Wageningen Academic Publishers

The influence of probiotic *Enterococcus faecium* strain I5 on the microbiota and cytokines expression in rats with dysbiosis induced by antibiotics

E. Tarasova[1], E. Yermolenko[1], V. Donets[2], Z. Sundukova[2], A. Bochkareva[1], I. Borschev[3], M. Suvorova[1], I. Ilyasov[1], V. Simanenkov[2] and A.N. Suvorov[1]

[1]Institute of Experimental Medicine, Ak. Pavlova st. 12, 197376 St. Petersburg, Russian Federation; [2]Medical Academy, Postgraduate School, St. Petersburg, Russian Federation; [3]Institute of Physiology, St. Petersburg, Russian Federation; alexander_suvorov1@hotmail.com

Received: 28 February 2010/ Accepted: 13 July 2010

Abstract

The animal model of intestinal dysbiosis induced by antibiotics was created. Dysbiotic condition was confirmed by the changes in titre of the indigenous microbiota (excessive growth of opportunistic microorganisms and reduced number of lactobacilli, bifidobacteria and enterococci) and the appearance of dyspeptic symptoms. Consumption of the fermented milk product with probiotic strain *Enterococcus faecium* L5 led to the rapid disappearance of dysbiosis symptoms, normalisation of the microbiota, increase in expression of IL-10 and decrease in IL-8 expression.

Keywords: fermented milk products, colonic microflora/microbiota, *Enterococcus faecium*, immune modulation

1. Introduction

Implementation of probiotics have been proved to be useful in many clinical conditions including antibiotic-associated diarrhoea, travellers' diarrhoea, lactose intolerance, prophylactic and treatment of infection diseases (Elmer, 2001; Foulquie Moreno *et al.*, 2006). Probiotics are the 'friendly bacteria' usually containing lactic acid bacteria (LAB) including *Lactobacillus* spp., *Lactococcus* spp. or *Enterococcus* spp. strains. After being taken orally by the patients, probiotics interact with indigenous intestinal flora. They are expected to protect the host from infection induced by the pathogenic bacteria, to participate in the host metabolic processes and positively influence the host's immunity (McFarland, 1998; Sheil *et al.*, 2007; Tamboli *et al.*, 2004).

Immunological effects of LAB include the changes in production of immunoglobulins (Haghighi *et al.*, 2005; Scharek *et al.*, 2007), activation of complement system (Beltiukov *et al.*, 2009), macrophage activity (Perdigon *et al.*, 2001) and expression of cytokines, chemokines and their receptors (Blum *et al.*, 1999). These effects have been proven to be strain specific. (Galdeano *et al.*, 2007; Lan *et al.*, 2005; Wang *et al.*, 2008). However, the immunomodulatory effects of probiotic cultures on the host after taking antibiotics have been studied insufficiently. *In vivo* models studying the effects of probiotics on mammals with artificially induced dysbiotic condition are very limited. These kinds of experiments are usually conducted on animals injured by various chemical and physical factors or after bacterial infection (Rambaud *et al.*, 2006; Rolfe, 1984). Previously, we studied the effects of *Enterococcus faecium* L5 (an erythromycin-resistant derivative of the probiotic strain *E. faecium* L3). It was shown that this strain possesses antimicrobial features and does not have any toxic effects on the experimental animals (Beltiukov *et al.*, 2009).

The purpose of this study was to create a model of intestinal dysbiosis and to study the influence of *E. faecium* L5 on cytokine expression.

2. Materials and methods

Bacterial strains and culture conditions

E. faecium L5 used in this study is an *ermB*-labelled derivative of the probiotic strain *E. faecium* L3 ('Laminolact', Russia). This strain was obtained by insertional mutagenesis employing the plasmid pPR512 with glutamine synthetase gene from group B streptococci and *erm*B gene (Suvorov *et al.*, 1997). *E. faecium* L5 was grown in tryptose broth (Difco, BD, Franklin Lakes, NJ, USA) and tryptose agar (Ferax Laborat GmbH, Berlin, Germany) containing erythromycin (10 μg/ml, Sigma, St. Louis, MO, USA) for 24 hours at 37 °C aerobically.

