Development of *Bacillus subtilis* MP and Effective Utilization on Productivity and Microorganisms in Feces of Suckling Piglets

Chamroon Maneewan,[1]

Koh-en Yamauchi,[2]

Mongkol Thirabunyanon,[3]

Suthut Siri,[1]

Apichai Mekbungwan,[1]

Narin Thongwittaya[1]

[1]Faculty of animal science and technology, Maejo University, Sansai Chiangmai 50290, THAILAND, TEL: 053-873440, FAX: 053-353830, E-mail: chamroon@mju.ac.th

[2]Laboratory of animal science, Faculty of agriculture, Kagawa University, Miki-cho, Kagawa-Ken, 761-0795 JAPAN, TEL, FAX: (087)891-3053, E-mail: yamauchi@ag.kagawa-u.ac.jp

3Department of Biology, Faculty of Science, Maejo University, Sansai Chiangmai 50290, THAILAND, TEL: 053-873535, FAX: 053-873518, E-mail: mongkols@mju.ac.th

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ABSTRACT

To investigate the effects of newly developed *Bacillus subtilis* MP9 and MP10 from fermented soybean on the productive performance and number of microorganisms in feces, 80 suckling piglets (Large White x Landrace x Duroc) were randomly divided into four groups. Three-day-old piglets were consecutively hand-fed 10 ml/pig/day of phosphate-buffered saline (control group), *Bacillus subtilis* MP9 (10^{11} CFU/ml.) (MP9 group), and *Bacillus subtilis* MP10 (10^{11} CFU/ml.) (MP10 group) until 10-day-old for 7 days. Another group of 3-day-old piglets were consecutively hand-fed 1 g of an antibiotic (Chlortetracycline)/pig/day until 6 -day-old for 3 days (antibiotic group).

Body weight was recorded at 3, 7, 14, 21, and 28 days of age. Diarrhea incidence was observed from 3 to 21 days of age. A diarrhea score was calculated using a scale from 0 to 1 (0 = no diarrhea, 1 = diarrhea). At 7 and 11 days of age, fresh feces were collected to be analyzed for *Escherichia coli, Salmonella* spp, *Lactobacillus* spp, and *Bacillus subtilis* MP (total of MP9 and MP10).

Compared with the control, the average daily weight gain from 1 to 28 days of age was increased in the MP9 and MP10 groups (P<0.05), not increased in the antibiotic

Table 1. *Body weight at 3-, 7-, 14, 21 and 28 days of age in piglets hand-fed a 10 ml/pig/day of phosphate buffered saline (control), Bacillus subtilis MP9 (1011 CFU/ml.) (Bacillus subtilis MP9) and Bacillus subtilis MP10 (1011 CFU/ml.) (Bacillus subtilis MP10) from three- to ten-day-old or 1 g antibiotic (Chlortetracycline)/pig/day from three- to six-day-old (antibiotic) (n = 20).*

Item	Control	*Bacillus subtilis* MP9	*Bacillus subtilis* MP10	Antibiotic	Pooled SEM	P-value
Body weight (kg)						
3 days of age	1.76	1.88	1.87	1.75	0.03	0.32
7 days of age	2.73	3.00	3.05	2.81	0.07	0.32
14 days of age	3.64^{b}	4.24^{a}	4.33^{a}	3.83^{ab}	0.10	0.04*
21 days of age	4.78^{b}	5.74^{a}	5.92^{a}	5.15^{ab}	0.15	0.03*
28 days of age	5.95^{b}	7.18^{a}	7.45^{a}	6.48^{ab}	0.17	0.01**

a,b Means having different superscripts within the same row are significantly different at P<0.05 (*) and P<0.01 (**).

group, and better in MP groups than the antibiotics group. Mean diarrhea score and numbers of piglets with diarrhea were better in the MP9 and MP10 groups than in the control. Periods of diarrhea were shorter in all the experimental groups except the control P<0.05). The MP9 and MP10 groups tended to have shorter periods than the antibiotic group. *Escherichia coli* and *Salmonella* spp. were suppressed, and *Lactobacillus* spp. and *Bacillus subtilis* MP were increased at 11 days of age in the MP9 and MP10 groups (P<0.05). However, *Escherichia coli* was not suppressed, and *Bacillus subtilis* MP was not increased in the antibiotics group.

