1   Effects of probiotics on loperamide-induced constipation in rats

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3   Takio Inatomi[1]*

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5   [1] Inatomi Animal Hospital, 1-1-24, Denenchofu, Ota-ku, Tokyo 145-0071, Japan

6

7   *Corresponding author

8   takato.inatomi@gmail.com (TI)

9

Abstract

10

11   Constipation, a common health problem, causes discomfort and affects quality of life. This study

12   aimed to evaluate the potential effect of probiotics on loperamide (LP)-induced constipation in

13   Sprague-Dawley (SD) rats, a well-established animal model of spastic constipation. In total, 100

14   male SD rats (age, 8 weeks; Japan SLC, Inc., Hamamatsu, Japan) were used in the experiments

15   following a 12-day acclimatisation period. They were randomly divided into two treatment groups

16   (groups 1 and 2) of 50 rats each. Spastic constipation was induced via oral administration of LP (3

17   mg/kg) for 6 days 1 hour before the administration of each test compound. Similarly, a probiotics

18   solution (4 ml/kg body weight) was orally administered to the rats once a day for 6 days in group 2.

19   In group 1, phosphate buffer solution was orally administered once a day for 6 days 1 hour after each

20   LP administration. The changes in body weight, faecal parameters, short-chain fatty acid

21   concentration in faeces, and faecal immunoglobulin (Ig)-A concentration were recorded. In the

22   present study, the oral administration of probiotics improved faecal parameters, short-chain fatty

23   acid concentration in faeces, and faecal IgA concentration. Our results indicate that probiotics

24   increase the levels of intestinal short-chain fatty acids, especially butyric acid, thereby improving

25   constipation and intestinal immunity.

26

Introduction

27

28 Constipation is a common problem, and probiotics have been reported to improve bowel motility

29 [1,2]. Symptoms of constipation include the following: a decrease in the frequency of bowel

30 movements, decrease in the amount of faeces, painful bowel movements, dry faeces, and

31 dissatisfaction after a bowel movement [3]. Constipation has a significant impact on quality of life,

32 and prebiotics and probiotics are expected to help in the treatment of constipation [4,5]. Therefore,

33 there is a growing interest in better understanding the effects of probiotics on constipation.

34 Probiotics are defined as living microorganisms that enter the gastrointestinal tract in their active

35 form in sufficient numbers to exert positive effects [6,7]. Probiotics may, in fact, facilitate a return to

36 normal status after a perturbation of the microbiota (e.g. because of the use of antibiotics or illness)

37 or may reduce the degree of change invoked by such challenges. A few studies have measured a

38 probiotic-enhanced return to baseline levels after antibiotic use in humans [8]. The concept of

39 stabilising intestinal microorganisms via the associated probiotics for health improvement dates to

40 the beginning of the last century. Many studies have been conducted to determine the effect of

41 probiotic cultures on health [9-14]. The ingestion of probiotics is beneficial for treating different

42 diarrhoea-like disorders and lowering the levels of metabolites that are harmful to health, including

43 cancerous markers in the colon [15,16]. Probiotic microorganisms promote various

44 immunomodulatory effects by modulating the gut microbiota [17,18]. Thus, probiotic bacteria could

45   be used for the treatment of constipation because of their health-promoting benefits.

46   Recently, advances in medical treatments have led to increased numbers of immunocompromised

47   patients; some patients contract opportunistic infections caused by some bacterial species, which are

48   considered non-pathogenic bacteria [19]. However, there are only few studies on intestinal immunity

49   during constipation [20]. Therefore, determination of the safety of probiotics is crucial.

50   Bio-three H (Takeda Consumer Healthcare Company Limited, Tokyo, Japan) has been approved for

51   medical use and is widely used in Japan, China, and India for the treatment and prevention of

52   infectious diseases. In terms of safety, Bio-three H is considered suitable for the treatment of

53   constipation [21].