Fermented milk product was prepared by growing *E. faecium* L5 in the milk. Inoculum (0.5 ml 10^8 cfu/ml) was added to the milk (10 ml) and bacterial culture was incubated for 18 hours at 37 °C aerobically.

Rat model of antibiotic associated dysbiosis

Wistar rats of both sexes (weight 200-250 g) were randomly divided into 2 groups (A and B) with 12 animals in each group. Experimental intestinal dysbiosis in rats was induced by 15 mg ampicillin (Orgenica, Novokuznetsk, Russia) and 10 mg metronidazole (Nycomed, Roskilde, Denmark) which were fed to the animals for three days. After three days of antibiotic consumption rats belonging to group A were fed with 0.5 ml of fermented milk product containing 5.5×10^8 cfu/ml of *E. faecium* L5 for 5 days. Rats in group B received 0.5 ml milk. The control group of rats was not taking any antibiotics or probiotics.

The rats were kept under similar conditions (in separate cages and with the same temperature, light and humidity; they also received the same type of food). Physical activity, body weight, appetite (measured as the weight of eaten food), presence of dyspeptic symptoms, and consistency of the excrements were monitored throughout the whole experiment. Pathomorphological changes in the bodies of the animals were studied at the end of the experiment.

Microbiological studies

Quantitative and qualitative contents of the intestinal microbiota were determined at the different periods of the experiment (before and after exposure to antibiotics and at the end of the experiment). Changes in the gut microbiota (first, third and eighth day) were tested by analysis of the faecal samples. At the end of the experiment chyme samples were taken from the different parts of the gut. These samples were diluted in phosphate buffer and then plated on the differential diagnostic media and cultivated at 37 °C aerobically. Chyme samples taken from group A rats (receiving *E. faecium* L5) were examined for the presence of erythromycin resistant enterococci.

RNA isolation and reverse transcription

Expression of IL-8, IL-10, IL-1β and IL-18 were quantified by the reverse transcription polymerase chain reaction (RT-PCR) technique. On the eighth day of the experiment rats were sacrificed in accordance with all the necessary ethical requirements and regulations. The pieces of mucosa from different parts of the small and large intestine were immediately subjected to mRNA isolation. Intestinal biopsies were homogenised with a mechanical tissue homogeniser. Total RNAs from the samples were prepared by guanidinium thiocyanate-phenol-chloroform extraction (Sambrook *et al.*, 1989). RNA concentration was determined by spectrophotometer and the purity of RNA from DNA was tested by PCR. Total RNA was heated at 70 °C for 5 min and reverse transcribed employing the M-MLV reverse transcriptase (Promega, Madison, WI, USA) (20 u/μl), dNTP (Medigen, Novosibirsk, Russian Federation) 0.5 мМ each, oligo-dT primers (Promega) and RNA inhibitor (5 u/μl) (Promega). cDNA synthesis was performed with 2 μg total RNA in a final volume of 40 μl. Reverse transcription was carried out at 37 °C during 70 min, and then reaction was stopped after incubation for 10 min at 70 °C.

Gene-specific cDNA amplification was performed employing Taq DNA polymerase (Silex, Moscow, Russian Federation), cDNA and 15 pmol of each primer in a final

Table 1. Oligonucleotide primers used for PCR.

Cytokines	Oligonucleotides sequences		Size of PCR products (bp)
	Forward primer 5'3'	Reverse primer 5'3'	
IL-1β	CTCCATGAGCTTTGTACAAGG	TGCTGATGTACCAGTTGGGG	245
IL-8	CCACGCCACAAGTACACTGAT	TGGTTCTCATGAGGGTGTCTG	393
IL-10	TGGGTTGCCAAGCCTTGT	ATCGATGACAGCGTCGCA	152
IL-18	ACTGTACAACCGCAGTAATAC	AGTGAACATTACAGATTTATCCC	320
β-actin	GAAGATCCTGACCGAGCGTG	AGCACTGTGTTGGCATAGAG	326

reaction volume of 25 µl. cDNA was used as a template for PCR reactions employing DNA primers listed in Table 1. The RT-PCR products were analysed on 2% agarose gels stained with ethidium bromide. DNA bands were visualised under UV light. Only samples which were positive for β-actin gene amplification have been included in this study. Digitised gel electrophoresis data were analysed in a semi-quantitative fashion by calculating the ratio of cytokine expression to constitutive β-actin expression. The density of each band in the agarose gels was quantified using Scion Image software and standardised against the amount of β-actin.

