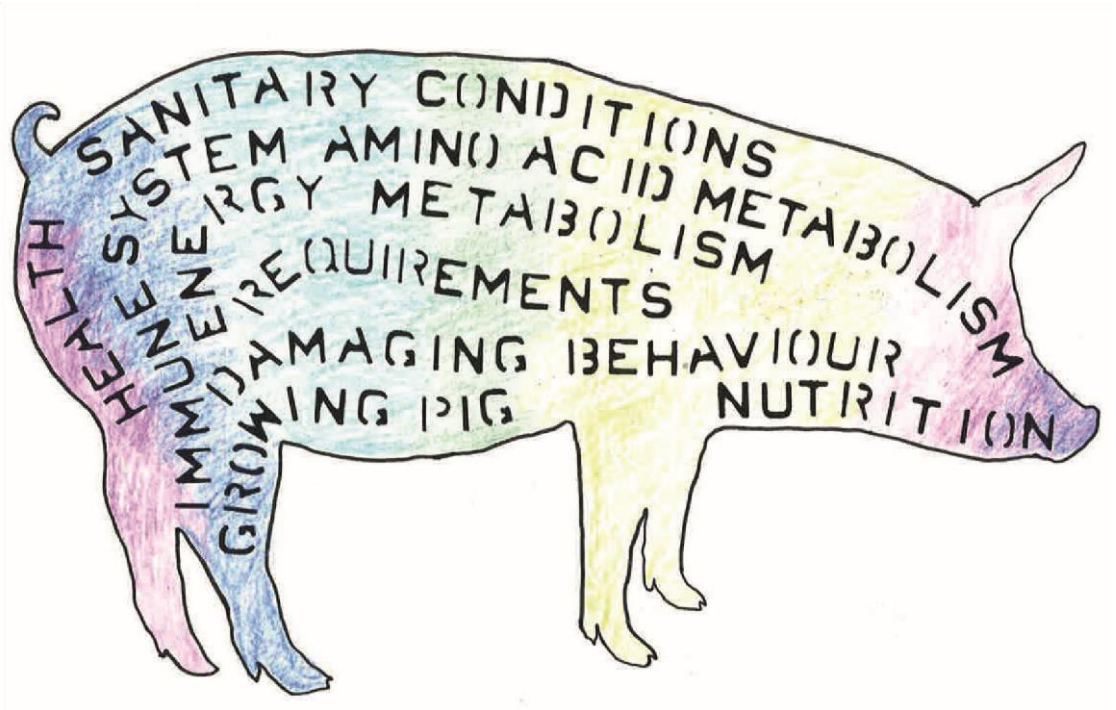
Nutrition of pigs kept under low and high sanitary conditions

Effects on amino acid and energy metabolism and damaging behaviour



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Thesis

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Chapter 1

General introduction

DIETARY REQUIREMENTS OF GROWING PIGS

Feed costs are a large proportion of the total costs for pig production. It is therefore of economic importance to match the dietary nutrient supply to the nutrient requirements of a pig as closely as possible. Recommendations for nutrient requirements of a pig are presented using several nutrient and energy evaluation systems (ARC, 1981; Whittemore et al., 2003; Santioga Rostagno et al., 2011; NRC, 2012; CVB, 2016). Mostly a concept is used that splits requirements for growing pigs in a part for maintenance and production (growth) purposes. The definition of maintenance is the level of feeding at which the requirements for nutrients are just met to ensure the continuity of vital processes so that there is no net gain or loss of nutrients in tissue and animal products (ARC, 1981). Requirements for maintenance and growth are both measured under experimental conditions.

Protein requirements of growing pigs. In practice, the dietary protein level of growing pigs typically decreases during the life of a pig when phase feeding is used. This reduction in dietary protein level follows the efficiency of a pig to use dietary protein for depositing protein tissue in the body. In Figure 1.1 is indicated how efficient the dietary protein is used by the pig to deposit protein during its life. This 3-dimensional representation by van Milgen and Noblet (2003) illustrates that the efficiency of a growing pigs reduces when it becomes more heavy/aged and is also depending on the intake of metabolizable energy (ME).

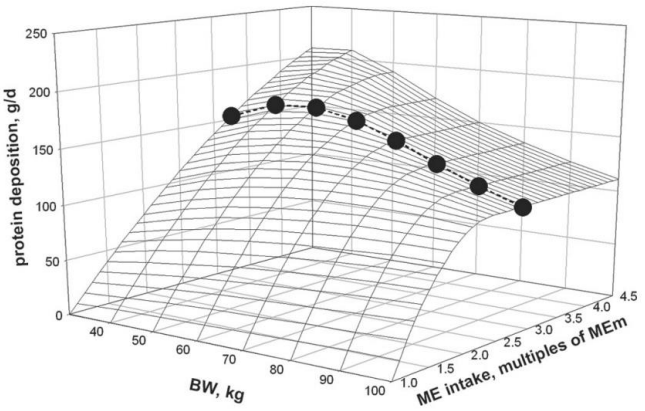

Figure 1.1 The response of protein deposition as a function of ME intake and body weight (**BW**). The connected points indicate the feed intake capacity (ad libitum intake) of a Large white barrow and corresponding protein deposition. Mem = ME intake for maintenance. Source: van Milgen and Noblet (2003).

Requirement values for dietary protein do not exist in the evaluation systems. Instead, recommendations for dietary amino acid (AA) intake are published (NRC, 2012; CVB, 2016). These AA requirements are determined in pig experiments that use a titration set up or are calculated from experiments with a factorial approach. In titration studies typically the response in performance of pigs to a stepwise increasing or decreasing dietary dose of AA is evaluated using different treatment groups or within the same individual pig in time. For studies with a factorial approach nutrient requirements for maintenance and protein deposition in the body can be calculated by using data obtained in pig experiments.

The ileal digestibility of AA is measured in AA requirement studies to evaluate the bio-availability of AA for the pig. There are different expressions for Ileal digestibility, namely apparent, true and standardized ileal digestibility (AID, TID and SID, respectively). The expression used is depending on which proportion of AA outflow is used in the digestibility calculation (Stein et al., 2007). The AID is calculated by subtracting the AA inflow by the total AA outflow including endogenous losses. The endogenous losses can be split into basal (not influenced by feed ingredient composition) and specific (influenced by ingredient composition) endogenous losses. Correcting for total endogenous losses results in the TID and correcting digestibility for basal endogenous losses only, results in the SID (Stein et al., 2007).
The AA and nitrogen (N) requirements for maintenance were described by Moughan (2003) as requirements for turnover of body protein, skin and hair AA losses, basal endogenous intestinal AA losses, synthesis of non-protein N-containing compounds, and urinary AA losses. The first three processes are considered to be quantitatively important for requirements for maintenance. The AA composition of the whole body protein can be used as the basis for an ideal AA profile for protein deposition as was suggested by Whittemore (1983). Several pig growth models have been developed, which simulate the AA uptake, metabolism and nutrient partitioning for growing pigs (Whittemore and Fawcett, 1974; Moughan, 1981; Whittemore, 1983). To estimate the biological maximal rate of body protein retention (Pdmax) net rates of body protein retention are determined under optimal dietary and environmental conditions (Moughan, 2003). Under practical farming conditions, which can be less optimal, this Pdmax is not that commonly reached, due to factors such as subclinical disease, thermal environment, and social conditions (Burrin et al., 2001).

Energy requirements of growing pigs. The energy that is ingested by an animal as feed is deposited as fat or protein, excreted in faeces and urine, or dissipated as heat. The dissipation of heat by a pig might represent more than 50% of the ingested energy (van Milgen and Noblet, 2000), which can be seen as the major part of inefficiency. It is therefore of major importance to better understand and quantify the energy requirements of pigs. Quantification of heat production helps to better understand the daily energy

balance of an animal. The dissipation of heat or the heat production of a pig can be measured by indirect calorimetry. When nutrients are oxidized, O_2 is consumed and CO_2 produced. Heat production can be calculated from the combination of O_2 consumption, CO_2 production (and urinary N and CH_4 production) according to Brouwer (1965). These components can be measured by placing the animals in respiration chambers, were the gas exchanges can be measured and used for calculation of heat production. The total heat production of an animal can be split in heat production due to physical activity, thermic effect of feeding (**TEF**) and fasting heat production.

production (Figure 1.2). The TEF can be split in a short and a long TEF. The short TEF is the heat production associated with a meal, related to nutrient digestion and absorption (van Milgen et al., 1998; van Milgen and Noblet, 2000) and the long TEF is the heat production due to "long-term" metabolic processes, including protein and lipid synthesis (van Milgen et al., 1998; van Milgen and Noblet, 2000). The fasting heat production (**FHP**) also called 'basal' metabolism is defined as the rate of energy expenditure of a non-reproductive, healthy, fasting, and a resting adult in thermo-neutral zone, in an inactive phase of the day (McNab, 1997; Labussière et al., 2008). The FHP is used as a proxy for energy requirement for maintenance. Overall it remains difficult to measure FHP in growing animals.

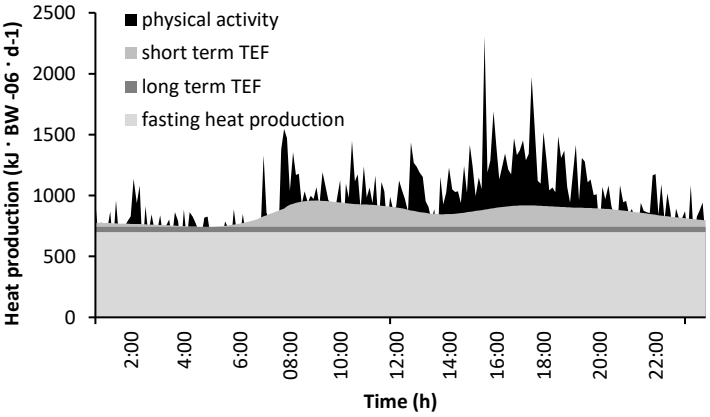

Figure 1.2 Heat production of a group of restricted fed growing pigs partitioned in different components of heat. Feed was offered in two different meals at 7:30 and 15:30. TEF = thermic effect of feeding. Based on own data.

There is already a lot of knowledge about the requirements for AA and energy in growing pigs. This information is based on pig experiments were pigs are kept under experimental conditions for the mentioned measurements, which means a clean environment, low animal density, and high attention of caretakers. These experimental conditions can therefore be considered as optimal conditions for a pig. There is not much known, however, about nutrient requirements of pigs kept in suboptimal conditions. In practice,

the environmental and management conditions are not always optimal. There is a large variation in pig performance on growing-finishing pig farms (Figure 1.3). The range in average daily gain (ADG) of 654 growing-finishing pig farms in the Netherlands was 630 until 1050 g · pig^{-1} · day^{-1}. A part of this variation might be affected by the health status / conditions (Textbox 1.1) of the farm (van der Peet-Schwering and Jansman, 2007; Pastorelli et al., 2012a). It is therefore of major importance to determine the effect of sub-optimal conditions on AA and energy requirements in growing pigs.

IMMUNE SYSTEM STIMULATION AND THE EFFECT ON NUTRIENT REQUIREMENTS

Pigs housed in sub-optimal conditions have an increased frequency of exposure to antigens resulting in a stimulation of their immune system. Immune system stimulation (ISS) in general leads to a series of responses in the body, such as production of cytokines, acute phase proteins (APP), blood cells, fever, increased muscle protein catabolism, alterations in sleep patterns, and reduction in feed intake (Heegaard et al., 2011).

Immune system stimulation by challenge studies. Several challenge studies have been performed to evaluate the effects of clinical immune system stimulation on nutrient metabolism. An overview of several acute challenge studies in pigs in this context is shown in Table 1.1. These acute challenge models are often used to stimulate the immune system or to mimic an infection in a specific part of the body. A model using *E. coli* results for instance in intestinal inflammation, whereas a challenge with Complete Freund's Adjuvant is leading to chronic lung inflammation. As a consequence these ISS models have very different effects in the animal, and the challenge should therefore be chosen carefully based on the objective of the study.