The present data suggest that *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10 have a growth promoting activity due to their suppressing diarrhea during the new-born to weaning period in piglets by competing with pathogenic gut flora, and by stimulating effective microorganisms. The *Bacillus subtilis* MP9 and MP10 are promising alternatives to antibiotics for use as a feed supplement in piglet diets.

INTRODUCTION

Piglets are usually weaned at 3 to 4 weeks of age, and are subjected to several stressors such as environmental, nutritional, and microbial unbalances at the time of weaning.[1] These stresses induce diarrhea and decreased growth performance.[2] To avoid these problems, dietary antibiotics are commonly used in diets. However, the use of antibiotics induces an increase in the number of microorganisms that become resistant to antibiotics.[3] Thus, the development of alternatives to antibiotics as growth promoters is needed in pig production. Probiotics seem to be a good alternative to antibiotics as growth promoters, as probiotics have tended to enhance average daily weight gain,[4] but decreased the occurrence and the severity of diarrhea in piglets.[5]

A probiotic containing *Bacillus licheniformis* and *Bacillus subtilis* spores improved average daily weight gain significantly.[6] A single probiotic (*Bacillus cereus* var. toyoi) elevated the concentration of short-chain fatty acid and lactic acid.[7] After supplementing the probiotic *Bacillus cereus* var. toyoi, Taras et al[8] reported that piglet digestion was improved, especially during pre-starter feed; that sows increased the body weight gain of their 35-day-old piglets due to greater lactation than the control; and that probiotic supplementation induced a 59% decrease in diarrhea and a 38% decrease in fecal consistency. *Bacillus subtilis* MA 139 was also reported as a promising alternative to antibiotics for use as a feed supplement in piglet diets.[9] Recently, fermented soybeans were found to have potential benefits in the control of diarrhea in weaned piglets.[10] From these reports, we hypothesized that some kinds of *Bacillus subtilis* useful in pig production could be obtained from fermented soybeans. We found that such was the case for two kinds of Bacillus subtilis, MP9 and MP10.

In this study, effects of *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10 on growth performance, diarrhea, and microorganisms in feces were observed in piglets.

MATERIALS AND METHODS

Preparation of *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10

From fermented soybean, 689 gram-positive microorganisms were collected, tested according to a probiotics characteristic determination method using pathogenic bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus*, and *Aeromonas hydrophil*, and isolated into two kinds of microorganisms. These microorganisms were gram-positive and spore-forming bacteria, and have high resistance ability for acidity (pH 3.0) and bile salts (3%). By comparing basal sequences of 16S rRNA gene sequence database in the bank base genes (GenBank), they can be classified into *Bacillus subtilis* 98% and 97%, respectively. We termed these two kinds of microorganisms *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10. The *Bacillus subtilis* MP 9 and 10 were cultured in a liquid medium (Nutrient Broth), and cured at 37ºC for 18 to 24 hours on a shaker at 150 rpms. The *Bacillus subtilis* MP was adjusted to 1011 CFU/ml with liquid medium, and their concentrations were measured with a spectrophotometer at a wavelength of 600 nm (OD=0.8). After the *Bacillus subtilis* MP was spun in a centrifuge at 5,000 rpms for 2 minutes at 4ºC, the upper clear part was left, and the remaining lower layer was washed in 0.85% saline (twice). Finally, 100 ml of saline was added to the bottom layer, and shaken together. This *Bacillus subtilis* MP was kept in an icebox for 30 minutes until introduced to the piglets.

Experiment Design

Eighty hybrid piglets (Large White x Landrace x Duroc) were divided to four groups of 20 piglets each. From 3-day-old, piglets were hand-fed 10 ml/pig/day phosphate buffered saline (control group), *Bacillus subtilis* MP9 (1011 CFU/ml.) (MP9 group), and *Bacillus subtilis* MP10 (1011 CFU/ml.) (MP10 group) until 10-day-old for 7 days. Another group of 3-day-old piglets were consecutively hand-fed 1 gram of an antibiotic/pig/day until 6-dayold for 3 days (antibiotic group).

