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Effect of Probiotics and α-Tocopherol Applications on Microbial Flora of Rat Gastrointestinal Tract

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Abstract: This study reports the effects of probiotics and α-tocopherol administration on microbial flora of rat gastrointestinal tract in a model system containing aspirin, ethanol and ammonia which are causative agent of gastric mucosal injury in animal and clinical studies. Sixty rats were used and randomly divided into three groups as Control (C), Probiotic mix culture (P) and α-Tocopherol (T). C, P and T groups received skim milk, probiotic mix culture and α-tocopherol for 14 days, respectively. Then, each group was also divided into four subgroups as aspirine, ethanole, ammonia and non-treated group. On day 15, aspirin, ammonia and ethanol were administrated to three subgroups, respectively. Non-treated subgroup just received saline in equal volume. In the 1, 5, 10 and 14th day of the feeding, fecal samples were taken from rats and lactic acid and coliform bacteria were determined. On day 15, rat intestine was taken out and examined for microbial flora. The results indicated that probiotic mix culture and α-tocopherol application had no significant effects on microbial flora of rat intestinal tract through 14 days of intake. In addition, there was not considerable difference in microbial flora in the jejunum, ileum and caecum for C, P and T groups with or without administration of aspirin, ethanol and ammonia.

Key words: Probiotics, α-tocopherol, rat, microbial flora, gastrointestinal tract

INTRODUCTION

Probiotics are microorganisms and presumed to be alive to exert a positive effect on the health and the well-being of the host (Guarner and Schaafsma, 1998). The probiotic culture should survive through the upper part of the gastrointestinal tract. High tolerance to enteric or pancreatic enzymes, low pH, bile salts and antibiotics corresponding to the conditions of the human gastrointestinal tract has been considered as important selection criteria (Fuller, 1992; Basyigit *et al*., 2006). It is important that probiotics are able to survive passage through the gastrointestinal tract irrespective of gastric acidity, pancreatic enzymes and bile acids so that they may reach the ileum and colon can colonize the intestinal mucosa (Holzapfel *et al*., 1998). There is evidence that the oral consumption of probiotics might have beneficial effects on several microbial disorders of the gut and produces a protective effect on the gut flora (Dembele *et al*., 1998; Gismondo *et al*., 1998).

The most commonly used strains are belong to the genera *Lactobacillus* and *Bifidobacterium* (Ouwehand *et al*., 2002). Lactic Acid Bacteria (LAB) are generally regarded as safe and widely used in fermentation of variety of food for the flavor, texture and preservation purposes. A certain strains can be used as probiotic organisms possess some important properties to improve human health. Previous studies showed that probiotic bacteria could maintain the healthy intestinal microbiota through competitive exclusion and antagonistic action against pathogenic bacteria in the animal intestine (Fuller, 1989).

It is well documented that probiotic bacteria inhibit the growth of various pathogenic bacteria due to the production of organic acids such as lactic and acetic acid (Gilliland and Speck, 1977), hydrogen peroxide, bacteriocins, bacteriocin like substances and possibility biosurfactants (Velraeds *et al*., 1998; Chang *et al*., 2001). In addition, probiotic bacteria could prevent the attachment of pathogens and stimulate their removal from

