PATHOPHYSIOLOGICAL EFFECTS OF ORAL INOCULATION OF GROWING PIGS WITH SALMONELLA ENTERICA SEROVARS TYPHIMURIUM OR CHOLERAESUIS

by

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ABSTRACT

Enteric pathogens are responsible for major economic losses in the swine industry. In the U.S., *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) and serovar Choleraesuis (SC) account for essentially all cases of salmonellosis in swine. Previous studies documented that oral ST eroded growth and produced unmistakable changes in the endocrine stress and somatotropic axis of young growing pigs. However, these effects occurred in the absence of elevated systemic inflammatory cytokines that were previously thought to accompany disease-associated growth retardation. In the current study, it was hypothesized that SC would produce very different systemic inflammatory cytokine responses compared to ST given the likelihood of SC to produce systemic disease in pigs. Weaned pigs were housed two per pen with free access to feed and water during a 14 d experiment. On d 0, pigs were fed either 10^8 CFU SC or 10^8 ST, and bacteria were re-fed twice weekly through the course of the experiment. Control pigs were fed dough without bacteria. Serum was collected on d 0, 7, and 14 for determination of tumor necrosis factor alpha (TNFα), interleukin-1beta (IL-1β), and insulin-like growth factor-I (IGF-I) were determined. Rectal temperatures (RT) were monitored daily beginning 2 d prior to challenge with bacteria and until 7 d following the first bacterial feeding. Pigs were weighed initially, and at the conclusion of the study. Daily body weight gain was reduced by 25.4% in pigs fed SC ($P<.0001$) compared to control, while growth was similar between control pigs and those fed ST. Pigs fed SC had increased RT beginning on d 2 and continuing though d 7 ($P < 0.05$) with the greatest elevation spike on d 3 ($P < 0.001$) when compared to controls. On d 7, pigs fed SC had reduced IGF-I when compared to both control ($P < 0.01$) and ST pigs ($P = 0.01$). Despite the obvious

febrile response, and the reductions in body weight gain and serum IGF-I, circulating TNFα and IL-1β were not affected by treatment. It was concluded that elevated TNFα and IL-1β are not obligatory correlates of SC-induced pathology and growth retardation in weaned pigs.

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Chapter 1

Pathogenesis and Concepts of Transmission of *Salmonella enterica* serovar Typhimurium and serovar Choleraesuis in Swine

ABSTRACT

Enteric pathogens are responsible for major economic losses in the swine industry. According to the National Animal Health Monitoring Survey *Salmonella* was responsible for $100 million in losses in the swine industry in the United States in 1990. These losses come in two different ways. The first being production losses as a direct result of Salmonellosis and the second being *Salmonella* contamination of pork products. In the U.S., *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) and serovar Choleraesuis (SC) account for essentially all cases of salmonellosis in swine. However, these pathogens produce very different clinical outcomes, with ST producing mainly self-limiting enteritis, whereas SC, a so-called swine host adapted pathogen, is more likely to result in a more serious and occasionally fatal septicemia. The severity of disease that *Salmonella* causes in the pig also depends on other factors. These factors not only include the serotype, but also the virulence and quantity of the ingested bacteria, the route of infection, and any natural and/or acquired immunity the host might possess. This report will review published material involving ST and SC, the differences and similarities between the two serovars and the disease they each cause in pigs.

KEYWORDS: Swine, Salmonella, Salmonella Typhimurium, Salmonella Choleraesuis, nomenclature

INTRODUCTION

All bacteria with in the genus *Salmonella* belong to the family *Enterobacteriaceae*. They are rod shaped, gram negative, non-spore forming, facultative anaerobic bacteria[1, 2]. Most serovars of *Salmonella* are motile and flagellated[2]. With over 2400 different serovars, *Salmonella* has a broad host range with a few serovars being uniquely adapted to certain host species[1]. *Salmonella* infection of swine is a concern for two major reasons. It is a cause of major production loses to the swine industry and is a potential source of pork contamination, which in turn makes it a possible source of human infection[1].

In the U.S., salmonellosis in swine is almost always caused by one of two serovars of *Salmonella*, *S.* Choleraesuis (SC) or *S*. Typhimurium (ST), with SC being the most frequent[1] and a swine adapted serovar[1, 2]. The aim of this review is to focus on contrasting pathophysiologic effects of SC and ST in pigs. Relevant ancillary discussion is also provided to establish current norms of nomenclature and factors affecting virulence and transmission of these swine pathogens.

NOMENCLATURE OF THE GENUS *SALMONELLA*

In recent years the nomenclature of the genus *Salmonella* has been a source of conflict among bacteriologists. Part of this conflict is due to the shear number of serotypes within the genus and how to classify them. Originally, each serovar of *Salmonella* was considered a separate species[1-4], but due to findings at the molecular level in the early 1970s, it was concluded that all serovars appear, in fact, to be a single species[2, 4].

When SC first appeared on the approved list of bacterial names, more confusion followed. This confusion was due to the fact that the name choleraesuis was also shared by a specific serovar. It was Le Minor and Popoff[4] that proposed *Salmonella enterica* as the only

species name of the genus *Salmonella*. The use of *Salmonella enterica* first encountered widespread criticism and then eventually became accepted by the scientific community prior to becoming accepted by the Judicial Commission of the International Committee for Systematics of Prokaryotes in 2005[4]. In 2000, before the Judicial Commission of the International Committee for Systematics of Prokaryotes accepted *Salmonella enterica* as the species name, a taxonomic note was written and published by Yabuuchi and Ezaki arguing against the change in name and suggested that *choleraesuis* the species be retained and that Choleraesuis the serovar be changed to Hogcholera[5]. This argument was not accepted.

Another source of confusion has been how to properly write the names of these individual serovars. There have been many different forms used by different scientists. According the Centers for Disease Control (CDC) and the American Society for Microbiology, when writing individual serovar names, these should be indicated with a single capital letter and not italicized[2, 4]. For example, *Salmonella enterica* subsp. *enterica* serovar Choleraesuis for the initial reference and any of the following references can be abbreviated *Salmonella* serovar Choleraesuis or more simply *Salmonella* Choleraesuis[2-4].

According to the *Salmonella Annual Summary, 2003*[6], there are only two species with in the genus *Salmonella*, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* includes six subspecies. These subspecies are referred to by name or Roman numerals and include the following, *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *and indica* (VI)[2, 3, 6]. Subspecies V of *Salmonella enterica* was originally occupied by *Salmonella bongori* but it has since been designated a separate species[6].

Two other species have since been described, *Salmonella bongori* described by Reeves in 1989[4] and *Salmonella subterranean* described by Shelobolina in 2004[4, 7]. The majority of

Salmonella serovars belong to the species *S. enterica* with 60% of the serovars belonging to *S. enterica* subspecies *enterica* and the majority of disease causing serovars belong to this subspecies[2].

