The effects of probiotic, prebiotic and synbiotic diets containing Bacillus coagulans and inulin on rat intestinal microbiota

Abhari, Kh.\(^1\); Shekarforoush, S. S.\(^2\); Sajedianfard, J.\(^3\); Hosseinzadeh, S.\(^2\) and Nazifi, S.\(^4\)

\(^1\)Ph.D. Student in Food Hygiene, Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; \(^2\)Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; \(^3\)Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; \(^4\)Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

*Correspondence: S. S. Shekarforoush, Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: Shekar@shirazu.ac.ir

(Received 12 Jan 2015; revised version 28 Apr 2015; accepted 19 May 2015)

Summary

An in vivo experiment was conducted to study the effects of probiotic Bacillus coagulans spores, with and without prebiotic, inulin, on gastrointestinal (GI) microbiota of healthy rats and its potentiality to survive in the GI tract. Forty-eight male Wistar rats were randomly divided into four groups (n=12) and fed as follows: standard diet (control), standard diet supplied with 5% w/w long chain inulin (prebiotic), standard diet with 10\(^5\)/day spores of B. coagulans by orogastric gavage (probiotic), and standard diet with 5% w/w long chain inulin and 10\(^5\) spores/day of B. coagulans by orogastric gavage (synbiotic). Rats were fed the diets for 30 days. At day 10, 20 and 30 of experiment, 24 h post administration, four rats from each group were randomly selected and after faecal collection were sacrificed. Small intestine, cecum, and colon were excised from each rat. The effects on microbial population in the gastrointestinal tract (except small intestine) in synbiotic, probiotic and prebiotic fed groups were also seen. The obvious decline in spores count through passing GI tract and high surviving spore counts in faecal samples showed that spores are not a normal resident of GI microbiota and affect intestinal microbiota by temporary proliferation. In conclusion, the present study clearly showed probiotic B. coagulans was efficient in beneficially modulating GI microbiota and considering transitional characteristics of B. coagulans, daily consumption of probiotic products is necessary for any long-term effect.

Key words: Bacillus coagulans, Intestinal microbiota, Prebiotic, Probiotic, Synbiotic

Introduction

In recent times, the use of probiotics in various food products has been increased in addition to suggesting their use as a food supplement for therapeutical purposes. Probiotic therapy is very attractive because it is an effective and noninvasive low cost approach which attempts to recreate natural flora rather than its disruption (Hammerman et al., 2006; Sorokulova et al., 2008).

The bacterial flora of the GI tract play major roles in human physiology by modulating metabolic and immunological processes to prevent overgrowth of opportunistic microorganisms (Plummer et al., 2005). Many disorders of the gut are believed to be correlated with disturbance in this distribution of normal bacterial species choosing the growth of pathogenic strains (Kalman et al., 2009).

The balancing action of probiotics upon the intestinal microbiota involves an increase in bacterial components, especially Lactobacilli and Bifidobacteria that may be beneficial to the host and a reduction in the potentially harmful microorganisms such as coliforms and clostridia, thus reducing risk of diarrhea, inflammatory and allergic diseases (Marzotto et al., 2006).

Microorganisms used as a probiotic for human are mainly Gram-positive bacteria belonging to the Lactobacillus and Bacillus spp. Bacillus probiotics differ in many characteristics from those based on Lactobacillus spp. While Lactobacillus represents a normal resident GI microflora of humans, Bacillus belongs only to the transitory GI bacteria (Sorokulova et al., 2008).

The members of genus Bacillus are endospore forming bacteria that make it extremely heat-stable and resistant to adverse gastrointestinal tract conditions and, when germinate in GI tract, cause positive effects for the host (Hoa et al., 2001; Losada and Oller, 2001; Casula and Cutting, 2002). Recently probiotic strain of Bacillus coagulans that can withstand the low pH of stomach acid and is activated in the intestines to modulate the gut microbiota has been granted self-affirmed GRAS status by the FDA (Cutting, 2011).

Prebiotics are food ingredients that cannot be digested by the human digestive system but are metabolized by discrete enteric microbes, thus
stimulating proliferation of selected GI bacteria species thought to be beneficial for human health. Fructooligosaccharides (FOS), which are extracted from chicory root in the form of inulin is a recommended prebiotic compound (Mikkelsen et al., 2003; Ogawa et al., 2005).

