**Title:** **Does pre-slaughter transportation and lairage affect *Salmonella enterica* shedding prevalence and levels in market pigs? - #07-025**

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**Industry Summary:**

This research was conducted with the objective of determining the effect of pre-slaughter transportation and lairage on *Salmonella enterica* shedding prevalence, shedding levels of total *Enterobacteriaceae* and *Lactobacilli*, and proportion of the sub-populations of antimicrobial resistant *Enterobacteriaceae* and *Lactobacilli* in market pigs, under commercial conditions. Five replicates of the experiment were conducted with 30 pigs each. Each animal was individually identified and fecal samples collected immediately before moving the animals from their resident pens to a transport trailer (pre-transport), immediately upon arrival at the abattoir (post-transport), and after approximately 2 hours of pre-slaughter resting (post-lairage). *Salmonella* prevalence increased significantly between each one of the defined sampling points (11.3% pre-transport, 20% post-transport, and 42% post-lairage). The total *Lactobacilli* population enumerated was significantly higher than the total *Enterobacteriaceae* population at all sampling points. No effect on the total numbers of total *Enterobacteriaceae* or total *Lactobacilli* was observed. No quantitative effect was observed on sub-populations of *Enterobacteriaceae* resistant to ampicillin, tetracycline, and gentamicin. Also, no quantitative effect on sub-populations of *Lactobacilli* resistant to tetracycline and erythromycin was observed. However, a significant quantitative increase of the ampicillin-resistant *Lactobacilli* was observed from the pre-transport to the post-lairage sampling. This research suggests that effects of stress on the microbial ecosystem of the gastrointestinal tract of swine differ between groups of bacteria, and also between its sub-groups. Our findings reveal a critical need for further research to advance our knowledge on the quantitative effects of stress on microbial populations of the gastrointestinal tract of swine and its potential food safety implications.

**Scientific Abstract:**

This research was conducted with the objective of determining the effect of pre-slaughter transportation and lairage on *Salmonella enterica* shedding prevalence, shedding levels of total *Enterobacteriaceae* and *Lactobacilli*, and proportion of the sub-populations of antimicrobial resistant *Enterobacteriaceae* and *Lactobacilli* in market pigs, under commercial conditions. Five replicates of the experiment were conducted with 30 pigs each. Each animal was individually identified and fecal samples collected immediately before moving the animals from their resident pens to a transport trailer (pre-transport), immediately upon arrival at the abattoir (post-transport), and after approximately 2 hours of pre-slaughter resting (post-lairage). *Salmonella* prevalence increased significantly between each one of the defined sampling points (11.3% pre-transport, 20% post-transport, and 42% post-lairage). The total *Lactobacilli* population enumerated was significantly higher than the total *Enterobacteriaceae* population at all sampling points. No effect on the total numbers of total *Enterobacteriaceae* or total *Lactobacilli* was observed. No quantitative effect was observed on sub-populations of *Enterobacteriaceae* resistant to ampicillin, tetracycline, and gentamicin. Also, no quantitative effect on sub-populations of *Lactobacilli* resistant to tetracycline and erythromycin was observed. However, a significant quantitative increase of the ampicillin-resistant *Lactobacilli* was observed from the pre-transport to the post-lairage sampling. This research suggests that effects of stress on the microbial ecosystem of the gastrointestinal tract of swine differ between groups of bacteria, and also between its sub-groups. Our findings reveal a critical need for further research to advance our knowledge on the quantitative effects of stress on microbial populations of the gastrointestinal tract of swine and its potential food safety implications.

**Introduction:**

Food safety is a defining issue in the global pork market today, and *Salmonella* contamination is a major concern for the pork industry all over the world. Foodborne pathogens are being considered as another measure of overall pork quality and a challenging issue. For the pork industry (producers and abattoirs) to be able to respond to this challenge, sound knowledge about the ecology and epidemiology of foodborne pathogens in general, and *Salmonella* in particular, is essential.

