

Histomorphologic Effects of *Bacillus clausii* Spores in Enteropathogenic *E. coli* O127:H21-infected Mice: A Pilot Study

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Abstract

Objective: The study aimed to determine the basic histomorphologic effects of *Bacillus clausii* (*B. clausii*) spores in enteropathogenic *Escherichia coli* (*E. coli*) O127:H21-infected mice by evaluating the spleen, mesenteric lymph nodes, and intestinal mucosa.

Methods: The study involved 46 apparently healthy Balb/c mice (*Mus musculus*) which were acclimatized for 19 days prior to any intervention. Sixteen mice were used to determine the sublethal dose of *E. coli*, which was performed by administering serially-diluted solutions and subsequent generation of a standard curve. From the remaining 30 mice, ten served as normal controls while the remaining 20 were randomized to receive either *B. clausii* or placebo of sterile water for a week. All mice were then challenged with *E. coli* for another week and euthanized, and the spleen, mesenteric lymph nodes, and small intestine harvested and examined microscopically. All study personnel were blinded of the treatment assignments.

Results: Histologic evaluation of the small intestine in *E. coli* only-fed mice exhibited prominent attachment-

effacement lesions, with severely denuded mucosa, lymphocytic infiltration, and debris in the intestinal lumen. However, mice given *B. clausii* prior to *E. coli* infection displayed only minimal mucosal damage with less sloughing of villus tips, plus increased mucus-secreting goblet cells. In the spleen, *E. coli* only-fed mice showed moderate to severe lymphoid hyperplasia with blurred boundaries between red and white pulp. In contrast, mice which received *B. clausii* prior to *E. coli* infection had only mild degrees of lymphoid hyperplasia. Similar findings were seen in the mesenteric lymph nodes where *E. coli* only-fed mice showed moderate to severe lymphoid hyperplasia while those given *B. clausii* prior to *E. coli* infection merely had mild lymphoid hyperplasia.

Conclusion: *B. clausii* exerts a potential protective and immunomodulatory action in *E. coli* O127:H21-infected mice based on histomorphologic effects. However, additional studies are needed to fully characterize these mechanisms. mice based on histomorphologic effects.

Keywords:

Introduction

The World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host."¹ To be considered a probiotic, a microorganism must pass several criteria: survival in food and in the gastrointestinal tract (GIT), adhesion to intestinal epithelial cells, fast multiplication with either temporary or permanent colonization, stabilization of intestinal flora, non-pathogenicity, generic specificity, and being of human origin.^{2,3} Studies have demonstrated the therapeutic efficacy of probiotics in several gastrointestinal disorders including acute infectious diarrhea,^{4,5} one of the

leading causes of morbidity and mortality in the Philippines.⁶ In these patients, probiotic administration was associated with less severe diarrheal episodes,⁷ faster resolution of disease,⁸ and a decreased need for intravenous rehydration.⁹

Although the best-documented probiotic strains are the *Lactobacillus* and *Bifidobacterium* species, there is growing evidence supporting the role of the *Bacillus* species (*Bacillus cereus*, *Bacillus pumilus*, and *Bacillus clausii* (*B. clausii*)) both in the prevention and treatment of acute infectious diarrhea. The *Bacillus* spores are immunogenic and exert their actions through the mesenteric lymph nodes, spleen, and small intestine,¹⁰ where they are postulated to exhibit various effects such as immunomodulation and cytokine alteration,¹¹ competition with pathogenic microorganisms for adhesion sites,¹² and production of specific antimicrobial substances. In particular, *B. clausii* (formerly *B. subtilis*) has been found to suppress *E. coli* O78:K80 infection in a chick model,¹³ and has likewise impeded the growth of *Vibrio harveyi*¹⁴ and *Helicobacter pylori*¹⁵ in other studies.