Reduction in feed intake. Reasons for a reduction in feed intake during ISS are not completely understood. Some studies describe an effect of cytokines (produced as a consequence of immune stimulation) on the nervous system, causing a reducing effect on feed intake (Johnson, 1997; Langhans, 2000). Langhans (2000) reported that anorexia and consequent behavioural changes during infections have several beneficial short term effects for the mammal host. Suppression of hunger results in saved energy and reduced heat loss, as the need for searching food is reduced. In addition, lower nutrient intake leads to less nutrients available for the growth of pathogens involved in clinical or subclinical disease. Although reduced feed intake in response to ISS seems beneficial for an animal in the short term, chronic anorexia can delay recovery and inhibits host defence (Langhans, 2000). Sandberg et al. (2006) described that the origin and level of infection affect the rate and duration of the reduced feed intake. The length of the recovery phase after infection

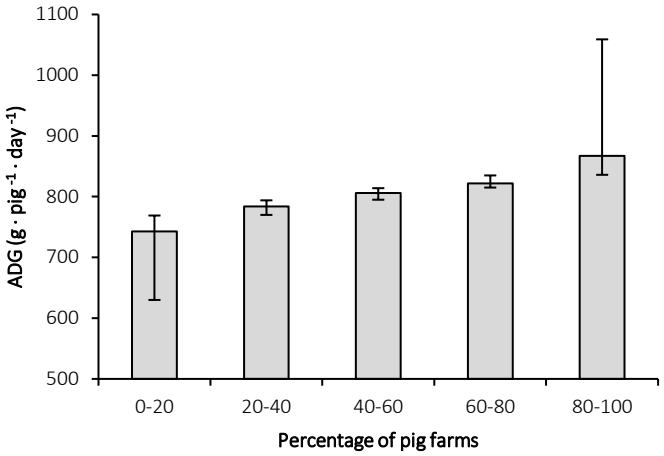

Figure 1.3 The average daily gain (**ADG**) of 654 growing-finishing pigs farms in The Netherlands from July 2015-June 2016, having pigs in a weight range of 25 – 119 kg. Each grey bar represents 20% of the farms and the bars are categorised at ADG level. The line bars represent the minima and maxima for each category. Whit permission of Agrovision (2016).

can be influenced by diet composition (Sandberg et al., 2006). Causal relationships between the composition of the diet and the rate of feed intake recovery after ISS are, however, not clearly described.

Immune stimulation and AA requirements. Studies carried out over the past years in pigs already showed that the requirement of tryptophan (**Trp**) (Melchior et al., 2004; Le Floc'h et al., 2008; Le Floc'h et al., 2012), methionine (**Met**) + cysteine (Rakhshandeh et al., 2010), and threonine (**Thr**) (Li et al., 2007; Ren et al., 2014) were increased in case of ISS by acute challenge models (Table 1.1). Increased muscle protein metabolism and increased production of several immune related metabolites, such as APP (Murata et al., 2004; Heegaard et al., 2011) during ISS, result in a redirection of protein or AA from development and tissue growth to immune related tissues in the body (Le Floc'h et al., 2009), thereby negatively influencing pig performance (Klasing and Johnstone, 1991; Pastorelli et al., 2012a). Besides the costs of AA as substrate for several immune related proteins in the pig, associated costs, such as increased cell turnover for tissue repair and influence on metabolism due to effects of ISS on hormonal regulations, might be even higher in case of ISS. Amino acids can also have a modulating effect on the immune system. Arginine, for example was described in a study of Bruins et al. (2002) to upregulate the immune system, while catabolism of glutamine seems to provide precursors for DNA and RNA synthesis of immune cells (Le Floc'h et al., 2004). These interactions between AA (metabolites) and the immune system are hitherto not fully understood. Although involvement of particular AA in the immune system is proven, many of these studies do not allow a conclusion whether or

not supplying additional quantities of the AA concerned is actually beneficial for the animal. The former hampers practical application of this research.

TEXTBOX 1.1. DEFENITIONS HEALTH STATUS AND SANITARY CONDITIONS

To characterize the health of an animal, two definitions are commonly used. The first one: "The absence of disease determined by clinical examinations combined with various diagnostic tests" (Petersen et al., 2004; CVB, 2016). The second one is more precise and is used by the World Health Organisation: "A state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (ARC, 1981; Santioga Rostagno et al., 2011; WHO, 2017).
Measurement of immune system activity alone is therefore not sufficient to evaluate animal health. Behavioural measurements to judge the animals mental and social well-being (Ullman-Culleré and Charmaine, 1999), should be included as well.

The definition health status is commonly used to characterize a herd of animals. Health status refers to the presence or the absence of infectious pathogens regardless whether animals are clinically healthy or not. Hence, a low health status herd can be clinically healthy and productive. To avoid this apparent contradiction, the term sanitary conditions is used throughout this thesis instead of health status. The term sanitary conditions is chosen to cover a combination of factors affecting health of the pigs. The word sanitary directly links to hygiene level, but can also cover other factors affecting health, such as application of vaccinations, medication, management, housing, and animal density.

Table 1.1. Overview of different acute challenges to stimulate the immune system in pigs in relation with nutrition

Immune challenge	Administration	Effect	Reference
Lipopolysaccharide, LPS	repeated intramuscular injection	increase in acute phase proteins, fever.	Rakhshandeh et al. (2010); de Ridder et al. (2012); Kim et al. (2012); Litvak et al. (2013); Campos et al. (2014).
Complete Freund's adjuvant, CFA, a mineral oil containing killed *M. tuberculosis*	intravenous injection	lung inflammation, pneumonia, increase in acute phase proteins, fever.	Melchior et al. (2004); Le Floc'h et al. (2008); Kampman-van de Hoek et al. (2015).
E. coli	oral administration	diarrhoea, intestinal infection, fever.	Marquardt et al. (2006); McLamb et al. (2013).
Turpetine oil	subcutaneous injection	increase in acute phase proteins, fever.	Carpintero et al.(2005); Heegaard et al. (2011); Kampman-van de Hoek et al. (2013).
A. pleuropneumoniae	voluntary inhalation of aerosol, or intranasal administration	lung inflammation, pneumonia, fever.	Heegaard et al. (1998); Heegaard et al. (2011); Skovgaard et al. (2009).

Immune stimulation and energy requirements. Apart from the effect of ISS on AA metabolism, energy metabolism is also found to be influenced during ISS (Benson et al., 1993; Humphrey and Klasing, 2004). Processes like fever and APP production in response to ISS consume energy (Lochmiller and Deerenberg, 2000; Parmentier et al., 2002). Immune challenge studies often result in reduced ADG for ISS treatment groups compared with control groups, which cannot be completely explained by the related decreased average daily feed intake (ADFI), as reported in a meta-analysis by Pastorelli et al. (2012b). Depending on the nature of the challenge, a substantial portion of the decrease in ADG is caused by changes in the energetic efficiency in these animals (Pastorelli et al., 2012b). A lower energetic efficiency might show that the animal has an increased energy requirement in the case of ISS (Williams et al., 1997; Le Floc'h et al., 2006). The change in energy efficiency during ISS might be due to an increased energy expenditure for the immune system, reflected in an increase in the FHP. Alternatively, ISS may reduce the maximum rate of protein deposition or change the priority of the animal for depositing protein and fat in the body. For example, Parmentier et al. (2002) found an increased fat deposition, in broilers repeatedly challenged with lipopolysaccharide (LPS).

Acute challenge studies versus a method mimicking on farm situations. Most evidence for increased AA requirements is obtained in studies in which pigs were repeatedly challenged with LPS (e.g., Kim et al., 2012; de Ridder et al., 2012; Rakhshandeh et al., 2010, Table 1.1), Complete Freund's Adjuvant (Le Floc'h et al., 2008; Kampman-van de Hoek et al., 2015), or turpentine oil (Kampman-van de Hoek et al., 2013), and it can be questioned to what extent these results can be extrapolated to pigs in commercial conditions (Pastorelli et al., 2012b). Instead, studies with a more practical approach, mimicking farm situations are needed to quantify the nutrient costs of suboptimal conditions. This approach should not focus on acute challenges and clinical infections but on factors that are encountered on farms in practice and influence the immune system of the pig without inducing severe clinical signs of disease or mortality. The needed model might for instance create a low-grade contrast in immune system stimulation by exposing pigs to different hygiene levels, medication, ventilation, and animal density. Use of such a model has the advantage that translation of the results to a farm situation is more easy than for a model with clinical infection. It might be a challenge, though, to maintain a similar standardized effect of the factors in the model on the pigs.

REDUCTION IN DIETARY PROTEIN: A RISK FOR PIG PRODUCTION?

Due to environmental issues and a growing world population there is a renewed focus to increase efficiency of livestock farming. In pig nutrition, reduction of the dietary crude protein (CP) level can contribute to this goal, as generally, protein intake is inversely related to protein efficiency (Batterham et al., 1990).

It is generally perceived that dietary deficiencies due to this CP reduction can be compensated for by supplementing essential AA to the diet, thus restoring normal growth at lower N intake (Kerr and Easter, 1995; Gloaguen et al., 2014). However, the ideal dietary AA profile differs depending on animal and environmental conditions, which can change the optimal AA profile (Le Floc'h et al., 2004). Suboptimal conditions may be an increasing challenge for future pig production, due to the restricted use of antibiotics in food animals (Cogliani et al., 2011).

Risk for behavioural problems? A reduction in dietary protein supply for pigs housed under suboptimal conditions may increase the risk on behavioural problems. It is known from literature that dietary AA deficiencies increase the risk of tail- and ear biting outbreaks (Jericho and Church, 1972; Taylor et al., 2010). Therefore, considering the large variation in conditions on today's pig farms, the challenge for an increasing protein efficiency on farms coincides with a lack of understanding of the dietary optimal AA profile and optimal dietary protein-energy ratio of pigs on these farms, which may aggravate behavioural problems. Behavioural characteristics are considered to be important indicators of animal health. Tail biting behaviour constitutes a large economical and welfare problem in the pig industry (Schrøder-Petersen and Simonsen, 2001; Moinard et al., 2003). The level of disease has already been identified as one of the multifactorial reasons influencing this behaviour (Schrøder-Petersen and Simonsen, 2001; Taylor et al., 2010). Respiratory disease and tail biting for example, seem to have an association with each other as found by Kritas and Morrison (2007) and Moinard et al. (2003). The background of the relationship between health/disease and biting behaviour is not completely understood. This relation might be influenced by the effect of ISS and the consequential change of nutrient requirements in the body. If some nutrients are limiting for pig performance or immune functioning, pigs might further increase their rooting behaviour to satisfy their nutritional needs. As biting behaviour is considered to be redirected rooting behaviour, consequently there can be an increased risk for biting behaviour in case of immune stimulation (Taylor et al., 2010).
Serotonin might play a key role in the effect of dietary nutrient deficiencies and ISS on biting behaviour. Serotonin is a neurotransmitter, synthesised from Trp, and is known to influence pig behaviour (Le Floc'h and Seve, 2007). Trp is one of the essential AA that might become limiting in case of ISS (Le Floc'h et al., 2009), and can, therefore, indirectly influence pig behaviour. Aggressive behaviour and stress, have been shown to be related to the concentration of dietary Trp and brain serotonin in several animal and human studies (Gibbons et al., 1979; Chamberlain et al., 1987; Salomon et al., 1994; Winberg et al., 2001; Lepage et al., 2002). It is very likely that Trp and serotonin play also a role in biting behaviour, however a direct link between, optimal or suboptimal health conditions, dietary Trp, serotonin, and biting behaviour has not yet been demonstrated. Ursinus et al. (2014) recently found that pigs that perform tail biting behaviour had lowered blood serotonin

levels. This can be due to the direct effect of serotonin on tail biting, however it can also be an indirect effect due to lowered AA level or lowered health status. Additionally, a reduced growth in case of low sanitary conditions, can have a direct effect on biting behaviour. Low growth rate due to poor health often results in a larger variation in size of animals within the same pen, influencing biting behaviour negatively (Taylor et al., 2010).
Overall, striving for a reduction in dietary protein content may increase the risk on health, performance, and behavioural problems, particularly so in pigs housed under low sanitary conditions.

In summary, AA metabolism and energy metabolism is altered in case of ISS. As the frequency of ISS is related to the sanitary conditions of the pig, altered protein and energy metabolism results in altered nutrient requirements of these pigs, when housed in optimal or suboptimal situations. Interactions between AA and energy metabolism, the immune system, and dietary AA and energy supply, are not fully understood and require further research. Future feeding strategies taking into account sanitary conditions of the farm might help to make pig production more efficient, specifically when striving for a reduction in dietary protein content and zero use of antibiotics. Development of such feeding strategies is only possible when nutritional needs affected by sanitary status are quantified, and the interactions between health, performance, behaviour and sanitary conditions are better understood.

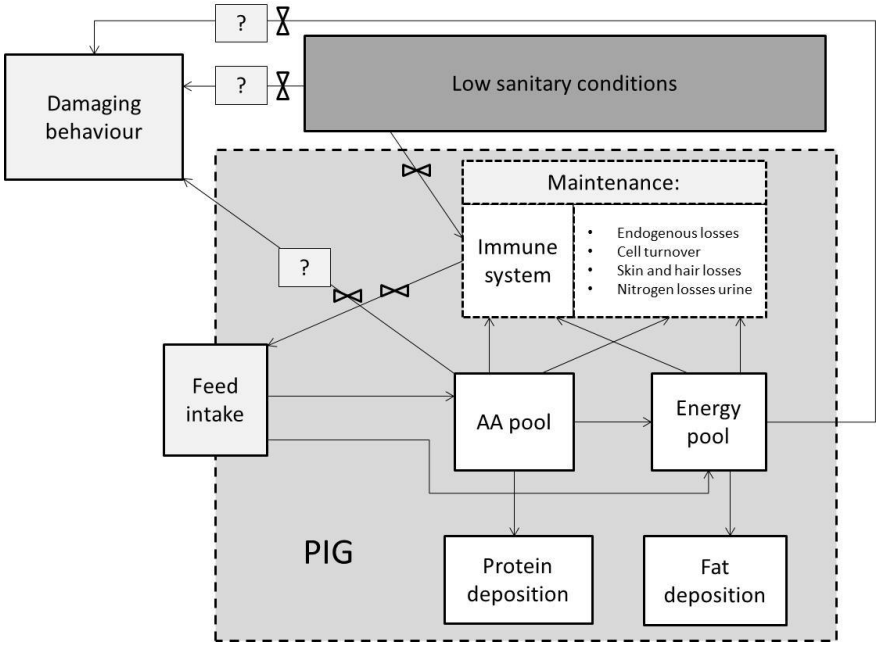

Figure 1.4. Schematic overview of the outline of this thesis. The light grey area represents a pig. The small squares represent pools or events. The arrows having a symbol represent effects. The question marks indicate likely effects. The arrows without symbols represent fluxes.

