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Effect of Surfactin on Growth Performance of Weaning Piglets in Combination with *Bacillus subtilis* BC1212

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Abstract : The aim of this study is to investigate the effects of surfactin in combination with *Bacillus subtilis* BC1212 isolated from Korean soybean paste, on feed utilization and growth performance during 4 weeks in weaning piglets. Eighteen weaning piglets (Landrace × Yorkshire × Duroc; weighing 7.68±0.97 kg) were divided into control (n=9) and experimental groups (n=9). The treatments included a control group consisting of the basal diet with no additives (control) and an experimental group consisting of the basal diet supplemented with 1 g of surfactin C and 1.0×10^9 CFU of *Bacillus subtilis* BC1212/kg feed. Piglets fed *Bacillus subtilis* BC1212 increased in average daily weight gain and feed efficiency. In comparison with the control group, the fecal *Bacillus subtilis* were significantly increased and the fecal coliform bacteria were markedly reduced in the experimental group. In addition, *Bacillus subtilis* BC1212 had excellent acid and bile tolerance. The treatment of surfactin (50 $\mu g\,ml^{-1}$) in lipopolysaccharide (LPS)-stimulated swine peripheral blood mononuclear cells (PBMCs) for 6 h showed a significant inhibitory effect on INF-γ, TNF-α and NO secretion ($p<0.05$) in comparison with LPS treatment alone but not on IL-10 secretion, with levels of secreted IL-10 similar to those secreted by PBMCs stimulated with LPS alone. Supplementation with surfactin in combination with *Bacillus subtilis* BC1212 in diets improved the ecosystem of gastrointestinal tract by increasing probiotic population and enhanced the systemic immune response in weaned piglets.

Key Words **:** Acid and bile tolerance, *Bacillus subtilis*, Probiotics, Surfactin, Peripheral blood mononuclear cells (PBMCs).

Introduction

The search for new probiotic strains has increased in recent years due to the necessity to find economic and effective substitutes for antibiotics used as feed additives. Probiotics present a promising alternative. A probiotic is a live microbial food supplement that benefits the host animal by improving its intestinal microbial balance (14). These health-promoting bacteria are increasingly being used in pig feed, as an alternative approach to controlling the growth of unfavorable microorganisms.

Bacillus species are able to synthesize antibiotics, proteases, amylases, amino acids, and other metabolites and exhibit immunomodulating activity (29,30), which account for their use in some therapeutic- and prophylactic-purpose commercial probiotic preparations. Moreover, *Bacillus* species, a type of exogenous spore-forming bacteria, are not normally found in the gastrointestinal tract but have also been shown to be effective in keeping a favorable balance of microflora in the gastrointestinal tract and in improving animal performance (34).

Surfactin isolated from *Bacillus subtilis* showed that it had a strong surface tension-lowering activity and showed antiviral, antitumor, fibrinolytic, anti-thrombotic and hypocholesterolemic activities (29). It is a natural compound of industrial importance and attains increasing biotechnological and pharmaceutical interests (28). Recently, it was reported to inhibit the lipopolysaccharide (LPS)-induced expression of inflammatory mediators (interleukin-1β, IL-1β; inducible nitric oxide synthase, iNOS) and reduce the plasma endotoxin, tumor necrosis factor-α (TNF-α) and nitric oxide levels in response to septic shock in rats (12,13). Surfactin was also shown to suppress the interaction of lipid A with LPS-binding protein (LBP) that mediated the transport of LPS to its receptors (12). Moreover, surfactin did not influence the viability of the eukaryotic cell lines tested. In addition, it was less toxic than other surfactants as judged from the results of an acute toxicity study in mice (26,31). Based on these findings, it is reasonable to assume that the inclusion of some

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combination of probiotics and surfactin may have considerable health promotion benefits for weaning piglets. The purpose of this study is to assess the effects of surfactin in combination with *Bacillus subtilis* BC1212 isolated from Korean soybean paste, on feed utilization and growth performance during 4 weeks in weaning piglets.