54   There have been many studies regarding the role of probiotics in the mitigation of constipation.

55   However, to the best of our knowledge, only few studies have investigated the role of

56   safety-guaranteed probiotics in the mitigation of constipation and gut immunity. Therefore, this

57   study aimed to investigate the effects of safety-guaranteed probiotics on constipation relief and gut

58   immunity in a rat model.

59

Materials and methods

Ethical approval

62   This study was conducted at Inatomi Animal Clinic in Tokyo Prefecture, Japan. It was performed

63   under the fundamental guidelines for the proper conduct of animal experiments and related activities

64   at academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports,

65   Science, and Technology. It was approved by the Ethics Committee of the Inatomi Animal Clinic

66   (Tokyo, Japan; approval number 2020-003).

67

Experimental animals

69   One hundred male Sprague-Dawley rats (age, 8 weeks; Japan SLC, Inc., Hamamatsu, Japan) were

70   used in the experiments following a 12-day acclimatisation period. They were randomly divided into

71   two treatment groups (groups 1 and 2) of 50 rats each. Animals were housed individually in a

72   polycarbonate cage, in a temperature (20-23°C)- and humidity (40-50%)-controlled room. The

73   light/dark cycle was 12/12 hours, and basic diet (Rodent Diet CL-2, CLEA JAPAN, Tokyo, Japan)

74   and water were supplied *ad libitum*. The probiotics solution used in this study was Bio-three H.

75   Bio-three H (1 g) contains 50 mg of *Bacillus mesentericus* TO-A, 10 mg of *Enterococcus faecalis*

76   T-110, and 50 mg of *Clostridium butyricum* TO-A. The probiotics solution was dissolved in

77   phosphate buffer solution (PBS) to a final concentration of 12.5 mg/ml. In group 2, the probiotics

78   solution (4 ml/kg body weight) was orally administered once a day for 6 days 1 hour after each

79   loperamide (LP) administration, as suggested by previously reported studies [22,23]. In group 1, a

80   PBS was orally administered once a day for 6 days 1 hour after each LP administration.

81

Induction of constipation in the rats

83 Constipation was induced in all rats through the oral administration of 3 mg/kg of LP, once a day for

84 6 continuous days at 1 hour before administration of each test material [22-25].

85

Changes in body weight

87 The body weights of individual rats were daily measured starting from 1 day before administration

88 of the test compounds through the sixth day of administration of the test compounds and LP.

89

Measurement of faecal parameters

91 The excreted faecal pellets of individual rats during a 24-hour period were collected 1 day before the

92 first administration of the test compound and immediately after the fourth administration for a

93 duration of 24 hours. The total number, water content, and wet weight of the faecal pellets were

94 measured. The collected faecal pellets were dried at 60°C in a general dry oven for 24 hours to

95 obtain faecal dry weights.

96

Short-chain fatty acid concentration in faeces

98 The short-chain fatty acid (SCFA) concentration in faeces of each rat was measured using gas

99   chromatography, as described previously [26]. Approximately 0.5 g of faeces from the dissections

100   described above was gently squeezed into a micro-centrifuge tube containing 1 mL of 10%

101   meta-phosphoric acid with 0.4 mL of 4-methyl valeric acid per mL added as an internal standard.

102   The solution was thoroughly mixed using a vortex mixer and centrifuged at 5,700 ×g for 20 minutes

103   at 4°C. The SCFA content of the supernatant was measured using an HP Agilent 6890 series gas

104   chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) fitted with an HP 5973 series

105   mass spectrometer (Agilent Technologies Inc.). The columns (Agilent Technologies) used were

106   HP-free fatty acid polyester stationary phase capillary columns of polyethylene glycol on Shimalite

107   TPA 60/80, measuring 30-m long with a 0.25-mm internal diameter.

108   The parameters of gas chromatography were as follows: 1-μl injection volume, 240°C injector

109   temperature, 12.15 psi pressure, and 1.1 mL min-1 constant flow using helium as a carrier. Fatty

110   acids were eluted with the following oven program: 80°C initial temperature hold for 5 minutes,

111   ramp 10°C min−1 to 240°C, and hold for 12 minutes. Individual SCFA concentrations were

112   expressed in mg/g wet faeces.