OBJECTIVES AND OUTLINES

The objective of the research in this thesis was to study the effects of low and high sanitary conditions on AA and energy metabolism in pigs. Also interactions between the immune system, metabolism and damaging behaviour were taken into account in this thesis. The relationships between low sanitary conditions, the immune system, AA and energy pool, feed intake, protein and fat deposition, and damaging behaviour are shown in a schematic overview in Figure 1.4.

In Chapter 2, an experiment is described revealing the effects of dietary protein level and dietary AA supplementation on the growth performance of pigs kept under different sanitary conditions. In Chapter 3, the effects of dietary protein level in combination with dietary AA supplementation on the damage behaviour of pigs kept under different sanitary conditions are evaluated. Chapter 4, describes the effects of different sanitary conditions on the energy requirement for maintenance, and on the incremental efficiencies for retention of dietary energy and protein in the body in the pig. In Chapter 5, diurnal patterns of heat production and carbohydrate and fat oxidation of pigs kept under different sanitary conditions are presented. Chapter 6 includes a general discussion with emphasis on different experimental models and methodologies used for immune stimulation studies and measurement of AA and energy metabolism. At the end of this general discussion the implications of the results found in this thesis for current feed industry are provided. Finally, general conclusions are presented.

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Chapter 2

Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation

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ABSTRACT

There is growing evidence that requirements for particular amino acids (AA) increase when pigs are kept under low sanitary conditions. The extent to which reduction in growth performance is related to these increased requirements is unclear. To evaluate this relationship, an experiment (2 × 2 × 2 factorial arrangement) was performed with 612 male pigs (9 per pen) kept under low sanitary conditions (LSC) or high sanitary conditions (HSC) and offered ad libitum access to either a normal crude protein (CP) concentration diet (NP; 17, 15, and 15% CP for the starter, grower, and finisher phase, respectively) or a low CP concentration diet (LP; 20% CP reduced relative to NP for each phase), each of which containing a basal AA profile (AA-B) or a supplemented AA profile (AA-S). The supplemented diet type contained 20% more methionine (Met), threonine (Thr), and tryptophan (Trp) relative to Lys on an apparent ileal digestible basis compared with the basal diet type. Pigs were followed for a complete fattening period and slaughtered at a targeted pen weight of 110 kg. Haptoglobin concentrations in serum (0.92 g/L for LSC and 0.78 g/L for HSC) and IgG antibody titers against keyhole limpet hemocyanin (3.53 for LSC and 3.08 for HSC) collected in the starter, grower, and finisher phases and pleuritis scores at slaughter (0.51 for LSC and 0.20 for HSC) were greater for LSC pigs compared with HSC pigs (P ≤ 0.01), illustrating that sanitary conditions affected health conditions. The average daily gain (ADG) and gain to feed (G:F) ratio were greater for HSC pigs compared with LSC pigs (*P* ≤ 0.01). The number of white blood cells (WBC) was higher in (AA-S)–fed pigs compared with (AA-B)–fed pigs when kept at LSC but not at HSC (sanitary conditions (SC) × AA interaction, *P* = 0.04). Pigs fed NP had a lower number of WBC compared with pigs fed LP (*P* = 0.02). The number of platelets in pigs fed AA-S diets was higher compared with pigs fed AA-B diets (*P* ≤ 0.01). A 20% reduction in dietary supplementation of Met, Thr, and Trp relative to Lys decreased G:F more in LSC pigs than in HSC pigs (interaction, *P* = 0.03), illustrating that dietary requirements for these AA differ depending on sanitary conditions. This study, performed under practical conditions, shows that AA requirements are dependent on sanitary conditions. Furthermore, supplementation of diets with particular AA may improve performance, especially under poor hygienic conditions. Dietary protein concentration as well as Met, Thr, and Trp supplementation can modify immune status, which may influence resistance to subclinical and clinical diseases.

Keywords: amino acid, immune system, performance, pig, protein, sanitary conditions

INTRODUCTION

A potential growth reduction with decreasing dietary protein intake can be ameliorated through the supplementation of limiting amino acids (AA) in the diet, thereby restoring growth at a lower CP intake (Kerr and Easter, 1995; Gloaguen et al., 2014). The optimal AA profile, however, differs depending on animal and environmental conditions (Le Floc'h et al., 2004). Activating the immune system can increase the requirements for nutrients, such as AA. If the enhanced requirements are not compensated for by greater intake, repartitioning of dietary protein or AA away from development and growth tissues occurs (Le Floc'h et al., 2009) toward use by the immune processes. This shift of nutrients, together with an often-observed reduction in feed intake in case of immune stimulation, reduces pig performance (Klasing and Johnstone, 1991; Pastorelli et al., 2012a). Despite the growing evidence that particular AA requirements are dependent on immune system activation, it remains unclear to what extent reduced performance during (subclinical) infections is related to changes in AA requirements and whether dietary supplementation of these AA can reverse the performance loss. Moreover, most evidence for increased AA requirements is obtained in studies in which pigs were repeatedly challenged with lipopolysaccharide (LPS; e.g., Kim et al., 2012; de Ridder et al., 2012; Rakhshandeh et al., 2010) or Complete Freund's Adjuvant (Kampman-van de Hoek et al., 2015; Le Floc'h et al., 2008), and it can be questioned to what extent these results can be extrapolated to pigs in commercial conditions (Pastorelli et al., 2012b). Therefore, we studied, under practical conditions, if the performance and immune status of pigs kept under different sanitary conditions is influenced by protein intake and AA supplementation. We hypothesized that increased provision of Met, Thr, and Trp would increase performance of pigs, particularly when kept under low sanitary conditions and low dietary protein intake.

MATERIAL AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee of Wageningen University, the Netherlands.

Experimental Design. In a 2 × 2 × 2 factorial arrangement, groups of pigs were allocated to either high sanitary conditions (HSC) or low sanitary conditions (LSC) and were offered ad libitum access to 2 different diets, a normal CP concentration diet (NP) or a low CP concentration diet (LP), each having either a basal dietary AA profile (AA-B) or supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile (AA-S).

Animals and Treatments. In total, 612 (Topigs 20 × Tempo; Topigs, Helvoirt, The Netherlands) newborn boar piglets were selected on a commercial nursery farm in the Netherlands and allocated to either the LSC or HSC treatment. Per nursery room, half of the boar piglets were selected for LSC and the other half for HSC treatment. Only HSC piglets received vaccinations in the first 9 wk of age. The HSC piglets were vaccinated, at 1 to 2 wk of age, against *Mycoplasma hyopneumoniae* (Porcilis M Hyo; MSD Animal Health, Boxmeer, the Netherlands); at 4 to 5 wk of age against *M. hyopneumoniae*, porcine circovirus type 2 (PCV2), and porcine reproductive and respiratory syndrome (PRRS; Porcilis M Hyo, Porcilis Circo, and Porcilis PRRS, respectively; MSD Animal Health) and *Lawsonia intracellularis* (Enterisol Ileitis Boehringer Ingelheim B.V., Alkmaar, the Netherlands); at 6 to 7 wk of age against *Actinobacillus pleuropneumoniae* (APP; Porcilis APP; MSD Animal Health) and influenza A virus (Gripovac3; Merial B.V., Velserbroek, the Netherlands); and at 8 to 9 wk of age against APP and influenza A virus (Porcilis APP and Gripovac3, respectively) by subcutaneous injection in the neck or, in the case of Enterisol, by oral drench. Piglets of both LSC and HSC treatments were housed in the same rooms until weaning (±24 d of age). After weaning, LSC and HSC pigs were group housed in different rooms to prevent cross-vaccination by the 2 living vaccines used in the HSC piglets (Enterisol Ileitis and Porcilis PRRS). The HSC and LSC pigs were separately transported to the experimental farm (Vlierbos V.O.F., Neerloon, the Netherlands). As it was not possible to obtain all measurements during the study on 612 pigs on a single day, the LSC and HSC groups were split into 2 batches of 324 (180 from the LSC treatment and 144 from the HSC treatment) and 288 pigs (144 from the LSC treatment and 144 from the HSC treatment) arriving 1 wk apart. Therefore, pigs of batch 1 and 2 arrived at the experimental farm at an age of 10 and 11 wk, respectively.

Upon arrival, all pigs were individually weighed and, within sanitary condition treatment and batch, allocated to their pen based on BW to minimize variation between pens and within pens (17.3 ± 0.06 kg for LSC batch 1, 18.1 ± 0.07 kg for LSC batch 2, 15.9 ± 0.07 kg for HSC batch 1, and 17.4 ± 0.07 kg for HSC batch 2). The LSC pigs of each batch were housed in 5 LSC rooms and the HSC pigs were housed in 4 HSC rooms located in the same building. Each room had separate manure pits and separate ventilation regulation and contained 8 pens with 9 pigs per pen (0.8 m^2 space/pig), except for 1 LSC room, where 4 out of 8 pens were left empty. In addition, the HSC and LSC rooms were separated by a wall in the central corridor. High sanitary condition rooms were intensively cleaned in 4 steps before arrival of the pigs: twice with foam (MS Topfoam LC Alk; MS Schippers, Bladel, the Netherlands) and high pressure washing and then treated twice with a disinfectant (MS Megades and MS Oxydes; MS Schippers). In addition, a strict hygiene protocol was adhered to when entering the HSC rooms, which included showering, change of clothes, and use of a hairnet and face mask. People were not allowed to have access to a pig farm 48 h before entering HSC rooms. High sanitary condition animals received a preventative antibiotic injection (Fenflor; AUV Veterinary Services B.V., Cuijk, the Netherlands; 1 mL/pig, intramuscular at Day 1 and

3 of the experiment) and were dewormed every 5 wk during the experiment starting at arrival (Flutelmium 0.6% premix; AUV Veterinary Services B.V.; topdressing, 1.5 mg Flubendazol/kg BW for 5 subsequent days). Rooms for the LSC pigs were not cleaned after a previous batch of commercial finisher pigs left the facility 2 d before, and no hygiene protocol was applied. Starting at 5 wk after arrival, fresh manure of another commercial pig farm was spread in the LSC pens every 2 wk until end of the experiment to enhance antigenic pressure. Low sanitary condition pigs did not receive any medication or preventive treatment. The experimental period lasted from December 11, 2013, until April 16, 2014. Animals were monitored for the complete fattening period, divided in 3 phases, that is, starter (0–34 d), grower (35–49 d), and finisher phases (from Day 50 until a target average pen weight of 110 kg BW). At the end of each phase, pigs were individually weighed.

Diets and Feeding. Pigs were allocated to 2 diets, NP (17, 15, and 15% CP for the starter, grower, and finisher phases, respectively) or LP (20% CP reduced relative to NP for each phase), each of which contained a basal or a supplemented AA profile. This resulted in 4 dietary treatments: Low protein - basal amino acid diet, low protein- supplemented amino acid diet, normal protein - basal amino acid die, and normal protein - supplemented amino acid diet. Each diet was fed to the pigs in both sanitary regimes resulting in 8 treatment groups. The apparent ileal digestible (**AID**) Lys to NE ratio of the diets was reduced in each subsequent phase of the experiment to follow a 3-phase feeding system. For the NP, the ratio was based on the Lys to net energy (**NE**) requirements for boars according to the NRC (2012). The values for Lys to NE requirements were multiplied by 0.95 to make sure that the dietary energy concentration was not limiting the growth performance of the pigs. This resulted in diets for the starter, grower, and finisher phases containing 0.90, 0.81, and 0.75 g AID Lys/MJ of NE. For the LP, the inclusion level of all protein-containing ingredients was decreased by 20% relative to the NP and replaced by maize starch and Opticell (Agromed Austria GmbH, Kremsmünster, Austria), resulting in 0.72, 0.65, and 0.60 g AID Lys/ MJ of NE. The basal AA profile (AA-B) was designed based on a factorial approach to cover the requirements for body protein deposition based on results from Bikker et al. (1994), Le Bellego and Noblet (2002), and the NRC (2012) and to cover losses associated with basal endogenous AA in ileal digesta based on results from Jansman et al. (2002) and the NRC (2012), related to losses of AA in skin and hair based on results from the NRC (2012), and AA losses related to cell turnover based on results from Moughan (1998). All values were expressed in the same units for a pig of 50 kg BW with an assumed protein deposition of 138 g/d. The Met + Cys (45% of AID Lys) and Trp (15% of AID Lys) concentrations in AA-B diets, obtained in this manner, were adjusted to 51% Met + Cys and 18% Trp based on results from Knowles et al. (1998) and Jansman et al. (2010), as we considered these to be far below the requirement values (CVB, 2011; NRC, 2012). The supplemented AA profile (AA-S) was derived from the AA-B profile by increasing the Met, Thr, and Trp ratio relative

to Lys by 20%. These AA were increased in particular as they are believed to be important as building blocks for proteins, for example, acute-phase proteins, synthesized in case of immune system activation (Melchior et al., 2004; Le Floc'h et al., 2008, 2012; Rakhshandeh et al., 2010), because of their function as precursors for important immune related components and antioxidants, and also because of their effects on several immune processes. Methionine is known to be an important methyl donor (Burke et al., 1951) and antioxidant (Wu, 2009), Thr plays an important role in mucus synthesis for gut integrity and immune function (Wu, 2009), and Trp is known as a precursor of melatonin and serotonin, both known to inhibit inflammatory cytokines (Wu, 2009). The ingredient and nutrient composition of the diets is shown in Tables 2.1, 2.2, and 2.3. All diets were isocaloric on a NE basis and contained TiO_2 as an indigestible marker. Diets were analyzed for AA composition by acid hydrolysis at 110°C for 23 h and ion-exchange chromatography with postcolumn derivatization with ninhydrin (ISO, 2005b) and Trp by alkaline hydrolysis at 110°C for 20 h ion-exchange chromatography with fluorescence detection (MOD.0094 version G; ISO, 2005c). Per pen (9 pigs), 1 feeder was used and feed and water were offered ad libitum. The feed was provided as pellets via a computerized automatic system (Fancom Multiphase; Fancom B.V., Panningen, the Netherlands), which registered the mass of feed delivered per pen per day. At the end of each phase (starter, grower, and finisher), remainders of the diet per pen were collected and weighed to determine the feed intake per pen per phase. The computerized feeding system was calibrated before the trial started and after each phase.