3. Results

In this study dysbiosis was caused by taking antibacterial drugs, confirmed by the dynamics of clinical symptoms and changes in gut microbiota.

Clinical symptoms

Before taking the probiotics all antibiotic-treated rats exhibited the following clinical symptoms: bloating, diarrhoea, with constipation in some rats (2 rats out of 24). Decreased appetite (Figure 1A) and weight (Figure 1B) was determined in most of the experimental animals in groups A and B. In group B the dysbiotic symptoms persisted almost until the end of the experiment. Animals treated with enterococcal fermented milk product quickly recovered their appetite and weight.

Microbiota of the intestinal tract

After bacteriological study analysis all the rats had dysbiosis (Figure 2A), which was reflected by decreased (1-2 lg cfu/ml) titres of bacteria belonging to the following genera: *Bifidobacterium, Lactobacillus*, *Escherichia* and *Enterococcus.* An increase in the quantity of lactase-negative and haemolytic *Escherichia coli* was determined. We could also determine the increase in the quantity (about 2.1-5.5 lg cfu/ml) of the following putative opportunistic bacteria: *Proteus* spp., *Staphylococcus aureus* and *Klebsiella* spp.

It was shown that only in group A was the recovery of microbiota back to normal at the end of the experiment (Figure 2B and Figure 2C). The bacteriological study revealed that the total amount of enterococci in the small intestine of rats belonging to group A was higher than in group B (Figure 3). It should be noted that the content of erythromycin resistant enterococci was approximately 10 times lower than erythromycin susceptible clones which reflects the proportion between the probiotic strain and indigenous *Enterococcus* spp. (data not shown). No bacteria were found in the organs of experimental animals (spleen, liver and heart) or the blood which reflected the absence of translocation.

Cytokine expression in the intestinal mucosa

mRNA expression level of IL-1β, IL-8, IL-10 and IL-18 was determined in the biopsy samples of the mucosa from colon or small intestine employing RT-PCR. There were no significant differences in the mRNA expression level between the samples taken from different parts of the small or large intestine. We also found no differences in IL-1β and IL-18 expression between the groups.

However, IL-10 expression was significantly increased in all the animals receiving *E. faecium* L5 as compared with other groups (Figure 4A). It should be noted that on day eight of the experiment, mRNA expression of IL-8 was significantly higher in rats belonging to the group B which were fed with milk (Figure 4B). This group of animals, unlike group A which was treated with probiotic, maintained clinical and laboratory features of dysbiotic condition during the entire experiment.