113

Faecal immunoglobulin A concentration

115   Faeces were suspended in six times the weight of PBS and extracted at 25°C for 24 hours. The

116   extract was centrifuged at 1500 ×g for 10 minutes to collect the supernatant and stored at -30°C, and

117   then, immunoglobulin (Ig) A was measured using an enzyme-linked immunosorbent assay (ELISA)

118   quantitation kit (Betyl Laboratories, Montgomery, Texas, USA). ELISA was conducted according to

119   the manufacturer protocol.

120   Briefly, 100 µl of the sample or standard was added to the wells of the plate and allowed to stand at

121   22°C for 1 hour; the plate was then washed 4 times. Next, a chicken IgA detection antibody was

122   added to each well and allowed to stand at 22°C for 1 hour; it was then washed 4 times. Horseradish

123   peroxidase solution A was allowed to stand at 22°C for 30 minutes, and the plate was then washed 4

124   times. One hundred microliters of 3,3',5, 5'-tetramethylbenzidine substrate solution was added to

125   each well, and the plate was developed in the dark for 30 minutes at 22°C. After stopping the

126   reaction by adding 100 µl of the stop solution to each well, the absorbance was measured at 45-nm

127   wavelength using a plate reader. Subsequently, a calibration curve was prepared, and the IgA

128   concentration was calculated.

129

130   Statistical analysis

131   The Kolmogorov-Smirnov test was used to assess the normal distribution of the data before

132   statistical analysis was performed. Mann-Whitney U tests were performed using EZR software

133   (Saitama Medical Center, Jichi Medical University); EZR is a graphical user interface for R (The R

134   Foundation for Statistical Computing, version 2.13.0). The significance level was set at $p<0.05$.

135

Results

Effect on body weight

138 Changes in body weight are shown in Table 1. There were no differences in body weight between

139 group 1 and group 2 during this study.

140

141 **Table 1. Changes in body weight of the rats (g).**

	Day -1	Day 1	Day 6
Group 1	290.1±1.641	287.3±1.776	290.9±2.236
Group 2	292.5±1.552	291.2±1.803	288.5±1.941

142

Changes in faecal parameters

144 Faecal parameters are shown in Tables 2 and 3.

145 **Table 2. Faecal parameters on day -1.**

	Pellet numbers (numbers/rat)	Wet weights of faeces (g/rat)	Dry weights of faeces (g/rat)	Water contents of faeces (%/rat)
Group 1	58.0±0.42	9.41±0.15	7.54±0.16	29.9±0.27
Group 2	57.7±0.45	9.34±0.15	7.41±0.2	30.1±0.24

9

146

147 **Table 3. Faecal parameters on day 4.**

	Pellet numbers (numbers/rat)	Wet weights of faeces (g/rat)	Dry weights of faeces (g/rat)	Water contents of faeces (%/rat)
Group 1	30.0 ± 0.19^a	4.12 ± 0.05^a	3.55 ± 0.04^a	13.9 ± 0.39^a
Group 2	58.3 ± 0.29^b	9.01 ± 0.10^b	6.70 ± 0.06^b	25.4 ± 0.75^b

148 [a, b] Different letters within columns indicate differences between the treatment groups ($p<0.01$).

149

150 At 1 day before treatment for 24 hours, there were no differences in pellet numbers, wet weights of

151 faeces, dry weights of faeces, and water contents of faeces between group 1 and group 2. After the

152 fourth administration for 24 hours, pellet numbers, wet weights of faeces, dry weights of faeces, and

153 water contents of faeces were significantly higher in group 2 than in group 1 ($p<0.01$).