Although much of the *Salmonella* contamination of pork and pork products occurs within abattoirs (i.e., in the slaughter and processing line), infected pigs leaving the farm are considered as the original source of abattoir contaminations (Botteldoorn et al.,2004; Namvar and Warriner, 2006). According to Berends et al.(1996), live animals that carry *Salmonella* at the time of slaughter are 3-4 times more likely to produce positive carcasses than *Salmonella*-free animals. In the U.S., our research team has shown through several studies that substantial numbers of pigs are carrying *Salmonella* when arriving in the abattoirs (Hurd et al.,2002; Rostagno et al.,2003; Hurd et al.,2005), with the intestinal tract and its associated lymph nodes being frequently contaminated/infected and providing a source from which *Salmonella* may be spread in the abattoir contaminating carcasses and other pork products. Considering the intestinal bacteria as the primary source of carcass contamination, it is reasonable to assume that the extent of carcass contamination is determined by the number (or levels) of these microorganisms entering the abattoir in the intestine of slaughtered pigs.

Bacteria levels in the gastrointestinal tract and shedding from unapparent carriers (in the case of *Salmonella*) may be affected by a long list of stressors. Stress represents the reaction of the body to stimuli that disturb its normal physiological equilibrium or homeostasis, which have significant impact on the immune system in general (Khansari et al., 1990). During loading, transport, unloading and commingling in lairage pens, pigs are exposed to various stressors before slaughter, including noise, unfamiliar smells, vibration, changes in temperature, breakdown of social groupings and food deprivation (Warriss et al.,1992). Consequently, many believe that the number of pigs shedding *Salmonella* as well as the levels of the bacteria in the intestines will be increased after all these stressors (as will their susceptibility to new infections). However, there is no conclusive data showing a direct association between stress or immune status and increased shedding or susceptibility to *Salmonella* infections in swine. Therefore, we decided to undertake the challenge of exploring this blurred link of the pre-harvest pork production chain.

Acute stress leads to a release of catecholamines, resulting in a decreased gastric acid production and an increased intestinal motility (Tache et al.,1980; Tache et al.,1999). The increase of the pH in the stomach leads to a greater probability that *Salmonella* will survive gastric passage (Tennant et al.,2008; Kanno et al.,2009). Consequently, animals become more susceptible to new infections, and animals that already excrete *Salmonella* can shed more with an increased defecation frequency. However, little is known about the effect of catecholamines released into the intestine on the carriage and virulence of bacterial pathogens *in vivo* (Vlisidou et al.,2004). Furthermore, some mediators of the nervous system released are known for their effect on the activity of immune cells (i.e., immune-suppression), resulting in increased translocation of *Salmonella* (Nyberg et al.,1988; Wood et al.,1989). The role of the neuroendocrine environment, particularly in the intestinal tract, in the pathogenesis of enteric bacterial infections is increasingly being recognized. It has been demonstrated (*in vitro*) that intestinal pathogens express virulence determinants in response to environmental signals indicating host stress (Alverdy et al.,2000).

We hypothesize that the physiological reactions to the stress imposed by transportation from the production farm to the abattoir and the pre-slaughter lairage environment affect the *Salmonella* status of the animals by inducing carriers to start shedding, increasing bacteria shedding levels, and also inducing *Salmonella*-free pigs to be more susceptible to infection.

**Objectives:**

This research was conducted to achieve the following objectives:

1) to determine the effect of pre-slaughter transportation and lairage on *Salmonella enterica* shedding prevalence;

2) to determine the effect of pre-slaughter transportation and lairage on the shedding levels of total *Enterobacteriaceae* and *Lactobacilli* in market pigs; and

3) to determine the effect of pre-slaughter transportation and lairage on sub-populations of antimicrobial resistant *Enterobacteriaceae* and *Lactobacilli* in market pigs, under commercial conditions.

**Materials & Methods:**

***Sampling:***

Multiple lots (or barns) of pigs during early grow-finish stage (75-125lb.) were sampled (N=64 individual fecal samples/lot; 2 samples/pen) to identify 5 *Salmonella*-positive lots to be used in this research. The 5 lots identified as *Salmonella*-positive were sampled again three times (total of 4 samplings/lot; N=64 individual fecal samples/sampling). These samplings allowed to determine the on-farm *Salmonella* shedding prevalence, and to identify pens with positive pigs. Upon reaching market weight, 30 pigs were randomly selected from the pens with positive pigs. By selecting pigs from these pens, we aimed to include in our study; 1) pigs shedding *Salmonella* at the time of the experiment, 2) pigs that were infected, but were not shedding *Salmonella*, and 3) negative pigs. Selected pigs were individually identified, and fecal samples were collected directly from the rectum as the animals were moved to be loaded for transportation. To eliminate the chance of contamination/infection of pigs from the transport trailer, a clean and disinfected trailer was used in this study (5 swab samples were collected prior to each replicate of the experiment). Pigs were transported for approximately 1 hour and 30 minutes to a commercial abattoir. During the process of unloading the pigs at the abattoir, individual fecal samples were collected directly from the rectum. Pigs were moved to a conventional pre-slaughter resting (i.e., lairage) pen. Lairage pens were sampled (5 swabs/pen) to verify pre-existing contamination. After approximately 2 hours of resting, individual fecal samples were collected directly from the rectum.