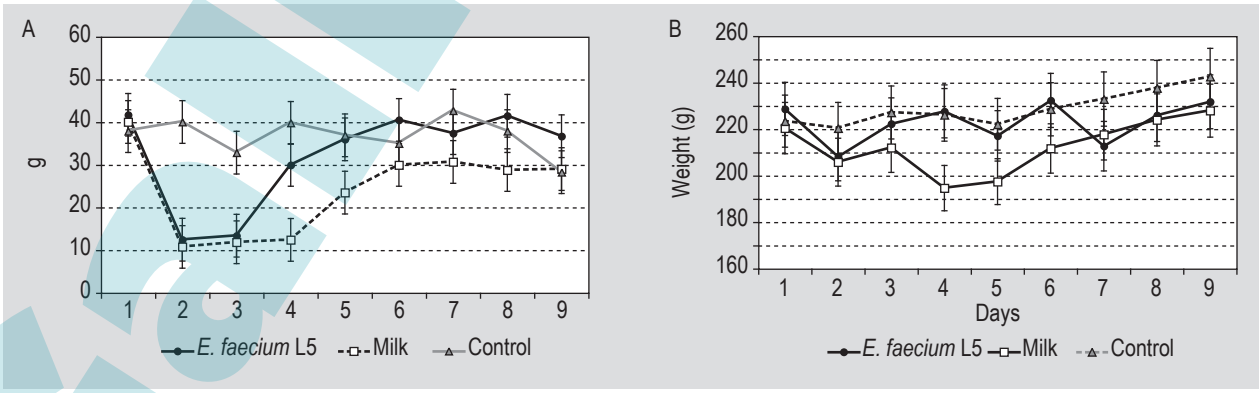

Figure 1. (A) Changes in rats' appetite during the experiment. (B) Changes in rats' weight during the experiment. * *P*<0.05.