Body weight was recorded at 3, 7, 14, 21, and 28 days of age. Diarrhea incidence was observed from 3 to 21 days of age. A diarrhea score was calculated using a scale from 0 to 1 (0 = no diarrhea, 1 = diarrhea). At 7 and 11 days of age, fresh feces was collected for analysis of *Escherichia coli*, *Salmonella* spp, *Lactobacillus* spp, and *Bacillus*

Table 2. *Average daily gain in piglets hand-fed a 10 ml/pig/day of phosphate buffered saline (control), Bacillus subtilis MP9 (1011 CFU/ml.) (Bacillus subtilis MP9) and Bacillus subtilis MP10 (1011 CFU/ml.) (Bacillus subtilis MP10) from three- to ten-day-old or 1 g antibiotic (Chlortetracycline)/pig/day from three- to six-day-old (antibiotic) (n = 20).*

Item	Control	*Bacillus subtilis* MP9	*Bacillus subtilis* MP10	Antibiotic	Pooled SEM	P-value
Average daily gain (g)						
3-7 days of age	194[b]	224 [ab]	236[a]	212[ab]	7	0.44
8-14 days of age	130	177	183	146	9	0.17
15-21 days of age	163	214	227	189	10	0.15
22-28 days of age	167	206	219	190	9	0.19
3-28 days of age	161[b]	204[a]	215[a]	182[ab]	6	0.01

[a,b]*Means having different superscripts within the same row are significantly different at P<0.01.*

subtilis MP (total of MP9 and MP10).

Statistical Analysis

All data were statistically analyzed using one-way analysis of variance (ANOVA) and significant differences among the treatments were determined with Duncan's multiple-range test. Differences were considered significant at P<0.05.[11]

RESULT AND DISCUSSION

In pig production, weaning is a critical phase that is associated with digestive disorders causing growth inhibition and diarrhea. The diarrhea is often associated with intestinal microorganisms such as *Escherichia coli* and *Salmonella* spp. Therefore, the main aim of this study was to investigate these factors after feeding of *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10.

Body Weight Gain

Body weight gain and average daily gain of the control, MP9, MP10, and antibiotic groups are shown in Tables 1 and 2, respectively. Compared with the control, the MP9 and MP10 groups showed a significant increase (P<0.05) in body weight at 14,- 21,- and 28-day-old, but the antibiotics group did not show a difference. Among the experimental groups, body weight was better in the MP groups than in the antibiotics' group, although the difference was not significant.

Average daily weight gain from 1- to 28-day-olds was increased in the MP9 and MP10 groups (P<0.05), but was not in the antibiotic group. Among the experimental groups, the average daily gain was better in the MP groups than the antibiotics' group, although with no significant difference. Similar results were reported. Probiotics tended to enhance mean daily body weight gain in weaned pigs.[4] Daily weight gain in piglets between 35 and 40 days old was enhanced by *Bacillus subtilis* MA139.[9] However, any significant increase in body weight due to probiotics has also been reported in piglets.[5,12]

The failure of probiotics to elevate body weight has been attributed to the inability of probiotics to survive in the intestinal tract or competitively exclude the pathogenic bacteria. The present result that *Bacillus subtilis* MP9 and MP10 increased body weight gain compared to the control and that the body weight gain value was better than with antibiotics suggests that these *Bacillus subtilis* MP would be useful probiotics for improving the efficiency of animal production.

Diarrhea

Kiers et al[10] reported that cooked and fermented soybeans could lower the incidence of diarrhea. The present *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10 isolated from fermented soybeans showed improvements in the mean diarrhea score and numbers of piglets with diarrhea than in the control, although the results were not significantly different (P>0.05) (Table 3). In addition, periods of diarrhea were shorter in the experimental groups than in the control (P<0.05). Periods for the MP9 and MP10 groups tended to be shorter than in the antibiotic group. This result means that the

Table 3. *Diarrhea sign in piglets hand-fed a 10 ml/pig/day of phosphate buffered saline (control), Bacillus subtilis MP9 (1011 CFU/ml.) (Bacillus subtilis MP9) and Bacillus subtilis MP10 (1011 CFU/ml.) (Bacillus subtilis MP10) from three- to ten-day-old or 1 g antibiotic (Chlortetracycline)/pig/day from three- to six-day-old (antibiotic) (n = 20).*

Item	Control	*Bacillus subtilis* MP9	*Bacillus subtilis* MP10	Antibiotic	Pooled SEM	P-value
Diarrhea level score*	0.55	0.35	0.30	0.35	0.05	0.39
(Numbers of diarrhea pig)	(11)	(7)	(6)	(7)		
Period of diarrhea (day)	4.70^{a}	1.60^{b}	1.50^{b}	2.00^{b}	0.41	0.02

^{a,b}*Means having different superscripts within the same row are significantly different at P<0.05.*
**Diarrhea level score; 0 = little---to---1 = strong*