However, probiotic evaluations using different studies produce different results given that there are no

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universal standards when it comes to the optimal dosage, frequency, or duration of intake. This reflects how probiotics have not been as thoroughly tested and regulated with set formulations compared to the more conventional medications being used, although they are generally recognized as safe (GRAS).³ Moreover, the immune response to probiotics is strain-dependent, making it imperative that different probiotic strains be studied independently.⁹ In this regard, animal models have been continually used to further elucidate their qualitative mechanisms and actions. This is a pilot study attempting to investigate the basic histomorphologic effects of *B. clausii* in mice challenged with *E. coli* O127:H21, the most common enteropathogenic *E. coli* (EPEC) serotype and a major etiologic agent of traveler's diarrhea in adults.¹⁶ As a pilot approach, the study focused on evaluating the characteristics of some important gut-associated lymphoid organs, namely the spleen and the mesenteric lymph nodes, as well as the intestinal mucosa.

Objective

The study aimed to determine the basic histomorphologic effects of *B. clausii* spores in enteropathogenic *E. coli* O127:H21-infected mice by evaluating the spleen, mesenteric lymph nodes, and intestinal mucosa.

Methods

This is a randomized, placebo-controlled study involving 46 apparently healthy Balb/c mice (*Mus musculus*). The mice were housed in a standard animal laboratory with access to standard rodent diet (Vitakraft®). All were acclimatized for 19 days, with feeding and temperature conditions kept constant, and were subjected to a standard 12-hour light/dark cycle. The pathologic strain used for the study, *E. coli* O127:H21, was obtained with permission from the National Institute of Molecular Biology and Biotechnology of the University of the Philippines (UP) Los Banos. Sixteen out of the 46 mice were used for the determination of the sublethal *E. coli* dose, which was performed by administering seven serially-diluted solutions via oral gavage from a 24-hour bacterial culture. Afterwards, generation of a standard curve for the solutions was done by reading the absorbances, obtaining a sublethal dose of 1×10^{11} CFU/ml.

From the remaining 30 mice, 10 were set aside to serve as normal controls; the rest were randomized to receive either 0.5 ml (2×10^9 spores) of *B. clausii* (Erceflora®) or placebo of sterile water once a day, every day for a week. The *B. clausii* spores were obtained with permission from Sanofi Aventis Philippines. Randomization was performed using software-generated random numbers while allocation concealment was done by the use of opaque envelopes. Each mouse received treatment using identical containers under trained study personnel. On the second week, all mice were then challenged with 0.5 ml of *E. coli* O127:H21 once

a day, every day for a week. The dose, timing, and duration for both *B. clausii* and *E. coli* administration were based on previous similar studies.^{17,18}

After the two-week intervention period, the mice were euthanized, and the spleen, mesenteric lymph nodes, and small intestine were harvested by a trained technician who was also blinded of the treatment assignments. Saline was used to rinse tissues, with avoidance of excessive manipulation in order to minimize artifacts during histologic examination. Representative samples were taken and trimmed to approximately 0.5 cm thickness in accordance with standard procedures and literature on tissue trimming,¹⁹ with recuts obtained as needed. Tissues were fixed by immersion in 10% buffered formalin for at least 24 hours and embedded in paraffin, after which they were processed into hematoxylin-eosin (H/E) stained sections/slides; special stains were used as needed.

The slides were read and interpreted by a pathologist who was also blinded of the treatment assignments. Microscopic findings were described and reported according to standard pathologic nomenclature and diagnostic criteria, including standard severity grades of "mild", "moderate" and "severe" to define the extent of tissue alteration.^{20,21}

Data were expressed as frequencies and percentages and reported according to findings per organ.

Results

Small Intestine

All mice remained alive after the two-week intervention period. Sections of the small intestine in controls showed normal undamaged mucosa, with continuous plicae circulares or folds of the mucosa and submucosa towards the lumen. The villi were intact and intestinal glands were also apparent (Figure 1, top.) In mice given *E. coli* only, the most prominent features were the sloughing off of villus tips and the formation of attachment-effacement (A/E) lesions, leading to the appearance of severely denuded mucosa and the presence of debris in the intestinal lumen. Moreover, lymphocytic infiltration was also observed (Figure 1, middle.) However, in mice given *B. clausii* prior to *E. coli* infection, only minimal mucosal damage was observed, with less sloughing of villus tips and an increase in mucus-secreting goblet cells. This was consistently found in all mice in the subgroup (Figure 1, bottom.).