Since the genus *Salmonella* has 2,463 different serovars[2, 3], a standard way of identifying these serovars is needed. Many use the Kauffmann-White scheme to identify the different serovars of *Salmonella*[1-3], and as of January 1, 2003 the CDC has officially adopted the Kauffmann-White Scheme. This was to ensure consistency and accuracy with reporting and surveillance[6]. The Kauffmann-White scheme is based on the H (fagellar) and O (somatic) antigens of each serovar and some serovars also have an Vi (capsular polysaccharide) antigen[2, 4]. An added source of confusion is that some serovars include variants[4]. Due to the complex and ever expanding nature of the genus *Salmonella* a standardization of nomenclature for the genus still needs to be formed.

VIRULENCE FACTORS AND TRANSMISSION OF SWINE *SALMONELLA* SEROVARS

There have been over 200 virulence factors associated with *Salmonella* but only a few of these are understood. Some of these virulence factors are as follows; adhesion, invasion, cytotoxicity, and the ability to resist intracellular killing[1]. Many of these virulence factors are encoded by *Salmonella* Pathogenicity Islands (SPI)[2]. SPIs are large clusters of conserved DNA in the genome of the species *Salmonella* that differ from the closest relative, *E. coli*[2]. Five SPIs have been identified within *Salmonella*[2]. Each are indicated with a number (e.g. SPI-1). More about SPIs and their contribution to the virulence of *Salmonella* will be discussed later.

Another important point to consider when considering the virulence of *Salmonella* is that subclinical infections are common as is environmental contamination[1]. These are the two main

sources of *Salmonella* infection in the pig, other pigs and the environment. Pigs that have a subclinical infection are also referred to as asymptomatic carriers. These asymptomatic carriers also play a role in the virulence of *Salmonella*. For example, ST has been isolated from fecal samples for up to 7 mo post infection[8] and at slaughter up to 7 mo post infection[1]. SC has been shown to be shed by pigs from 1d post infection[9] up to 12 wk post infection[10] and survive in dried feces for up to 6 mo[1]. Gray et al.[9] suggested that carrier pigs are in part responsible for the maintenance of this pathogen due to the fact that SC is not often isolated from the environment.

Fecal-oral route is the most likely route of *Salmonella* infection[1, 11]. It has also been speculated that virulence increases after *Salmonella* has been passed through the pig[1, 9]. The fact that salmonellosis outbreaks are usually spread in a pen to pen fashion[1] supports the fecal-oral route of infection. Fedorka-Cray et al.[11] demonstrated that ST could colonize the gut in esophagotomized pigs challenged intra-nasally (IN) and trans-thoracically. This observation demonstrated that the fecal-oral route is not the only route of *Salmonella* infection in pigs. Since ST was not found in the blood of the esophagotomized pigs, Fedorka-Cray et al.[11] speculated that it was the macrophages that transported the bacteria to the gut. Gray et al.[10] demonstrated that pigs challenged IN with ST exhibited a more severe disease response than pigs challenged intra-gastrically. These reports suggest that virulence may also depend on the route of infection.

The ability of Salmonella to survive in phagocytes is an important factor in the virulence of *Salmonella*[1, 2]. The role that both fimbriae and flagella contribute to virulence is unknown[2]. Flagella may be important in the ability of *Salmonella* to survive within macrophages[1], and possibly adhesion[12].

Insertion genes. The ability of *Salmonella* to invade host cells may be contributed to by the presence of serotype-specific plasmids[1]. SPI-1 contains multiple genes including the inv/spa

cluster[2]. It has been demonstrated that SPI-1 is required for intestinal invasion[2]. The Type III Secretion System (TTSS) is the mechanism by which both ST and SC enter non-phagocytic cells. The TTSS is encoded by SPI-1[2]. SPI-2 also contributes to the virulence of *Salmonella* in that SPI-2 is responsible for the prevention of the phagosomal-lysosomal fusion[2], and therefore survival in phagocytic cells.

Tissue entry. Intestinal invasion is thought to be a required virulence factor for all *Salmonella* serovars[11]. According to Van Diemen et al.[13], *Salmonella* invades and crosses the intestinal mucosa and can then become systemic and invade various other tissues. To be able to penetrate the gut epithelium, bacteria must be able to survive through the environment of the stomach and the gut lumen, including exposure to acid and proteolytic enzymes, and also to evade antigen presenting cells and other lymphocytes[14]. Within 10 min of exposure both ST and SC, these bacteria will adhere to the brush border of enterocytes and invade epithelial cells in porcine ileal and jejunal loops[12]. In 1 to 2 h after exposure both ST and SC can be detected in epithelial cells above the lamina propria[12].

Watson et al.[15] concluded that serovar host specificity is not dependent on translocation and intestinal colonization. ST has been found in the tonsils[8, 16, 17], esophagus[16], jejunum[16, 17], colon[8, 16, 17], cecum[8, 16], rectum[16, 17], and both mandibular[8] and mesenteric lymph nodes[16, 17] of infected pigs but were not found in or around M cells covering the Peyer's patches in the ileum[16]. ST was only found crossing the mucosal barrier in absorptive enterocytes within phagocytic vacuoles or between two adjacent absorptive enterocytes[16]. ST does not often invade the enteric mucosa and does not have a preference of location when it does invade[1]. Being the more invasive serovar, SC, preferentially invades in the colon and on the luminal surface of ileal M cells of Peyer's patches[16].

SC is more invasive than ST demonstrating the ability to infect via the pharyngeal tonsil[1]. Some studies indicate that the tonsil might be the primary site of invasion for SC in pigs[9]. SC has been found in the ileum, colon, cecum, jejunum, duodenum, and rectum, with the ileum and the colon being the sites where SC is most frequently found[16]. SC has been recovered from M cells covering Peyer's patches of the ileum and from within macrophages of the intestinal mucosa and related lymph nodes[16]. SC has a predilection for the enteric mucosa of the ileum and colon[16].

Antibiotic Resistance. Commensal bacteria do not cause an acute inflammatory immune reaction but do have important roles with respect to nutrition, immunology, and pathophysiology. This phenomenon is not completely understood[14] but the use of antibiotics can disrupt this balance and can also contribute to increasing antibiotic resistance. Antibiotic induced alterations in the intestinal flora may play a role in the virulence of *Salmonella*, for example, reducing the number of bacteria required for disease or making it easier for *Salmonella* to replicate[1]. Bacterial plasmids can contribute a variety of properties to the bacterial host and, some of these properties can include virulence properties, including antibiotic resistance[18]. Helmuth et al.[18] demonstrated that, among different serotypes of *Salmonella,* the highly virulent strains all contain serotype-specific plasmids and that avirulent strains are plasmid free. Furthermore, they showed that both SC and ST carry serotype-specific plasmids.