Materials and Methods

Bacterial strain

Bacillus subtilis BC1212 was isolated from Korean soybean paste (Korean Patent 10-2004-0092258) and surfactin was provided by B&C Biopharm (Yongin, Gyeonggi-do, Korea). *Bacillus subtilis* BC1212 was grown in a shaking incubator (150 rpm; 37°C) in liquid medium for another 48 h. The composition of the medium is as follows: glucose 10 g, corn starch 30 g, soybean meal 10 g, peptone 5 g, yeast extract 5 g, and $CaCO_3$ 2 g. CB442 (0.01%) (Nippon Oil & Fat Co. Ltd., Sumida-ku, Tokyo) was used as an antifoaming agent.

Acid and bile tolerance of *Bacillus subtilis*

The stability of *Bacillus subtilis* BC1212 to acid condition was assayed by the modified method of Duc et al (7). In short, each strain was diluted 1/100 in PBS at pH 1, 2, 3, 4 or 5, and incubated for 1, 2 and 4 h. Subsequently, the bacteria were transferred to trypticase soy broth (TSB, BD, NJ, USA) and incubated aerobically at 37°C overnight. The number of viable cells was counted by using the standard plate count method at 37°C for 48 h.

Bile tolerance of *Bacillus subtilis* BC1212 was evaluated by the method of Gilliland and Walker (8). The overnight cultures of *Bacillus subtilis* BC1212 were inoculated into TSB with or without 0.3% (*w/v*) oxgall (Sigma, Louis, USA). Bacterial cell in the culture broth was measured by reading the optical density (OD) at 550 nm for 12 h incubation at 37°C.

Inhibitory effect of surfactin on lipopolysaccharide (LPS)-induced cytokine production

Donor animals were 8-week-old healthy piglets (Landrace×Yorkshire×Duroc) weighing about 22 kg. Heparinized blood samples were collected by the jugular puncture and then mononuclear cells were separated by the gradient density centrifugation using Histopaque 1.077 (Sigma, MO, USA). Peripheral blood mononuclear cells (PBMCs) were washed twice with RPMI 1640 medium, counted and seeded at 1×10^6 cells ml^{-1} in 24-well microplates after detection of the cell activity with trypan blue (Sigma, MO, USA) dye exclusion. The RPMI 1640 was supplemented with 10% fetal calf serum (FCS, BioWhittaker, MD, USA), 100U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin.

To investigate the effect of surfactin on the IL-10, INF-γ, TNF-α and NO production of LPS-stimulated PBMC, cells (1×10^6 cells ml^{-1}) were incubated with simultaneous supplement of surfactin at different final concentrations (10, 20 and 50 μg ml^{-1}) and bacterial LPS from *Escherichia coli* serotype O111:B4 (Sigma, MO, USA) at a final concentration of 10 μg ml^{-1} at 37°C in 5% CO_2 for 24 h. After the incubation, cytokine concentrations in supernatants were separately determined by IL-10, INF-γ and TNF-α ELISA kits (Biosource, CA, USA). The sensitivity thresholds were <3.0 pg ml^{-1} for IL-10, <2.0 pg ml^{-1} for INF-γ and <3.0 pg ml^{-1} for TNF-α. NO concentrations were determined by the nitrite colorimetric assay kit (iNtRON Biotechnology, Seongnam, Korea). Standard curves generated with increasing amounts of nitrite were linear between 0.1 and 1000 μM. All samples were assayed in triplicate.