A symbiotic, on the contrary, represents a defined supplement comprised of a mixture of probiotics and prebiotics purposed to enhance the survival and colonization of the supplemented species in the GI tract (Rauch and Lynch, 2012).

The role of *B. coagulans* in changing GI flora of broilers has been studied by Lin et al. (2011). They reported a significant increase in cecal *Lactobacillus* population and decrease in *E. coli* counts in treatments fed diets supplied with *B. coagulans* (Lin et al., 2011). Also, studies suggest that *B. coagulans* decreases the symptoms of abdominal pain and bloating in subjects with inflammatory bowel disease. Another study showed that *B. coagulans*-based product was effective in improving the quality of life and reducing gastrointestinal symptoms in adults with post prandial intestinal gas related symptom in sixty-one adult healthy subjects (Kalman et al., 2009). Based on the results obtained in the above mentioned studies, the objective of this study was to determine the effect of oral administration of *B. coagulans* spores and inulin as single supplements as well as a dietary combination on the microbial population of different parts of GI of rats.

**Materials and Methods**

**Preparation of spore suspension of probiotic bacteria**

Lyophilized probiotic *B. coagulans* was donated by the Pardis Roshd Mehregan Co., Iran. It was grown aerobically in nutrient yeast extract salt medium (NYSM) agar (Russell et al., 1989) at 37°C for 24 h. A single colony from the NYSM plate was inoculated into 500 ml of NYSM broth and incubated at 37°C with shaking at 250 rpm for 48 h. The bacterial suspension was pelleted three times by centrifugation at 3000 × g for 20 min, and washed with sterile normal saline. Final pellet was resuspended in 100 ml sterile normal saline.

To determine the spore per ml of suspension, the solution was heated at 80°C for 15 min to kill the vegetative cells before appropriate serial dilution and plating in NYSM agar. Finally, the spore suspension was prepared at a concentration of 1 × 10^8 spore/ml in sterile saline and kept in the refrigerator until use.

**Animals and diets**

Forty-eight male Wistar rats (200 ± 8.4 g) were provided by the Animal Centre of Razi Research Institute, Shiraz, Iran. Animals were housed in groups of six rats per cage in a temperature controlled environment (22 ± 2°C) with 55 ± 10% relative humidity and controlled lighting (12 h light/dark cycle).

Rats were randomly divided into 4 groups and fed as follows: 1) standard diet (control), 2) standard diet supplemented with 5% w/w long chain inulin (Sensus, Netherlands) (prebiotic), 3) standard diet with 10^7 spores/day *B. coagulans* (gavage 1 ml of prepared spore suspension using a blunt ended needle) (probiotic), and 4) standard diet supplied with 5% w/w long chain inulin and 10^7 spores/day *B. coagulans* (symbiotic). The standard pellet feedstuff contained 14.5% protein, 4.7% ash, 51.2% starch, 4.3% sugar and 4% fat (3.2 kcal/g). Regarding micronutrients, the feedstuff contained 0.72% calcium, 0.6% phosphorus, 0.23% magnesium and 0.25% chloride among others. To add inulin, the standard pellet feedstuff was first broken to powder and after adding 5% inulin, carboxymethyl cellulose (CMC) 0.1% was used to rebind the ingredients and left at room temperature until dried. The inulin content in the rat diet was calculated based on food intake. The food intake of each rat with mean of 200 g body weight is 10 g/day, it means each rat received 0.5 g inulin/day. Food and tap water were provided *ad libitum*.

To assimilate the experimental conditions, the control and prebiotic group were gavaged with 1 ml of sterile normal saline once a day.

**Experimental design**

All animals were acclimatized for 2 weeks before the experimental session. Rats were fed the diets for 30 days and had free access to the experimental diets and tap water. At day 10, 20 and 30 of experiment (24 h post *B. coagulans* administration) 4 rats from each group were randomly selected and sacrificed after faecal collection. Following this, dissection and ligation of selected organs, small intestine (duodenum, jejunum and ileum), cecum, and colon were excised from each rat. Samples of organs were weighed including luminal contents and used for immediate microbiological analysis. This experiment was accomplished under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986 were followed, regarding the standards in the protection of animals used for experimental purposes.