Contamination at Lairage. An emerging area of focus on transmission of *Salmonella* is contamination of pigs in holding pens (lairage) at slaughter facilities. This mode of contamination is important mainly from a pork safety perspective and less so from a swine health perspective. There is now clear evidence that transport and holding of pigs prior to slaughter increases the incidence of isolation of *Salmonella* from pigs[19, 20]. The holding facilities in pork

slaughter plants, including the drinking water, appear to be significant potential sources for pre-slaughter contamination[21]. Moreover, cross-carcass contamination appears to occur even within the abattoir with carcasses being contaminated by *Salmonella* from previously slaughtered pigs[22].

Despite the evidence of cross contamination of pigs prior to and at slaughter, little information is available to suggest clear differences between ST and SC in the likelihood of transmission at lairage and slaughter. In one study of pre-slaughter contamination, SC (biotype Kunzendorf) was not among the 12 most frequently isolated servers from pigs, trailers, and pens, while ST accounted for nearly 19 % of the isolates from these locations[21]. However, it is generally recognized that SC presents greater culturing challenges than does ST[1] . This difficulty may contribute to the failure to isolate the serovar from transport trailers and abattoir holding pens in studies in which all samples are processed and enriched under similar conditions.

PATHOPHYSIOLOGY OF SWINE *SALMONELLA* SEROVARS

In an excellent review, Johnson[23] summarized the concept of sickness behavior as an adaptive response designed to facilitate host recovery to infection. A prominent theme of the review was that two centrally regulated processes contribute positively to host defense and recovery, namely the febrile response and pathogen driven innapetence. In addition, the review further advanced the concept that central, rather than peripheral inflammatory mechanisms play a major role in driving fever and reduced motivation to eat. This concept is important in that it is compatible with the paradoxical finding that ST failed to increase circulating tumor necrosis factor-α (TNFα) in pigs, while producing unmistakable febrile and anorectic responses in swine[24].

The level of disease that is caused by *Salmonella* depends on a number of factors. These factors include the route of infection, the serotype, dose, and any acquired immunity the pig

might have. The minimum dose of any serovar required to establish disease has not been defined with certainty[1]. Although experimental disease has been produced with challenges between 10^8 to 10^{11} CFU it should be recognized that experimental situations do not accurately represent natural field situtions[1]. Watson et al.[15] pointed out that naturally, SC usually only infects pigs, but experimentally SC can infect pigs, cattle, mice, rats, guinea pigs, and rabbits. Gray et al.[9] observed that for a natural infection by SC, the minimum dose that is required to elicit disease is lower than that of an experimental infection.

The swine adapted serovar SC, like most other host-adapted serovars, usually results in septicemia[2] but can also result in enterocolitis, pneumonia and hepatitis[1]. The second most frequent serovar that causes disease in swine is ST and usually results in enterocolitis[1]. Other serovars can cause disease in swine but are rare and are usually associated with either a suppressed or naïve immune system[1]. It is also important to note that since infection does not equal disease, many serovars that do not cause disease in swine can still serve as sources of pork contamination[1].

Outbreaks of Salmonellosis usually occur in intensively reared weaned pigs. This is due to the absence of an established normal flora in the gut of young pigs. Disease is not common in adult pigs. This is most likely due to the presence of an established normal flora although, *Salmonella* infections can still occur in mature pigs[1]. While the fecal-oral route of infection is the most likely route of infection to occur, there are other possibilities. Nose-nose transmission and aerosol transmission may also be possible with *Salmonella*[1]. Disease can also be transferred by handlers[1]. Some epidemiologists suggest that crowding and contact with infected feces can increases infection rates[9].

Pigs infected with SC usually do not show any clinical signs until 36 to 48 h after exposure[9]. These pigs usually are inappetent, lethargic, and febrile. They can also sometimes have a shallow, moist cough with sight expiratory dyspnea[1]. In extreme cases, cyanosis of the extremities may occur[1]. Diarrhea is usually not seen until the third or four day of infection. The fatality rate associated with SC is high but the morbidity rate is variable and usually less than 10%[1]. Disease is most often spread by ingestion of contaminated feces or nasopharyngeal secretions[1].

Fever. Fever is one of the common symptoms of *Salmonella* infection in the pig and is one of the two regulated systems that Johnson[23] proposes that contribute to host defense and recovery. A febrile response is very commonly observed following experimentally infection of pigs. Pigs challenged with ST develop fever[8, 24-27] as early as 12 h post infection[24]. The fever usually persists for 3 to 6 d[8, 24-27] following exposure to ST with peaks between d 1[8] and 2[24]. Watson et al.[15] observed a rapid but short-lived elevation of rectal temperatures in ST challenged pigs and a more prolonged elevation of rectal temperature in SC challenged pigs.

In a study evaluating natural resistance of pigs to *Salmonella,* Van Diemen et al.[13] observed a febrile response in pigs challenged gastrically with 8 x 10^8 CFU of SC. The febrile response in these pigs began 12 h after exposure and continued through d 7 post challenge, when the pigs were sacrificed. In a study reported by Gray et al.[9], pigs were challenged intra-nasally with 10^8 CFU SC and then commingled with a naïve group of pigs 1d following the initial challenge. The intra-nasally challenged pigs were considered the experimentally infected pigs, and the commingled pigs were considered naturally infected. A febrile response was observed in both groups 2 d post infection. In that study, the experimentally infected pigs peaked at 41.2 ºC 4 d post infection. These pigs maintained a fever until 11 d post infection. Where as the

naturally infected pigs peaked at 41.4 ºC 5 d post infection and their fever was maintained until 8 d post infection.

In a series of published reports from our group at Kansas State University using the same ST isolate across multiple studies[24-27], fever was consistently generated when 10^9 to 10^{10} CFU was orally administered. In general rectal temperatures exceeding 40ºC required at least 24 h to develop, but resolved with 4 to 5 d post exposure.

Feed Intake and Growth. A reduction in feed intake or pathogen driven inappetence is the other processes that Johnson[23] suggested contributed to host defense and recovery. Decreases in both body weight gain and feed intake in growing pigs in response to *Salmonella* challenges have been documented. Pigs challenged with ST usually have a transient reduction in feed intake and weight gain, but the ST challenge usually has little impact on future growth[25]. Van Diemen et al.[13]observed a decrease in weight gain 4 to 5 d post challenge, but they then returned to previous weight gain following that. Wood et al.[8] observed that pigs challenged with 1.4×10^{10} ST had a diminished appetite within 2 d post exposure. Experiments done completed by our group have also observed decreases in average daily gain, feed intake, and gain to feed ratios in pigs challenged with both ST and SC[24-28].