Digestion of dry matter and nitrogen. At wk 13, 18, and 24 of age, pigs in 4 pens per room and 4 rooms per sanitary treatment were sampled for feces by rectal stimulation. In 3 subsequent days, 1 sample per pig was collected and samples were pooled per pen and stored at −20°C. Sampling pens were equally distributed over dietary treatments. Frozen feces samples were dried at 103°C in an oven for 24 h to determine DM content (method 930.15; AOAC; ISO, 1999) and were analyzed for nitrogen (N) by the Kjeldahl method (ISO 5983; ISO, 2005a). Before Ti analysis (Short et al., 1996; Myers et al., 2004), samples were freeze-dried and ground to pass a 1-mm screen using a Retsch ZM 100 mill (Retsch GmbH, Haan, Germany). Apparent total tract digestibility (ATTD) for dry matter (DM) and N was calculated using TiO2 as an indigestible marker (Kotb and Luckey, 1972).

Blood Sampling. At the start of the experiment, 2 pigs with an average weight per pen were selected for blood sampling at 13, 18, and 24 wk of age from the vena cava. Selected pigs were sampled during each of the 3 phases. Per sampling moment, two 9-mL tubes per animal were filled: 1 EDTA tube for blood cell counts (Vacuette; Greiner Bio-One, Kremsmünster, Austria) and 1 serum tube for acute-phase protein and natural antibody (Nab) analysis (Vacuette). Blood samples collected in EDTA tubes were immediately stored on ice and transported to the lab where blood cell counts were performed using a Microcell

counter Sysmex pocH- iV Diff; Toa Medical Electronics Co., Ltd., Kobe, Japan). Blood samples in serum tubes were allowed to clot for 1 h at room temperature, after which serum was collected after centrifugation for 10 min at 5,251 × g at room temperature and stored at −20°C pending analysis of haptoglobin (Tridelta Phase Haptoglobin Assay, catalog number TP-801; Tridelta Development, Ltd., Maynooth, Ireland), pig major acute-phase protein (Cusabio Pig-MAP, ELISA, catalog number CSB-E13425p; Cusabio Biotech Co, Ltd., Wuhan, Hubei Province, China), and Nab titers against keyhole limpet hemocyanin (KLH) types IgG and IgM using ELISA.

Table 2.1. Ingredients and nutrient composition of the starter diets

	LP[1]		NP[1]	
Item	AA-B[2]	AA-S[2]	AA-B	AA-S
Ingredient, g/kg of feed				
Maize	320.00	320.00	400.00	400.00
Soybean meal	182.02	182.00	227.54	227.54
Barley	160.00	160.00	200.00	200.00
Wheat	45.53	45.53	56.91	56.91
Maize starch	206.79	204.64	40.65	37.90
Sugarcane molasses	20.00	20.00	20.00	20.00
Limestone	13.94	13.94	14.11	14.11
Monocalcium phosphate	9.99	9.99	8.93	8.93
Soybean oil	10.65	10.98	15.98	16.38
Vitamin + mineral mix[3]	5.00	5.00	5.00	5.00
Salt	3.19	3.19	3.83	3.83
L-lysine HCl	1.95	1.94	2.35	2.35
Titanium dioxide	2.50	2.50	2.50	2.50
Sodium bicarbonate	2.58	2.58	1.34	1.34
L-threonine	0.60	1.46	0.63	1.71
L-tryptophan	0.03	0.31	0.00	0.37
DL-methionine	0.23	0.94	0.23	1.13
Cellulose[4]	15.00	15.00	0.00	0.00
Nutrients calculated, g/kg				
NE, MJ/kg[5]	9.80	9.80	9.80	9.80
DM	889.60	889.80	893.20	884.90
Crude protein	138.00	136.00	168.00	167.00
Starch[4]	474.10	472.30	410.00	407.70
Lys[6]	8.60	8.60	10.50	10.50
Thr[6]	5.40	6.20	6.60	7.40
Trp[6]	1.70	1.90	2.00	2.30
Met + Cys[6]	4.30	4.80	5.20	5.90
Ile[6]	5.60	5.60	6.90	6.80
Arg[6]	8.30	8.40	10.50	10.30
Phe[6]	6.60	6.60	8.20	8.10
His[6]	3.40	3.40	4.20	4.20
Leu[6]	10.9	11.00	13.60	13.40
Tyr[6]	4.40	4.50	5.70	5.70
Val[6]	6.40	6.40	7.90	7.70

[1] LP = low CP concentration diet, NP = normal CP concentration diet.

[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile.

[3] Supplied the following per kilogram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[4] Opticell (Agromed Austri GmbH, Kremsmünster, Austria).

[5] Based on chemical composition, digestibility, and energy value for pigs from the Centraal Veevoeder Bureau livestock feed table (CVB, 2011).

[6] Analyzed values.

Table 2.2. Ingredients and nutrient composition of the grower diets

	LP[1]		NP[1]	
Item	AA-B[2]	AA-S[2]	AA-B	AA-S
Ingredient, g/kg of feed				
Maize	400.00	400.00	500.00	500.00
Soybean meal	138.15	138.15	172.69	172.69
Barley	171.36	171.36	214.19	214.19
Maize starch	199.19	197.24	43.34	40.86
Wheat	20.00	20.00	20.00	20.00
Sugarcane molasses	13.11	13.11	13.33	13.33
Limestone	8.62	8.62	7.67	7.67
Monocalcium phosphate	9.19	9.48	10.20	10.57
Soybean oil	5.00	5.00	5.00	5.00
Vitamin + mineral mix[3]	3.95	3.95	3.61	3.61
Salt	2.45	2.45	2.92	2.92
L-lysine HCl	2.50	2.50	2.50	2.50
Titanium dioxide	1.56	1.56	2.00	2.00
Sodium bicarbonate	3.87	3.87	1.52	1.52
L-threonine	0.72	1.49	0.74	1.72
L-tryptophan	0.21	0.46	0.22	0.55
DL-methionine	0.12	0.76	0.07	0.87
Cellulose[4]	20.00	20.00	0.00	0.00
Nutrients calculated, g/kg				
NE, MJ/kg[4]	9.84	9.84	9.84	9.84
DM	883.70	885.90	882.80	887.70
Crude protein	124.00	124.00	152.00	152.00
Starch[5]	497.10	495.50	448.90	446.90
Lys[6]	8.00	7.90	9.70	10.00
Thr[6]	5.10	5.90	5.90	7.00
Trp[6]	1.56	1.75	1.91	2.16
Met + Cys[6]	3.98	4.52	4.76	5.58
Ile[6]	4.80	4.70	5.90	5.80
Arg[6]	7.20	7.00	9.00	9.00
Phe[6]	5.80	5.70	7.10	7.10
His[6]	3.00	3.00	3.70	3.60
Leu[6]	10.0	9.90	12.3	12.3
Tyr[6]	3.80	3.90	5.00	5.00
Val[6]	5.70	5.80	6.90	6.90

[1] LP = low CP concentration diet, NP = normal CP concentration diet.

[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile.

[3] Supplied the following per kilopgram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[4] Opticell (Agromed Austri GmbH, Kremsmünster, Austria).

[5] Based on chemical composition, digestibility, and energy value for pigs from the Centraal Veevoeder Bureau livestock feed table (CVB, 2011).

[6] Analyzed values.

Table 2.3. Ingredients and nutrient composition of the finisher diets

	LP[1]		NP[1]	
Item	AA-B[2]	AA-S[2]	AA-B	AA-S
Ingredient, g/kg of feed				
Maize	360.10	360.10	450.10	450.10
Soybean meal	115.70	115.70	144.60	144.60
Barley	240.00	240.00	300.00	300.00
Maize starch	187.20	185.30	36.40	34.10
Wheat	20.00	20.00	20.00	20.00
Sugarcane molasses	12.20	12.20	12.50	12.50
Limestone	7.40	7.40	6.50	6.50
Monocalcium phosphate	14.00	14.30	13.20	13.60
Soybean oil	5.00	5.00	5.00	5.00
Vitamin + mineral mix[3]	2.30	2.30	2.60	2.60
Salt	2.40	2.40	2.80	2.80
L-lysine HCl	2.50	2.50	2.50	2.50
Titanium dioxide	5.30	5.30	3.00	3.00
Sodium bicarbonate	0.00	0.60	0.00	0.70
L-threonine	0.70	1.40	0.70	1.60
L-tryptophan	0.10	0.40	0.10	0.40
DL-methionine	0.10	0.10	0.00	0.00
Cellulose[4]	25.00	25.00	0.00	0.00
Nutrients calculated, g/kg				
NE, MJ/kg[5]	9.84	9.84	9.84	9.84
DM	885.90	886.50	881.60	887.70
Crude protein	132.00	126.00	151.00	148.00
Starch[5]	541.70	509.40	541.70	540.20
Lys[6]	8.00	7.60	8.90	8.90
Thr[6]	5.30	5.60	5.90	6.50
Trp[6]	1.51	1.62	1.68	1.91
Met + Cys[6]	4.03	4.22	4.68	5.04
Ile[6]	4.90	4.60	5.70	5.60
Arg[6]	7.30	6.80	8.20	8.20
Phe[6]	6.00	5.60	6.80	6.80
His[6]	3.10	2.90	3.50	3.50
Leu[6]	10.10	9.60	11.90	11.30
Tyr[6]	4.00	3.70	4.70	4.70
Val[6]	6.00	5.70	6.60	6.60

[1] LP = low CP concentration diet, NP = normal CP concentration diet.

[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile.

[3] Supplied the following per kilopgram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[4] Opticell (Agromed Austri GmbH, Kremsmünster, Austria).

[5] Based on chemical composition, digestibility, and energy value for pigs from the Centraal Veevoeder Bureau livestock feed table (CVB, 2011).

[6] Analyzed values.

Oral Fluid Sampling for Presence of Respiratory Pathogens. In each room with 8 pens of pigs, 2 pens with the normal protein - basal amino acid diet were selected for oral fluid sampling by using a swine oral fluid test kit (Tego oral fluids kit; ITL Corporation, Melbourne, Australia). At wk 14, 20, and 24 of age (1 time point per phase), a clean rope was hanged in the pen at pigs' shoulder height and securely tied. Pigs were allowed to chew on the rope for 30 min. Subsequently, the rope was removed from the pen (by wearing gloves) and placed in a clean pouch bag. The bag was closed and the fluid was extracted from the rope by squeezing the rope through the bag. Oral fluid was collected from the bag in a clean sample tube after the corner of the bag was torn off. Oral fluid was refrigerated until further analysis. Oral samples were analyzed with multiplex PCR for presence of *Mycoplasma hyorhinis*, porcine respiratory corona virus, PRRS virus, *M. hyopneumoniae*, influenza A virus, porcine cytomegalo virus, and PCV2 (IVD GmbH, Hannover, Germany).

Anti-Keyhole Limpet Hemocyanin IgM and IgG Assessment. Antibody titers were determined as described by de Koning et al. (2015) with the minor modification that a 4-step dilution (40, 160, 640, 2,560 times diluted) of the sera was made instead of a 3-step dilution.