154

155 **Short-chain fatty acid concentration in faeces**

156 Short-chain fatty acid concentrations in faeces are shown in Table 4.

157

158

159

160   **Table 4. Short-chain fatty acid concentration in faeces (mg/g).**

	Day -1		Day 4	
	n-butyric acid (mg/g)	acetic acid (mg/g)	n-butyric acid (mg/g)	acetic acid (mg/g)
Group 1	0.497±0.007	1.481±0.016	0.448±0.007[a]	1.480±0.015[a]
Group 2	0.489±0.007	1.519±0.015	0.523±0.007[b]	1.561±0.020[b]

161   [a, b] Different letters within the columns indicate differences between the treatment groups ($p < 0.01$).

162

163   At 1 day before treatment for 24 hours, there were no differences in n-butyric and acetic acid

164   concentrations in faeces between group 1 and group 2. After the fourth administration for 24 hours,

165   n-butyric and acetic acid concentrations in faeces were significantly higher in group 2 than in group

166   1 ($p < 0.01$).

167

168   # Faecal IgA concentration

169   Faecal IgA concentrations are shown in Table 5.

170

171

172

173   **Table 5. Faecal immunoglobulin A concentration (mg/g).**

	Day -1	Day 4
Group 1	2.05±0.04	2.04±0.04[a]
Group 2	2.04±0.04	2.36±0.03[b]

174   [a, b] Different letters within the columns indicate differences between the treatment groups ($p<0.01$).

175

176   At 1 day before treatment for 24 hours, there were no differences in IgA concentrations in faeces

177   between group 1 and group 2. After the fourth administration for 24 hours, IgA concentrations in

178   faeces were significantly higher in group 2 than in group 1 ($p<0.01$).

179

Discussion

181   In the present study, the oral administration of Bio-three H improved faecal parameters, short-chain

182   fatty acid concentration in faeces, and faecal IgA concentration. Constipation can arise owing to

183   various causes, including dietary habits; use of chemical compounds, such as morphine; and

184   psychological stress [27]. In this study, the dose of Bio-three H used in rats was determined based on

185   the dose used in humans. The administration of Bio-three H did not show any adverse effects on rats.

186   The results of the faecal parameters of group 1 24 hours after the fourth administration suggested

187   that constipation was properly induced using LP, in accordance with previous studies [22-25]. The

188   body weight of rats was not markedly different between groups 1 and 2 in this study. These results

189   are consistent with those of previous studies [22,23].

190   Shi et al. reported significantly reduced faecal levels of SCFA (acetic acid, propionic acid, and

191   butyric acid) in a population with constipation compared with those in a healthy population [28].

192   These observations indicate an association between the occurrence of constipation and the intestinal

193   levels of SCFAs. Therefore, SCFAs produced by intestinal flora or probiotics were believed to be

194   effective for constipation alleviation. Moreover, it was reported that the administration of

195   *Lactobacillus plantarum* NCU116 significantly improved symptoms of constipation in mice and led

196   to significant increases in acetic acid and propionic acid levels in their faeces [29].

197   SCFA stimulates the intestinal tract and enhances motility as one of the mechanisms of action of

198   SCFA on constipation [30,31]. In this study, Bio-three H significantly increased the faecal

199   concentrations of butyric acid and acetic acid in LP-induced constipation model rats. These results

200   are consistent with those of our previous study [32]. In support of past studies, the current study

201   findings suggest that three bacteria contained in Bio-three H produced SCFA. Bio-three H

202   significantly improved faecal parameters, such as pellet numbers, wet weights of faeces, dry weights

203   of faeces, and water contents of faeces, in this study. From the above results, it is considered that the

204   administration of Bio-three H improved constipation symptoms by increasing SCFA production, and

205   as a result, the faecal parameters were improved.