**Microbiological analysis**

Five g of samples (dissected organs or faeces) tissues were transferred into stomacher bags along with 45 ml of sterile buffered peptone water and homogenized in a stomacher for 2 min. A ten-fold serial dilution was performed and appropriate dilutions were pour plated using plate count agar (PCA) (Merck, Germany) and incubated at 37°C for 48 h to obtain the total aerobic and anaerobic bacterial counts. Anaerobic plates were placed in anaerobic jar using an anaerobic gas pack system (Anaerocult A, Merck, Germany). Lactic acid bacteria (LAB) were enumerated using Man Rogosa agar (Merck 5413, Darmstadt, Germany) after incubation in an anaerobic environment at 37°C for 48 h. Enterobacteriaceae were enumerated on MacConkey agar (Merck 5465, Darmstadt, Germany) after incubation at 37°C for 24 h. *B. coagulans* spore counts were determined on NYSM agar after incubation at 37°C for
was not changed in prebiotic fed group compared to control during experiment. There were no significant changes (P>0.05) seen in the number of aerobic bacteria in small intestine during feeding trials.

**Results**

**LAB counts**

The populations of LAB in the faecal samples and various segments of GI tract of rats were not affected by the experimental diets after 10 days of feeding (Figs. 1A-D). A significant effect was observed, 20 and 30 days post feeding trials. The number of LAB was significantly higher throughout small intestine of synbiotic and prebiotic fed rats as compared with probiotic fed and control diet at 20 (P=0.009) and 30 days (P=0.002) of feeding trials. Results showed that population of LAB in cecum, colon and faecal samples of rats fed with synbiotic and probiotic diets were significantly (P<0.05) higher than other experimental groups. Population of LAB in colon and faecal samples were 8.4-8.8 log CFU/g in synbiotic and probiotic group following 30 days, these values were 7.3-7.6 log CFU/g in prebiotic and control groups.

**Enterobacteriaceae counts**

The enterobacterial populations were not significantly different during first 10 days but then decreased in the supplemented groups (P<0.05) (Figs. 2A-D). At day 20 and 30 the numbers of Enterobacteriaceae in various segments of the GI tract (except small intestine) of synbiotic, probiotic and prebiotic fed groups were significantly lower compared to control. Highest level of decrease in enterobacterial population referred to synbiotic fed group then probiotic and prebiotic fed groups, respectively. Enterobacterial counts in cecum, colon and faecal sample of control group increased about 1 log CFU/g after 30 days. Results showed that feeding trials inhibited enterobacterial growth. After twenty days of feeding trials, enterobacterial count in cecum and colon of synbiotic fed groups were significantly (P<0.05) lower than prebiotic and probiotic fed groups.

**Total aerobic bacteria**

Results for number of total aerobic bacteria are shown in Figs. 3A-D. In groups fed with synbiotic and probiotic diet, significantly higher numbers of aerobic bacteria were observed in the caecum, colon and faecal samples (P<0.05) as compared with prebiotic fed group and control in the days 20 and 30. Feeding with synbiotic and probiotic diets led to 1 log CFU/g increase in total aerobic bacteria level of GI tract. Total aerobic bacteria population referred to synbiotic fed group then probiotic and prebiotic fed groups.
diets (P>0.05) (Figs. 4A-D). Results obtained showed that total anaerobic bacteria in caecum, colon and faecal samples of *B. coagulans* fed groups were significantly higher compared to prebiotic fed and control. The level of total anaerobic bacteria on days 20 and 30 in symbiotic and probiotic fed groups were 1 log CFU/g higher than prebiotic fed and control groups. An additive effect of inulin with *B. coagulans* on total anaerobic bacterial growth was seen in symbiotic fed group compared to probiotic group, which was not significant (P>0.05).

### Bacillus coagulans spores count

Results for the number and distribution of *B. coagulans* spores are shown in Figs. 5A-C. A significant increase in the mean level of *B. coagulans* spores was observed following passing through from small intestine to distal parts of GI tract. The highest and lowest levels of spores were found in faecal samples and small intestine, respectively, 24 h post dosing. The number of spores was significantly higher in symbiotic fed rats compared to probiotic fed in faecal sample and various segments of gastrointestinal tract except small intestine.