Spleen

All groups exhibited mild to moderate splenic congestion and hemosiderosis. Spleen sections of controls displayed normal follicles and mitogenic activity, with no evidence of lymphoid hyperplasia (Figure 2, top.) In mice administered with *E. coli* only, moderate to severe lymphoid hyperplasia

was observed, blurring boundaries between splenic red and white pulp (Figure 2, middle.) This degree of lymphoid hyperplasia was found in 70% of the *E. coli* group. In contrast, majority of the mice which received *B. clausii* prior to *E. coli* infection showed only a mild degree of lymphoid hyperplasia (Figure 2, bottom.) Table I summarizes the degrees of splenic lymphoid hyperplasia seen in the different subgroups.

Figure 1. Top: Jejunum segment of normal mouse small intestine showing an intact mucosa, with apparent villus and goblet cells; Middle: Jejunum segment of mouse given *E. coli* O127:H21 only, showing sloughed-off mucosa and debris formation in the intestinal lumen; Bottom: Jejunum segment of mouse given *B. clausii* prior to *E. coli* infection, showing less sloughed mucosa and an increase in mucus-secreting cells (100x.)

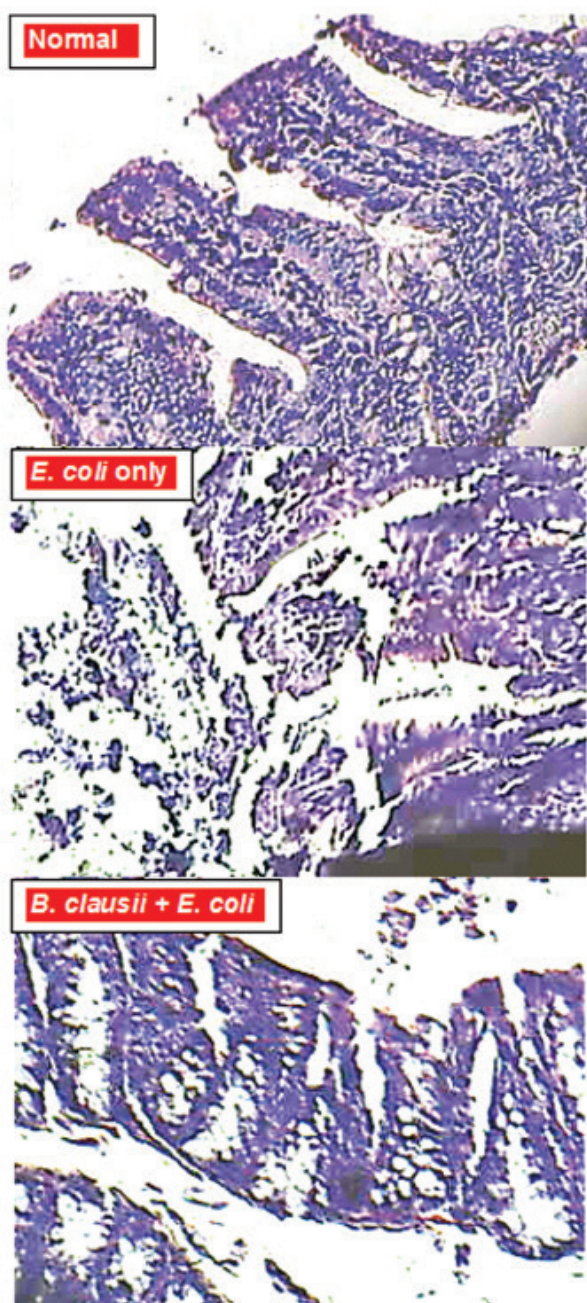
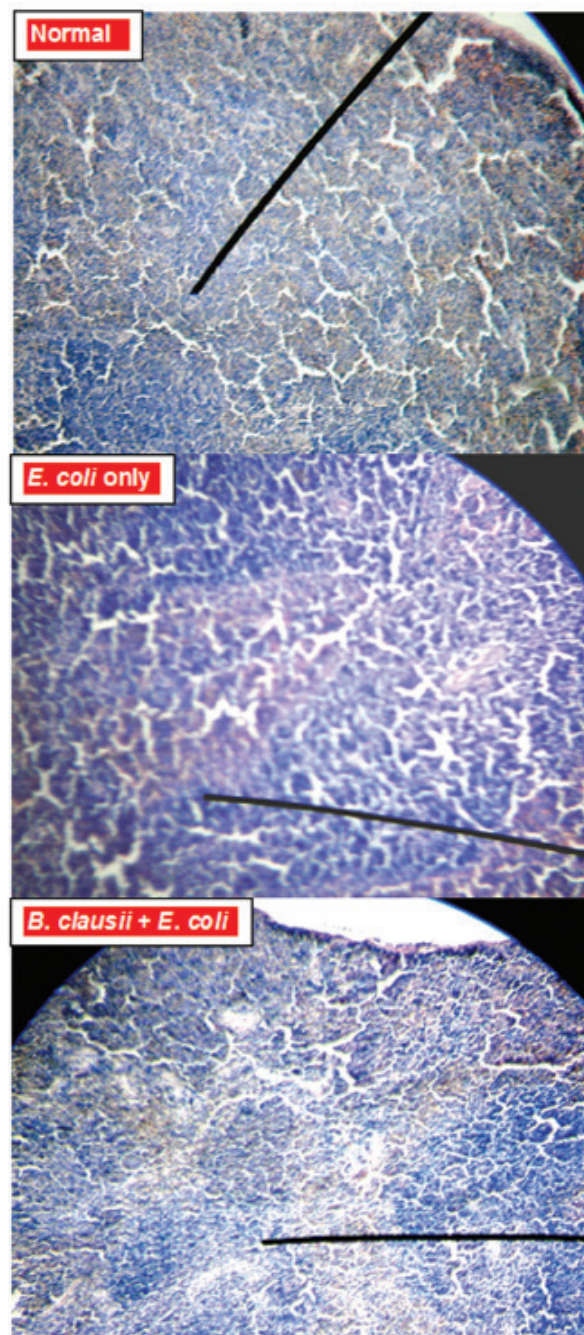


Figure 2. Top: Cross section of normal mouse spleen, with normal follicles and no lymphocytic hyperplasia; Middle: Spleen section of mouse given *E. coli* O127:H21 only, showing moderate to severe lymphocytic hyperplasia, blurring boundaries between red and white pulp; Bottom: Spleen section of mouse given *B. clausii* prior to *E. coli* infection, showing only mild lymphocytic hyperplasia (100x.)



Mesenteric Lymph Node

Evaluation of mesenteric lymph node sections in controls revealed an intact capsular domain with cellular components that were neither hypoplastic nor hyperplastic (Figure 3, top.) In mice administered with *E. coli* only, moderate to severe lymphoid hyperplasia was seen, which was found in 70% of the subgroup (Figure 3, middle.) On the other hand, an overwhelming majority (90%) of the mice that received *B. clausii* prior to *E. coli* infection exhibited only mild lymphoid hyperplasia (Figure 3, bottom.) Table II summarizes the degrees of mesenteric lymph node hyperplasia observed in the different treatment groups.