Insulin-like growth factor-I (IGF-I) is an anabolic growth factor. A decrease in serum IGF-I has been associated with acute parasitic infections[29]. This too has also been shown in pigs infected with ST[24-27] and has been used as an ancillary marker of clinical severity. Circulating IGF-I is also sensitive to acute changes in feed intake[27] and in most cases, changes in IGF-I following ST have been attributed to changes in intake associated with the enteric disease. For example, Balaji et al.[24] observed a decrease in serum IGF-I following challenge with ST without

an accompanying change in growth hormone and the authors suggested the inappetence was most likely the explanation for effects on IGF-I.

Peripheral Immune Markers of Inflammation. Watson et al.[15] challenged porcine alveolar macrophages in culture with both SC and ST and measured the amount of interlukin-1β (IL-1β) and TNFα released from the macrophages. Infection of these macrophages induced the release of both IL-1β and TNFα, with the magnitude of the release being similar between the different serovars. These observations suggested that, as a phagocytic cell, the alveolar macrophage, didn't distinguish between the serovars as measured by inflammatory cytokine production.

The release of pro-inflammatory cytokines is usually the result of the immune system recognizing the presence of an infectious pathogen[23]. Balaji et al.[24] observed a lack of elevation of peripheral TNFα following challenge with ST in spite of obvious signs of enteric disease. The lack of elevated systemic cytokines in these pigs might be an indicator that the gastrointestinal mucosal immune system of the pig can contain ST locally. This observation was confirmed subsequently in independent studies with ST in which neither systemic IL-6[27], IL-1β[28], nor TNFα[28] were elevated in pigs following oral exposure. A very recent report confirmed too that oral SC, sufficient to slow growth and stimulate a prolonged febrile response in growing pigs, did so in the absence of elevated systemic IL-1β or TNFα[28]. According to Johnson[23] inflammatory cytokines are responsible for causing fever and reducing an animal's motivation for food but, it was noted that reduced motivation to obtain food (and the accompanying slowing of growth) is likely stimulated by central (local) rather than systemic elaboration of inflammatory cytokines[23]. This observation is generally consistent with the failure of ST[24, 27] or SC[28] induced enteric disease to be accompanied by elevated systemic inflammatory cytokines.

CONCLUSION

In the past there has been considerable conflict over the nomenclature used for the genus *Salmonella*, and it is likely that the nomenclature may further evolve as the molecular basis of pathogenesis is further clarified. Standards need to be established to avoid any future conflicts or confusion. The two most common swine serovars, ST and SC, produce contrasting disease outcomes in pigs. ST produces enterocolitis with a fever usually lasting about 3-6 d and SC produces septicemia with generally longer lasting fever. *Salmonella* infections in pigs also cause a reduction in feed intake, and in turn a reduction in body weight gain. The reduction in feed intake is usually only transient in ST infected pigs. A reduction in IGF-I is also seen in both ST and SC infected pigs, most likely the result of the reduction in feed intake. In vitro studies have shown that ST and SC activate alveolar macrophages and stimulate similar changes in inflammatory cytokines. In vivo, pigs challenged with either ST or SC fail to exhibit a peripheral cytokine response, suggesting that the cytokine response maybe a local response only. Additional studies are needed to further define contrasting response to SC and ST in swine tissues and cells, and to provide additional insight into their pathogenesis of these serovars.

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Chapter II

Effects of Feeding *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis to Weaned Pigs on Growth Performance and Circulating Insulin-like Growth Factor-I, Tumor Necrosis Factor Alpha, and Interleukin-1 Beta

ABSTRACT: The most common Salmonella serovars causing clinical disease in pigs are *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC). Given that the swine host-adapted serovar SC was reported to cause systemic disease, a very different disease outcome than that of ST, our working hypothesis was that this serovar would likely engage systemic immune-inflammatory mechanisms that would result in elevated systemic cytokine secretion. Forty-eight weaned pigs were blocked by BW and sex, and randomly allotted to one of three treatments in a 14-d study. Each treatment had eight replicates (pens) with two pigs/pen. Treatments consisted of a negative control and pigs repeatedly fed either 10^8 CFU ST or SC. On d 0, pigs were fed SC or ST in dough balls, and bacteria were re-fed twice weekly throughout the experiment. Control pigs received dough balls without bacteria. All pigs were housed in temperature-controlled rooms under constant lighting and were fed a standard corn-soybean meal-based nursery diet. Pig BW and feed disappearance were used to determine ADG, ADFI, and G:F. Rectal temperatures were obtained daily from one pig/pen starting 2 d before the first bacterial feeding through d 7 using rapid-response digital thermometers. Serum was collected from a single pig/pen for analysis of IGF-I, tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) on d 0, 7, and 14. There was no change in the rectal temperature of both the control and the ST challenged pigs (when compared to day 0) or when comparing ST challenged pigs to controls. In contrast, pigs fed SC had increased rectal temperature beginning on d 2 and continuing though d 7 ($P < 0.05$) with the greatest elevation on d 3 ($P < 0.001$) when compared to controls. ADG and ADFI of pigs challenged with ST did not differ from those of the control pigs. Pigs fed SC had about a 25 % reduction in ADG ($P < 0.0001$) and ADFI ($P < 0.002$) when compared to control pigs. On d 7, pigs fed SC had reduced IGF-I when compared to both control ($P < 0.01$) and ST pigs ($P = 0.01$). Bacterial feeding did not affect serum TNFα and IL-1β

compared to control pigs at any time throughout the experiment. We conclude that repeated exposure of weaned pigs to SC eroded growth performance in the absence of changes in systemic inflammatory cytokines.

Key Words: IGF-I, IL-1β, Salmonella enterica, Swine, TNFα,

INTRODUCTION

Enteric disease erodes growth performance in pigs, and salmonellae organisms are important swine enteric pathogens that are implicated in reduced growth performance of pigs. The most common salmonellae serovars causing clinical disease in pigs are *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC) (Fedorka-Cray et al., 2000). These salmonellae serovars produce very different patterns of disease in growing pigs. Pigs infected with ST are more likely to develop mild enteritis and self-limiting diarrhea, whereas pigs infected with SC, a so-called swine host adapted serovar, usually develop systemic disease such as septicemia (Schwartz, 1999). Although oral exposure of weaned pigs to ST caused fever and growth suppression (Balaji et al., 2000), ST did not stimulate changes in systemic concentrations of the inflammatory cytokines TNFα (Balaji et al., 2000) or IL-6 (Burkey et al., 2004). Based upon these results, we concluded that ST was largely contained by the mucosal immune system without provoking systemic inflammatory cytokine secretion. This is in contrast to swine lipopolysaccharide (LPS) models of bacterial infection which generally result in major elevations in inflammatory cytokines (summarized in Johnson et al., 2005). Given that the swine host-adapted serovar SC was reported to cause systemic disease, a very different disease outcome than that of ST, our working hypothesis was that this serovar would likely engage systemic immune-inflammatory mechanisms that would result in elevated systemic cytokine secretion.