Animals and experimental diets

Piglets (Landrace×Yorkshire×Duroc), obtained from the Swine Breeding Center of Chungnam National University, were weaned at average 28 days of age. A total of 18 piglets were used, with each treatment group consisting of 9 piglets. The treatments included a control group consisting of the basal diet with no additives (control) and experimental group consisting of the basal diet supplemented with 1 g of surfactin C and 1.0×10^9 CFU of *Bacillus subtilis* BC1212/kg feed. The basal diet formulated to provide digestible energy 13.90 MJ/kg, 20.69% crude protein, 0.90% calcium, 0.73% phosphorus and 1.42% lysine. Sufficient vitamins and minerals were added to meet the nutrient requirements for the growth of piglets of the weight range studied based on NRC (25). Reference to the NRC (25) is imperative for a thorough evaluation of all swine diets, including the breeding herd, on a commercial operation. Feed consumption per piglet was monitored and piglets were weighed weekly. Average daily gain (ADG), average daily feed intake (ADFI) and gain:feed ratio (G/F) were calculated. Fresh fecal samples of each treatment were collected in sterile centrifuged tubes at the day before the end of the experiment.

Bacterial counts

At 10 : 00 a.m., the day before the end of the experiment, fecal samples of 2 groups were collected in sterile centrifuged tubes containing 9 ml of phosphate buffered saline (pH 7.2, Sigma). Samples were brought to the laboratory within 2 h after defecation. The fecal samples were put into bottles filled with CO_2 and were stored at -70°C until used.

Dilution series of collected fecal samples in PBS were plated to CDC anaerobe blood agar (Oxoid, Hampshire, UK) and incubated anaerobically at 37°C for 48 h for total anaerobe bacterial count determinations. Polymixin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA, Oxoid, Hampshire, UK) and MacConkey agar with aerobic incubation at 37°C for 24 h were used for the determination of *Bacillus subtilis* and total coliform counts.

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical comparisons for the production of cytokines and NO in LPS induced swine PBMC were made using the analysis of variance (ANOVA) among doses. Post hoc com-

Table 1. Survival of different *Bacillus subtilis* BC1212 + surfactin C after incubation at various pH values

pH	Survival (%) after incubation at		
	1 h	2 h	4 h
1	76.2	70.3	64.0
2	93.0	91.1	85.7
3	100	97.2	98.4
4	99.7	96.8	96.1
5	99.9	95.9	97.9

parisons were performed by Duncan's multiple comparison tests. The difference between data of groups was considered significant at the level of $p<0.05$.

Growth performance and colonization of the intestinal tract with the administered surfactin in combination with *Bacillus subtilis* BC1212 were monitored and expressed with mean values and standard deviations in triplicate trials. Data generated from the experiments were analysed for significance by the Student's *t*-test. Differences were considered significant at $p<0.05$.

Results

Acid and bile tolerance

The results on acid tolerance (survival at various pH values) showed that *Bacillus subtilis* BC1212 survived an incubation period of 4 h at pH 1.0 to pH 5.0 (Table 1). Fig 1 shows its growth curves in TSB with or without bile. The addition of 0.3% oxgall bile made the growth of *Bacillus subtilis* BC1212 delay for the first 2 h incubation, increase slightly for another 2 h incubation and augment rapidly thereafter.

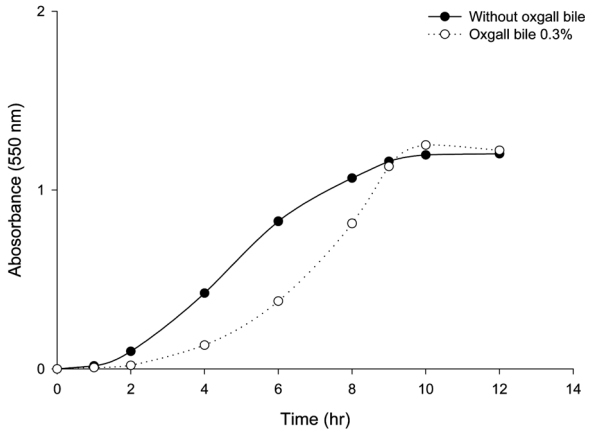

Fig 1. Effect of oxgall bile on the growth of *Bacillus subtilis* BC1212 cells for 12 h. Experimental conditions were as described in materials and methods. The cultural broth used was TSB with or without 0.3% oxgall bile. The growth rate of *Bacillus subtilis* BC1212 was obtained by measuring the increment of absorbance per hour at 550 nm from the third to the 12 h after incubation.