***Laboratory Procedures:***

In the laboratory, individual fecal samples were homogeneized and divided in 2 aliquots, which were processed for the isolation of *Salmonella enterica* and enumeration of *Enterobacteriaceae* and *Lactobacilli*, or frozen at -80$^{o}$C (with 30% glycerol) for further analyses (e.g., qPCR and DGGE). The isolation and identification of *Salmonella* was accomplished through sequential enrichment in Tetrathionate broth (1:10), Rappaport-Vassiliadis broth containing novobiocin (20μg/mL), and subsequent plating on XLT-4 agar. Suspect colonies were selected and streaked on Rambach agar for identification as *Salmonella* or non-*Salmonella*. For the enumeration of total *Enterobacteriaceae* and *Lactobacilli* populations, fecal samples were serially diluted (10-fold) and plated on MacConkey and Rogosa agars, respectively. With the objective of determining potential changes in different sub-groups (or sub-populations) within these two large groups of bacteria inhabiting the gastrointestinal tract of the pigs, additional agar plates were prepared to contain selected antibiotics. MacConkey agar plates containing ampicillin (32 μg/mL), tetracycline (16 μg/mL), and gentamycin (16 μg/mL), and Rogosa agar plates containing ampicillin (32 μg/mL), tetracycline (16 μg/mL), and erythromycin (8 μg/mL) were inoculated with selected serial dilutions of the samples. All MacConkey agar plates were incubated under aerobic conditions, whereas all Rogosa agar plates were incubated under microaerophilic conditions (5% $N_2$, 10% $O_2$, 85% $CO_2$).

***Statistical Analysis:***

The experimental design applied was based on individually paired samples (i.e., individually matched fecal samples collected at different time points; pre-transportation, post-transportation and post-lairage from the same pigs). Qualitative data (i.e., prevalence) was analyzed by comparison of proportions using McNemar's Chi-square test, whereas quantitative data (i.e., levels or CFU/g) was subjected to logarithmic transformation ($\log_{10}$), and analyzed using paired t-test. All statistical inferences were based on $P<0.05$.

**Results:**

A significant increase of the *Salmonella* prevalence was observed from the pre-transport to post-transport sampling points (P<0.05; Figure 1). An additional significant increase of the *Salmonella* prevalence was observed from immediately post-transport to 2 hours post-lairage (P<0.05; Figure 1).

There was no effect of transportation or lairage on total *Enterobacteriaceae* counts (P>0.05; Figure 2). Sub-populations of *Enterobacteriaceae* resistant to ampicillin, tetracycline and gentamicin were found in all 5 lots of pigs used in this study. However, no effect of transport or lairage in any of these sub-populations was observed (P>0.05; Figure 2).

A trend for increased counts of *Lactobacilli* was observed from pre-transport to post-lairage (P=0.05; Figure 3). Additionally, a significant increase in the counts of ampicillin-resistant *Lactobacilli* was observed from pre-transport to post-lairage (P<0.05), whereas no effect on sub-populations of *Lactobacilli* resistant to tetracycline and erythromycin (P>0.05; Figure 3).

When comparing the total counts of *Enterobacteriaceae* versus *Lactobacilli*, it was observed that levels of *Lactobacilli* were 104.7 and 141.3 times higher than of *Enterobacteriaceae*, pre-transportation and post-lairage, respectively (P<0.05 for both sampling points; Figure 4). The significantly higher counts of *Lactobacilli* compared to *Enterobacteriaceae* resulted in higher levels of *Lactobacilli* sub-populations resistant to ampicillin and tetracycline, both pre-transportation and post-lairage (Figures 2 and 3).

