

## Evaluation of Manganese Ion on Controlling Harmful Microorganisms *In vitro* and *In vivo* for the Early-Weaned Pig

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**ABSTRACT:** Two experiments were conducted to determine the effects of MnSO<sub>4</sub> on controlling harmful microorganisms *in vitro* and *in vivo*. The *in vitro* experiment was conducted to examine the effects of manganese sulfate (MnSO<sub>4</sub>) on the reduction of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) by growth stimulation of *Pediococcus acidilactici* (*P. acidilactici*; lactic acid bacteria). Manganese ion (0.003 %) was found to stimulate the growth of *P. acidilactici* in the *In Vitro* system. When *E. coli* and *S. aureus* were grown in a mixture with *P. acidilactici*, their numbers were reduced. This may be the result of a reduction of pH in the medium as a result of better growth of *P.*

*acidilactici* due to stimulation by the Mn ion. The *in vivo* experiment was conducted to determine the effects of MnSO<sub>4</sub> in diets on controlling harmful microorganisms in fecal samples of pigs. There were no significant differences for the microbial numbers (i.e., total microorganisms, *E. coli*, lactic acid bacteria and *S. aureus*) in feces of pigs fed MnSO<sub>4</sub> compared to feces of pigs fed the control diet through 7 days. However, on day 7 of experiment, the pH of feces in pigs fed MnSO<sub>4</sub> (0.1%) decreased faster than pigs fed the control diet.

(Key Words: Manganese, Microorganisms, *In vitro*, *In vivo*, Feces, Pig)

### INTRODUCTION

At birth, the piglet is essentially devoid of circulating antibodies, since placental transfer of maternal immunoglobulins to the fetus is minimal (Kim et al., 1966). Consequently, the piglet receives immunological protection from the sow via passive transfer of immunoglobulins contained in the colostrum. Typically, the pig's passive immunity is maximized 24-36 hours postpartum, and decreases logarithmically thereafter (Miller et al., 1962). At 3 weeks of age, humoral immunity is low, presenting potential problems in the early-weaned pig. Blecha et al. (1983) reported that the pig's ability to mount an immune response was suppressed as weaning age was decreased from 5 to 2 weeks of age.

In pigs, postweaning diarrhoea causes great economic loss due to the reduced growth, occasional death, and cost of medical treatment and prophylaxis (Svensen et al., 1974; Jahn and Uecker, 1987; Svensmark et al., 1989). Strains of *Escherichia coli* (*E. coli*) are the primary cause of diarrhoeic diseases at weaning and the period immediately thereafter (Kenworthy and Crabb, 1963;

Sojka, 1965; Svendsen et al., 1977; Sarmiento et al., 1988).

Manganese ion was found to accelerate the growth and bacteriocin production of lactic acid bacteria (LAB) (Biswas et al., 1991). According to Raccach (1983), manganese ion appears to be essential to the growth and metabolic activities of LAB. The effects of manganese ion on LAB include enhancement of lactic acid fermentation and protection from oxygen toxicity. Furthermore, the rapid increase in LAB number can attribute to the inhibition of harmful microorganisms such as *E. coli* or *Staphylococcus* spp. by antimicrobial agents (i.e., lactic acid, bacteriocin or enzyme etc.) (Bruno et al., 1992; Kone and Fung, 1992).

Therefore, the objectives of the experiments reported herein were to determine the effects of manganese sulfate (MnSO<sub>4</sub>) on the reduction of *E. coli* and *Staphylococcus aureus* (*S. aureus*) by growth stimulation of *Pediococcus acidilactici* (*P. acidilactici*) for the *in vitro* experiment and the effects of MnSO<sub>4</sub> in diets on controlling harmful microorganisms in fecal samples of pigs for the *in vivo* experiment.

### MATERIALS AND METHODS

#### 1. *In vitro* experiment

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### (1) Cultures tested

*Pediococcus acidilactici* isolated from HP starter culture (Diversitech Inc., Duncanville, TX) was transferred into Brain Heart Infusion (BHI, Difco) broth and incubated at 40°C for 48 hours. A 1% inoculum (0.75 ml) of the suspension was transferred into a Klett flask containing 75 ml of BHI broth with 1.5% sucrose added. *Escherichia coli* and *S. aureus* obtained from the culture collection at Kansas State University was inoculated into the same BHI medium either individually or in combination with *P. acidilactici*.