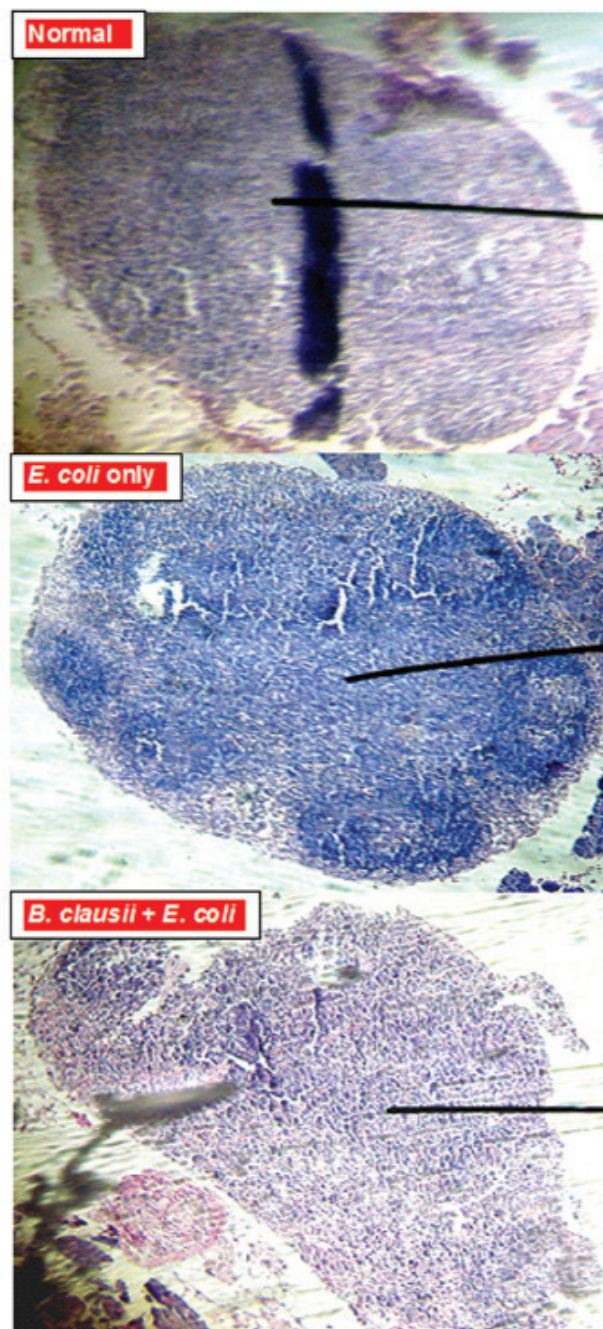
Table I. Incidence of splenic lymphoid hyperplasia among the treatment groups.

Group	No hyperplasia (n)	Mild hyperplasia (n)	Moderate-severe hyperplasia (n)
Control (n=10)	10	0	0
<i>E. coli</i> only (n=10)	0	3	7
<i>B. clausii</i> + <i>E. coli</i> (n=10)	0	7	3

Table II. Incidence of mesenteric lymph node hyperplasia among the treatment groups.

Group	No hyperplasia (n)	Mild hyperplasia (n)	Moderate-severe hyperplasia (n)
Control (n=10)	10	0	0
<i>E. coli</i> only (n=10)	0	3	7
<i>B. clausii</i> + <i>E. coli</i> (n=10)	0	9	1

Figure 3. Top: Cross section of normal mouse mesenteric lymph node with intact capsule; Middle: Lymph node of mouse given *E. coli* O127:H21 only, showing moderate to severe lymphocytic hyperplasia; Bottom: Lymph node of mouse given *B. clausii* prior to *E. coli* infection, showing only mild lymphocytic hyperplasia (100x.)



Discussion

This is the first study documenting the histomorphologic effects of *B. clausii* in a standard healthy mouse model infected with enteropathogenic *E. coli* O127:H21. The results generally suggest a potential protective effect of the probiotic against the pathogenic consequences of *E. coli* infection, in particular through reduced mucosal denudation and increased mucin secretion in the small intestine, and through reduced lymphocytic hyperplasia in both the spleen and mesenteric lymph nodes.

