different organs (Catanzaro & Green 1997; Gill & Guarner 2004). Probiotics are living creatures in food and are essential for human health. These living microorganisms naturally exist in fermented food (such as yogurt, milk, fermented cheese, etc.), and they are useful for the human body, especially in the digestive system (De Vuyst, Falony & Leroy, 2008; Gill & Guarner 2004).

Previous studies have demonstrated microorganisms with probiotic activities in the dairy products of different parts of the world (Chiquette, 2009; Vélez *et al.*, 2007). The advantageous effects of the probiotics in the food have been proven in various studies. Epidemiologic studies have shown that consumption of food with active probiotics decreases the chance of cancer, improves the health of the heart, sharpens the immune system, lessens the symptoms of menopause, increases the health level of the gut and urinary systems,

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and has antibacterial and antiviral effects (Corthésy, Gaskins & Mercenier, 2007; Makras *et al.* 2006; Wollowski, Rechkemmer & Pool-Zobel, 2001).

The most commonly known probiotic microorganisms are divided into 3 categories: bacteria, fungi and yeast (Lourens-Hattingh & Viljoen 2001; Shah 2000; Varsha *et al.*, 2014). Among the three categories, the focus is more on bacteria to identify and produce probiotic products. Previous studies have shown the probiotic activities of some bacteria,such as the selected strains of *Lactobacillus*, *Bifidobacterium* and *Enterococci* existing in dairy products (Madsen *et al.*, 2001; Ohland & MacNaughton 2010).

Enterococci are a part of the standard flora of fermented dairy products and of cheese; *Enterococci* cause a particular smell and taste in different traditional cheeses in various regions (Vahjen & Männer 2003; Zeyner & Boldt 2006). Although most researchers of bacteria have focused on *Lactobacillus*, studies have shown that *Enterococci can* also be considered a probiotic bacterium (Efthymiou *et al.*, 1974; Zeyner & Boldt 2006).

The current study aims to isolate *Enterococcus* in the local cheese of the Ardebil province and illustrate the probiotic activity of the isolated bacteria.

METHODS

Sample collection

Ten samples of local cheese (from the Ardebil province, Iran) were collected and kept at -20°C.

Sample preparation

Cheese samples of 10 g were weighed in a hygienic and sterile situation and placed on a sterile plate with 90 ml of water, and 2% of sodium citrate; then it was mashed and diluted to different concentrations up to 10^{-6} with the sodium citrate (Du Toit *et al.*, 1998; Nguyen, Kang & Lee, 2007). In addition, 0.1 ml of prepared samples were cultured in the KF-*Streptococcus* Agar or MRS agar culture media. The liberal media were maintained in the heating room for 24-48 h. *Enterococci* colonies were selected according to catalyse test and gram staining. Selected colonies were cultured again in blood agar, and the type of *Enterococci* was identified based on growing capability in 10-45°C in the presence of 6.5% salt and 40% bile salt and bile aesculin hydrolysis. Finally,the *Enterococci* serotypes were identified with sugar fermentation tests (Nguyen *et al.*, 2007).

Anti-pathogenic study

In order to study anti-pathogenic activates of the isolated *Enterococci,* we used gastrointestinal tract pathogens of *Shigella dysenteriae* (PTCC1188), *Pseudomonas aeruginosa* (PTCC1430), *Escherichia coli* (PTCC1399) and *Salmonella typhimurium* (ATCC 1596). All pathogens were cultured in BHI medium for 24 h. Five smooth colonies of bacteria were transferred and cultured in nutrient broth medium and incubated for 1-2 h in 37°C to obtain a concentration of 0.5 McFarland. Suspension samples were transferred to Mueller Hinton agar medium and homogenised uniformly in the medium.

Using sterile punch, 6 mm wells were created in the medium and filled with 200 ml of isolated *Enterococci* suspension. After 24 h of incubation in 37°C, the inhibition zone diameter was measured for each pathogen.