### (2) Laboratory medium with MnSO<sub>4</sub>

The BHI+MnSO<sub>4</sub> media (BHIM: 0.003% MnSO<sub>4</sub>) broth was prepared as follows. A stock solution of MnSO<sub>4</sub> was prepared by adding 0.5 g of MnSO<sub>4</sub> to 10 ml sterilized water. After mixing, the stock solution was filter sterilized through 0.2 µm membrane filter and added 0.06 ml to 100 ml BHI broth. The control (0%) and 0.003% of MnSO<sub>4</sub> in BHI broth were tested for acid production and growth of *P. acidilactici*.

### (3) Effect of MnSO<sub>4</sub> on inhibiting *E. coli* by *P. acidilactici*

*Escherichia coli* (10<sup>4</sup> CFU/ml) was inoculated into BHIM broth, BHI broth with each individual supplement, or BHI broth with no supplement along with *P. acidilactici* (10<sup>6</sup> CFU/ml). The Klett flasks with cultures were incubated without agitation at 40°C. Klett units and viable cell counts of *E. coli* were obtained using Violet Red Blue (VRB, Difco) agar and *P. acidilactici* using Lactobacilli deMan Rogosa and Sharp (MRS, Difco) agar. *Escherichia coli* without *P. acidilactici* was evaluated as a control.

### (4) Effect of MnSO<sub>4</sub> on inhibiting *S. aureus* by *P. acidilactici*

*Staphylococcus aureus* (10<sup>4</sup> CFU/ml) was inoculated into BHIM broth and BHI broth along with *P. acidilactici* (10<sup>6</sup> CFU/ml). Viable cell counts of *S. aureus* using Baird-Parker (Difco) agar and *P. acidilactici* using MRS agar were measured at regular intervals (after 0, 6, 12, 18 and 24 hours) incubation at 40°C. *Staphylococcus aureus* without *P. acidilactici* were evaluated as control.

### (5) Statistical analysis

Data from this experiment were analyzed using the General Linear Model (GLM) procedures of SAS (1988) with LSD (Steel and Torrie, 1980) to compare treatment means.

## 2. *In vivo* experiment

### (1) Animals

Eighteen pigs (initially 5.1 kg body weight and 21 days of age) were used in 7 days to determine the effect of MnSO<sub>4</sub> in diets on controlling harmful microorganisms in fecal samples. The pigs were housed in individual cages (46 cm × 46 cm) equipped with woven-wire flooring. Each cage contained a one-hole self feeder and one nipple waterer to allow ad libitum consumption of feed and water. Room temperature was maintained at 28°C.

### (2) Treatments and diets

Treatments were: 1) corn-soybean meal based control diet, 2) corn-soybean meal based diet with 0.01% MnSO<sub>4</sub> and 3) corn-soybean meal based diet with 0.1% MnSO<sub>4</sub>. The concentrations of MnSO<sub>4</sub> were diluted with distilled water and sprayed diluted MnSO<sub>4</sub> into the diets. All diets were formulated to contain 1.50% lysine, 0.42% methionine, 0.9% Ca and 0.8% P and contained 25% dried whey, 9% lactose and 7.5% porcine plasma protein (table 1). The diets were fed in pellet form and were

Table 1. Composition of basal diet<sup>a</sup>

Ingredient	%
Corn	31.78
Dried whey	25.00
Soybean meal, 48.5%	15.03
Lactose	9.00
Spray-dried plasma protein	7.50
Soybean oil	5.00
Fish meal	3.00
Monocalcium phosphate	1.56
Antibiotic <sup>b</sup>	1.00
Limestone	0.38
Vitamin premix <sup>c</sup>	0.25
L-Lysine · HCl	0.15
Trace mineral premix <sup>d</sup>	0.15
DL-Methionine	0.12
Copper sulfate	0.08
Total	100.00

<sup>a</sup> Diets were formulated to contain 1.50% lysine, 0.42% methionine, 0.90% Ca and 0.80% P.

<sup>b</sup> Provided 165 µg/kg of apramycin.

<sup>c</sup> Premix provided per kilogram of complete diet: vitamin A, 11,025 IU; vitamin D<sub>3</sub>, 1,103 IU; vitamin E, 44 IU; menadione (menadione sodium bisulfate complex), 4.4 mg; riboflavin, 8.3 mg; d-pantothenic acid, 29 mg; niacin, 50 mg; choline, 166 mg; and vitamin B<sub>12</sub>, 33 µg.