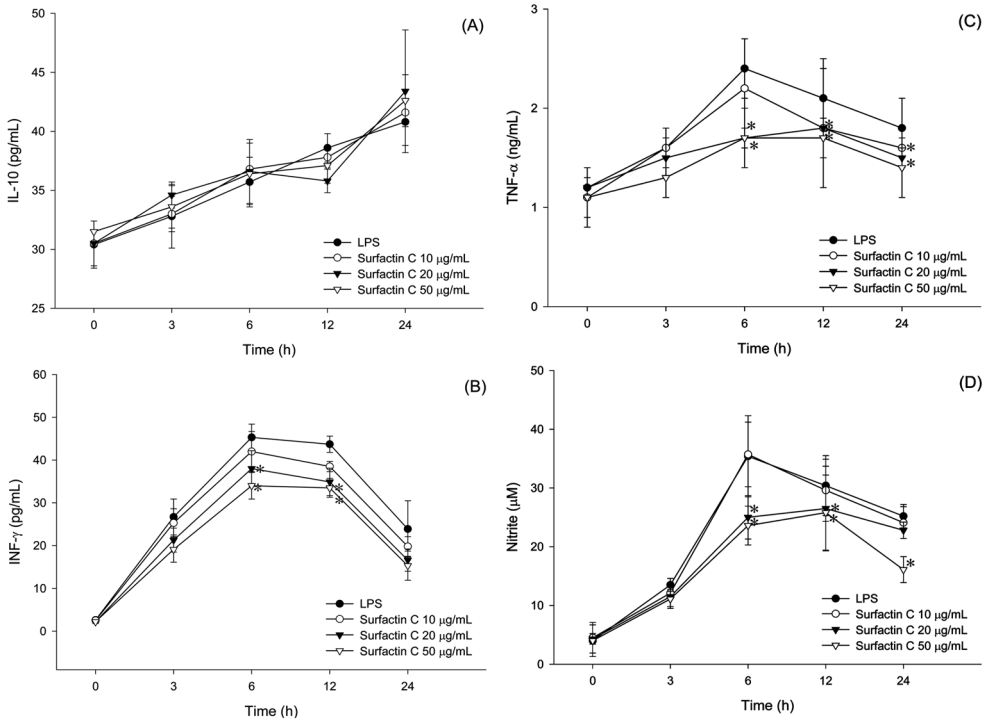

Fig 2. The kinetics of IL-10, INF-γ, TNF-α, and NO production by swine PBMC, activated by LPS. The production of IL-10 (A), INF-γ (B), TNF-α (C), and NO (D) was determined in the culture supernatants harvested at the times indicated by the cytokine specific ELISA and nitrite colorimetric assay kit. Results are expressed as mean with SD. Values were considered significantly different in the case of $p<0.05$ by Duncun's multiple comparison test, *is $p<0.05$.

Inhibitory effect of surfactin on LPS-induced cytokine production

Time course experiments showed the optimal incubation time for the detection of IL-10, INF-γ and TNF-α production from LPS-stimulated swine PBMCs to be 6 h except for IL-10. Surfactin significantly inhibited the production of IFN-γ, TNF-α and NO with the dose dependent fashion (Fig 2). The treatment of surfactin (50 μg ml^{-1}) in LPS-stimulated swine PBMCs for 6 h showed a significant inhibitory effect on INF-γ, TNF-α and NO secretion (p<0.05) in comparison with LPS treatment alone but not on IL-10 secretion, with levels of secreted IL-10 similar to those secreted by PBMCs stimulated with LPS alone (Fig 2). The levels of INF-γ, TNF-α and NO were decreased at 12 h after stimulation with LPS, but IL-10 secretion was increased with time

Bacterial counts

Fig 3 presents the profiles of the intestinal microorganisms in each group. There was no change in the number of anaerobic bacteria between two groups. *Bacillus subtilis* were significantly increased in the treatment group in comparison with the control group (p<0.05). The number of coliform bacteria was significantly decreased in the treatment group as compared to the control group (p<0.05).