MATERIALS AND METHODS

Experimental Design. The experimental protocol used in this study was approved by the Kansas State University Institutional Animal Care and Use Committee. A total of 48 weaned pigs were blocked by BW and sex, and randomly allotted to one of three treatments in a 14-d study. Each treatment had eight replicates (pens) with two pigs/pen. Treatments consisted of a

negative control and pigs repeatedly fed either *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC). On d 0 pigs were fed 10^8 CFU SC or ST in dough balls, and bacteria were re-fed twice weekly throughout the experiment. The control pigs received dough balls without bacteria. Because the dough contained uncooked eggs, the dough itself was cultured for the presence of salmonellae bacteria by standard microbiological techniques (detailed below) and found to be free of culturable organisms.

Bacteria were cultured for feeding as needed, using ST and SC that had been transformed with green and red fluorescent protein, respectively, as described previously from our laboratory (Burkey et al., 2006; Skjolaas et al., 2006). Importantly, these transformed bacteria were confirmed to retain their inflammatory signaling in swine gastrointestinal epithelial cells and to affect the relative expression of toll-like receptors and selected chemoattractive cytokines and chemokines (Burkey et al., 2006; Skjolaas et al., 2006). The fluorescence and kanamycin resistance conferred by the transformed plasmids provided two phenotypic markers from which to isolate and distinguish the serovars from potential environmental salmonellae. On days of bacterial feeding, bacteria were washed and diluted in PBS to deliver 10^8 CFU/100 μL. A small disposable pipet tip was used to make a depression in the dough ball, and the 100 μL containing the desired bacteria count was pipetted into the depression. The depression was then pinched closed.

Bacteria for the initial two bacterial feedings were from colonies grown in the laboratory following transformation. Subsequently, bacteria were obtained following passage through the pigs. For this, fecal samples were collected and pooled among all pens within day and treatment. Fecal samples were obtained and cultured on d 1 to 8, and again on d 11. Then, all pigs within a treatment were subsequently fed bacteria from a fecally isolated colony.

Fecal samples were pre-enriched in tetrathionate broth (Sigma, St. Louis, MO; Cat. no. T-1938) for 24 h at 37°C. Selective enrichment was performed by transferring 1% of the tetrathionate broth/fecal culture to Rappaport's medium (BD Biosciences, Sparks, MD; Cat. no. 218581) for an additional 24 h at 37°C. Selective agar plating was then performed by streaking 100 μL of the Rappaport's medium onto Luria-Bertani plates containing 50 μg/mL of kanamycin followed by overnight incubation at 37°C. Bacteria were isolated and deemed the appropriate salmonellae serova, based upon growth in the presence of kanamycin, as well as by either green (ST) or red (SC) fluorescence.

This level of bacterial feeding was arrived at empirically from our previous experience with this same ST isolate (Balaji et al., 2000; Burkey et al., 2004; Jenkins et al., 2004; Turner et al., 2002b; Turner et al., 2002a) and from published work that suggested this level of oral exposure would be expected to produce only mild clinical effects (Schwartz, 1999). We had not used this isolate of SC in our previous research, but like the ST isolate, SC was derived from a swine clinical case and was a gift from Dr. Jerome Nietfeld (Department of Diagnostic Medicine/Pathobiology, Kansas State University). The identity of both the wildtype and transformed isolates was confirmed by the National Veterinary Services Laboratory (Ames, IA).

All pigs were housed in temperature-controlled rooms under constant lighting. Each pen contained a single nipple waterer and a single self-feeder to facilitate ad libitum access to water and feed. Pigs were fed a standard corn-soybean meal-based nursery diet formulated to exceed NRC requirements for growth. To ensure that the diet itself was not antimicrobial, it was formulated to be free of growth promoting antibiotics, zinc oxide or copper sulfate. To ensure that pigs began the study free of clinical salmonellosis, fecal samples were cultured before beginning the study, and confirmed to be negative for salmonellae organisms.

Pig BW and feed disappearance were recorded initially and at the conclusion of the study to determine ADG, ADFI, and G:F. Rectal temperatures were obtained daily from one pig/pen starting 2 d before the first bacterial feeding through d 7 using rapid-response digital electric thermometers. Blood sampling (detailed below) and rectal temperature measures were consistently obtained early in the morning, generally between 0500 and 0700 and were obtained from the same pig within a pen across days.

Serum Analysis. Serum was collected from a single pig/pen for analysis of tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) on d 0, 7, and 14. Blood was collected into glass tubes containing no anticoagulant, and was allowed to clot at room temperature and stored overnight at 4ºC before harvest of serum by centrifugation. An immunoradiometric assay, described previously for use in pigs (Balaji et al., 2000) was utilized to analyze serum IGF-I concentrations. A swine specific ELISA was used for determination of TNFα (R&D Systems, Minneapolis, MN; Quantikine Porcine TNF-α/TNFSF2 Immunoassay; Catalog number PTA00). A swine specific ELISA was also used for determination of IL-1β (R&D Systems, Minneapolis, MN; Quantikine Porcine IL-1β Immunoassay; Catalog number PLB00).

Statistical Analyses. Data were analyzed by the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) as a randomized complete block design with repeated measures over time on each experimental unit (individual pens). The model included terms for the fixed effects of disease challenge, time, and the interactions, and block and pen were considered random effects. Comparisons between bacterial challenges and/or sampling times were made only when a significant ($P < 0.05$) *F*-test for the main effect or interaction was found using the least significant difference procedure. All means reported are least square means.

RESULTS

In the course of collecting fecal samples to isolate bacteria for subsequent re-feeding, we confirmed that feces from pens containing control pigs were always negative for culturable ST and SC. Moreover, SC was never isolated from pens of pigs fed ST, or vice-versa. Thus, our qualitative assessment was that cross-contamination of pens did not occur through the 14 d study.

Rectal temperatures were monitored daily beginning 2 d prior to challenge with bacteria and until 7 d following the first bacterial feeding (Figure 1). There was no change in rectal temperature of either the control or the ST challenged pigs (when compared to day 0) and no change in the ST challenged pigs when compared to the controls. In contrast, pigs fed SC had increased rectal temperature beginning on d 2 and continuing though d 7 ($P < 0.05$) with the greatest elevation on d 3 ($P < 0.001$) when compared to controls.

Average daily gain, ADFI, and G:F were monitored through the 14 d experiment by weighing pigs and feeders (Figure 2). The ADG and feed intake of pigs challenged with ST did not differ from those of the control pigs. Pigs fed SC had about a 25 % reduction in ADG ($P < 0.0001$) and ADFI ($P < 0.002$) when compared to control pigs. There were no differences in G:F among the treatment groups.

Concentrations of insulin like growth factor-I (IGF-I) in serum were measured on d 0, 7, and 14 (Figure 3). Concentration of IGF-I was similar between the treatment groups on d 0 and d 14. On d 7, pigs fed SC had reduced IGF-I when compared to both control ($P < 0.01$) and ST pigs ($P = 0.01$).