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the infected intestinal tract (Lee *et al*., 2000). The mechanisms of these benefical effects are related to exclusion of pathogenic bacteria by direct antogonism, competition for nutrients, adhesion reseptors and stimulation of host immunity (Elmer *et al*., 1996; Fuller and Gibson, 1997). Tocopherol (vitamin E) is a fatsoluble vitamin and widely recognized as an antioxidant and stabilizer of membranes (Wang and Quinn, 2000). Therefore, it is essential for human and animal health. The predominant isomer found in the body is α-tocopherol. It protects the polyunsaturated membrane lipids against free radical attack as a lipid antioxidant. α-tocopherol is an efficient scavenger of lipid peroxyl radicals, thus it is able to break peroxyl chain propagation reactions (Wang and Quinn, 2000). Other functions are believed to be act as membrane stabilizers by forming complexes with the products of membrane lipid hydrolysis, such as lysophospholipids and free fatty acids (Wang and Quinn, 1999; Quinn, 2004). The intestinal microflora and their metabolite have a vital role in human health by protecting the host from pathogenic bacteria (Cummings and Macfarlene, 1991). The ecological balance of the microflora may be distrupted by various diseases of the host (Salminen *et al*., 1995). Aspirin is widely used as antiinflammatory and analgesic agents. Despite its therapeutic benefits, aspirin can cause inflammation and ulceration in gastrointestinal tract (Whittle, 2004). Aspirin damages gastrointestinal mucosa by suppression of endogenous prostaglandin production (Wang *et al*., 1989) and exerting direct topical damage (Kauffman, 1989; Wallace *et al*., 1990). Ethanol has also been recognized as causative agent of gastrointestinal system in animal and clinical studies. Ethanol induced damage is associated with the depletion of gastric mucus content, decreased mucosal blood flow and mucosal cell injury (Jaarin *et al*., 2000). Ammonia is one of the pathogenic factors in *Helicobacter pylori* induced mucosal injury. Ammonia produced from urea by urease activity of *H. pylori* cause mucosal damage by decreasing mucosal cell viability (Tsujii *et al*., 1992; Murakami *et al*., 1993; Mori *et al*., 1998). Even though aspirin, ethanol and ammonia play roles in gastric mucosal damage, their effects on the microflora in gastrointestinal tract is not well established.

The aim of this study was to investigate the effects of probiotic mix culture (*L. fermentum*, *L. plantarum* and *E. faecium*) and α-tocopherol administration on microbial flora in rat feces during 14 days of feeding period. The role of probiotic mix culture and α-tocopherol on microbial flora in the gastrointestinal tracts of rats with or without aspirine, ethanol and ammonia administration was also investigated.

Animals: Male Wistar albino rats (200-250 g) were fed on standard laboratory diet and water *ad libitum* and kept in cages at a temparature (22 +/- 2°C) with a 12 h dark-light cycle before and during experiments. Experiments were approved by Suleyman Demirel University School of Medicine Ethical Commity. During this experimental study, we acted according to the principles of guide for the care and use of laboratory animals.

MATERIALS AND METHODS

Probiotic mix culture: In this study, a probiotic mix culture consisting of *L. fermentum* (BB16-75, AK2-8, AK5-22, AK6-26), *L. plantarum* (AA17-73, AK7-28, AK8-31B) and *E. faecium* (AB6-21, AB16-68, AK-4-120, AK7-31, BK9-40, BK13-54) was used. These strains were isolated from feces samples taken from nineteen adult volunteers at Suleyman Demirel University Research Hospital, Turkey. Some probiotic properties of these isolates were determined (Basyigit *et al*., 2006). These strains were also identified with 16S rRNA analysis (Basyigit *et al*., 2006). Each strain was inoculated in MRS broth medium and incubated at 37°C for 24 h. Growth rate was adjusted at the level of 10^9 cfu mL^{-1} for each strain. The cells were pelleted by centrifugation at 5000×g for 10 min at 20°C. Pellets were washed in phosphate Buffered Saline (PBS, pH 7.4) twice. Finally probiotic mixture was adjusted to 1.3×10^{10} cfu mL^{-1} in 10% reconstitute sterile skim milk.

Study design: Sixty animals were randomly assigned to three treatment groups (twenty rats in each group) as control (C), Probiotic mix culture (P) and α-tocopherol (T). C, P and T groups received 0.2 mL skim milk, 0.2 mL of $1,3\times10^{10}$ cfu mL^{-1} of probiotic mix culture and 100 mg kgL^{-1} in a volume of 0.2 mL day^{-1} of α-tocopherol by oral gavage once a day for 14 days, respectively. Then each group was also divided into four subgroups (five rats in each group) as Aspirine (ASP), Ethanole (ETH), Ammonia (AM) and Non-Treated group (NT). On day 15, aspirin (200 mg kg^{-1}), ammonia and 98% ethanol (1 mL) were administrated to three subgroups, respectively. NT subgroup just received saline (1 mL) instead of aspirin, ammonia and ethanol in equal volume. Rats were fasted for 12 h before the experiment but they had free access to the drinking water. Coprophagy was avoided. All treatments were administered orally by gavage through an intragastric tube.