<sup>d</sup> Premix provided per kilogram of complete diet: Mn, 12 mg; Fe, 165 mg; Zn, 165 mg; Cu, 16 mg; I, 0.3 mg; and Se, 0.3 mg.

formulated to meet or exceed all other nutrient concentrations as recommended by the NRC (1988).

### (3) Feces sampling

On day 0, 3 and 7 of the experiment, fecal samples were collected from six pigs per treatment by rectal massage to measure microbiological change. Fecal samples were transferred to filter stomacher bags (Model SFB 0410, Spiral Biotech Inc., Bethesda, MD) to make  $10^{-1}$  dilution with 0.1% sterile peptone water and pummeled for 2 minutes in a stomacher (Model 400, Seward Medical, London, UK). Serial dilutions were prepared with 9 ml dilution blanks of 0.1% sterile peptone water. Dilutions were plated with spiral plater (Model DV2, Spiral Systems, Inc., Cincinnati, OH) on the appropriate media (plate count agar for total microorganisms, MRS for LAB, VRB for *E. coli* and Baird-Parker for *Staphylococcus* spp.).

### (4) Statistical analysis

Data collected were analyzed as a randomized complete block design with pen as the experimental unit and initial weight as the blocking factor. Data were analyzed using the General Linear Model (GLM) procedures of SAS (1988). Duncan's multiple range test was used to separate means (Steel and Torrie, 1980).

## RESULTS AND DISCUSSION

### 1. *In vitro* experiment

#### (1) Inoculum of *E. coli* with *P. acidilactici*

Figure 1 shows the growth (CFU/ml) of inoculated *E. coli* ( $10^4$  CFU/ml) in the laboratory medium containing *P. acidilactici*. Without  $MnSO_4$ , *E. coli* increased to 8.34 log CFU/ml at 40°C incubation with *P. acidilactici*. However, the final numbers of *E. coli* in the medium with the mixture of  $MnSO_4$  with *P. acidilactici*, after 16 hours of incubation at 40°C, increased to only 7.41 log CFU/ml ( $p < 0.05$ ). In the presence of  $MnSO_4$  and *E. coli*, the numbers of *P. acidilactici* increased from 6.2 log to 9.23 log CFU/ml, whereas with *E. coli* alone, *P. acidilactici* increased from 6.2 log to 8.5 log CFU/ml ( $p < 0.05$ ; figure 2). The suppression of growth of *E. coli* by *P. acidilactici* in the presence of supplement was great. In conclusion, 0.003% of  $MnSO_4$  was a good stimulator for the growth of *P. acidilactici*. In the mixed culture with *E. coli* ( $10^4$  CFU/ml) and *P. acidilactici* ( $10^6$  CFU/ml), Mn ion was a good stimulator to control *E. coli* by *P. acidila*

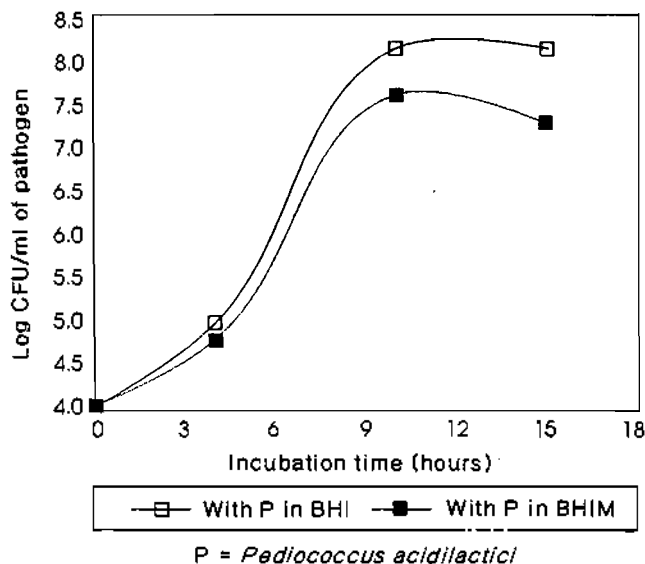


Figure 1. The growth of *Escherichia coli* in BHI and BHIM with *Pediococcus acidilactici* (P); (BHI=Brain Heart Infusion broth; BHIM = BHI +  $MnSO_4$ ).