Serum TNFα and IL-1β concentrations are depicted in Figure 4. Bacterial feeding did not affect either cytokine compared to control pigs.

DISCUSSION

Our laboratory has used the same isolate of ST used in the current experiment in numerous other studies that targeted a variety of experimental objectives (Balaji et al., 2000; Burkey et al., 2004; Jenkins et al., 2004; Turner et al., 2002b; Turner et al., 2002a). In those experiments, we used a single oral dose of approximately 10^9 to 10^{10} bacteria. In general, in those experiments, we utilized rectal temperature, feed intake and circulating IGF-I as indicators of the clinical effects of the enteric pathogen. The current experiment differed in two important ways. First, the current experiment included the so-called swine host adapted serovar SC (discussed in more detail below). Secondly, here, we elected to use a lower level of bacterial exposure, but provide it repeatedly in order to more closely mimic the oral-fecal transmission that likely occurs within nursery pens under production conditions. Because we had never evaluated the low dose, repeated exposure to ST in nursery pigs, nor had we previously used the SC serovar in this disease model, we considered the rectal temperature, feed intake and IGF-I data to be vital to the thorough characterization of this experimental model.

Within 2 d of SC exposure, rectal temperature was elevated in pigs fed this serovar. It peaked at d 3, and remained elevated above control pigs through d 7. We elected a priori not to continue daily rectal temperature measurements through the entire study to reduce undue stress of continued animal handling and temperature measurement. So, we are not certain if pigs fed SC remained somewhat febrile through the conclusion of the study. We were somewhat surprised however that ST failed to elevate rectal temperature above controls. On the other hand, the lack of a rectal temperature response to ST may be related to the lower numbers of bacteria provided in the current experiment. We favor this interpretation, because even though ST stimulated a strong febrile response to a single oral dose of 10^9 to 10^{10} ST (Balaji et al., 2000;

Burkey et al., 2004; Jenkins et al., 2004; Turner et al., 2002b; Turner et al., 2002a) the response has varied among studies with pigs given 10^{10} CFU ST and only resulted in a single day of elevated rectal temperature in one study (Jenkins et al., 2004). Moreover, 10^{8} oral salmonellae are reported to be at the low end of bacteria producing clinical signs (Schwartz, 1999). However, it is clear from the current experiment that the response to small oral doses of salmonellae organisms is very much serovar dependent, making it difficult to predict a minimum oral exposure to produce clinical symptoms across all important swine serovars.

Oral exposure to SC resulted in approximately 25% reduction in growth that likely is explained by a reduction in feed intake of similar magnitude. But, oral exposure to an identical oral dose of ST did not result in erosion of growth performance. Although the reduction in growth in response to SC is generally consistent with other published reports of young growing pigs carrying SC (Gray et al., 1995), it differs from our previous findings with a single exposure to ST. In our other studies (Balaji et al., 2000; Burkey et al., 2004; Jenkins et al., 2004; Turner et al., 2002b; Turner et al., 2002a) ST challenge resulted in reduced feed intake. In the current study the ST challenged pigs did not have a significant reduction in feed intake compared to the controls. Again, we suspect the lack of effect of ST on intake likely reflects the reduced oral dose compared to our previous studies, and yet this observation may have more far reaching implications. Namely, the current dogmatic view is that pigs respond to low level antibiotic feeding with improved growth performance because doing so controls pathogens in the gastrointestinal tract (Dritz et al., 2002). Our data here with ST, compared to control pigs, suggest that the mere presence of invasive enteric pathogens is not in itself sufficient to slow growth and that it appears to be a dose-dependent effect.

The association between level of intake and circulating IGF-I is unmistakably coupled in young pigs and circulating IGF-I declines rapidly following feed deprivation (Salfen et al., 2003). We have previously evaluated circulating IGF-I as an ancillary marker of the inappetence associated with carriage of an enteric pathogen (Johnson et al., 2005). Indeed pigs fed SC in the current study demonstrated the expected reduction in circulating IGF-I and this reduction was likely the result of SC-reduced feed intake. However, IGF-I did not remain reduced in pigs fed SC in that the growth factor was similar among all treatments by the conclusion of the study. The return of IGF-I in pigs fed SC compared to that of controls and pigs fed ST by the conclusion of the study suggests that the majority of the reduction in intake (Figure 2) probably occurred within the first week of SC feeding.

Models of immune/inflammatory challenge based upon injection of pigs with LPS, without exception, demonstrated unmistakable elevations in the inflammatory cytokine trio TNFα, IL-1β, and IL-6 following LPS treatment. These models of LPS generally helped to shape the dogmatic view that circulating inflammatory cytokines associated with systemic inflammatory processes participated in slowed growth in sick animals (Fossum, 1998; Fossum et al., 1998; Johnson, 1997; Spurlock, 1997). However, in our previous studies with ST, the bacteria failed to affect circulating TNFα (Balaji et al., 2000) or IL-6 (Burkey et al., 2004). Therefore, that neither TNFα nor IL-1β was affected by ST in the current study is generally consistent with those reports. In this regard, it is important to again point out that, although the dose of ST was lower than we've used previously, the transformed bacteria produced unmistakable changes in tissue expression of toll-like receptors,IL-8, macrophage migration inhibitory factor, osteopontin (Burkey et al., 2006) and CC chemokine ligand 20 (Skjolaas et al., 2006). Nevertheless, this is our first evaluation of IL-1β in response to ST. Here, we evaluated

peripheral cytokines after 7 and 14 d following first exposure to the bacteria. Moreover, in the current study, we re-exposed pigs throughout the study. We cannot rule out the possibility that both TNFα and IL-1β may have been affected prior to d 7, although we feel this is not likely. Of greater interest however, relative to a primary objective of the current study, is that, although SC reduced growth and produced fever, these effects occurred in the absence of changes in TNFα and IL-1β. So, despite the documented likelihood of SC to produce systemic disease in pigs, we failed to gather data to support our working hypothesis that SC, in contrast to ST, would result in elevated peripheral inflammatory cytokines.

In conclusion, we report a model of re-feeding transformed ST and SC that may mimic fecal-oral exposure in production settings and perhaps offer advantages over that of our previously used model of a single intragastic inoculation of bacteria. Although SC reduced growth by approximately 25 % and transiently decreased IGF-I, neither swine salmonellae serovar produced changes in systemic TNFα or IL-1β.