Determination of viable bacterial counts in feces: In the 1, 5, 10 and 14th day of the feeding, fecal samples were taken from the rats. Fecal samples, obtained by manually

pressing the lower abdomen of rats were analyzed individually by suspending 50 mg with a glass rod in 0.5 mL of 0.1 % (w/v) sterile peptone water to obtain a concentration of 100 mg mL^{-1}. The suspensions were serially diluted 10-fold and appropriate dilutions were plated in duplicate on MRS agar for LAB and EMB agar for fecal coliforms. All plates were incubated at 37°C for 24-48 h (Du Toit *et al*., 1998).

Microbiological analyses of intestine system: To determine effect of the aspirine, ethanol and ammonia administrations on the bacterial levels in intestinal system, tissue samples from jejunum, ileum and caecum were aseptically taken for bacterial counting. Briefly, tissue samples were weighed and suspended in 10 fold PBS (Elliott *et al*., 2000). The tissue were homogenised (LG-10640 Tissue Grinder, Glass Pestle, Labglass, US) for 60 sec and serial dilutions were plated onto MRS agar for lactic acid bacteria and EMB agar for coliform groups of bacteria. All plates for LAB were incubated for 48 h at 37°C in anaerobic incubator. Coliform groups were incubated in aerobic conditions for 48 h at 37°C.

Statistical analysis: The significance of differences in quantitative variables between groups was performed by one-way analysis of variance (ANOVA) and Tukey's test was used for pairwise comparisons between means by using the MINITAB V.14.1 (MINITAB Inc. USA, 2003).

RESULTS AND DISCUSSION

Microbiological analyses of intestine system: The present study compared the effect of skim milk, probiotic mix, α-tocopherol on rats' intestinal microbial flora in conditions where aspirin, ethanol and ammonia are existing. The results are shown in Table 1. The results of this study indicated that there was not significant difference in the numbers of LAB and coliform bacteria in the jejunum, ileum and caecum for C, P and T groups with or without administration of aspirin, ethanol and ammonia ($p>0.05$). There was also not any significant difference in aspirin, ammonia and ethanol applied groups for coliform level in the jejunum, ileum and caecum among the all treatment groups (C, P and T). However, LAB count in caecum in Non-Treated (NT) groups of C, P and T were higher than that of jejenum and ileum ($p< 0.05$). Minor differences in LAB population levels from jejunum, ileum and caecum of rats received skim milk or probiotic or α-tocopherol indicated that administration of probiotics did not alter the gross composition of bacterial ecosystem in the intestinal tract.

The aspirin, ammonia and ethanol administrations did also not affect the number of lactic acid bacteria which were treated with α-tocopherol. The lowest LAB count in gastrointestinal tract administrated with ammonia was determined in groups treated with α-tocopherol. This indicated that α-tocopherol was not able to prevent negative effect of ammonia well on growth of bacteria in intestinal tract. In all groups administrated with aspirin, LAB and coliform counts of jejenum were the lowest compared to those of ileum or caecum.

The highest LAB and coliform counts were obtained in caecum. However, differences among jejenum, ileum and caecum was statistically not significant ($p>0.05$). Hayashi *et al*. (2005) mentioned that caecal microbiota were more complex than jejunal and ileal microbiota. On the other hand, Marteau *et al*. (2001) found that the LAB and *Escherichia coli* were more prevalent in the caecum. Similar to our results Mangell *et al*. (2006) reported that the total number of lactobacilli in the intestine did not increase when the rats were fed with *L. plantarum* 299v at the level of 1.1×10^{10} cfu mL^{-1} for 8 days.