**Fig. 2:** Entrobacterial counts of small intestine (A), cecum (B), colon (C) and faeces (D) of Wistar rats fed with symbiotic (●), probiotic (●), prebiotic (●) and control (●) during 30 days trial. Values not sharing the same superscript are significantly different (P<0.05). Bars represent standard error values

**Fig. 3:** Total aerobic counts of small intestine (A), cecum (B), colon (C) and faeces (D) of Wistar rats fed with symbiotic (●), probiotic (●), prebiotic (●) and control (●) during 30 days trial. Values not sharing the same superscript are significantly different (P<0.05). Bars represent standard error values

Fig. 4: Total anaerobic counts of small intestine (A), cecum (B), colon (C) and faeces (D) of Wistar rats fed with synbiotic (■), probiotic (■), prebiotic (■) and control (■) during 30 days trial. Values not sharing the same superscript are significantly different (P<0.05). Bars represent standard error values

Discussion

The ability of probiotic strains to survive, transit and to colonize the GI tract is considered as an important factor for providing potential health benefits. Thus, the use of Bacillus products raised a number of questions, including their mode of action. In the present study, response of rat microbiota at different times during oral intake of B. coagulans spores was investigated. There has been limited information on probiotic activity of B. coagulans spores on GI tract flora. Studies on the effects of B. coagulans on animals have been limited to those who investigated the effects of administration of B. coagulans spores on the chicken growth performance and composition of intestinal microflora. Cavazzoni et al. (1998) reported significant improvement in chicken performance treated with Bacillus as compared with chicken receiving no additive with highest mean body weights and daily weight gains. Another study by Lin et al. (2011) examined the effect of B. coagulans spores on the intestinal microbiota of broiler chicken.

Fig. 5: Bacillus coagulans spore counts after ten (A), twenty (B), thirty (C) days of different organs of Wistar rats fed with synbiotic (■), and probiotic (■), during 30 days trial. Values not sharing the same superscript are significantly different (P<0.05). Bars represent standard error values
The present study is the first study where the effect of *B. coagulans* spores alone and in combination with inulin was studied on GI flora of rat as an animal model.

*B. coagulans* spores, when administered at a level of 10^6 spores/day for 30 days, caused an increase in LAB number. Results of LAB count showed that *B. coagulans* spores were activated in cecum and promote the LAB growth in distal segments of gastrointestinal tract. The numbers of LAB were significantly higher throughout small intestine of synbiotic and probiotic fed rats as compared with probiotic fed and control diet (P<0.05). This could be due to the fact that *B. coagulans* spores in small intestine were still not activated and proliferated and only inulin promoted the LAB growth. It is in agreement with data shown by Casula and Cutting (2002) who indicated that spore germination was not detected in the duodenum but was readily detectable in the ileum. The small intestine contains regions of different physicochemical conditions, and the high content of stomach acids and bile salts in duodenum may support spore germination (Casula and Cutting, 2002). Higher LAB count in synbiotic fed rats indicates additive effect between inulin and *B. coagulans*. This idea was supported by Gallaher and Khil (1999) who showed that administration of probiotic oligofructose with probiotic *Bifidobacterium* to rats resulted in additive effects compared to their individual administration.

In the present study, enterobacterial count of control group increased over time (P<0.05), while their number decreased in other treatment groups. Strongest effect of different diets in decreasing enterobacterial population referred to synbiotic fed group then probiotic and probiotic fed group, respectively. Enterobacteriaceae family contains many potentially pathogenic/harmful bacteria. Thus, reduction or control of proliferation of these bacteria plays a role in maintaining health and well-being and in reducing the risk of some diseases including antibiotic associated diarrhea, acute gastroenteritis, inflammatory bowel disease, food allergy, and colon cancer (DeRoos and Katan, 2000).

The results obtained in this study are in agreement with a previous study by Lin et al. (2011) who found an increase in LAB counts and a decrease in *E. coli* counts in the duodenum and cecum of broilers fed with *B. coagulans* spores.