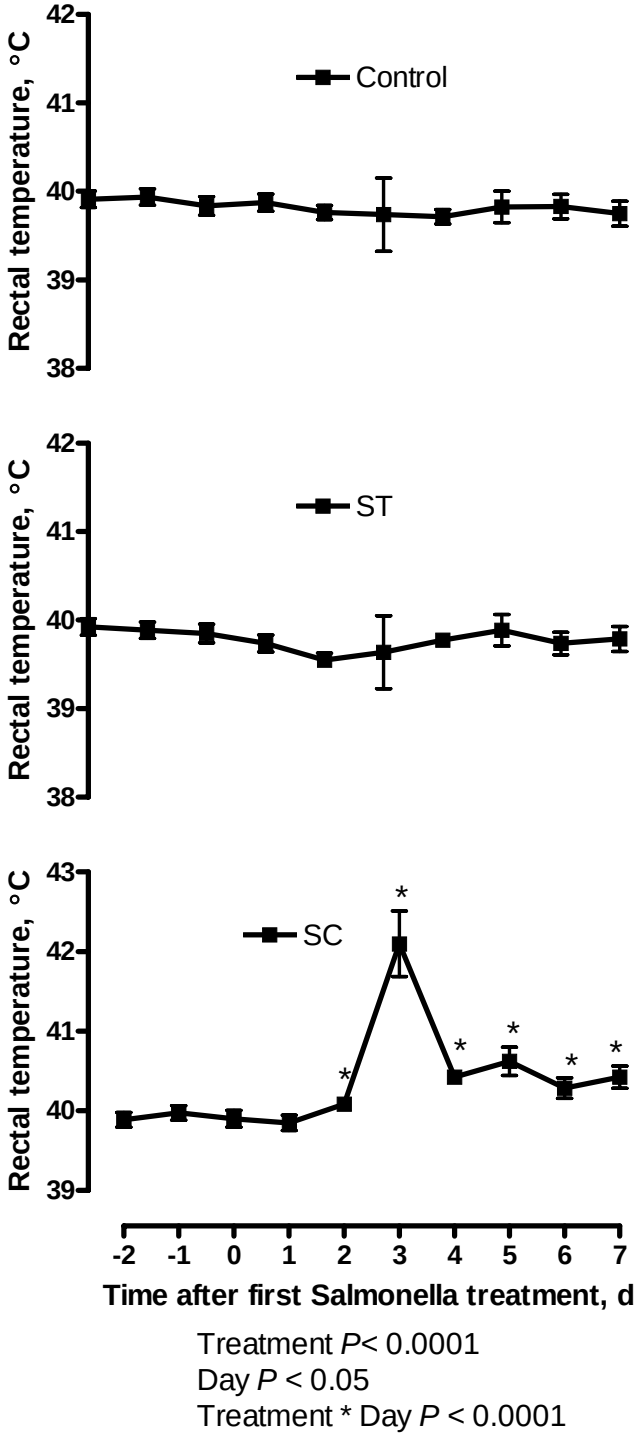

Figure 1. Rectal temperatures of pigs treated orally with *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC) through the initial 7 d of the experiment. Control pigs received uninfected dough balls used to deliver bacteria. Pigs received 10^8 CFU bacteria twice weekly through the 14 d experiment. Asterisks denote days when SC pigs had elevated rectal temperatures compared to ST and control ($P < 0.05$).

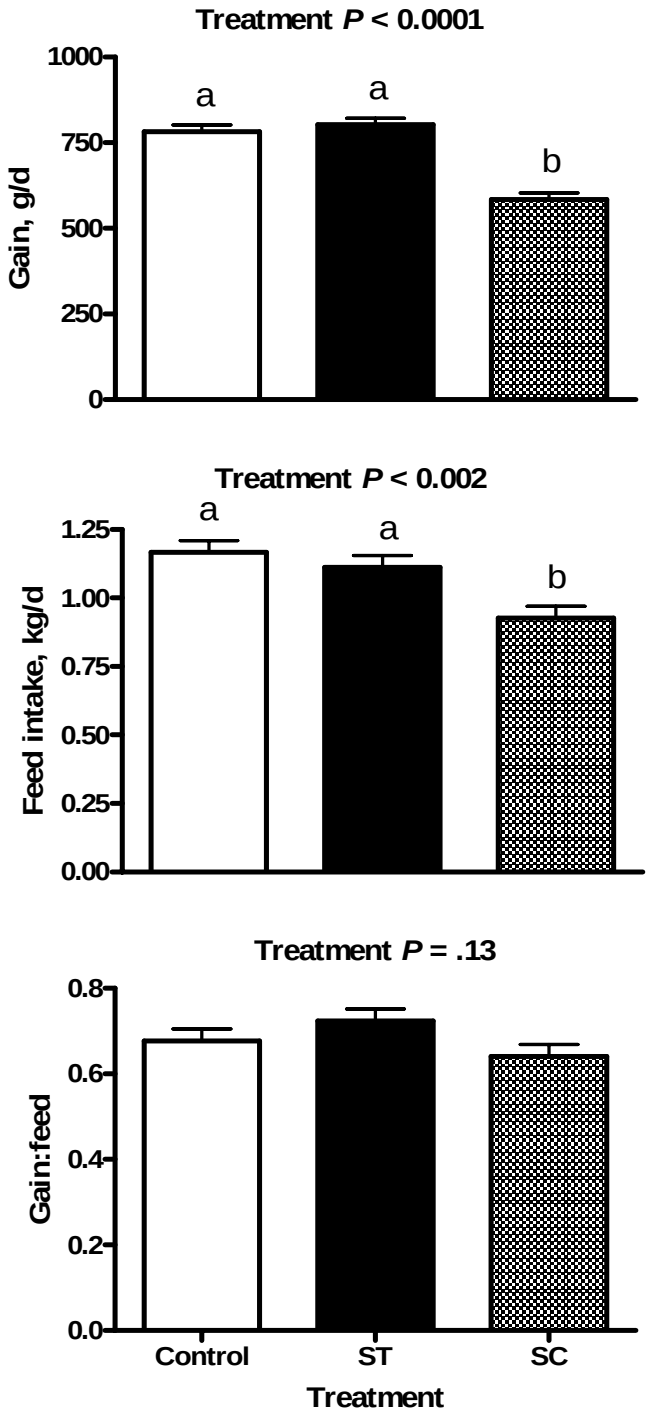

Figure 2. Growth performance of pigs treated orally with *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC). Control pigs received uninfected dough balls used to deliver bacteria. Pigs received 10^8 CFU bacteria twice weekly through the 14 d experiment. Bars without common letters differ ($P < 0.05$).