Table 1: The effects of probiotics and α-tocopherol with or without aspirin, ethanol and ammonia administration on microbial flora of rat gastrointestinal tract (log_{10} cfu g^{-1})

	Lactic acid bacteria			Coliform bacteria		
Groups	J	I	C	J	I	C
C+NT	5.47±0.50	5.62±0.54	8.37±0.33	4.54±0.14	5.07±0.12	7.06±0.67
P+NT	3.92±0.10	6.29±1.15	7.81±0.26	4.16±0.60	7.08±1.84	8.02±0.73
T+NT	4.17±0.4	6.57±0.28	7.52±0.40	5.26±0.54	7.92±0.26	7.52±0.74
C+ASP	4.47±0.08	5.97±0.32	7.56±0.22	5.32±0.12	6.81±1.74	7.41±0.36
P+ASP	3.86±0.05	6.92±0.03	7.42±0.74	6.11±0.15	5.20±0.25	6.03±0.85
T+ASP	4.05±0.07	6.19±0.50	7.31±0.37	5.98±0.69	5.10±0.69	6.59±0.58
C+AM	5.84±0.08	7.47±0.08	7.90±1.07	6.38±0.72	8.68±0.76	7.91±0.79
P+AM	4.51±0.44	6.07±0.78	7.51±0.68	5.28±0.08	6.81±0.97	7.49±0.24
T+AM	3.90±0.66	4.77±047	6.71±0.38	4.89±0.28	6.10±0.11	7.11±0.28
C+ETH	5.63±0.90	7.90±0.57	7.73±0.73	6.78±0.81	8.05±0.21	7.56±0.33
P+ETH	4.57±0.75	5.12±0.24	6.74±0.75	4.62±0.96	4.38±0.40	7.31±0.81
T+ETH	4.93±0.15	6.08±0.58	7.51±0.10	5.23±0.22	6.28±0.66	7.03±0.38

C: Control, P: Probiotic, T: α-tocopherol, ASP: Aspirine, AM: Ammonia, ETH: Ethanole, J: Jejenum, I: Ileum, C: Caecum

The factors important for colonization of the indigenous lactobacilli in the gastrointestinal tract are largely unknown (Deplancke *et al*., 2002).

However, it is a well known fact that lactobacilli constitute a significant part of the indigenous intestinal microflora (Wang *et al*., 2001). Some other researches about intestinal microflora showed that it can be difficult to differentiate and enumarate the exogenous lactobacilli from native species (Gorbach, 2002).

Herias *et al*. (1999) compared two different diets (one with *E. coli* alone and another one with *E. coli* and *L. plantarum*) on rats. They reported that *E. coli* established in the caecum at 10^9-10^{10} cfu g^{-1} of contents while the numbers in the small intestine were lower and more variable.

The researchers indicated that *L. plantarum* reduced the *E. coli* levels, both in the caecum and in the small intestine. They showed that this difference was significant 1 week after colonization but not after 5 weeks.

They also reported that *L. plantarum* colonized the small intestine and the caecum; the population levels increased between 1 and 5 weeks after colonization but did not reach as high levels as *E. coli*.

Ichikawa *et al*. (1999) reported that the major genera such as *Bacteroides*, *Eubacterium* and *Enterococcus* in the cecum were not affected by the administration of probiotics. They also reported that the total number of lactobacilli did not vary between control and probiotics applied groups. It was reported that the concentration of the bacterial species in the intestinal tract varied with the age and with the diet of rats (De LeBlanc *et al*., 2008). With increasing the age of rats, microbial flora becomes stable and changing microbial flora with oral feeding administrations is more difficult. Fak *et al*. (2008) reported that a 10-fold increase in the mean number of lactobacilli was obtained in the young rats whereas, there was only a 2-fold increase in the older groups due to probiotic *L. plantarum* treatment. This may explain insignificant increase in the number of microbial flora in the study since mature rats were used in this study.

On the other hand, Johnson-Henry *et al*. (2004) reported that the higher lactic acid bacterial populations were determined in mice treated with probiotic mixture consisting of *Lactobacillus rhamnosus* R0011 and *Lactobacillus acidophilus* R0052 compared with untreated controls.

The results of this study clearly demostrated that consuming milk, probiotic and alpha-tocopherol may stabilize the LAB population in intestinal tract in presence of aspirin, ethanol and ammonia.