Observations at Slaughter. All pigs per pen were slaughtered in the week in which the average BW of the pigs in that pen was close to the target weight of 110 kg. In the slaughterhouse, lungs were collected, examined, and scored by a pathologist for pleuritis (0 to 2 scale, in which 0 = absence of pleuritis, 1 = adhesion of lung tissue with film-like tissue, and 2 = lung tissue completely grown together) and pneumonia lesions (0 to 3 scale, in which 0 = absence of pneumonia, 1 = one spot of pneumonia, 2 = a few spots of pneumonia, and 3 = diffuse deviation of pneumonia spots). Carcass weight, backfat thickness, and muscle thickness were measured in the slaughterhouse. Both fat and muscle thickness were measured at 6 cm from the back midline between the third and fourth last rib. Lean meat and dressing percentages were calculated per pig from the parameters obtained at slaughter using the following formulas:

lean meat (%) = 66.86 − 0.6549 × (fat, mm) + 0.0207 × (muscle, mm) (Engel et al., 2012), and dressing (%) = (carcass weight/live weight) × 100% (Watkins et al.,1990).

Table 2.4. Performance parameters of growing pigs kept under different sanitary conditions and fed diets differing in protein content and amino acid supplementation, (bold *P*-values are significant and underlined *P*-values are considered as tendency)

	LSC[1]				HSC[1]											
	LP[2]		NP[2]		LP		NP			*P*-values[5]						
Item	AA-B[3]	AA-S[3]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SEM[4]	SC	CP	AA	SC×CP	SC×AA	CP×AA	SC×CP×AA
No. of pens[6]	9	9	9	9	8	8	8	8								
Start, kg BW	17.6	18.0	17.6	17.6	16.6	16.6	16.7	16.6	0.1	**<.0001**	0.23	0.43	0.15	0.21	0.10	0.41
ADG, g/d																
0-34 d	602	591	655	652	618	660	697	724	16	**0.01**	**<.0001**	0.22	0.53	<u>0.06</u>	0.86	0.60
35-49 d	962	992	1023	1088	979	1024	1113	1132	33	0.25	**<.0001**	**0.02**	0.22	0.65	0.89	0.39
17-110, kg BW	825	846	864	884	860	886	925	969	18	**0.03**	**<.0001**	**0.01**	<u>0.10</u>	0.49	0.66	0.65
ADFI, g/d																
0-34 d	1246	1225	1218	1198	1201	1280	1230	1259	20	0.17	0.39	0.21	0.27	**0.01**	0.42	0.40
35-49 d	2122	2123	2120	2070	2100	2222	2220	2244	51	0.11	0.45	0.49	0.17	0.17	0.27	0.69
17-110, kg BW	1950	1924	1901	1890	1910	1989	1940	1998	37	0.25	0.66	0.24	0.13	**0.04**	0.90	0.63
G:F, g/g																
0-34 d	0.48	0.48	0.54	0.54	0.52	0.52	0.57	0.57	0.009	**0.003**	**<.0001**	0.62	0.75	0.94	0.59	0.96
35-49 d	0.46	0.47	0.48	0.52	0.47	0.46	0.5	0.51	0.013	0.93	**<.0001**	0.11	0.91	<u>0.09</u>	0.60	0.81
17-110, kg BW	0.42	0.44	0.46	0.48	0.45	0.44	0.48	0.48	0.004	**0.002**	**<.0001**	**0.01**	0.70	**0.03**	0.32	0.15

[1]LSC = low sanitary conditions; HSC = high sanitary conditions. [2]LP = low CP concentration diet; NP = normal CP concentration diet. [3]AA-B = basal dietary AA profile, AA-S = supplemented ietary AA profile containing 20% more Met, Thr, and Trp compared with basal profile. [4]SEM = pooled SEM, Means are presented as least square means. [5]SC = sanitary conditions. Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$. [6]A pen contained 9 pigs.

Statistical Analysis. Data were analyzed as a 2 × 2 × 2 factorial arrangement using the GLM procedure for parameters measured at slaughter and the Mixed Model procedure for other performance parameters and serum and blood data (SAS 9.3; SAS Inst. Inc., Cary, NC) with pen as experimental unit for all parameters. For all data, the normality of the distribution of Studentized residuals was assessed by the Shapiro–Wilk statistic. If required, transformation of data was performed to obtain normal distribution of residuals. Values are presented as least squares means ± SEM, and effects were considered significant at $P \leq 0.05$ and a trend was defined as $0.05 < P \leq 0.10$. Sanitary condition, dietary CP level, and AA profile, batch, and their interactions were used as fixed effects. Phase was added in the model as a fixed effect and phase × sanitary condition as an interaction for all blood parameters and ATTD of N and DM. The effect of room within sanitary status was used as a random effect to correct for differences between rooms. The Kenward–Roger statement was used to correct for the degrees of freedom for batch. The difference between individual start BW and average BW of the treatment group (sanitary conditions and batch) was used as covariate in the model in the first statistical evaluations but finally omitted because of absence of statistical significance.

RESULTS

Two pigs selected for blood sampling died during the grower phase and. as such. the data of these pigs are missing for the finisher phase. Data of another pig selected for blood sampling was omitted from the data set as this pig was treated with antibiotics in the starter phase due to lung problems. No other clinical signs of illness were observed during the experiment. All results are presented in Tables 2.4 through 2.8. For clarity, selected treatment interactions are represented in Fig. 2.1A through 2.1I.

Performance. Mean BW at start of the experiment was greater for LSC pigs (17.7 ± 0.1 kg) compared with HSC pigs (16.6 ± 0.1 kg; $P \leq 0.01$; Table 2.4). The ADG was (52 g/d) lower for LSC pigs compared with HSC pigs for the starter phase ($P \leq 0.05$) and (55 g/d) during the complete fattening period ($P \leq 0.01$) but not during the grower phase ($P > 0.10$). The LP pigs had (56 g/d) lower ADG in the complete fattening period compared with NP pigs (all, $P \leq 0.05$). The AA-B pigs tended to have (28 g/d) lower ADG compared with AA-S pigs in the starter phase when kept under HSC but not under LSC [sanitary conditions (SC) × AA, $P \leq 0.10$].

For ADFI, an interaction was present for SC × AA in the starter phase ($P \leq 0.01$) and over the complete fattening period ($P \leq 0.05$; Fig. 2.1A) but not in the grower phase ($P > 0.10$). The AA-B pigs had (54 g/d in the starter phase and 69 g/d over the complete fattening period) lower ADFI compared with AA-S pigs when kept under HSC but not under LSC. The G:F was (0.035 g/g) lower for pigs kept under LSC compared with HSC for the starter phase ($P \leq$

0.01) and (0.013 g/g) for the entire grower–finisher period ($P \leq 0.01$) but not for the grower phase ($P > 0.10$).

The G:F was 0.038 g/g lower for LP pigs compared with NP pigs in all phases (all, $P \leq 0.01$). The AA-B pigs had a (0.008 g/g) lower G:F compared with AA-S pigs in the entire grower–finisher period ($P \leq 0.01$) but not for the grower phase ($P > 0.10$). The G:F was 0.038 g/g lower for LP pigs compared with NP pigs in all phases (all, $P \leq 0.01$). The AA-B pigs had a (0.008 g/g) lower G:F compared with AA-S pigs in the entire grower–finisher period ($P \leq 0.01$). The greater G:F for AA-B pigs compared with AA-S pigs for the entire experimental period was (0.025 g/g) greater for LSC pigs compared with HSC pigs (SC × AA, $P \leq 0.05$; Fig. 2.1B). A tendency for a similar interaction was found for the G:F in the grower phase ($P \leq 0.10$).

Acute-Phase Proteins and Natural Antibodies against Keyhole Limpet Hemocyanin in Serum. In LSC pigs but not HSC pigs, reduction of dietary CP concentration reduced serum haptoglobin by 0.24 g/L (CP × SC, $P \leq 0.01$; Table 2.5; Fig. 2.1I). The LSC pigs showed lower haptoglobin concentrations over time whereas HSC pigs had lower concentrations during the grower phase compared with the starter phase and showed greater concentrations again in finisher phase (SC × phase, $P \leq 0.05$; Fig. 2.1E). The LSC pigs had (0.29 g/L) greater serum haptoglobin concentrations compared with the HSC pigs ($P \leq 0.01$). The LSC pigs had (0.03 g/L) lower PigMAP concentrations in the grower phase compared with the starter phase and (0.09 g/L) greater concentrations again in the finisher phase compared with the grower phase, whereas the HSC pigs had (0.09 g/L) lower concentrations in the grower phase compared with the starter phase (SC × phase, $P \leq 0.05$; Fig. 2.1F). Keyhole limpet hemocyanin–specific IgM antibody titers in serum tended to be (0.04) lower for AA-S–fed pigs compared with AA-B–fed pigs ($P \leq 0.10$). Keyhole limpet hemocyanin–specific IgG antibody titers were (0.45) greater for LSC pigs compared with HSC pigs ($P \leq 0.05$).

Blood Cell Counts. The number of white blood cells (**WBC**) was (1.8×10^9/L) greater (Table 2.6) in AA-S–fed pigs compared with AA-B–fed pigs when kept under LSC but not under HSC (SC × AA, $P \leq 0.05$; Fig. 2.1C). Over time, the concentration of WBC in pigs decreased (by 4.1×10^9/L) but the number of red blood cells consistently increased (with 0.5×10^{12}/L) in all treatment groups ($P \leq 0.01$). Pigs fed the NP had a (3.1%) lower number of WBC compared with pigs fed the LP ($P \leq 0.05$). Hemoglobin concentration was (0.1 mmol/L) lower in AA-S–fed pigs compared with AA-B–fed pigs, particularly under HSC (SC × AA, $P \leq 0.05$, and $P \leq 0.05$ for AA). Hemoglobin concentrations increased with age in all treatment groups (0.74 mmol/L); however, this increase was greater in HSC pigs compared with LSC pigs (SC × phase, $P \leq 0.05$). Pigs fed NP had a (0.1 mmol/L) greater hemoglobin concentration than pigs fed LP ($P \leq 0.05$). Mean cell volume was (0.7×10^{15}L) greater for AA-S–fed pigs compared with AA-B–fed pigs in LSC but this was reversed in HSC (SC × AA, $P \leq 0.01$; Fig. 2.1D). The mean cell volume was (0.4×10^{15}L) greater in pigs fed NP compared with pigs

fed LP (P ≤ 0.05). The number of platelets decreased (by 258 × 10^9/L) in pigs over time for all treatments, but the decrease was (60%) greater in HSC pigs compared with LSC pigs (interaction, P ≤ 0.05). The concentration of platelets in pigs fed AA-S diets was (121 × 10^9/L) greater compared with pigs fed AA-B diets (*P* ≤ 0.01).

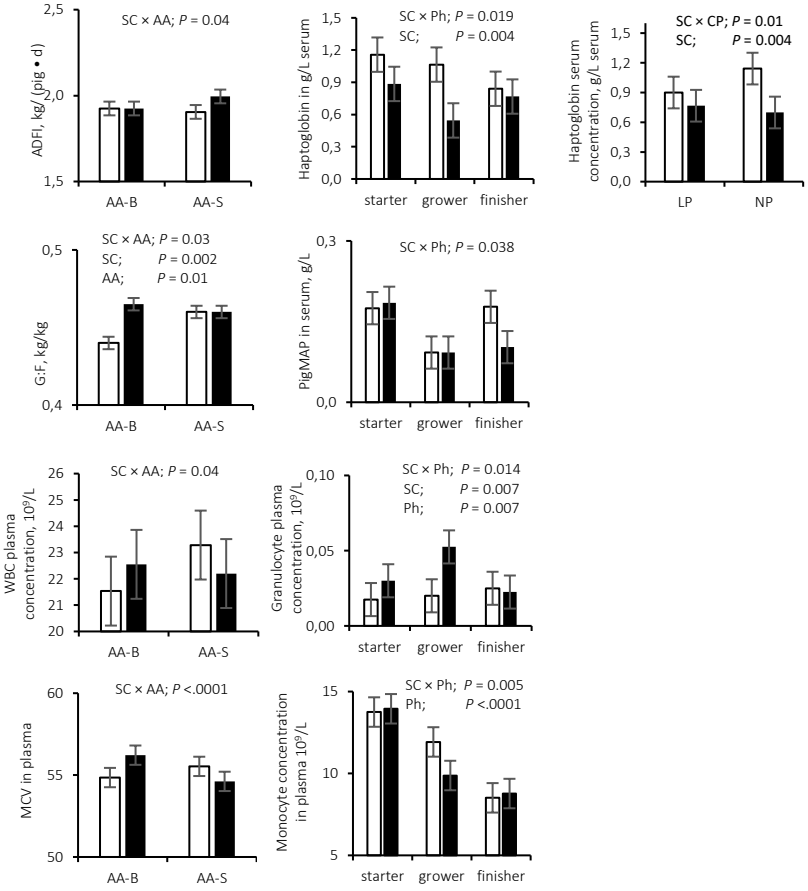

Figure 2.1. Interactions between low sanitary condition (**LSC**) and high sanitary condition (**HSC**) pigs and a basal dietary AA profile (**AA-B**) or a supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile (**AA-S**), for ADFI (**A**), G:F (**B**), white blood cell (**WBC**) number (**C**), and mean cell volume (**MCV**; **D**). SC = sanitary conditions. Interactions between LSC and HSC pigs and phase (**Ph**) for serum haptoglobin concentration (**E**), serum PigMAP concentration (**F**), plasma granulocyte number (**G**), and plasma monocyte number (**H**). Interaction between LSC and HSC pigs and a low CP concentration diet (**LP**) or a normal CP concentration diet (**NP**) for serum haptoglobin concentration (**I**). The open bars represent the LSC treatment and the filled bars represent the HSC treatment. Bars represent least squares means ± SEM. *P*-values were considered significant when *P* ≤ 0.05.