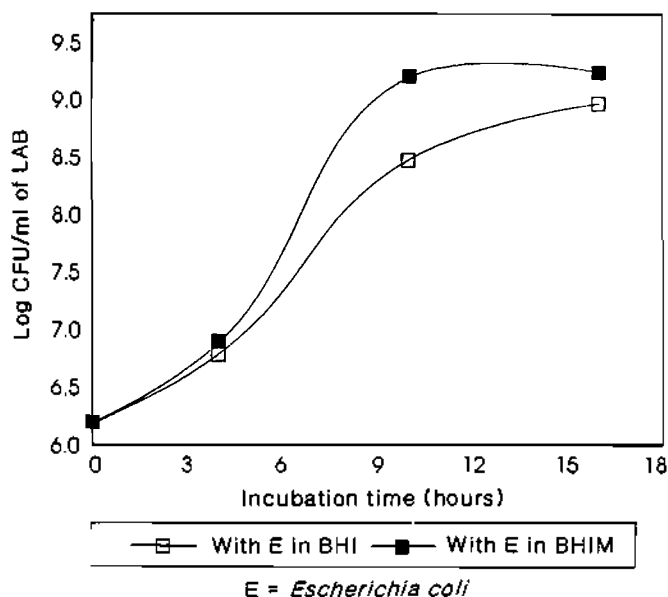
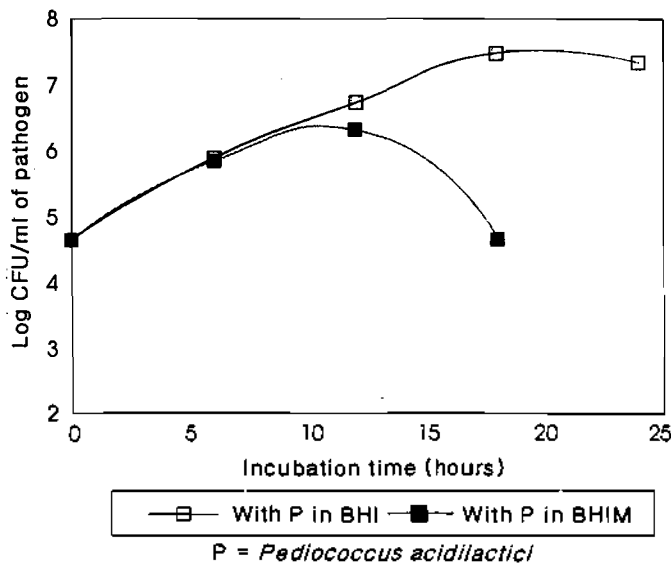


Figure 2. The growth of *Pediococcus acidilactici* in BHI and BHIM with *Escherichia coli* (E); (BHI=Brain Heart Infusion broth; BHIM = BHI +  $MnSO_4$ ).

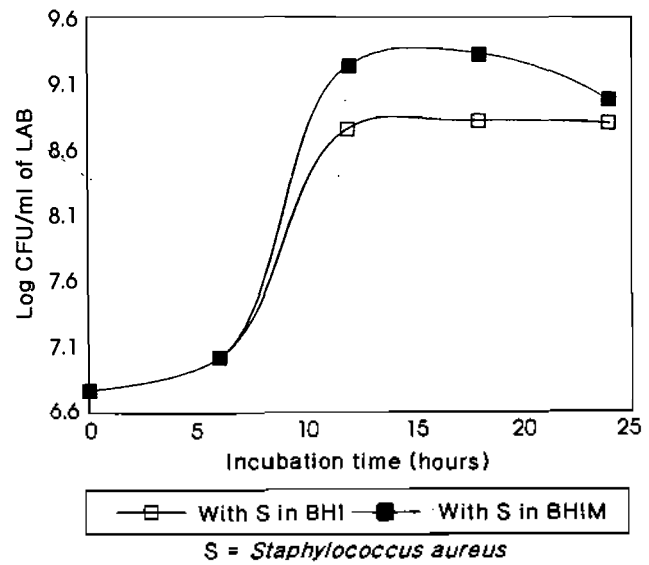
*ctici* during fermentation.