**Discussion:**

Increased bacteria shedding frequency and levels caused by stress (in general) is commonly assumed. However, solid scientific and conclusive evidence are lacking, and in some cases, observations have not confirmed that this general assumption is correct. Studies of the effects of transporting pigs to slaughter are scarce. This study shows that a significant increase of *Salmonella* prevalence from the farm to the abattoir occurs. Although others have already shown an increase of *Salmonella* prevalence occurring due to rapid infections during the pre-slaughter lairage (Hurd et al.,2001; Hurd et al.,2002; Rostagno et al.,2003), this study shows that the process of moving market pigs from the production farm to the abattoir (i.e., transportation) also causes a prevalence increase, under commercial conditions.

An early study by Williams and Newell (1970) showed, in a small group of pigs, that shipment (transport) of pigs led to increased shedding of *Salmonella*. More recently, Isaacson et al.(1999a) showed that pigs experimentally infected (i.e., inoculated) with *Salmonella* Typhimurium exhibit increased shedding after transportation. The same authors however, reported conflicting results in another study to determine the effect of feed withdrawal and transportation on the shedding of *Salmonella* Typhimurium in experimentally infected pigs (Isaacson et al,1999b). Collectively, the results of these two studies suggest that there is an interaction between feed withdrawal and transportation that can lead to increased shedding of *Salmonella* by pigs. This study corroborates that transportation of market pigs is capable of increasing *Salmonella* prevalence, and therefore, the associated food safety risk. The key value of this study is based on the fact that it was conducted under natural infection and commercial conditions, differently than others. However, further research is required to clarify these observations, particularly as the process of moving market pigs from the production farms to the abattoirs is actually a multifactorial complex that includes a variety of potential stressors (e.g., handling, mixing, floor space, thermal conditions, vibration, noises, and time). The identification of the specific factors determining the observed effect, and understanding the effectors' mechanism(s) will provide insights for the development of targeted and effective intervention strategies.

Additional research suggests that transportation of pigs and its associated stress may cause changes in bacterial species, biotypes, and antimicrobial resistance phenotypes (Molitoris et al.,1987; Langlois and Dawson, 1999) inhabiting their gastrointestinal tract. Moro et al.(1998, 2000), Jones et al.(2001), and Mathew et al.,(2003) showed that changes occur in the intestinal populations of *Escherichia coli* upon exposure to various stressors. However, our knowledge on the potential effects of stress on the gastrointestinal microbial ecology continues to be limited at best. This study sheds some light on the occurrence of qualitative changes in the balance of the gastrointestinal microbial ecology of market pigs, which in some cases, may have some food safety implications. For instance, a wide dominance of the bacterial group *Lactobacilli* over the *Enterobacteriaceae* group was demonstrated by this study. Moreover, it shows that although the process of moving pigs from the production farms to abattoirs does not seem to affect this dominance, as it persists from the farm (i.e., pre-transportation) until immediately prior to slaughter (i.e., post-lairage). Although no differences in the proportions of *Enterobacteriaceae* resistant to the evaluated antibiotics (i.e., ampicillin, tetracycline and gentamicin) were observed, a significant increase in resistance to ampicillin was observed for the *Lactobacilli* group from the farm to the post-lairage sampling. This observation suggests that effects of stress on the gastrointestinal microbial populations are not the same for all bacterial groups and its respective sub-populations. Therefore, further research is critically needed to advance our knowledge in this very complex arena.

Only by knowing the cause and effect relationship between different stressors applied on the animals (as hosts) and their microbial ecosystem (quantitative and qualitatively), and subsequently, the mechanism(s) involved in stress-induced changes, can the pork industry develop and implement specific and effective solutions (i.e., intervention strategies). Although many can argue that transporting animals from the farm to the abattoir is a process that cannot be avoided, it is important to keep our minds open to potential changes or modifications. For instance, transportation conditions can be easily manipulated to minimize stress, and/or the gastrointestinal microbial ecosystem can be made more resilient to changes (i.e., stabilized) through the use of probiotics, prebiotics or other alternatives.