206   Havenaar and Spanhaak demonstrated that probiotics stimulate the immunity of animals in two

207   ways: 1) flora from the probiotic migrate throughout the gut wall and multiply to a limited extent;

208   and 2) antigens released by dead microorganisms are absorbed, thus stimulating the immune system

209   [33]. Probiotics containing *B. mesentericus* TO-A, *C. butyricum* TO-A, and *S. faecalis* T-110 cause

210   an increase in immunoglobulin production from the mesenteric lymph nodes in rats [34], stimulate

211   the T-helper 1 immune response in peripheral blood mononuclear and dendritic cells [35], and cause

212   an influx of CD8+ T cells into the intestinal mucosa. These changes may enhance intestinal

213   immunity by CD8+ T cells in young chicks [36] and increase IgA concentrations in the jejunum and

214   ileum in broiler chickens [37]. IgA plays an important role in intestinal immunity by binding to and

215   neutralising pathogens and toxins in the intestinal tract [38]. Intestinal IgA is mainly produced by

216   Peyer plate plasmatic cells, and IgA production by plasmatic cells is induced by follicular helper T

217   cells [39]. The follicular helper T cells differentiate from regulatory T cells in the intestinal tract, and

218   regulatory T cells differentiate from naive T cells [40]. Recently, it has also been shown that butyric

219   acid promotes the differentiation of naive to regulatory T cells in the intestinal tract [41]. In this

220   study, the administration of probiotics increased faecal IgA concentrations. This is because

221   probiotics promoted the differentiation of naive T cells into regulatory T cells by producing butyrate

222   in the intestinal tract, and the increased regulatory T cells differentiated into follicular T cells, which

223   may have indirectly increased IgA production. In support of past studies, the current study findings

224   suggest that three bacteria contained in Bio-three H increased faecal IgA concentrations. However,

225   the mechanism by which this probiotic contributes to the increase in the butyrate concentration in the

226   intestinal tract and stimulates IgA production, as well as the relationship between constipation and

227   IgA production, needs to be studied in detail in the future.

228

229   Conclusions

230   Our results indicate that probiotics containing *B. mesentericus* TO-A, *E. faecalis* T-110, and *C.*

231   *butyricum* TO-A increase the levels of intestinal SCFA, especially butyric acid, thereby improving

232   constipation and intestinal immunity. In this study, the probiotics improved constipation symptoms

233   and immune status in rats; however, their effect in humans is unknown. In addition, this study

234   showed the effect of the probiotics on loperamide-induced constipation, but the effect on

235   constipation caused by other factors is unknown. Further studies in humans are needed, but the

236   results of the current study show the potential of this probiotic in improving constipation and

237   immune status in immunosuppressed humans.

238

239   **Author Contributions**

240   The authors have performed everything related to this study.

241

242   **Competing Interests**

243   The authors declare no competing interests.

244

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References

248

249 1. Gallagher PF, O'Mahony D, Quigley EM. Management of chronic constipation in the elderly.

250 Drugs Aging. 2008;25: 807-821.

251 2. Müller-lissner S, Rykx A, Kerstens R, Vandeplassche L. A double-blind, placebo-controlled study

252 of prucalopride in elderly patients with chronic constipation. Neurogastroenterol Motil.

253 2010;22: 991.

254 3. Beery RMM, Chokshi RV. Overview of constipation. New York: Springer; 2014.

255 4. Rothbarth J, Bemelman WA, Meijerink WJ, Stiggelbout AM, Zwinderman AH,

256 Buyze-Westerweel ME, et al. What is the impact of fecal incontinence on quality of life?

257 Dis Colon Rectum. 2010;44: 67-71.

258 5. Whitehead WE, Wald A, Norton NJ. Treatment options for fecal incontinence. Dis Colon Rectum.

259 2001;44: 131-142.

260 6. Heller KJ. Probiotic bacteria in fermented foods: Product characteristics and starter organisms.