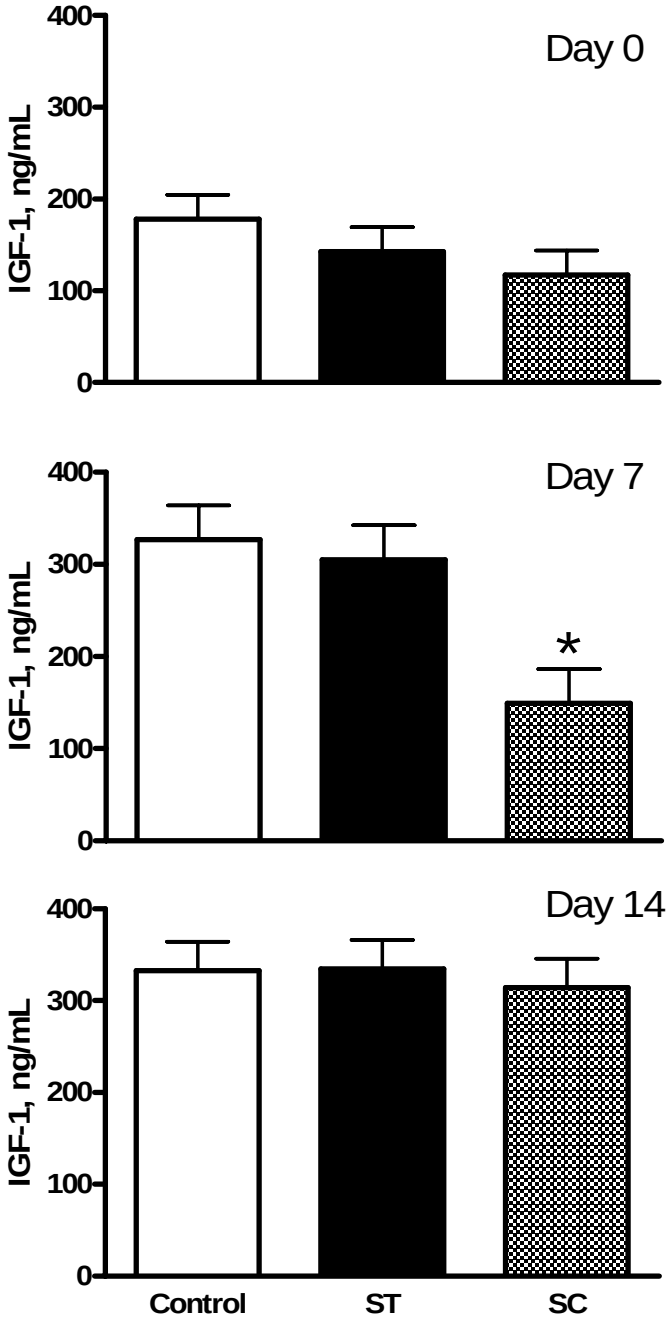

Figure 3. Serum IGF-I concentrations in of pigs treated orally with *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC). Serum was collected on day 0, 7, and 14 following treatment. Control pigs received uninfected dough balls used to deliver bacteria. Pigs received 10^8 CFU bacteria twice weekly through the 14 d experiment. Asterisks denote a significant reduction in IGF-I in SC compared to Control and ST ($P < 0.05$).

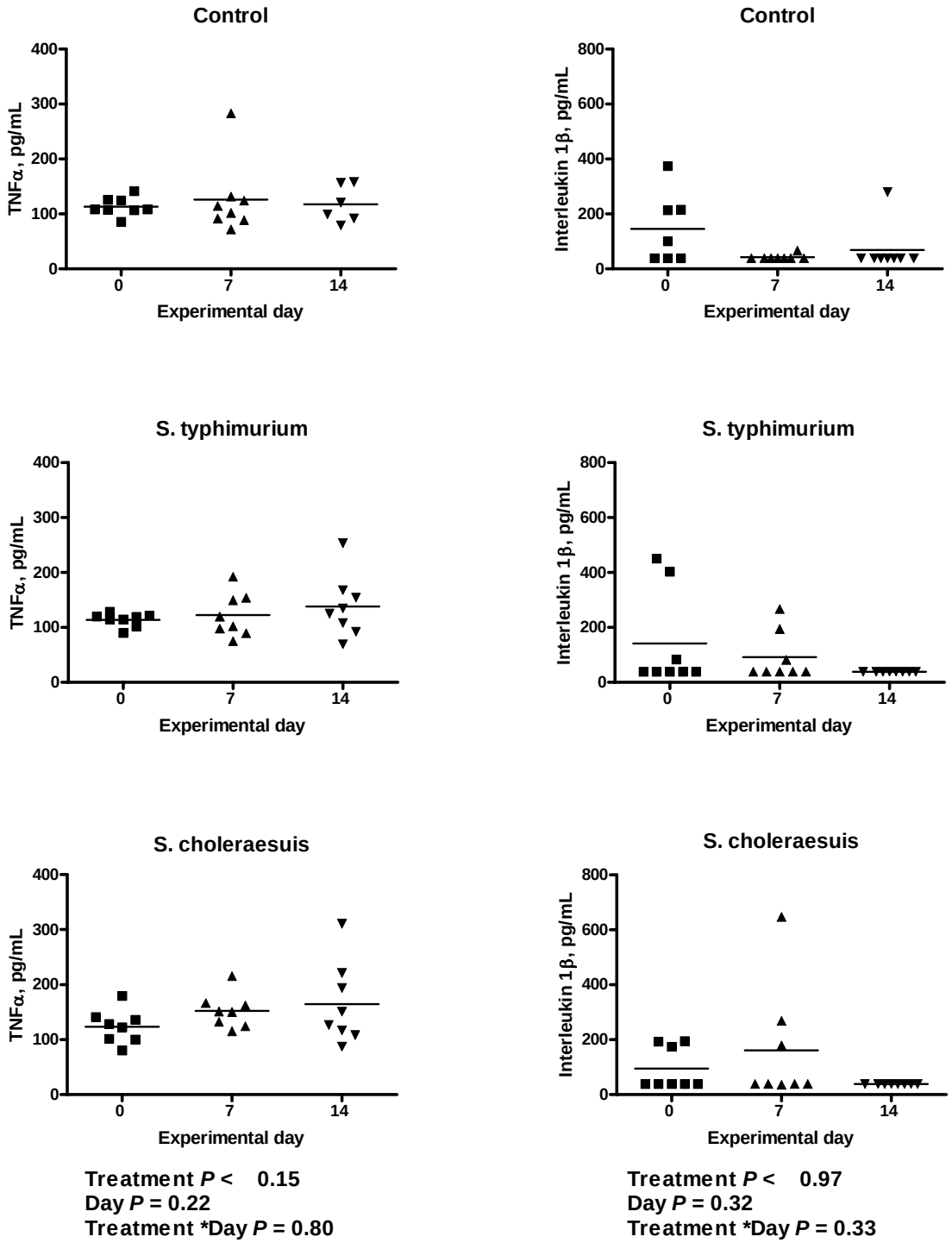

Figure 4. Serum TNF-α and IL-1β levels in pigs treated orally with *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis. Serum was collected on day 0, 7, and 14. Control pigs received uninfected dough balls used to deliver bacteria. Pigs received 10^8 CFU bacteria twice weekly through the 14 d experiment.

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