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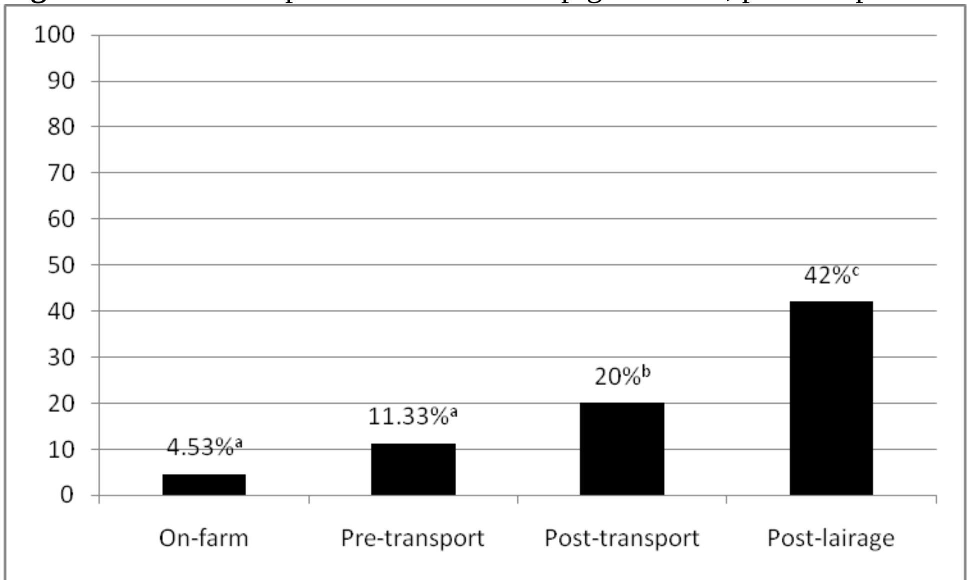
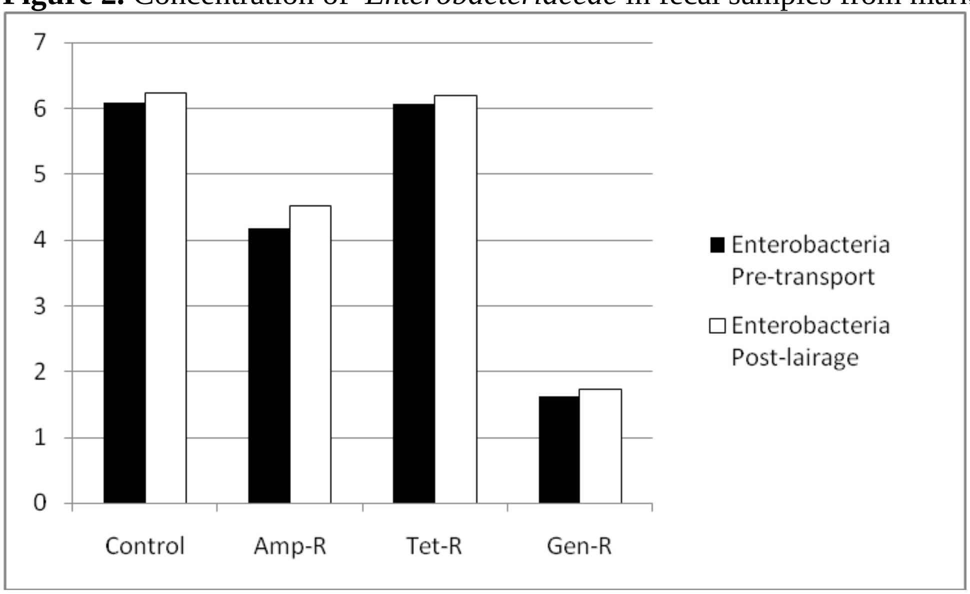
**Figure 1.** *Salmonella* prevalence in market pigs on-farm, pre- and post-transportation, and post-lairage.