In the small intestine of *E. coli* only-fed mice, prominent A/E lesions were seen as a result of bacterial attachment, leading to dissolution of the intestinal brush border membrane, loss of microvillar structures (effacement), and consequent secretory diarrhea. The bacteria, aided by plasmids and bundle-forming pili, are known to preferentially attach to cuplike projections or pedestals at the apical membrane.^{22,23} This amount of damage was ameliorated in mice fed with *B. clausii* prior to *E. coli* infection, as manifested through minimal mucosal denudation plus an increase in mucus-secreting goblet cells. The latter is consistent with studies showing the association of antigen exclusion with the capacity of the mucous membrane to produce IgA and mucus.²⁴ These findings are also consistent with literature citing the ability of probiotics to competitively exclude pathogens in the GIT and produce antimicrobial agents such as biosporin.¹²

In the spleen, the degree of lymphoid hyperplasia is a significant indicator of infection and inflammatory activity and is generally dependent on the pathogenicity of an antigen. Splenic mitogenic activities are also important properties of immune modulators and have been used to evaluate the immunomodulating activities of probiotics.²⁵ In *E. coli* only-fed mice, histologic evaluation revealed moderate to severe lymphoid hyperplasia, which could be explained by the activated lines of cell-mediated and humoral immunity against EPEC, producing increased numbers of circulating lymphocytes. A study done in murine trypanosomiasis hypothesized that this is probably a T-cell dependent phenomenon.²⁶ In contrast, *B. clausii* reduced the degree of lymphoid hyperplasia in murine spleens when administered first before *E. coli*, suggesting the effectiveness of its antimicrobial and immunomodulatory action.²⁷ The relatively more intact mucosa and increased mucin production in the small intestine of *B. clausii*-fed mice may have also served as a collective protective barrier, reducing *E. coli* entry and translocation to more distant organs such as the spleen and mesenteric lymph nodes. In addition to the above findings, mild to moderate splenic congestion was observed in all subgroups including the control group, most likely due to the pooling of blood in the splenic parenchyma after cervical dislocation (the method used for rodent euthanasia.) Hemosiderosis was also found in all groups and can be due to the increased composition of iron and ascorbic acid in the rodent feeds.

Similar to the spleen, moderate to severe mesenteric lymph node hyperplasia was seen in *E. coli* only-fed mice and can be explained by heightened immune responses to active infection. The accompanying severe denudation of the intestinal epithelia in the same subgroup may have facilitated large waves of bacterial translocation to the mesenteric lymph nodes.²⁸ Milder degrees of lymphoid hyperplasia were observed in mice given *B. clausii* prior to *E. coli*, probably for the same reasons cited as in the spleen. In this regard, the ability of *B. clausii* to regulate the pro-inflammatory response in both the spleen and mesenteric lymph nodes is quite striking and affirms its wide host of immunomodulatory properties. These include the ability to alter antigen proteolysis, reduce the secretion of gut inflammatory mediators, reverse increased intestinal permeability, enhance enteral antigen degradation, and normalize the composition of intestinal microflora.³

The study was limited by its small sample size, although as a pilot study, the number of mice used was sufficiently comparable to similar studies. Hence, it is relatively difficult to derive definite conclusions from findings observed in such a small group. The study was also limited by its short intervention period of only two weeks, precluding the investigation of any additional *B. clausii* effects given that some studies advocate its long-term therapeutic usage or as prophylaxis in high-risk populations.²⁹ Since the investigators were primarily after the characterization of microscopic changes in select organs, a complete necropsy of the mice was not performed and no macroscopic evaluations were done in the harvested tissues. Clinical findings such as fever, diarrhea, and weight loss were also not included in the scope of the study since the main objective was to determine basic histomorphologic mechanisms. Finally, due to limited resources, the study was unable to include the evaluation of other gut-associated lymphoid tissues (GALT) such as the tonsils, adenoids, and lymphoid aggregates in the esophagus, stomach, appendix, and large intestine, as well as other quantitative parameters of immune function including B and T cell counts, immunoglobulin titers, and cytokine levels.