Growth performance

ADG and ADFI during the total period did not show statistically significant difference between the treatment group and the control group (Table 2). However, piglets fed surfactin in combination with *Bacillus subtilis* BC1212 showed significantly increased feed efficiency of 9.23 and 15.05% in the third and fourth week, respectively (p<0.05).

Discussion

In animal studies with various strain of *Bacillus* spp, positive effects such as increased weight gain, improved feed conversion ratios and lowered mortality rates of piglets have been reported (Cenci 20,21). Two possible mechanisms for the beneficial effects of *Bacillus* strains on gastrointestinal disturbances are: (i) production of antimicrobial substances

Table 2. Growth performance data[1]

	Control	BC 1212 + Surfactin	S.E.M.[a]	p-value
Initial BW (kg)	7.32	7.95	0.459	0.173
Final BW (kg)	22.28	22.47	0.134	0.853
ADG (g)	534.05	540.9	3.450	0.812
ADFI(g)	715	663*	26.00	0.142
G/F	0.748	0.820*	0.051	0.106

[1]Mean±SD of 9 piglets for each group. [a]Standard error of the mean. *P<0.05 vs control.

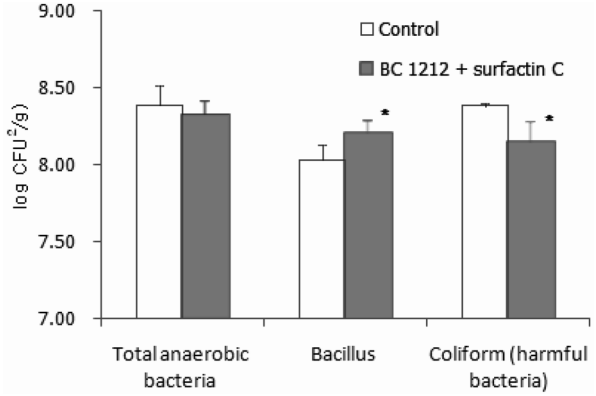

Fig 3. Effect of probiotics on fecal microorganisms in pigs[1]. [1]Mean±SD of 9 piglet for each group. [2]CFU : colony forming unit. *P<0.05 *vs* control.

such as surfactin or subtilisin; and (ii) adherence to the mucosa and co-aggregation to form a barrier which prevents colonization by pathogens (22). Other factors, such as tolerance to acid and bile, also need to be considered if the desired results are to be obtained from the use of *Bacillus* spp as growth promotants. Recently, emphasis has been placed on the selection and preparation of *Bacillus* spp. as probiotics (7).

Resisitance to acid and bile of probiotics are of great importance in their survival and growth in the intestinal tract (11). Spore formed by bacillus probiotic strain copes with the adverse environment in connection with bile and acid (6,9). In this study, *Bacillus subtilis* BC1212 was tolerant to acid in the wide range of pH between 1 and 5. It had a moderate survival rate even at pH 1.0 and 2.0 but much better survival rate at pH 3.0, 4.0 and 5.0. The result of present study was in agreement with those of previous study. (9). Bile tolerance of *Bacillus subtilis* BC1212 showed no growth for the first 2 h, a slow growth for another 2 h, but a rapid growth, thereafter in the presence of bile, demonstrating its bile tolerance. In addition, the counts of *Bacillus subtilis* were significantly increased in fecal samples fed with *Bacillus subtilis* BC1212. These results supported that *Bacillus subtilis* BC1212 was well tolerated against acid and bile.