Table 2.5. Concentrations of acute-phase protein and natural antibody titres against keyhole limpet hemocyanin (KLH) in serum of pigs kept under different sanitary conditions and fed 1 of 4 diets, with a low or normal protein level and a basal or supplemented AA profile (bold *P*-values are significant and underlined *P*-values are considered as tendency)

		LSC[2]				HSC[2]											
		LP[2]		NP[2]		LP		NP			*P*-values[6]						
Item	Phase[1]	AA-B[4]	AA-S[4]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SEM[5]	SC	CP	AA	SC×Ph[7]	SC×CP	SC×AA	CP×AA	SC×CP×AA
No.of pens[7]		9	9	9	9	8	8	8	8									
Haptoglobin,	Starter	1.15	0.75	1.36	1.37	0.91	0.79	0.86	0.98	0.16	**0.004**	0.25	0.91	**0.02**	**0.01**	0.42	0.12	0.74
g/L	Grower	0.97	0.97	1.21	1.11	0.63	0.47	0.46	0.62									
	Finisher	0.83	0.73	0.85	0.95	0.85	0.95	0.58	0.69									
PigMAP,	Starter	0.17	0.14	0.14	0.25	0.18	0.16	0.19	0.21	0.03	0.25	0.19	0.55	**0.04**	0.99	0.35	0.22	0.39
g/L	Grower	0.07	0.08	0.10	0.12	0.08	0.09	0.11	0.09									
	Finisher	0.19	0.19	0.16	0.17	0.11	0.10	0.09	0.11									
KLH[8]-IgM	Starter	6.59	6.29	6.68	6.60	6.40	6.78	6.59	6.42	0.20	0.68	0.47	<u>0.07</u>	0.32	0.92	0.32	0.47	0.20
	Grower	7.50	7.27	7.47	7.09	7.06	7.03	7.31	6.99									
	Finisher	7.85	7.46	7.80	7.69	7.78	7.65	7.90	7.84									
KLH-IgG[7]	starter	3.12	3.30	3.08	2.97	2.78	2.46	2.47	3.02	0.28	**0.04**	0.75	0.62	<u>0.09</u>	0.42	0.87	0.81	<u>0.08</u>
	grower	3.64	4.13	3.67	3.75	3.10	2.98	3.15	2.99									
	finisher	3.62	3.70	3.82	3.52	3.46	3.59	3.36	3.59									

[1]The experiment consisted of different phases: starter, grower, and finisher. [2]LSC = low sanitary conditions, HSC = high sanitary conditions. [3]LP = low CP concentration diet, NP = normal CP concentration diet. [4]AA-B = basal dietary AA profile; AA-S = supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile. [5]SEM= pooled SEM, Means are presented as least square means. [6] SC = sanitary conditions. Considered significant when $P \leq 0.05$, considered a tendency when $0.05 < P \leq 0.10$. [7] All animals were group housed in pens with 9 animals per pen. Two animals per pen were selected for blood sampling. [8] Keyhole limpet hemocyanin is a protein produced by a sea snail. Pigs have natural antibodies against this protein.

White blood cell distribution. The AA-S–fed pigs had (0.7 × 10^9/L) a greater number of blood lymphocytes compared with AA-B– fed pigs when provided a LP, but this was reversed (–0.5 × 10^9/L) when pigs were provided a NP (CP × AA, $P \leq 0.05$). The number of lymphocytes increased (0.9 × 10^9/L) with age for all treatment groups ($P \leq 0.01$). The number of monocytes (2.9 × 10^9/L) decreased over time for all treatment groups, particularly for LSC pigs (SC × phase, $P \leq 0.01$; Fig. 2.1H). The AA-S–fed pigs had a (0.6 × 10^9/L) greater monocyte number compared with AA-B–fed pigs ($P \leq 0.05$). The NP-fed pigs had a (0.8 × 10^9/L) lower monocytes number compared with LP-fed pigs ($P \leq 0.05$). The concentration of granulocytes increased (by 0.01 × 10^9/L) over time for LSC pigs and, in the HSC pigs, increased (0.02 × 10^9/L) from the starter to the grower phases but decreased (0.03 × 10^9/L) again in the finisher phase (SC × phase, $P \leq 0.05$; Fig. 2.1G). The concentration of granulocytes was (0.01 × 10^9/L) greater for HSC pigs than for LSC pigs ($P \leq 0.01$).

Carcass observations at slaughter and lung scores. Results obtained at slaughter are presented in Table 2.7. Carcass weight was (3.8 kg) lower for LP pigs compared with NP pigs but not dressing percentage ($P \leq 0.01$ and $P > 0.05$, respectively). The LP pigs had (1.2 mm) greater backfat thickness ($P \leq 0.01$) and (0.8%) lower lean meat percentage ($P \leq 0.01$) compared with NP pigs. The AA-B pigs had (1.5 mm) lower muscle thickness ($P \leq 0.01$; 0.5 mm), greater backfat thickness ($P \leq 0.01$), and a (0.4%) lower lean meat percentage ($P \leq 0.01$) compared with AA-S pigs. Pleuritis scores were (0.3) greater for LSC pigs compared with HSC pigs ($P \leq 0.01$). Percentage of lung surface with pleuritis was also (1.2%) greater for LSC pigs compared with HSC pigs ($P \leq 0.01$). The AA-B pigs had a (1%) greater percentage of lung surface with pleuritis compared with AA-S pigs when kept in LSC, but for HSC pigs, this was reversed (SC × AA, $P \leq 0.05$). The LP pigs had (0.2) greater pneumonia scores compared with NP pigs when kept under HSC; however, when kept under LSC, this was reversed (SC × CP, $P \leq 0.05$). The AA-B pigs tended to have greater pneumonia scores compared with the AA-S pigs in all cases except in LSC pigs fed a LP (CP × AA, $P \leq 0.01$).

Dry matter and N digestion. The effect of experimental treatments on the ATTD of DM and N were consistent over the duration of the experiment and are presented averaged over all phases (Table 2.8). In general, treatment differences, albeit significant, were small. Apparent total tract digestibility for DM was (0.3%) lower for LSC pigs compared with HSC pigs during all phases (all, $P \leq 0.05$). The AA-B pigs had (0.4%) lower ATTD of DM compared with AA-S pigs ($P \leq 0.01$). Apparent total tract digestibility for N was (0.98%) greater for HSC pigs compared with LSC pigs during all phases (all, $P \leq 0.01$), regardless the dietary CP content or AA profile. Apparent total tract digestibility for N increased from 80.6 (starter phase) to 83.9 (grower phase) and 84.7% finisher phase ($P \leq 0.01$). There were no interactions between phase and other independent variables.

Table 2.6. Cell count in fresh blood of pigs kept under different sanitary conditions and fed one of four diets; with a low or normal protein level, and a basal or supplemented AA profile (bold *P*-values are significant and underlined *P*-values are considered as tendency)

		LSC[2]				HSC[2]													
		LP[3]		NP[3]		LP		NP			*P*-values[6]								
Item[1]	Phase	AA-B[3]	AA-S[3]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SEM[5]	SC	CP	AA	phase	SC×Ph	SC×CP	SC×AA	CP×AA	SC×CP×AA
No. of pens[7]		9	9	9	9	8	8	8	8										
WBC, 10^9/L	Starter	24.3	26.6	20.8	25.0	26.0	25.0	25.1	23.4	1.3	0.89	**0.02**	0.15	**<.0001**	0.08	0.60	**0.04**	0.18	0.46
	Grower	22.7	24.9	22.5	22.0	21.9	22.7	21.3	19.9										
	Finisher	19.4	21.2	19.5	20.0	19.6	21.4	21.4	20.8										
Lymphocytes, 10^9/L	Starter	10.7	11.3	9.4	10.3	11.0	11.3	11.2	10.4	0.7	0.43	0.10	0.69	**0.005**	0.88	0.13	0.05	**0.02**	0.30
	Grower	11.0	11.9	10.9	10.8	11.4	11.8	11.7	10.9										
	Finisher	11.2	12.5	11.2	11.1	11.1	11.6	13.2	10.9										
Monocytes, 10^9/L	Starter	13.6	15.3	11.3	14.8	15.0	13.9	13.9	13.0	0.9	0.34	**0.01**	**0.05**	**<.0001**	**0.005**	0.55	0.13	0.69	0.56
	Grower	11.7	13.1	11.6	11.3	10.4	10.8	9.5	8.8										
	Finisher	8.1	8.7	8.4	8.9	8.2	9.7	8.1	9.1										
Granulocytes, 10^9/L	Starter	0.02	0.01	0.01	0.03	0.02	0.03	0.04	0.03	0.0	**0.007**	0.27	0.88	**0.007**	**0.01**	0.39	0.61	0.74	0.10
	Grower	0.02	0.02	0.01	0.03	0.05	0.07	0.05	0.04										
	Finisher	0.04	0.02	0.02	0.02	0.04	0.03	0.01	0.01										
RBC, 10^{12}/L	Starter	5.7	5.6	5.6	5.5	5.4	6.0	5.5	5.4	0.1	0.83	0.83	0.95	**<.0001**	0.30	0.95	0.08	0.44	0.36
	Grower	5.9	5.8	5.9	5.6	5.7	6.0	5.9	5.8										
	Finisher	6.0	5.9	6.0	6.1	6.1	6.1	6.0	6.3										
Hb, mmol/L	Starter	6.4	6.3	6.5	6.4	6.3	6.3	6.5	6.0	0.1	0.13	**0.01**	**0.03**	**<.0001**	**0.05**	0.62	**0.05**	0.18	0.07
	Grower	6.6	6.6	6.8	6.6	6.8	6.7	7.2	6.7										
	Finisher	6.8	6.9	6.9	7.3	7.2	7.0	7.3	7.2										
Ht, %	Starter	47.8	30.9	31.4	30.9	30.3	31.0	31.0	29.3	3.7	0.49	0.45	0.28	0.64	0.23	0.41	0.40	0.48	0.29
	Grower	31.6	31.6	32.3	31.2	31.9	32.3	33.0	31.6										
	Finisher	32.9	32.9	33.4	34.3	34.0	33.5	34.3	34.5										
MCV, 10^{-15}L	Starter	54.8	55.1	55.8	56.1	56.0	54.4	56.0	54.1	0.59	0.48	**0.04**	0.06	0.13	0.24	0.31	**<.0001**	0.52	0.95
	Grower	54.0	54.7	54.8	55.3	56.0	54.3	56.4	54.9										
	Finisher	54.5	56.0	55.2	56.0	56.2	55.0	56.7	55.0										

PTL, 10^9 /L	Starter	614	712	562	721	571	703	744	1219	94.8	0.92	0.23	**0.002**	**<.0001**	**0.02**	0.14	0.46	0.60	0.78
	Grower	522	606	520	652	438	551	439	559										
	Finisher	489	555	480	494	405	508	447	401										

[1] WBC = white blood cells; RBC = red blood cells; Hb = haemoglobin; Ht = Haematocrit; MCV = mean cell volume; PTL = platelets. [2] LSC = low sanitary status; HSC = high sanitary status. [3] LP = low CP concentration diet, NP = normal CP concentration diet.[4] AA-B= basal dietary AA profile; AA-S = supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile. [5]SEM = pooled SEM, Means are presented as least square means. [6] SC=sanitary conditions. Considered significant when [1] WBC = white blood cells; RBC = red blood cells; Hb = haemoglobin; Ht = Haematocrit; MCV = mean cell volume; PTL = platelets. [2] LSC = low sanitary status; HSC = high sanitary status. [3] LP = low CP concentration diet, NP = normal CP concentration diet.[4] AA-B= basal dietary AA profile; AA-S = supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile. [5]SEM = pooled SEM, Means are presented as least square means. [6] SC=sanitary conditions. Considered significant when $P \leq 0.05$ and considered a tendency when $0.05 < P \leq 0.10$. [7]All animals were group housed in pens with 9 animals in total. Two animals per pen were selected for blood sampling.

Oral fluid samples. Oral fluid samples of pigs in all sampled pens (18 pens in total) were positive for M. hyorhinis and negative for PRRS, M. hyopneumoniae, influenza A virus, and porcine cytomegalo virus in all phases. All pens were negative for porcine respiratory corona virus in the starter and grower phases, but in the finisher phase, 4 out of 9 LSC pens and 3 out of 9 HSC pens became positive. All pens were negative for PCV2 in the starter phase, but LSC pigs became positive in grower phase (7 out of 9 pens) and finisher phase (all, 9 pens). Only 1 out of 9 HSC pens became positive for PCV2 in the grower phase and was negative again in the finisher phase. The rest of the sampled HSC pigs were negative for PCV2 in all phases.