261 Am. J Clinic Nutr. 2001;73: 374s-379s.

262 7. Sanders ME. Probiotics: Definition, sources, selection, and uses. Clin Infect Dis. 2008;46:

263 S58-S61.

264 8. Engelbrektson AL, Korzenik JR, Sanders ME, Clement BG, Leyer G, Klaenhammer TR, et al.

265 Analysis of treatment effects on the microbial ecology of the human intestine. FEMS

266   Microbiol Ecol. 2006;57:239-250.

267   9. Bezkorovainy A. Probiotics: determinants of survival and growth in the gut. Am J Clinical Nutr.

268   2001;73: 399s-405s.

269   10. Fuller R. History and development of probiotics. New York: Springer; 1992.

270   11. Isolauri E. Probiotics in human disease. Am J Clinic Nutr. 2001;73: 1142S-1146S.

271   12. Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary

272   prevention of atopic disease: A randomised placebo-controlled trial. Lancet. 2001;357:

273   1076-1079.

274   13. Kopp-Hoolihan L. Prophylactic and therapeutic uses of probiotics: a review. J Am Diet Assoc.

275   2001;101: 229-241.

276   14. Spanhaak S, Havenaar R, Schaafsma G. The effect of consumption of milk fermented by

277   Lactobacillus casei strain Shirota on the intestinal microflora and immune parameters in

278   humans. Eur J Clin Nutr. 1998;52: 899-907.

279   15. De Roos NM, Katan MB. Effects of probiotic bacteria on diarrhea, lipid metabolism, and

280   carcinogenesis: A review of papers published between 1988 and 1998. Am J Clinical Nutr.

281   2000;71: 405-411.

282   16. De Vrese M, Marteau PR. Probiotics and prebiotics: Effects on diarrhea. J Nutr. 2007;137;

283   803S-811S.

284   17. Isolauri E, Kirjavainen P, Salminen S. Probiotics: A role in the treatment of intestinal infection

285   and inflammation? Gut. 2002;50: iii54-iii59.

286   18. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal

287   inflammatory disease. Nature. 2008;453: 620.

288   19. Di Rosa R, Creti R, Venditti M, D'Amelio R, Arciola CR, Montanaro L, et al. Relationship

289   between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical

290   isolates of Enterococcus faecalis and Enterococcus faecium. FEMS Microbiol Lett.

291   2006;256: 145-150.

292   20. Khalif IL, Quigley EMM, Konovitch EA, Maximova ID. Alterations in the colonic flora and

293   intestinal permeability and evidence of immune activation in chronic constipation Dig Liver

294   Dis. 2005;37: 838-849. doi: 10.1016/j.dld.2005.06.008.

295   21. Noguchi N, Nakaminami H, Nakase K, Sasatsu M. Characterization of *Enterococcus* strains

296   contained in probiotic products. Biol Pharm Bull. 2011;34: 1469-1473.

297   22. Choi JS, Kim JW, Cho HR, Kim KY, Lee JK, Sohn JH, et al. Laxative effects of fermented rice

298   extract in rats with loperamide-induced constipation. Exp Ther Med. 2014;8: 1847-1854.

299   23. Choi JS, Kim JW, Kim KY, Lee JK, Sohn JH, Ku SK. Synergistic effect of fermented rice

300   extracts on the probiotic and laxative properties of yoghurt in rats with loperamide-induced

301   constipation. Evid Based Complement Alternat Med. 2014;2014: 878503.

302   24. Bustos D, Ogawa K, Pons S, Soriano E, Banji JC, Bustos FL. Effect of loperamide and bisacodyl

303   on intestinal transit time, fecal weight and short chain fatty acid excretion in the rat. Acta

304   Gastroenterol Latinoam. 1991;21: 3-9.

305   25. Wintola OA, Sunmonu TO, Afolayan AJ. The effect of Aloe ferox mill. In the treatment of

306   loperamide-induced constipation in Wistar rats. BMC Gastroenterol. 2010;10: 95.