Sepsis is a complex clinical syndrome that may arise due to the activation of host immune responses by LPS (32). Septic shock results in the induction of activation of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12, IL-10 and IFN-γ, which may damage cells and lead to organ injury (19,10). Especially, IFN-γ is the prototypic macrophage-activating factor (15), thus innate IFN-γ produced following LPS is a part of a positive back loop which amplifies antimicrobial immune responses by inducing phagocytosis and respiratory burst, antigen presentation, and cytokine secretion by antigen-presenting cells (4). In this study, surfactin C significantly inhibited the production of IFN-γ, TNF-α and NO with the dose dependent fashion (Fig 3). In the previous study, the plasma levels of TNF-α and NO *in vivo* rat septic

shock model were significantly decreased by the administration of surfactin C. Moreover, surfactin suppressed the interaction of lipid A with LBP and had a LPS-neutralizing activity by its strong surface tension-lowering activity (12,31). The result of present study was in agreement with those of our previous studies (12,31).

The improved growth performance of domestic fowl fed with probiotics (5,18,27) is thought due to its probiotic actions, such as the maintenance of normal intestinal microflora and increased digestive enzyme activity (16,17). Kiers *et al* (20) and Ushakova *et al* (33) reported increased average daily weight gain and feed utilization improvement in piglets fed *Bacillus* spp. In this study, piglets fed surfactin in combination with *Bacillus subtilis* BC1212 showed increased G/F ratio, which was in agreement with other reports (6,33).

Bacillus subtilis BC1212 had excellent acid and bile tolerant properties enough to be used as probiotics. *Bacillus subtilis* BC1212 improved the ecosystem of the intestinal tract by increasing the probiotic population and surfactin enhanced the systemic immune response of piglets. In conclusion, surfactin in combination with *Bacillus subtilis* BC1212 increased G/F ratio and feed efficacy. Therefore, we assume surfactin in combination with *Bacillus subtilis* BC1212 isolated from Korean soybean paste is a favorable candidate for probiotics.

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바실러스 섭틸리스 BC1212와 설팩틴의 병용투여가 이유돈의 성장에 미치는 영향

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요 약 : 한국된장에서 분리한 바실러스섭틸리스 BC1212와 설팩틴을 함유한 사료첨가제가 이유돈의 성장에 미치는 영향을 연구하였다. 총 18마리의 이유돈 (랜드레이스×요크셔×듀록; 체중, 7.68±0.97 kg)을 9마리씩 두 그룹으로 배치하여 실험을 실시하였다. 실험군은 사료 kg 당 설팩틴 C 1 g과 1.0×10^{9} CFU 바실러스섭틸리스 BC1212를 급여하였고, 대조군은 일반사료를 4주간 급여하였다. 그 결과 바실러스섭틸리스 BC1212와 설팩틴을 급여한 군에서는 일평균 증체량과 사료효율이 증가하였으며, 무처리 대조군과 비교해서, 분변 중 바실러스-섭틸리스의 생존률이 현저히 높았다. 또한 바실러스섭틸리스는 산과 담즙에 대한 높은 내성을 보였다. 세포 내 독소 투여 후 말초혈액단액세포에 설팩틴 ($50\ \mu g\ ml^{-1}$) 처리 후 약 6 시간까지 INF-γ, TNF-α 과 NO 분비량을 설팩틴 무처리군에 비해 현저히 감소시켰다 ($p<0.05$). 그러나 IL-10의 분비량은 설팩틴 무처리군과 유사했다. 결론적으로 바실러스섭틸리스 BC1212와 설팩틴을 병용하여 이유돈에 급여할 경우, 이유돈의 위장관내 생균수 증가와 함께 면역반응을 증대시킬 수 있다.

주요어 : 산・담즙내성, 바실러스섭틸리스, 생균제, 설팩틴, 말초혈액단액세포 (PBMCs).