Table 2.7. Slaughter results of fattening boars kept under different sanitary conditions and fed diets with 2 CP levels and 2 AA profiles (bold *P*-values are significant and underlined *P*-values are considered as tendency)

	LSC[1]				HSC[1]					*P*-values[5]						
	LP[2]		NP[2]		LP		NP									
Item	AA-B[3]	AA-S[3]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SEM[4]	SC	CP	AA	SC×CP	SC×AA	CP×AA	SC×CP×AA
No. of pens[6]	9	9	9	9	8	8	8	8								
BW, in kg[7]	109.7	108.0	112.8	114.3	108.1	111.9	113.1	116.3	1.85	0.40	**0.0007**	0.20	0.98	0.17	0.62	0.48
Carcass weight, kg	84.7	82.3	86.4	88.2	82.0	85.7	86.3	88.8	1.55	0.78	**0.001**	0.20	0.95	0.12	0.49	0.23
Muscle, mm[7]	55.4	55.6	55.3	59.1	55.3	56.6	55.8	56.6	0.90	0.78	0.12	**0.008**	0.19	0.42	0.18	0.07
Backfat, mm[7]	15.3	13.9	13.3	12.9	14.7	14.3	13.4	13.7	0.33	0.45	**<.0001**	**0.047**	0.25	0.07	0.08	0.88
Lean meat, %[7]	58.1	58.9	59.3	59.7	58.4	58.7	59.2	59.1	0.23	0.53	**<.0001**	**0.03**	0.26	0.11	0.13	0.90
Dressing, %	77.1	76.2	76.6	77.2	75.9	76.6	76.3	76.4	0.35	0.18	0.51	0.63	0.73	0.25	0.32	**0.02**
Pleuritis score[8]	0.44	0.50	0.59	0.43	0.15	0.21	0.12	0.33	0.08	**<.0001**	0.43	0.51	0.96	0.10	0.87	0.09
Pleuritis lung %[9]	1.82	1.26	2.55	1.04	0.39	0.96	0.06	0.36	0.60	**0.004**	0.39	0.77	0.83	0.49	0.66	0.58
Pneumonia score[8]	0.53	0.65	0.79	0.74	0.94	0.80	0.86	0.57	0.15	0.32	0.65	0.33	**0.02**	0.07	0.16	0.83
Pneumonia lung, %[9]	1.42	1.26	3.17	2.14	1.81	1.93	2.48	0.70	1.40	0.78	0.22	0.15	0.12	0.27	0.16	0.46

[1]LSC = low sanitary conditions, HSC = high sanitary conditions. [2]LP= low CP concentration diet; NP = normal CP concentration diet. [3]AA-B = basal dietary AA profile; AA-S = supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile. [4]SEM= pooled SEM, means are presented as least square means. [5]SC = sanitary conditions. Considered significant when $P \leq 0.05$ and considered as tendency when $0.05 < P \leq 0.10$. [6]A pen contained 9 pigs. [7] Body weight expressed 1 d before slaughter day; muscle, backfat, and lean meat expressed as corrected for carcass weight, by including carcass weight as a covariate in the statistical model. [8]Pleurits was scored on a scale of 0 to 2 and pneumonia was scored on a scale of 0 to 3.

Table 2.8. Apparent fecal digestibility (%) of DM and N in fattening boars kept under different sanitary conditions and fed 1 of 4 experimental diets with either a low or normal protein level and a basal or supplemented AA profile (bold *P*-values are significant)

	LSC[1]				HSC[1]												
	LP[2]		NP[2]		LP		NP			*P*-values[5]							
Item	AA-B[3]	AA-S[3]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SEM[4]	SC	CP	AA	Ph[6]	SC×CP	SC×AA	CP×AA	SC×CP×AA
No. of pens[7]	4	4	4	4	4	4	4	4									
DM	88.1	88.5	87.9	88.6	88.8	88.9	88.3	88.5	0.16	**0.05**	0.10	**0.03**	**<.0001**	0.21	0.19	0.51	0.70
N	82.3	82.1	82.9	83.1	83.6	83.4	84.2	83.1	0.43	**0.02**	0.14	0.28	**<.0001**	0.31	0.30	0.62	0.31

[1]LSC = low sanitary conditions; HSC = high sanitary conditions. [2]LP = low CP concentration diet; NP = normal CP concentration diet. [3]AA-B = basal dietary AA profile; AA-S = supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile. [4]SEM = pooled SEM. Means are presented as least square means. [5] SC = sanitary conditions. Considered significant when $P \leq 0.05$. [6]Results are presented as average values of measurement of digestibility at 3time points, once in each of the 3 experimental phases. [7]A pen contained 9 pigs.

DISCUSSION

The main objective of the present experiment was to evaluate, under practical conditions, if diets with low or normal CP level and basal or supplemented Met, Thr, and Trp have differential effects on pig performance and immune status at different sanitary conditions.

Effect of sanitary conditions on performance and immune status. A contrast in sanitary conditions was generated by imposing a combination of differences in hygiene, antibiotic treatment, deworming, and a vaccination protocol, all applied to piglets originating from the same farm. Over the duration of the experiment, HSC pigs showed greater ADG (50 g/d) and G:F (0.013 kg/kg) than LSC pigs. Low sanitary condition pigs had greater serum haptoglobin levels and greater KLH–IgG titers than HSC pigs. This indicates that LSC pigs had a more active immune system compared with HSC pigs. In addition, at slaughter, higher pleuritis scores were observed in LSC pigs compared with HSC pigs. Taken together, the absence of clinical signs of illness of pigs in either treatment group and the lower ADG and G:F, elevated haptoglobin concentrations, increased KLH-specific IgG titers, greater pleuritis occurrence, and oral fluid samples positive for PCV2 in the grower and finisher phases in the LSC groups illustrate a difference in subclinical health status between the LSC and HSC pigs (Le Floc'h et al., 2006; van den Berg et al., 2007; Piñeiro et al., 2009). We expected that the sanitary regimes used in our study would affect PigMAP and haptoglobin concentrations in a similar way, as these are both considered to be positive acute-phase proteins and are expected to increase in response to immune stimulation (Murata et al., 2004). Haptoglobin appeared more responsive to the difference in sanitary conditions than PigMAP, which is in line with Heegaard et al. (1998) and Kampman-van de Hoek et al. (2016). In addition, PigMAP levels found here (0.14 g/L serum) were low compared with the values found by Piñeiro et al. (2009) and Kampman-van de Hoek et al. (2016; 0.9 and 1.4 g/L serum, respectively), whereas the haptoglobin concentrations reported by these authors were similar to values reported in the present study.

The KLH antigen is a relevant antigen to measure Nab levels as pigs had no previous exposure to KLH. Natural antibodies are defined as antigen-specific antibodies that are present in the absence of intentional immunization with specific antigens (KLH, in this case; Star et al., 2007). The IgG titer against KLH was greater for LSC pigs than for HSC pigs. Because Nab play a role in the first line of defense against pathogens, the increase in Nab levels might be an adaptive response of the pigs to the higher infection pressure at LSC. Keyhole limpet hemocyanin–specific antibodies were shown to be cross-reactive with antigens of pathogens (Hamilton et al., 1999). Therefore, it can not be excluded that antigen specific Nab are products of adaptive immune responses and cross-reactive with structurally related antigens. Specific memory B cells were shown to be activated in a polyclonal but antigen-independent way (Lanzavecchia et al., 2006). This way of activation

is triggered by nonspecific microbial-associated molecular patterns, such as lipopolysaccharide or bacterial or viral nucleotide motifs. This B cell activation mechanism is likely responsible for the lifelong presence of circulating specific antibodies, which forms an important part of the first line of defense. It can easily be envisaged that memory B cells of pigs under LSC become more activated by microbial associated molecular patterns than under HSC, resulting in higher levels of KLH-specific IgG levels. For IgM titers against KLH, there were no differences between LSC and HSC pigs. In the study of Ploegaert et al. (2010), a similar result in IgM and IgG Nab was found. Ploegaert et al. (2010) studied genetic and phenotypic correlation of Nab titers in dairy cattle and found greater estimates for environmental variation in the IgG than in the IgM isotype of Nab. Most probably, the LSC in the present study, as an environmental factor, stimulated KLH-specific IgG responses. Immunoglobulin M titers are believed to be influenced by genetic factors (Ploegaert et al., 2010), which explains the absence of differences in IgM against KLH between sanitary regimes.

As the HSC pigs were vaccinated against several pathogens in their first 10 wk of life, vaccination and/ or antibiotic treatment might have modulated immune functions of these animals. Our results show that despite the vaccination of the HSC pigs, LSC pigs had significantly higher values for haptoglobin and IgG antibodies against KLH, showed reduced ADG and G:F, and had significant higher pleuritis scores, indicating that the effects of the LSC conditions outweighed the potential effects of the vaccinations or antibiotic treatment of the HSC pigs. However, the vaccinations of the HSC pigs may have reduced the immunological contrasts between the pigs kept under the different sanitary conditions. If this is the case, the observed interactions between diet and sanitary conditions could even be larger when vaccinations are omitted. In addition, vaccinations or antibiotic treatment may have affected other (undetermined) immune parameters as well.

Sanitary conditions and growth performance. At start of the experiment, the BW of LSC pigs was 1.1 kg greater compared with that of HSC pigs, likely related to the vaccination program of the HSC pigs prior to arrival at the experimental farm. During the starter phase, HSC pigs compensated for this lower starting weight, or the ADG of the LSC pigs was negatively influenced by the LSC.

A lower ADG of LSC pigs was also found in other studies evaluating a contrast in sanitary conditions (Williams et al., 1997; Le Floc'h et al., 2009; Pastorelli et al., 2012a). This decreased ADG can be explained by the competition for use of nutrients between the immune system and for use in deposition in organs and body tissues. In general, it is perceived that use by the immune system has a high priority (Humphrey and Klasing, 2004; Le Floc'h et al., 2004). From a meta-analysis, Pastorelli et al. (2012b) concluded that themajor portion of the reduction in ADG (12.2% lower ADG of the total 16.3% lower ADG in pigs in poor housing conditions compared with unchallenged animals) was related to a

decreased feed efficiency (in Pastorelli et al. (2012b), indicated as "maintenance") rather than to a decrease in feed intake. This was also observed in the present experiment, where G:F was affected but ADFI was not influenced by sanitary conditions. The former indicates a greater maintenance requirement or a lower growth efficiency for LSC pigs as suggested by Pastorelli et al. (2012b).

Signs of respiratory problems, such as pleuritis or pneumonia, are related to reduced performance (Saco et al., 2011). The LSC pigs had greater scores for pleuritis and greater percentage of lung surface with pleuritis at slaughter compared with HSC pigs, which might have negatively affected growth and G:F of LSC pigs. From the meta-analysis of Pastorelli et al. (2012b), it appeared that a reduction in ADFI is the major contributor to reduced ADG in case of respiratory problems. In contrast, in our study, ADFI is not the major contributor to reduced ADG, indicating that respiratory problems are likely not the cause of the decrease in G:F in LSC pigs or that conclusions about the respiratory problems drawn by Pastorelli et al. (2012b) are not representative for our study. Pastorelli et al. (2012b) used many different challenge studies for the meta-analysis, differing from studies conducted under more practical conditions such as the present study.

The greater G:F of the HSC pigs might partly be due to a greater apparent total N digestion in these animals compared with LSC pigs. When assuming similar postabsorptive efficiencies for absorbed AA, the observed increase in N digestibility would typically explain approximately 20% of the observed increase in ADG, hence leaving 80% unaccounted for. Differences in ATTD for N correspond with results by Kampman-van de Hoek et al. (2016), who found a reduction in ATTD for N of 3.7% in LSC growing pigs compared with HSC pigs ($P \leq 0.01$). The reduced ATTD for N might be due to intestinal infections, intestinal damage, or an increased digesta passage rate (Sandberg et al., 2006; Pastorelli et al., 2012b). The small difference found in ATTD of DM might suggest that the ATTD of GE also differed to a similar extent.

Greater ADG and G:F were expected for NP-fed pigs compared with the LP-fed pigs. It is well known that additional AA under conditions where AA are limiting but sufficient energy is available lead to greater ADG and improved G:F (Noblet et al., 1987). Supplementation of extra AA by increase in dietary CP (LP vs. NP) or by specifically supplementing Met, Thr, and Trp (AA-B vs. AA-S diet type) resulted in increased ADG, suggesting that one of these AA was limiting. The increased G:F in AA-S pigs vs. AA-B pigs, particularly in LSC pigs (SC × AA interaction; Fig. 2.1B), confirms our hypothesis that the dietary AA-S profile better matches the AA requirements of pigs under LSC. It should be noted, however, that this effect was not yet present in the starter phase. Our results are in accordance with other studies that show that immune stimulation by different challenges lead to increased requirements for specific AA compared with unchallenged pigs (Grimble and Grimble, 1998; Le Floc'h et al., 2004; Klasing, 2007; Rakhshandeh et al., 2014). As a consequence, these animals require more AA for their immune system. The increased demand for these AA (Met, Thr, and Trp)

for LSC pigs was expected to be greater for pigs fed the LP. This interaction, however, was absent. Therefore, the results of our study do not confirm our hypothesis that at low levels of protein intake, the requirements for Thr, Trp, and Met relative to Lys are increased. As illustrated by the meta-analysis by Pastorelli et al. (2012b), the type of challenge has a major impact on the response of pigs. The contrast in sanitary conditions, as applied in our study, illustrates that also in absence of clinical disease, requirements for Met, Thr, and Trp are persistently affected over the entire weight range. This is in agreement with observations by Kim et al. (2012) for Met, using a repeated LPS model, but not in a study by de Ridder et al. (2012) for Trp, also using a repeated LPS model, in which the response to incremental intake of Trp was demonstrated to be transient. Dietary protein concentration did not affect ADFI in our study. Apparently, the exchange of protein for starch did not affect the satiating potential of the diets. This is in agreement with Le Bellego and Noblet (2002) and Kerr et al. (2003); however, research in humans has indicated satiating effects of dietary proteins to exceed that of carbohydrates and fats (Andersen and Moore, 2004).