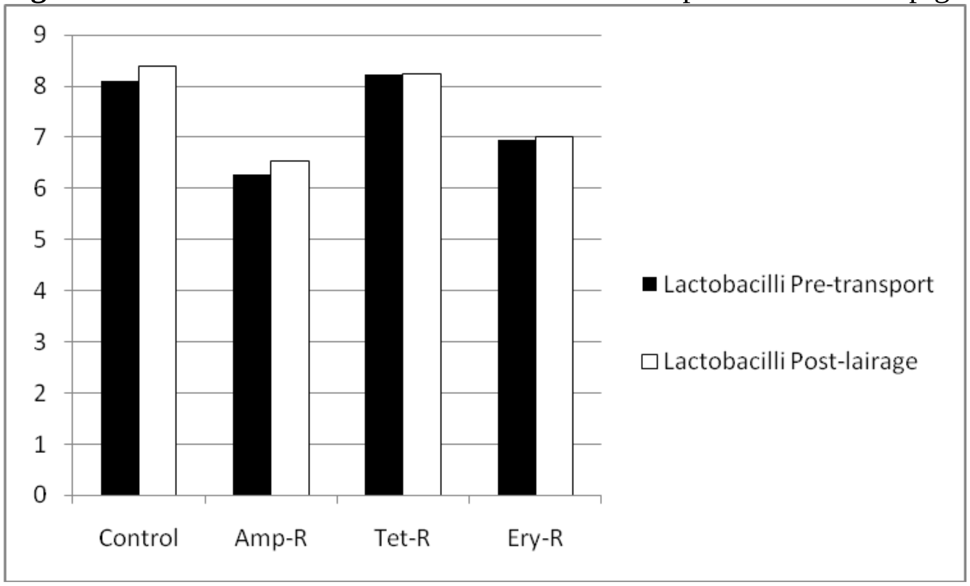

[a,b,c]: Different superscript letters indicate statistically significant difference ($P<0.05$).

**Figure 2.** Concentration of *Enterobacteriaceae* in fecal samples from market pigs ($\log_{10}$ cfu/g).



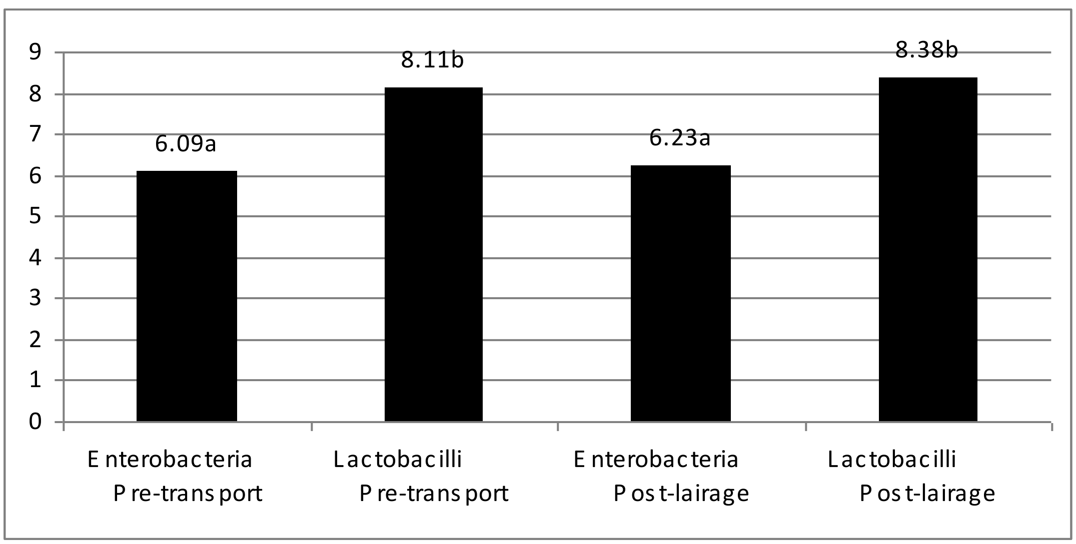

Control: Total *Enterobacteriaceae*; Amp-R: Ampicillin-resistant; Tet-R: Tetracycline-resistant; Gen-R: Gentamicin-resistant.

**Figure 3.** Concentration of *Lactobacilli* in fecal samples from market pigs ($\log_{10}$ cfu/g).




Control: Total *Lactobacilli*; Amp-R: Ampicillin-resistant; Tet-R: Tetracycline-resistant; Ery-R: Erythromicin-resistant.

**Figure 4.** Comparison between concentrations of total *Enterobacteriaceae* and total *Lactobacilli* in fecal samples from market age pigs pre-transport and post-lairage ($\log_{10}$ cfu/g).




a,b: Different letters indicate statistically significant difference ($P<0.05$).