It is important to note that in our study, *B. clausii* was given prior to *E. coli* challenge and was subsequently discontinued throughout exposure to the pathogen – a methodology derived from similar studies.^{17,18} Thus, it served more as a preventive measure rather than as treatment. Different results may be obtained if the study design was altered with regards to the duration, timing, and intensity of both probiotic and pathogen exposure (for instance, if both were given concurrently or if *B. clausii* was administered after the mice were already infected with *E. coli*.) In general, however, the results reflect the potentially positive effects of *B. clausii* in EPEC infection, although the exact molecular mechanisms underlying this as well as its safety and long-term behavior warrant additional investigation before being recommended for broader application.

Conclusion

B. clausii exerts a potential protective and immunomodulatory effect in *E. coli* O127:H21 infected mice through increased mucin production and reduced mucosal denudation in the small intestine, and through regulation of lymphoid hyperplasia in the spleen and mesenteric lymph nodes. The study recommends the evaluation of additional immunologic parameters to fully gauge and characterize these mechanisms, and hereby shed more light on the role of probiotics in combating the scourge of acute infectious diarrheal diseases in the country.

Ethical Considerations and Disclosures of Interest

The study protocol was approved by an institutional technical review and animal studies committee prior to field work. We declare no conflicts of interest. The authors are not connected to Sanofi Aventis in any way. No financial compensation was involved in the conduct of this study.