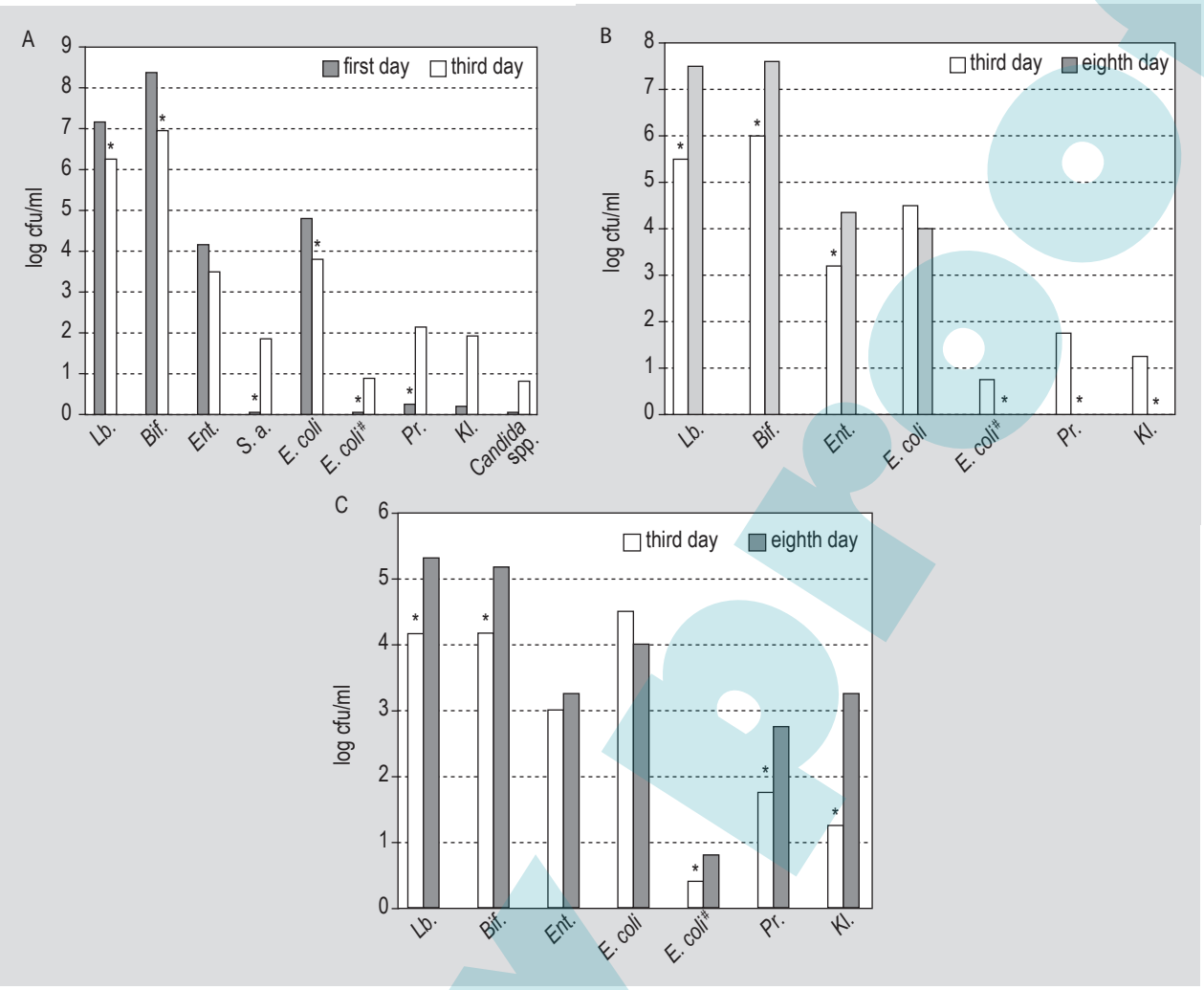

Figure 2. (A) Quantitative characteristics of microorganisms in the faeces of rats with dysbiosis after administration of antibiotics. (B) Changes in the gut microbiota of rats with antibiotic associated dysbiosis after administration of *E. faecium* L5. (C) Changes in the gut microbiota of rats with antibiotic-associated dysbiosis after administration of milk.
***Lb.* – *Lactobacillus* spp.; *Bif.* – *Bifidobacterium* spp.; *Ent.* – *Enterococcus* spp.; *S.a.* – *Staphylococcus aureus*; *Pr.* – *Proteus* spp.; *Kl.* – *Klebsiella* spp.; *E. coli* – *Escherichia coli*; *E. coli* # – lactase-negative and haemolytic *Escherichia coli*. * *P*<0.05.**

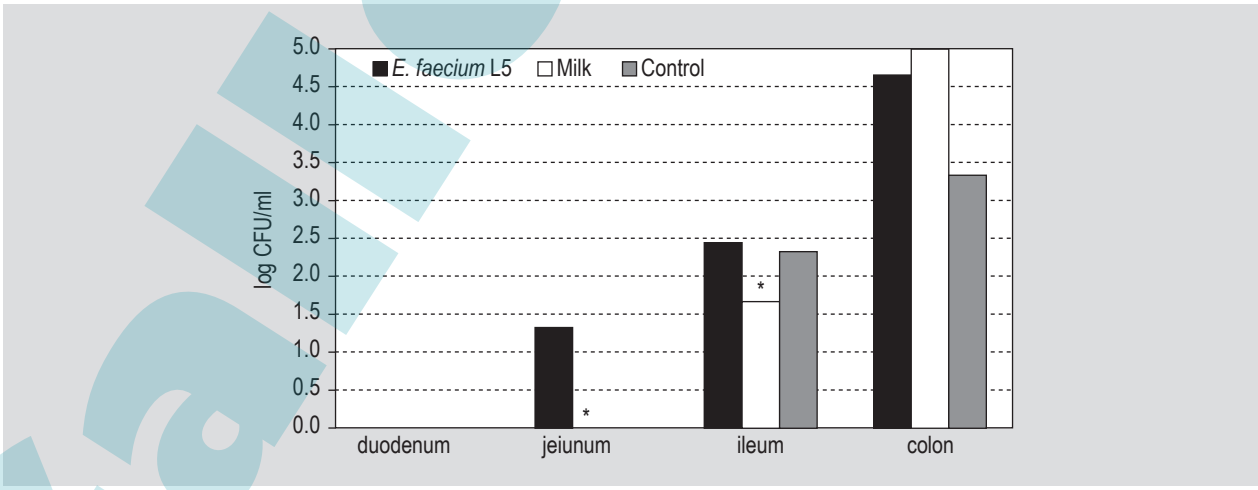

Figure 3. Numbers of enterococci in different parts of the gastrointestinal tract of the rats after administration of milk with or without *E. faecium* L5 (* *P*<0.05 between groups A and B).