Supplementation with *B. coagulans* spores significantly increased total anaerobic bacteria in faecal sample and various segments of GI tract (except small intestine). The reason for the increase in the total bacterial counts at the follow up period is not clear, it may be due to facultative anaerobic characteristic of *B. coagulans* (De Vecchi and Drago, 2006), which is proved by Ripamonti et al. (2009) who showed the ability of *B. coagulans* to grow in anaerobic conditions. Thus anaerobic conditions of small intestine and presence of suitable nutritional environment (e.g., fructose, L-alanine) stimulated spores germination (Casula and Cutting, 2002).

It is possible that higher number of total aerobic bacteria in *B. coagulans* spores fed groups (synbiotic and probiotic) compared with probiotic fed and control was correlated to increase in *Lactobacillus*. This result is similar to results shown by Matsumoto et al. (2010) who reported an increase in total aerobic bacteria following *Lactobacillus casei* consumption. These data are in agreement with earlier studies that showed probiotics supplementation increases faecal total *Lactobacilli*, anaerobic counts and *Bifidobacteria* concentrations significantly (Kuisma et al., 2003). Perhaps the characteristics of *B. coagulans* probiotic in changing GI microbiota prevent GI disorders, considering previous studies indicated that the total faecal obligate anaerobe, Bacteroidaceae, *Bifidobacterium*, and *Lactobacillus* counts in patients with acute diarrhea were lower than in healthy adults (Hong et al., 2005). Moreover, another study specified that, in patients with irritable bowel syndrome characterized by diarrhea (IBS-D), the *Bifidobacterium* spp. counts were lower than in a healthy control group (Malinen et al., 2005).

One of the important findings of this study was the high concentration of *B. coagulans* spores (9 log spores/g) in faecal samples that was equal to inoculum dose. This data shows *B. coagulans* spores are stable to bile and acidic condition of GI tract. Previous studies showed the ability of *Bacillus* species to survive in host GI tract (Hoa et al., 2001; Duc et al., 2004; Ripamonti et al., 2009). Another study indicated *B. coagulans* GanedenBC30 highly survived (70%) in a dynamic, validated, *in vitro* model of the stomach and small intestine, although germination of the spores was minimal (<10%) under the conditions tested (Maathuis et al., 2010).

The obvious decline in spores count through passing rat GI tract while affecting GI microbiota supports the fact that *Bacillus* spp. could have an endosymbiotic relationship with the host, being able to temporarily survive and proliferate within the GI tract (Hong et al., 2005). An *in vivo* study reported *B. coagulans* being lost one week after administration (Cavazzoni et al., 1998).

The results of this study showed that use of inulin caused significant lowering effect on enterobacterial population. Inulin, which was used in combination with *B. coagulans* as symbiotic, showed an increase, but not a significant effect, in promoting both LAB and *B. coagulans* populations. Several studies showed significant differences in the microbiota of rats fed with inulin. Most studies reporting inulin-derived benefits have assayed relatively high inulin doses, especially in animals. However, the present study showed a low inulin intake could also exert some benefits by exploring differential effects on intestinal microbiota (Azorin-Ortuno et al., 2009).

In conclusion, using an animal model, the present study clearly showed that *B. coagulans* was efficient in beneficially modulating GI microbiota and considering transitional characteristic of probiotic *B. coagulans* that are not able to colonize the intestine and are quickly eliminated in faeces, daily consumption of probiotic products is necessary for any long-term effect.
Acknowledgement

The work was financially supported by School of Veterinary Medicine, Shiraz University.

References


Matsumoto, K; Takada, T; Shimizu, K; Moriyama, K; Kawakami, K; Hirano, K; Kajimoto, O and Nomoto, K (2010). Effects of a probiotic fermented milk beverage containing Lactobacillus casei strain Shirota on defecation frequency, intestinal microbiota, and the intestinal environment of healthy individuals with soft stools. J. Biosci. Bioeng., 110: 547-552.


Plummer, SF; Garaiova, I; Sarvotham, T; Simon, L; Cottrell, SL; Scouiller, SL; Weaver, MA; Tang, J; Dee, P and Hunter, J (2005). Effects of probiotics on the composition of the intestinal microbiota following antibiotic therapy. Int. J. Antimicrob. Agents. 26: 67-74.