Table 4. *Microorganism count (log 10/CFU/g) at 7 and 11 days of age in piglets hand-fed a 10 ml/pig/day of phosphate buffered saline (control), Bacillus subtilis MP9 (1011 CFU/ml.) (Bacillus subtilis MP9) and Bacillus subtilis MP10 (1011 CFU/ml.) (Bacillus subtilis MP10) from three- to ten-day-old or 1 g antibiotic (Chlortetracycline)/pig/day from three- to six-day-old (antibiotic) (n = 20).*

Microorganism types	**Control**	***Bacillus subtilis* MP9**	***Bacillus subtilis* MP10**	**Antibiotic**	**Pooled SEM**	**P-value**
7 days of age						
Escherichia coli	4.78^{a}	4.30^{b}	4.48^{ab}	3.62^{c}	0.11	0.01**
Salmonella spp.	3.71^{a}	2.73^{c}	3.24^{b}	2.81^{c}	0.10	0.01**
Lactobacillus spp.	9.63^{b}	11.27^{a}	11.04^{a}	10.54^{a}	0.19	0.01**
Bacillus subtilis MP	6.64^{b}	8.28^{a}	7.97^{a}	6.52^{b}	0.20	0.01**
11 days of age						
Escherichia coli	7.74^{a}	6.22^{b}	6.35^{b}	7.06^{a}	0.15	0.01**
Salmonella spp.	4.05^{a}	3.31^{b}	3.37^{b}	3.54^{b}	0.09	0.02*
Lactobacillus spp.	9.23^{c}	11.09^{a}	10.76^{ab}	10.40^{b}	0.17	0.01**
Bacillus subtilis MP	3.58^{b}	4.68^{a}	4.46^{a}	3.71^{b}	0.11	0.01**

[a, b, c]*Means having different superscripts within the same row are significantly different at P<0.05 (*) and P<0.01 (**).*

experimental groups recovered more quickly from diarrhea than the control, suggesting that *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10 would be beneficial in controlling diarrhea.

Microorganism in the Feces

Antibiotics used as growth promoters act by reducing the pathogenic bacteria and modifying the intestinal microflora in the animal gut.[13] However, the transfer of antibiotic resistance to pathogens is reported by such applications.[14] Therefore, probiotics are known to be good alternatives to the antibiotics as growth promoters.[15] Also in this study on pathogenic bacteria, compared at 7 days of age with the control microorganism, most experimental groups showed decreased numbers of *Escherichia coli* and *Salmonella* spp. (P<0.01). There was, however, no change in the numbers of *Bacillus subtilis* MP10 for *Escherichia coli*.

At 11 days of age, the antibiotic group showed decreased numbers of *Salmonella* spp. (P<0.02), but lost effectiveness for *Escherichia coli*. The *Bacillus subtilis* MP groups decreased both *Escherichia coli* (P<0.01) and *Salmonella* spp. (P<0.02). Compared with the antibiotic group, the *Bacillus subtilis* MP groups showed a lower value of both pathogens. These results suggest that the period of effectiveness of chlortetracycline as an antibiotic was not long, and that *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10 would be more beneficial in the control of *Escherichia coli* and *Salmonella* spp.

On the other hand, compared with the control antibiotic group, beneficial microorganisms could elevate *Lactobacillus* spp. (P<0.01) in piglets at 7 days of age, but could not increase *Bacillus subtilis* MP. *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10 increased these beneficial microorganisms (P<0.01). At 11 days of age, the antibiotics group could elevate numbers of *Lactobacillus* spp. (P<0.01), but could not increase *Bacillus subtilis* MP. *Bacillus subtilis* MP, *Bacillus subtilis* MP9, and *Bacillus subtilis* MP10 increased these beneficial bacteria (P<0.01), and increased them much more than in the antibiotic group, with the exception of *Bacillus subtilis* MP10. This result suggests that *Bacillus subtilis* MP9 and *Bacillus subtilis* MP10 would be beneficial in the control of microorganisms.

CONCLUSION

Bacillus subtilis MP9 and *Bacillus subtilis* MP10 have a growth promoting effect by depressing the incidence of diarrhea during the new-born to weaning period in piglets, by competing with pathogenic gut flora, and by stimulating effective microorganisms. *Bacillus subtilis* MP9 and MP10 are promising alternatives to antibiotics for use as a feed supplement in piglet diets.

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