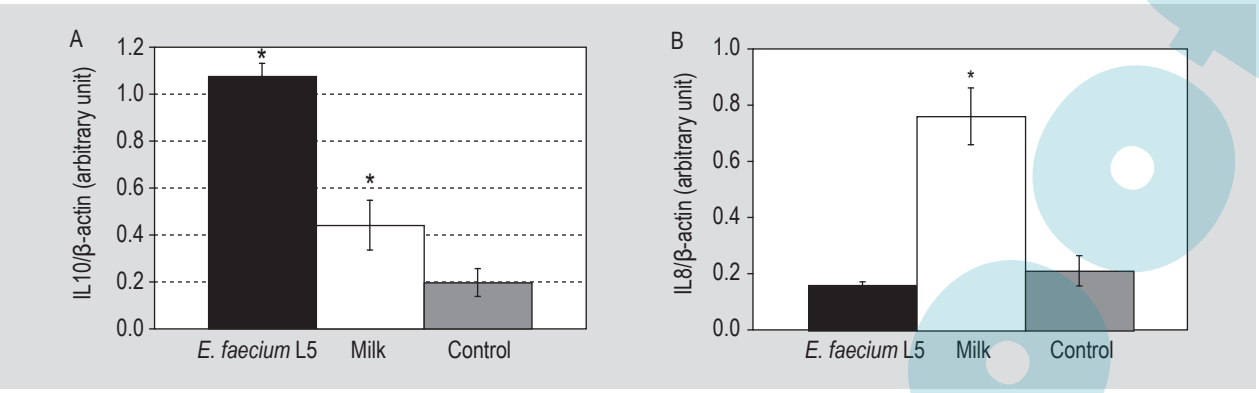

Figure 4. (A) Expression of IL-10 in the intestinal mucosa. (B) Expression of IL-8 in the intestinal mucosa. Densitometer readings and following statistical analyses of the bands obtained after RT-PCR. * *P*<0.05.

4. Discussion

Based on available research and clinical data, there are believed to be four common causes of intestinal dysbiosis: putrefaction (the result of diet high in fat or animal flesh); fermentation dysbiosis resulting from inefficient host digestion, which causes carbohydrate intolerance and fatigue due to an overgrowth of bacteria or fungi in the intestines; deficiency dysbiosis caused by antibiotic exposure; sensitisation dysbiosis as the result of abnormal immune responses caused by an alteration in the normal intestinal flora (Kao *et al.*, 2009).

Regardless of the possible causes of dysbiotic condition in practical clinical situations dysbiosis is usually caused by antibiotic treatment. Consumption of antibiotics leads to a deficiency of normal intestinal flora and overgrowth of opportunistic bacteria. In previous studies the increase in the numbers of *Clostridium difficile, S. aureus, Proteus* spp., *Klebsiella* spp, *E. coli, Candida* spp., and the decrease in concentrations of *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp. in the intestines was determined (Gibson, 2002; Salminen *et al.*, 1995).

In this work we have presented the model of antibiotic-associated intestinal dysbiosis and described the changes in microbiota and the innate immune system after administration of probiotic enterococci. The use of strain *E. faecium* L5, labelled by the resistance to erythromycin, allowed the settlement of probiotic enterococci in the gastrointestinal tract to be traced. After the administration of antibiotics the numbers of bifidobacteria, lactobacilli and enterococci in the gut decreased. After the addition of probiotic enterococci the increase in their content was determined. The use of probiotic enterococci leads to the displacement of pathogenic bacteria with the increased numbers of lactobacilli and bifidobacteria. After taking the probiotic the healthy condition of the animals with dysbiosis was quickly restored (normalisation of appetite, weight gain and loss of the symptoms of dyspepsia). No changes of this kind were determined in the group of animals taking milk. Perhaps the increased expression of the IL-8 in the animals with pathological changes in microbiota composition can be considered a marker of the adverse development of dysbiotic process. We can also suppose that the positive physiological effects of probiotic enterococcal strain were caused by the reaction of the innate immune system, characterised by the decrease in the production of the pro-inflammatory cytokine IL-8 and the increase in anti-inflammatory cytokine IL-10.

5. Conclusions

Administration of *E. faecium* L5 enables faster recovery from dysbiotic condition caused by antimicrobial therapy with antibiotics. Thus, the use of *E. faecium* L5 may promote the treatment of infection by eliminating clinical symptoms and restoring the original composition of the intestinal microbiota.