Effects of separated bacteria on blood lipids, glucose, and cytokines in mice

Male mice (NMRI species), weighing 28±3 g, were provided from the Pasteur Institute (Tehran, Iran) and separated into the following groups:

- Treated with *E. faecalis* with 2 McFarland (6×10^8CFU/ML)
- Treated with *E. faecalis* with 3 McFarland(9×10^8CFU/ML)
- Treated with *E. faecalis* with 4McFarland(12×10^8CFU/ML)

Table 1. Effects of faecalis and faecium enterococcus on lipid levels of serum

		Triglycerides	Cholesterol	HDL	LDL
	Control	94±7.1	110.3±4.3	44± 2.1	38.3±1.8
Enterococci faescium	McFarland2	90.5±6.1	102.5±6.3	40.3±3.2	36.2±2.4
	McFarland 3	88.6±5	81.1±6.4*	39±4.6	31.6±2.1
	McFarland 4	96.2±6.4	80.6±5.2*	46.7±5.2	26.4±1.9*
Enterococci faecalis	McFarland	95.7±5.2	108.5±8.3	41.3±2.8	38.1±2.1
	McFarland 3	89.6±6.6	102.1±7.2	42±4.1	38.4±2.2
	McFarland 4	92.2±4.9	104.6±6.3	44.7±3.2	26.3±1.8

- Treated with *E. faecium* with 2 McFarland(6×10^8CFU/ML)
- Treated with *E. faecium* with 3 McFarland(9×10^8CFU/ML)
- Treated with *E. faecium* with 4 McFarland(12×10^8CFU/ML)

Each mouse was orally treated with 1 ml of the respective bacteria dilution for 2 weeks. After 14 days of continuous treatment, blood samples were collected from the retro-orbital sinus of animals and immediately centrifuged, and serum samples were provided. ELISA kits (Pars Gene Company, Tehran, Iran) were used to measure the level of cholesterol, LDL, HDL, and triglycerides in the serum samples. Serum cytokines level was measured via ELISA kits (R&D systems, USA) in accordance with the producer's manual.

RESULTS

The results showed the growth and existence of *Enterococci* clones in the cheese samples from Ardebil. We successfully isolated 6 *E. faecalis* and 5 *E. faecium* serotypes. The results obtained showed that different concentrations of *E. faecalis* did not have any significant effect on the serum cholesterol level. However, *E. faecium* with concentrations of 3(F 3, 36 = 5.42, $p < 0.05$), and 4(F 5, 86 = 2.60, $p<$ 0.05) McFarland significantly decreased the total cholesterol level, and McFarland 4 (F 3, 36 = 6.20, $p < 0.05$) decreased the LDL level of the mice serum (Table 1). The anti-bacterial test showed that none of *the Enterococci* serotypes significantly affected gut pathogen growth.

Continuous treatment of the *E. faecalis* serotypes considerably increased the concentration of interleukin 6 (F 3, 36 = 5.94, $p < 0.05$) and interleukin 10 (F 3, 36 = 73.45, $p < 0.001$) in the blood of the mice but did not have a significant effect on interleukin 2 (F 3, 36 = 2.60, $p > 0.05$) level (Figures 1, 2 & 3). Continuous treatment with various concentrations of *E. faesium* serotype significantly increased the level of interleukin 10 (F 3, 36 = 4.47, $p < 0.01$) but not the levels of interleukin 6(F 3, 36 = 3.01, $p<$ 0.05) and interleukin 2 (F 3, 36 = 2.40, $p<$ 0.05) (Figures 1, 2 & 3).