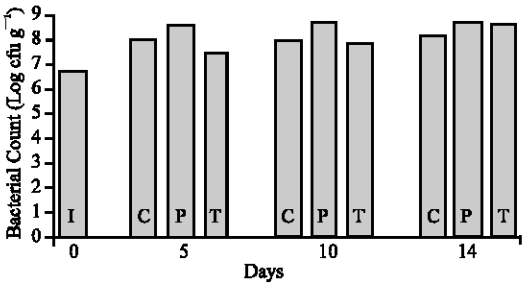

Fig. 1: The effects of probiotics and α-tocopherol administration on LAB in rat feces during 14 days of feeding period, I: Initial LAB count, C: Control, P: Probiotic, T: α-tocopherol

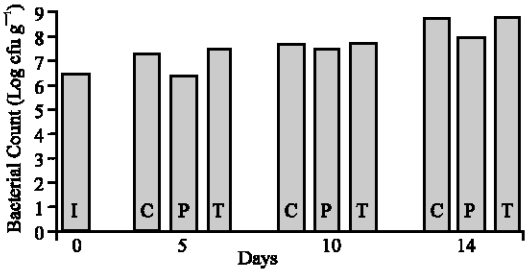

Fig. 2: The effects of probiotics and α-tocopherol administration on coliform level in rat feces during 14 days of feeding period, I: Initial coliform level, C: Control, P: Probiotic, T: α-tocopherol

Determination of viable bacterial counts in feces: The number of LAB and coliform in feces during 14 days of feeding period are shown in Fig. 1 and 2. The results showed that initial number of LAB in feces was 6.3×10^6 cfu g^{-1}. Then LAB level reached 1.2×10^8 on day 14 while it was 4.4×10^8 and 4.2×10^8 cfu g^{-1} for P and T, respectively. There was a 2 log difference between C and other groups (P and T).

Hovewer, there was no considerable difference between P and T groups for LAB counts. In a similar study, Chang *et al*. (2001) reported an increase in the *Lactobacilli* count in feces of rat that was fed basal diet devoid of probiotic agent.

Another study indicated that twice daily intake of the probiotic *L. plantarum* 299v for 2 weeks significantly increased the number of faecal lactobacilli from log 4.4 to log 7.9 cfu g^{-1} feces (Goossens *et al*., 2005). On the other hand, there was a significant increase in the number of coliforms after 14 days in both C, P and T groups ($p<0.05$) while the difference between groups was not significant ($p> 0.05$). Coliform level in control group was 3.1×10^6 at the beginning of feding. Then it increased to 5.8×10^8, 8.5×10^8, 6.1×10^8 on day 14 for C, P and T groups, respectively.

The increase in the number of coliform group bacteria may indicate that the ability of the lactobacilli to inhibit translocation is not through reduction of the number of potentially pathogenic bacteria present in the intestine (Mangell *et al*., 2006). Animal feed is an important factor that influences the composition of the intestinal microflora. In this research, the nutritional compositon of skim milk may propogate the growth of enteric bacteria in the gut, causing high numbers of coliforms in feaces. An earlier report showed that a selected probiotic strain *L. reuteri* and *L. acidophilus* showed increasing effect on numbers of enterobacteria in piglets. Moreover, it was also thought that feeding the rats with non-sterile feed may also cause the increase of the coliforms as well as some other enteric bacteria.

Minor differences in faecal bacterial population levels were found among experimental groups. The results indicated that diet with skim milk, probiotic and α-tocopherol did not alter the population level of fecal bacteria. Even though addition of probiotic did not change the levels of LAB in intestinal system, it affected the nature of intestinal system. Addition of probiotic mix culture resulted in lower coliform level in intestinal system compared to the control.

CONCLUSION

In this study the results showed that probiotic mix culture and α-tocopherol administration had no significant effects on microbial flora of rat gastro intestinal tract through 14 days of intake. In addition, there was not significant difference in microbial flora in the jejunum, ileum and caecum for skim milk, probiotic mix culture and α-tocopherol applied rat groups with or without application of aspirin, ethanol and ammonia. More research with longer period of feeding time is needed to determine the effects of probiotics on increasing LAB count in gastrointestinal tract.

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