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References

1. **Nomoto, K.** Prevention of infections by probiotics: Review. *J Biosc and Bioeng.* 100 (6): 583-92, 2005
2. **Tomasik, PJ and Tomasik, P.** Probiotics and prebiotics. *Cereal chem;* 80(2): 113-7, 2003
3. **Isolauri, E; Sutas, Y; Kankaanpaa, P; Arvilommi, H; Salminen, S.** Probiotics: effect on immunity. *Am J Clin Nutr.* 2001, 73(2):444S-450S, 2001
4. **Mazza, P.** The use of *Bacillus subtilis* as an antidiarrheal microorganism. *Boll Chim Farmaceutico;* 133: 3-18, 1994
5. **Rolfe, RD.** The role of probiotic cultures in the control of gastrointestinal health. *J Nutr.* 130: 396S-402S, 2000
6. **Leading causes of morbidity and mortality.** [Online] [Cited: September 26, 2015.] Can be accessed at: www.doh.gov.ph/node/198.html.
7. **Shornikova, A; Casas, I; Isolauri, E; Mykkanen, H; Vesikari, T.** *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. *J Ped Gastroenterol Nutr.* 24: 399-404, 2007
8. **Kelleher, S; Casas, I; Carbajal, N; Lonnerdal, B.** Supplementation of infant formula with the probiotic *Lactobacillus reuteri* and zinc: Impact on enteric infection and nutrition in infant rhesus monkeys. *J Ped Gastroenterol Nutr;* 35: 162-8, 2002
9. **Szymanski, H; Pejcz, J; Jawien, M; Chmielarczyk, A; Strus, M; Heczko, P, et al.** Treatment of acute infectious diarrhea in infants and children with a mixture of three *Lactobacillus rhamnosus* strains - a randomized, double-blind, placebo-controlled trial. *Aliment Pharmacol Ther.* 23: 247-53, 2006
10. **Duc, LH; Hong, HA; Fairweather, N; Ricca, E; Cutting, SM.** Bacterial spores as vaccine vehicles. *Infect Immun.* 71: 2810-18, 2003
11. **Duc, H, Hong, HA and Cutting, SM.** Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. *Vaccine.* 21: 4215-24, 2003
12. **Fuller, R.** Probiotics in human medicine. *Gut.* 32: 439-42, 1991
13. **La Ragione, RM; Casula, G; Cutting, SM; Woodward, SM.** *Bacillus subtilis* spores competitively exclude *Escherichia coli* 070:K80 in poultry. *Vet Microbiol.* 79: 133-42, 2001
14. **Rengpipat, S; Phianphak, W; Piyatiratitivorakul, S; Menasveta, P.** Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture.* 167: 301-13, 1998
15. **Pinchuk, IV; Bressollier, P; Verneuli, B; Fenet, B; Sorokulova, IB; Megraud, F, et al.** In vitro anti-*Helicobacter pylori* activity of the probiotic strain *Bacillus subtilis* 3 is due to secretion of antibiotics. *Antimicrob Agents Chemother.* 45: 3156-61, 2001
16. **Ghilardi, ACR; Gomes, TAT; Elias, WP; Trabulsi, LR.** Virulence factors of *E. coli* strains belonging to serogroups O127 and O142. *Epidemiol Infect.* 131: 815-21, 2003
17. **Cheng, JC, Bahaman, AR and Zakaria, Z.** Efficacy of a commercial probiotic in protecting mice against *Salmonella* infection. Conference proceedings. 5th Seminar on Veterinary Sciences. 2010. Serdang, Selangor, Malaysia.
18. **Andino, A; Zhang, N; Diaz-Sanchez, S; Yard, C; Pendleton, S; Hanning, I, et al.** Characterization and specificity of probiotics to prevent salmonella infection in mice. *Functional Foods in Health and Disease.* 4 (8): 370-80, 2014
19. **Ruehl-Fehlert, C; Kittel, B; Morawietz, G; Bube, A; Keane, K; Halm, S, et al.** Revised guides for organ sampling and trimming in rats and mice-part 1. *Exp Toxicol Pathol.* 55 (2-3): 91-106, 2003
20. **Bregman, CL; Adler, RR; Morton, DG; Regan, KS; Yano, BL.** Recommended tissue list for histopathologic examination in repeat-dose toxicity and carcinogenicity studies: a proposal of the Society of Toxicologic Pathology (STP). *Toxicol Pathol* 31 (9): 252-3, 2003
21. **Frame, SR and Mann, PC [ed.]** Principles of pathology for toxicology studies. In: Principles and methods of toxicology, 5th edition. Wallace, AH. [ed.] New York : Informa Healthcare. 591-610, 2008
22. **Knutton, S; Baldwin, T; Williams, PH; McNeish, AS.** Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun.* 57: 1290-98, 1989
23. **Donnenberg, MS and Kaper, JB.** Enteropathogenic *Escherichia coli*. *Infect Immun.* 60: 3953-61, 1992
24. **Slomiany, BL, Sarosiek, Y and Slomiany, A.** Gastric mucus and the mucosal barrier. *Dig Dis Sci.* 5: 125-45, 1987

25. **Fang, H, Morita, H and Ouwehand, AC.** Bifidobacteria and Lactobacilli exhibited different mitogenic activity on murine splenocytes. *Int J Probiot and Prebiot.* 1(1): 77-82, 2006
26. **Robinett, JP and Rank, RG.** Splenomegaly in murine trypanosomiasis: T cell-dependent phenomenon. *Infect Immun.* 23 (2): 270-5, 1979
27. **Urdaci, MC, Bresollier, P and Pinchuk, I.** *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *J Clin Gastroenterol.* 38: S86-90, 2004
28. **Narioka, J; Nishi, M; Ogata, Y; Kuwahara, T; Nakayama, H; Tashiro, S, et al.** Promotion of bacterial translocation by major liver resection in obstructive jaundice in rats colonised predominantly with indigenous *Escherichia coli*. *J Med Microbiol.* 51: 687-94, 2002
29. **Costa-Ribeiro, H; Ribeiro, T; Mattos, A; Valois, S; Neri, D; Almeida, P, et al.** Limitations of probiotic therapy in acute, severe dehydrating diarrhea. *J Ped Gastroentero Nutr.* 36: 112-5, 2003