DISCUSSION

The current study demonstrated the existence of *E. faecalis* and *E. faesium* in Ardebil local cheese. The results showed that *E. faesium* did not have any effect on the serum level of glucose and triglycerides but significantly decreased the level of total cholesterol and the LDL level. Moreover, the *E. faecalis* serotype did not make any significant changes in the factors mentioned above. Some of the previous reports have shown the useful effects of the probiotics on decreasing blood cholesterol. Although few studies have been done on the effects of *Enterococci* on the metabolism of the

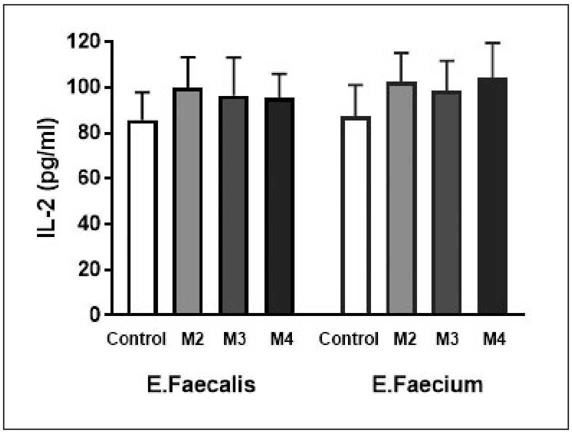

Figure 1. Effects of *faecalis* and *faecium* serotypes on serum IL-2 level in control and *enterococcus* treated mice

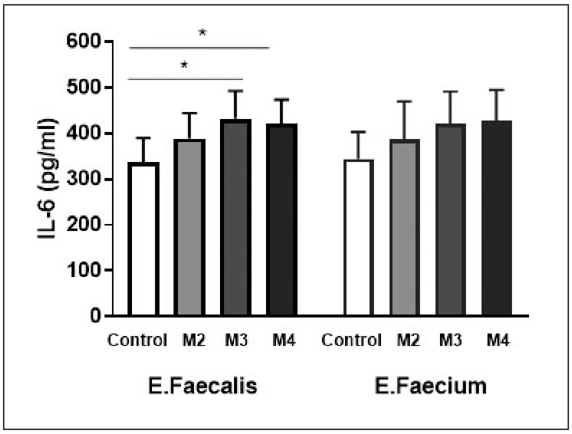

Figure 2. Effects of *faecalis* and *faecium* serotypes on serum IL-6 level in control and *enterococcus* treated mice

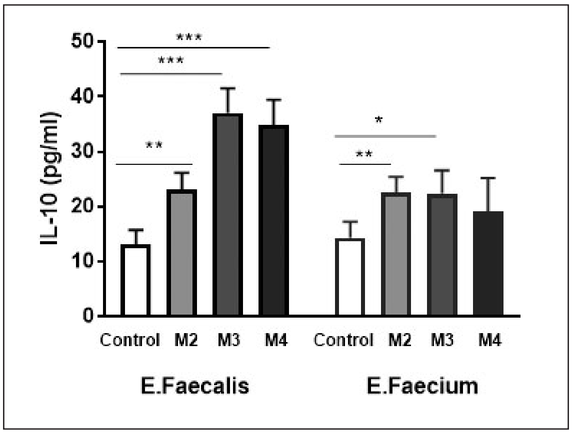

Figure 3. Effects of *faecalis* and *faecium* serotypes on serum IL-10 level in control and *enterococcus* treated mice

lipids, some of the articles show the effects of *Enterococci* on cholesterol metabolism. Studies have shown that bacteria can intake cholesterol from the culture media, and due to this characteristic of the bacteria, a lot of attention has been paid to the decreasing effects of probiotic bacteria on blood cholesterol over the past few years (Agerholm-Larsen *et al.,* 2000; Mohan *et al.*, 1995; Mohan *et al.*, 1996). De-Feng *et al.* have shown that isolated *Enterococci* from a pig's gut can decrease the level of cholesterol in the culture media (Ooi & Liong 2010). Several studies also have shown *in vivo* effects of probiotic bacteria on the reduction of serum cholesterol levels in animals. A study by Pereira and her colleagues illustrated that probiotic *Bifidobacterium* decreases cholesterol levels in mice. Moreover, the cholesterol-lowering effects of *Lactobacilli* have been shown in several studies (Nguyen, Kang & Lee 2007; Pereira & Gibson 2002; Pereira, McCartney & Gibson, 2003).