307   26. Murugesan GR, Romero LF, Persia ME. Effects of protease,phytase and a Bacillus sp. direct-fed

308   microbial on nutrient and energy digestibility, ileal brush border digestive enzyme activity

309   and caecal short-chain fatty acid concentration in broiler chickens. Plos One. 2014;9:

310   p.e101888. doi: 10.1371/journal.pone.0101888.

311   27. Kakino M, Tazawa S, Maruyama H, Tsuruma K, Araki Y, Shimazawa M, et al. Laxative effects

312   of agarwood on low-fiber diet-induced constipation in rats. BMC Complement Altern Med.

313   2010;10: 68.

314   28. Shi Y, Chen Q, Huang Y. Function and clinical implications of short-chain fatty acids in patients

315   with mixed refractory constipation. Colorectal Dis. 2016;18: 803-810. doi:

316   10.1111/codi.13314.

317   29. Li C, Nie SP, Zhu KX, Xiong T, Li C, Gong J, et al. Effect of Lactobacillus plantarumNCU116

318   on loperamide-induced constipation in mice. Int J Food Sci Nutr. 2015;66: 533-538. doi:

319   10.3109/09637486.2015.1024204.

320   30. Kamath PS, Phillips SF. Initiation of motility in canine ileum by short chain fatty acids and

321   inhibition by pharmacological agents. Gut. 1988;29: 941-948. doi: 10.1136/gut.29.7.941.

322   31. Kamath PS, Phillips SF, Zinsmeister AR. Short-chain fatty acids stimulate ileal motility in

323   humans Gastroenterology. 1988;95: 1496-1502. doi: 10.1016/s0016-5085(88)80068-4.

324   32. Inatomi T. Laying performance, immunity and digestive health of layer chickens fed diets

325   containing a combination of three probiotics. Science Postprint. 2016;1: e00058. doi:

326   10.14340/spp.2016.03A0001.

327   33. Havenaar R, Spanhaak S. Probiotics from an immunological point of view. Curr Opin

328   Biotechnol. 1994;5: 320-325. doi:10.1016/0958-1669(94)90036-1.

329   34. Okabe M, Matsuo A, Nishida E, Tachibana H, Yamada K. Dietary effect of a live-bacterial drug

330   on lipid metabolism and immune function of sprague-dawley rats [article in Japanese].

331   Nippon Shokuhin Kagaku Kogaku Kaishi (J Jpn Soc Food Sci). 2003;50: 224-229.

332   35. Hua MC, Lin TY, Lai MW, Kong MS, Chang HJ, Chen CC. Probiotic Bio-Three induces Th1

333   and anti-inflammatory effects in PBMC and dendritic cells. World J Gastroenterol. 2010;16:

334   3529-3540.

335   36. Huang A, Shibata E, Nishimura H, Igarashi Y, Isobe N, Yoshimura Y. Effects of probiotics on

336   the localization of T cell subsets in the intestine of broiler chicks. J Poult Sci. 2013;50:

337   275-281.

338   37. Inatomi T. Growth performance, gut mucosal immunity and carcass and intramuscular fat of

339   broilers fed diets containing a combination of three probiotics. Sci Postprint. 2015;1:

340   e00052. doi: 10.14340/spp.2015.10A0001.

341   38. Xiong N, Hu S. Regulation of intestinal IgA responses. Cell Mol Life Sci. 2015;72: 2645-2655.

342   39. Winstead CJ. Follicular helper T cell-mediated mucosalbarrier maintenance. Immunol Lett.

343   2014;162: 39-47.

344   40. Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, et al. Preferential

345   generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches.

346   Science. 2009;323: 1488-1492.

347   41. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal

348   microbe-derived butyrate induces the differentiation of colonic regulator y T cells. Nature.

349   2013;504: 446-450.

350