References

Beltiukov, P.P., Abdurasulova, I.N., Tarsova, E.A., Suvorov, A.N., Zakharova, E.T., Sokolov, A.V., Karpenko, M.N. and Ermolenko, E.I., 2009. Influence of probiotic *Escherichia coli* and enterococci on the immune system of healthy rats. Scientists note Saint-Petersburg State Medical University acad. I.P. Pavlov XVI (2): 54-58.

Blum, S., Alvares, S., Haller, D., Perez, P. and Schiffrin, E.J., 1999. Intestinal microflora and the interaction with immunocompetent cells. Antonie van Leeuwenhoek 76: 199-205.

Elmer, G.W., 2001. Probiotics: 'living drugs'. American Journal of Health-System Pharmacy 58: 1101-1109.

Foulquie Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E. and De Vuyst, L., 2006. The role of appplication of enterococci in food and health. International Journal of Food Microbiology 106: 1-24.

Galdeano, C.M., De Moreno de Blance, A., Vinderola, G., Bonet, M.E. and Perdigon, G., 2007. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clinical and Vaccine Immunology 4: 484-492.

Gibson, G.R., 2002. Human gut microbiology: the end of the food chain or the start of good health. Microbiology Today 29: 229-254.

Haghighi, H.R., Gong, J., Gyles, C.L., Hayes, M.A., Sannei, B., Parvizi, P., Gisavi, H., Chambers, J.R. and Sharif, S., 2005. Modulation of antibody-mediated immune response by probiotics in chickens. Clinical and Diagnostic Laboratory Immunology 12: 1387-1392.

Kao, C.-H. and Levytam, S., 2009. The role of the eubiotic diet in intestinal dysbiosis and hypertension. International Journal of Naturopathic Medicine 4: 42-48.

Lan, J.-G., Cruicshank, S.M., Singh, J.C.I., Farrar, M., Lodge, J.P.A., Felsburg, P.J. and Carding, S.R., 2005. Different cytokine response of primary colonic epithelial cells to commensal bacteria. World Gastroenterology 11: 375-384.

McFarland, L., 1998. Epidemiology, risk factors and treatments for antibiotic associated diarrhea. Digestive Diseases 16: 292-307.

Perdigon, G., Fuller, R. and Raya, R., 2001. Lactic acid bacteria and their effect on the immune system. Current Issues in Intestinal Microbiology 2: 27-42.

Rambaud, J.-C., Buts, J.-P., Corthier, G. and Flourie, B., 2006. Gut microflora: digestive physiology and pathology. John Libbey Eurotex, Paris, France. 247 pp.

Rolfe, R.D., 1984. Interactions among microorganisms of the indigenous intestinal flora and their influence on the host. Reviews of Infectious Diseases 6: 73-79.

Salminen, S., Isolauri, E. and Onnela, T., 1995. Gut flora in normal and in disordered states. Chemotherapy 41: 5-15.

Sambrook, J., Fritsch, F. and Maniatis, T., 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA. 560 pp.

Scharek, L., Guth, J., Filter, M. and Schmidt, M.F., 2007. Impact of the probiotic bacteria *Enterococcus faecium* NCIMB 10415 (SF68) and *Bacillus cereus* var toyoi NCIMB 40112 on the development of serum IgG and faecal IgA of sows and their piglets. Archives of Animal Nutrition 61: 223-234.

Sheil, B., Shanahan, F. and O'Mahony, L., 2007. Probiotic effects on inflammatory bowel disease. The Journal of Nutrition 137: 819-824.

Suvorov, A.N., Flores, A.E. and Ferrieri, P., 1997. Cloning of the glutamine synthetase gene from group B streptococci. Infection and Immunity 65: 191-196.

Tamboli, C.P., Neut, C., Desreumaux, P. and Colombel, J.F., 2004. Dysbiosis in inflammatory bowel disease. Gut 53: 1-4.

Wang, S., Ng, L.H., Chow, W.L. and Lee, Y.K., 2008. Infant intestinal *Enterococcus faecalis* down-regulates inflammatory responses in human intestineal cell lines. World Journal of Gastroenterology 14: 1067-1076.