On the other hand, the other results of this study show that *E. faesium* has significant effects on the interleukin 10 level and that *E. faecalis* can noticeably increase interleukin 6 and 10. The current results showed that both *Enterococci* serotypes, especially *E. fecalis* were effective on the interleukin 10 level but only *E. faecalis* with the concentration of 3 McFarland was able to increase the interleukin 6 level. Interleukin 6 is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory cytokine. Interleukin 10 is an anti-inflammatory cytokine, also known as human cytokine synthesis inhibitory factor (CSIF). Considering anti-inflammatory activities of both interleukin 6 and 10, we conclude that treatment with *E. Faesium* may result in decreased immunologic reactions in the body, via increased production of anti-inflammatory interlukines.

The effects of the probiotics, including probiotic *Enterococci,* on the immune system of the body have been conveyed in many reports (Galdeano & Perdigon, 2006; Perdigon *et al.,* 1995). Studies by Zheng and his colleagues examined the anti-inflammatory effects of the dietary probiotic, *E. faecium*, on the liver of broiler chickens (Zheng *et al*., 2016). Molina and his colleagues have reported immune-stimulatory effects of *E. faecalis* probiotic strains (Molina *et al.,* 2015). Considering the fact that interleukin 2, 6, and 10 are related to both TH1 and TH2 lymphocytes, it can be concluded that the isolated *Enterococci* from Iranian traditional cheese can cause the increase of lymphocyte numbers in both TH1 and TH2 (Kiely, 1998).

CONCLUSION

This study shows that *Enterococci* in Iran traditional Ardebil cheese has potential to decrease serum total cholesterol and LDL-chol. More studies are needed to confirm this finding and the role of *Enterococci* in strengthening the immune system of the body.

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Conflict of interest

There is no conflict of interest to decare in this paper.

REFERENCES

Agerholm-Larsen L, Bell M, Grunwald G & Astrup A (2000). The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. *Eur J Clin Nutr* 54: 856-860.

Catanzaro JA & Green L (1997) Microbial ecology and probiotics in human medicine (Part II). *Alt Med Rev* 2: 296-305.

Chiquette J (2009). Evaluation of the protective effect of probiotics fed to dairy cows during a subacute ruminal acidosis challenge. *Anim Feed Sci Technol* 153: 278-291.

Corthésy B, Gaskins HR & Mercenier A (2007) Cross-talk between probiotic bacteria and the host immune system. *J Nutr* 137: 781S-790S.

De Vuyst L, Falony G & Leroy F (2008). Probiotics in fermented sausages. *Meat Sci* 80: 75-78.

Du Toit M, Franz C, Dicks L, Schillinger U, Haberer P, Warlies B, Ahrens F & Holzapfel W (1998). Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. *Int J Food Microbiol* 40: 93-104.

Efthymiou C, Baccash P, Labombardi V & Epstein D (1974). Improved isolation and differentiation of *enterococci* in cheese. *Appl Microbiol* 28: 417-422.

Galdeano CM & Perdigon G (2006). The probiotic bacterium *Lactobacillus case*i induces activation of the gut mucosal immune system through innate immunity. *Clin Vaccine Immunol* 13: 219-226.

Gill H & Guarner F (2004). Probiotics and human health: a clinical perspective. *Postgraduate Med J* 80: 516-526.

Kiely PD (1998). The Th1-Th2 model—what relevance to inflammatory arthritis? *Ann Rheum Dis* 57: 328-330.

Lourens-Hattingh A, & Viljoen B (2001). Growth and survival of a probiotic yeast in dairy products. *Food Res Int* 34: 791-796.

Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, Doyle J, Jewell L & De Simone C (2001). Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterol* 121: 580-591.

Makras L, Triantafyllou V, Fayol-Messaoudi D, Adriany T, Zoumpopoulou G, Tsakalidou E, Servin A & De Vuyst L (2006). Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Res Microbiol* 157: 241-247.

Mohan B, Kadirvel R, Bhaskaran M & Natarajan A (1995). Effect of probiotic supplementation on serum/yolk cholesterol and on egg shell thickness in layers. *Bri Poul Sci* 36: 799-803.

Mohan B, Kadirvel R, Natarajan A & Bhaskaran M (1996). Effect of probiotic supplementation on growth, nitrogen utilisation and serum cholesterol in broilers. *Bri Poul Sci* 37:395-401.

Molina MA, Díaz AM, Hesse C, Ginter W, Gentilini MV, Nuñez GG, Canellada AM, Sparwasser T, Berod L & Castro MS (2015). Immunostimulatory effects triggered by *Enterococcus faecalis* CECT7121 probiotic strain involve activation of dendritic cells and interferon-gamma production. *PloS One* 10: e0127262.

Nami Y, Haghshenas B, Abdullah N, Barzegari A, Radiah D, Rosli R & Khosroushahi AY (2015). Probiotics or antibiotics: future challenges in medicine. *J Med Microbiol* 64: 137-146.

Nguyen T, Kang J & Lee M (2007). Characterisation of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *Int J Food Microbiol* 113: 358-361.

Ohland CL & MacNaughton WK (2010). Probiotic bacteria and intestinal epithelial barrier function. *Am J Physiol Gastrointest Liver Physiol* 298: G807-G819.

Ooi L-G & Liong M-T (2010). Cholesterol-lowering effects of probiotics and prebiotics: a review of *in vivo* and *in vitro* findings. *Int J Mol Sci* 11: 2499-2522.

Perdigon G, Alvarez S, Rachid M, Agüero G & Gobbato N (1995). Immune system stimulation by probiotics. *J Dairy Sci* 78: 1597-1606.

Pereira DI & Gibson GR (2002). Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. *Appl Environ Microbiol* 68: 4689-4693.

Pereira DI, McCartney AL & Gibson GR (2003). An *in vitro* study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. *Appl Environ Microbiol* 69: 4743-4752.

Shah N (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. *J Dairy Sci* 83: 894-907.

Vahjen W & Männer K (2003). The effect of a probiotic *Enterococcus faecium* product in diets of healthy dogs on bacteriological counts of *Salmonella* spp., *Campylobacter* spp. and *Clostridium* spp. in faeces. *Arch Anim Nutr* 57: 229-233.

Varsha KK, Priya S, Devendra L & Nampoothiri KM (2014). Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. *Appl Biochem Biotechnol* 172: 3402-3413.

Vélez MP, Hermans K, Verhoeven T, Lebeer S, Vanderleyden J & De Keersmaecker S (2007). Identification and characterization of starter lactic acid bacteria and probiotics from Columbian dairy products. *J Appl Microbiol* 103: 666-674.

Wollowski I, Rechkemmer G & Pool-Zobel BL (2001). Protective role of probiotics and prebiotics in colon cancer. *The Am J Clin Nutr* 73: 451s-455s.

Zeyner A & Boldt E (2006). Effects of a probiotic Enterococcus faecium strain supplemented from birth to weaning on diarrhoea patterns and performance of piglets. *J Anim Physiol Anim Nutr* 90: 25-31.

Zheng A, Luo J, Meng K, Li J, Bryden WL, Chang W, Zhang S, Wang L, Liu G, Yao B (2016) Probiotic (*Enterococcus faecium)* induced responses of the hepatic proteome improves metabolic efficiency of broiler chickens (*Gallus gallus*). *BMC Genomics* 17: 1.