#### (2) Inhibition of *S. aureus* by *P. acidilactici* with and without growth factor

Figure 3 shows the growth of *S. aureus* in laboratory media containing *P. acidilactici*. Without  $MnSO_4$ , *S. aureus* increased from 4.7 log to 7.42 log CFU/ml after 16 hours of incubation with *P. acidilactici*. However, the final numbers of *S. aureus* in the medium with the mixture of  $MnSO_4$  plus *P. acidilactici* after 16 hours were



**Figure 3.** The growth of *Staphylococcus aureus* in BHI and BHIM with *Pedococcus acidilactici* (P); (BHI=Brain Heart Infusion broth; BHIM=BHI + MnSO<sub>4</sub>).



**Figure 4.** The growth of *Pedococcus acidilactici* in BHI and BHIM with *Staphylococcus aureus* (S); (BHI=Brain Heart Infusion broth; BHIM=BHI + MnSO<sub>4</sub>).

reduced from 4.7 log to under 1.0 log CFU/ml ( $p < 0.05$ ). In the presence of MnSO<sub>4</sub> and *S. aureus*, the numbers of *P. acidilactici* increased from 6.8 log to 9.3 log CFU/ml, whereas with *S. aureus* alone, *P. acidilactici* increased from 7.2 log to 8.75 log CFU/ml ( $p < 0.05$ ; figure 4). The suppression of growth of *S. aureus* by *P. acidilactici* in the presence of supplement was great (reduction of about 6-7 log CFU/ml units).

In conclusion, 0.003% of MnSO<sub>4</sub> was a good stimulator for growth and acid production of *P. acidilactici* in cultures with *E. coli* or *S. aureus* in the *in vitro* system.

## 2. *In vivo* experiment.

Table 2 shows the effect of MnSO<sub>4</sub> on microbial changes in feces of pigs. There were no significant differences for the microbial numbers (i.e., total microorganisms, *E. coli*, lactic acid bacteria and *S. aureus*) in feces of pigs fed MnSO<sub>4</sub> compared to feces of pigs fed the control diet through 7 days. Table 3 shows the effects of MnSO<sub>4</sub> on pH changes in feces of pigs. For day 0 and 3 of experiment, there was no significant difference for the pH change among the treatments. However, after 7 days, the pH of feces in pigs fed MnSO<sub>4</sub> (0.1%) decreased faster ( $p < 0.05$ ) than pigs fed the control diet (8% less). Manganese ion contributes to the rapid decrease in pH through stimulation for LAB. Low pH is important to control harmful microorganisms and

bacteriocin production from LAB (Biswas et al., 1991). From these data, MnSO<sub>4</sub> did not show the significant effect as in the short term (7 days) *in vivo* experiment. However, on the basis of the rapid pH decrease of treated pig's feces, MnSO<sub>4</sub> might affect the microbial numbers in a long term experiment.

**Table 2.** Effects of MnSO<sub>4</sub> on controlling harmful microorganisms in feces of pigs<sup>a</sup>

Item	MnSO <sub>4</sub>			SE
	0%	0.01%	0.1%	
Total microorganisms	... log CFU/ml ...			
day 0	8.06	8.13	8.07	0.11
day 3	8.77	8.53	8.62	0.18
day 7	8.58	8.67	8.66	0.25
<i>Escherichia coli</i>				
day 0	6.80	6.61	6.73	0.12
day 3	6.95	6.75	6.80	0.26
day 7	6.93	6.90	6.89	0.17
<i>Lactic acid bacteria</i>				
day 0	7.36	7.36	7.42	0.25
day 3	7.43	7.63	7.59	0.31
day 7	7.60	7.71	7.74	0.17
<i>Staphylococcus</i> spp.				
day 0	4.37	4.23	4.17	0.22
day 3	4.13	4.10	4.07	0.15
day 7	3.93	4.10	3.80	0.20

<sup>a</sup> A total of 18 pigs were allotted with one pig per pen and six pens per treatment.

**Table 3.** Effects of MnSO<sub>4</sub> on pH changes in feces of pigs<sup>a</sup>

Item	MnSO <sub>4</sub>			SE
	0%	0.01%	0.1%	
day 0	6.89	6.91	6.92	0.12
day 3	6.82	6.84	6.66	0.05
day 7	6.90 <sup>b</sup>	6.95 <sup>b</sup>	6.34 <sup>c</sup>	0.02

<sup>a</sup> A total of 18 pigs were allotted with one pig per pen and six pens per treatment.

<sup>b,c</sup> Means with different superscripts in the same row differ significantly ( $p < 0.05$ ).

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