A remarkable result is the interaction between SC and AA profile in ADFI, particularly observed during the starter phase. High sanitary condition AA-S pigs had greater ADFI compared with HSC AA-B pigs, whereas pigs in LSC ate the same amount, regardless of AA supplementation. The greater ADFI for HSC AA-S pigs resulted in a tendency for ADG in the same direction as the change in ADFI. This result illustrates that pigs in HSC were probably more limited in their growth by the AA-B profile than LSC pigs receiving the same profile, which was expected to be the other way around due to the effect of immune stimulation of pigs housed in LSC. We speculate that 1 of the 3 supplemented AA, probably Met, has restricted growth, particularly inhibiting HSC pigs to exploit their full ADG potential following a period of restricted growth before the start of the trial. Methionine is thought to be the limiting AA because the Met:Lys ratio deviates most from recommended values (e.g., CVB, 2011) compared with Thr and Trp. The growth restriction in HSC pigs before the start of the trial was possibly caused by the vaccination strategy and illustrated by a lower BW of HSC pigs at the onset of the trial. The AA profile of the basal diets may not have been sufficient to support compensatory growth following a period of growth restriction.

Dietary protein effect on immune status. Increasing the dietary protein concentration increased serum haptoglobin concentrations in LSC pigs but not in HSC pigs. The effect of protein scarcity on total serum protein concentrations has been demonstrated in mice (Cooper et al., 1974). Houdijk et al. (2007) found a reduced C-reactive protein and haptoglobin response for infected pigs fed a low protein diet. This may reflect a sensitive response of acute-phase proteins to protein scarcity (Houdijk et al., 2007) or a high priority for the use of AA for protein gain in young pigs, occurring at the expense of the synthesis of haptoglobin. It is unexpected that this interaction was not observed for WBC counts, which were consistently higher for LP pigs compared with NP pigs (P = 0.02), being unaffected by

SC. Possible differences in migration of WBC into the lymphatic system or tissues complicates clear conclusions on this point (Ganusov and Auerbach., 2014; Marelli-Berg et al., 2010).

Dietary AA effect on immune status. Supplementation of AA in diets has been shown to influence the immune system by increased variation of lymphocytes, increased production of antibodies and cytokines, and activation of lymphocytes, natural killer cells, and macrophages (Daly et al., 1990; Li et al., 2007; Negro et al., 2008). Although increasing the dietary protein concentration reduced monocyte counts, supplementation of Met, Thr, and Trp increased monocyte counts, regardless of SC. Elevated levels of Trp in the AA-S–fed pigs might have stimulated production of monocytes, as Trp is known to play a role in functionality of monocytes and lymphocytes (Melchior et al., 2004). The administration of 300 mg Trp to rats increased monocyte phagocytosis and the innate immune response (Esteban et al., 2004). Overall, there was no clear interaction for sanitary conditions and AA profile present on the measured blood parameters.

In summary, poor sanitary conditions imposed in our study, in a practical setting, reduced ADG over the entire BW range of 17 to 110 kg. Low sanitary conditions increased pleuritis scores at slaughter and increased indicators for the innate immune response and serum haptoglobin concentrations. The vaccination strategy for the HSC pigs in early life may have triggered a compensatory performance response (particularly ADFI) during the starter phase. Dietary supplementation of Met, Thr, and Trp improved the G:F, particularly under LSC, illustrating that dietary requirements for these AA are affected by sanitary conditions. Furthermore, this study provides indications that dietary protein concentration and Met, Thr, and Trp supplementation modify immune status. Our study suggests that dietary supplementation of Met, Thr, and/or Trp, in addition to provision of AA for covering basal requirements for protein deposition, is beneficial for animal performance, particularly under poor sanitary conditions. Further research should focus on defining determinants of sanitary status and identification of the requirement of individual AA to be supplemented.

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SUPPLEMENTAL TABLE

Supplemental Table I. Dietary amino acid profiles relative to lysine on apparent ileal digestible level, fed in combination with different dietary protein levels to pigs kept under different sanitary conditions

Amino acid	AA-B profile[1]	AA-S profile[1]
Lysine	100	100
Methionine + cysteine	51	61
Threonine	59	71
Tryptophan	18	22
Arginine	87	87
Histidine	43	43
Isoleucine	53	53
Leucine	101	101
Phenylalanine	54	54
Valine	69	69

[1] AA-B= basal AA profile based on Bikker, 1994; Moughan, 1998; Jansman et al., 2002; NRC, 2012; AA-S profile =supplemented profile contains basal profile with 20% extra Met, Thr, and Trp in ratio to Lys.

Chapter 3

A link between damaging behaviour in pigs, sanitary conditions, and dietary protein and amino acid supply

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ABSTRACT

The tendency to reduce crude protein (**CP**) levels in pig diets to increase protein efficiency may increase the occurrence of damaging behaviours such as ear and tail biting, particularly for pigs kept under suboptimal health conditions. We studied, in a 2×2×2 factorial design, 576 tail-docked growing-finishing entire male pigs in 64 pens, subjected to low (**LSC**) vs. high sanitary conditions (**HSC**), and fed a normal CP (**NP**) vs. a low CP diet (**LP**, 80% of NP) ad libitum, with a basal amino acid (**AA**) profile or supplemented AA profile with extra threonine, tryptophan and methionine. The HSC pigs were vaccinated in the first nine weeks of life and received antibiotics at arrival at experimental farm at ten weeks, after which they were kept in a disinfected part of the farm with a strict hygiene protocol. The LSC pigs were kept on the same farm in non-disinfected pens to which manure from another pig farm was introduced fortnightly. At 15, 18, and 24 weeks of age, prevalence of tail and ear damage and of tail and ear wounds was scored. At 20 and 23 weeks of age, frequencies of biting behaviour and aggression were scored for 10×10 min per pen per week. The prevalence of ear damage during the finisher phase (47 vs. 32 % of pigs, $P < 0.0001$) and the frequency of ear biting (1.3 vs. 1.2 times per hour, $P = 0.03$) were increased in LSC compared with HSC pigs. This effect on ear biting was diet dependent, however, the supplemented AA profile reduced ear biting only in LSC pigs by 18% (SC × AA profile, $P < 0.01$). The prevalence of tail wounds was lower for pigs in LSC (0.13 ± 0.02) than for pigs in HSC (0.22 ± 0.03) in the grower phase ($P < 0.007$). Regardless of AA profile or sanitary conditions, LP pigs showed more ear biting (+20%, $P < 0.05$), tail biting (+25%, $P < 0.10$), belly nosing (+152%, $P < 0.01$), other oral manipulation directed at pen mates (+13%, $P < 0.05$), and aggression (+30%, $P < 0.01$) than NP pigs, with no effect on ear or tail damage. In conclusion, both LSC and a reduction of dietary protein increase the occurrence of damaging behaviours in pigs and therefore may negatively impact pig welfare. Attention should be paid to the impact of dietary nutrient composition on pig behaviour and welfare, particularly when pigs are kept under suboptimal (sanitary) conditions.

INTRODUCTION

In the pig industry, reduction of dietary protein contributes to the goal of making livestock farming more nutrient efficient. Reducing dietary protein level seems possible without compromising the growth performance of pigs if supplementary dietary essential amino acids (AA) are provided (Kerr and Easter, 1995; Gloaguen et al., 2014). It has been suggested, however, that feeding diets with low dietary protein levels may increase the occurrence of damaging behaviours, such as ear and tail biting (Jericho and Church, 1972; Jensen et al., 1993). If particular nutrients are limiting for growth or immune functioning, pigs might increase their foraging behaviour and alter their feed preferences to satisfy their nutritional needs (Jensen et al., 1993). Indeed, pigs provided a low-protein diet, although fed ad libitum, showed more foraging activities than pigs fed a diet with adequate protein levels (Jensen et al., 1993). Pigs may, in turn, redirect their natural foraging and exploratory behaviour to pen mates, particularly when suitable rooting substrates are not or only sparsely available. The nosing, chewing, rooting and sucking directed at the tails, ears and other body parts of conspecifics may culminate in vigorous biting and, as a consequence might lead to wounds (Beattie et al., 2001; Zonderland et al., 2008). Once wounds have developed, biting behaviour may escalate, particularly in pigs fed low-protein diets, which are even more attracted to blood (Fraser et al., 1991).

Dietary AA imbalances have also been associated with injurious biting behaviour. Several studies suggest that pigs respond to a shortage of specific essential AA, including methionine (Met), threonine (Thr), and tryptophan (Trp), by adjusting their feed selection behaviour accordingly (Ettle and Roth, 2004, 2005; Roth et al., 2006; Ettle and Roth, 2009). Due to a relative shortage in AA, foraging behaviour and redirected biting behaviour might increase, and blood may become more attractive. In line with the latter, McIntyre and Edwards (2002) found that the preference to chew on a blood-soaked rope as opposed to a water-soaked rope increased when pigs were fed a diet low in Trp. The increased preference for blood in pigs fed low-Trp diets may, consecutively, influence injurious biting. In support of this, weanling pigs fed diets supplemented with free Trp in excess to requirement bit less on the tails and ears of their pen mates (Martínez-Trejo et al., 2009).

Dietary protein deficiencies or AA imbalances may influence behaviour through their effects on brain neurotransmitters, as many of these are synthesized from particular AA. The effects of dietary Trp concentration, for instance, on aggressive behaviour and stress as reported in numerous species (Gibbons et al., 1979; Chamberlain et al., 1987; Salomon et al., 1994; Winberg et al., 2001; Lepage et al., 2002) have been linked to its role as a precursor of serotonin (5-hydroxytryptamine; 5-HT). Recent studies have provided evidence for a link between damaging biting behaviour and 5-HT. Pigs had lowered blood platelet 5-HT storage and higher blood platelet 5-HT uptake velocities in phases of life during which they were classified as tail biters (Ursinus et al., 2014a). Additionally, Valros and Heinonen

(2015) reported that the Trp and central 5-HT metabolism of tail biters was different from that of victims and non-biters.

Apart from dietary influences on injurious biting behaviour, a poor health status has been identified as one of the risk factors for this multifactorial problem (Schrøder-Petersen and Simonsen, 2001; Taylor et al., 2010). Respiratory disease and tail biting for example, seem to be associated at animal level (Kritas and Morrison, 2007) and at farm level (Moinard et al., 2003). The exact nature of the relationship between health and biting behaviour is not completely understood. On the one hand, a low health statusF or poor sanitary conditions could lead to increased requirements for specific nutrients, particularly those involved in immune system activation, such as Trp, and in this way contribute to biting behaviour. On the other hand, biting can also cause health problems, as it frequently leads to inflammatory responses (Munsterhjelm et al., 2013) as supported by Heinonen et al. (2010) who found higher acute phase concentrations in blood in tail bitten pigs compared with non tail-bitten control pigs. Finally, poor health and injurious biting could also partly reflect suboptimal management, feed or climatic conditions, all of which are known to exacerbate both health and behavioural problems (Moinard et al., 2003; Edwards, 2006; Zonderland et al., 2010), without necessarily being causally related. Cause and effect are thus difficult to disentangle in the health-biting relationships reported so far, and, to the best of our knowledge, no studies have been executed on the topic in which health status was experimentally manipulated rather than only assessed.

Reducing dietary protein concentrations on pig farms with poor health conditions may therefore aggravate behavioural problems. Therefore we studied the combined effects of diverging sanitary conditions, dietary protein level and AA profile on redirected biting behaviours and tail and ear damage of pigs. Performance and health parameters of the pigs in the present study have been published elsewhere (van der Meer et al., 2016).

ANIMALS, MATERIALS AND METHODS

The experiment was approved by the Animal Care and Use Committee of Wageningen University.

Experimental design. A 2 × 2 × 2 factorial design was applied with sanitary conditions (high: HSC, or low: LSC), dietary crude protein level (low: LP, or normal: NP), and dietary AA profile (basal: AA-B or supplemented: AA-S) as experimental factors at pen level.

Animals and treatments. In total 576, entire male tail-docked Topigs 20 × Tempo (Topigs, Helvoirt, The Netherlands) piglets were selected on a commercial farm. Half of the piglets involved were subjected to the LSC and the other half to the HSC treatment. The piglets for both LSC and HSC were selected from the same farrowing rooms and were allocated per

litter; all boar piglets of a sow were selected for either LSC or HSC. After weaning (mean age 24 d), LSC and HSC pigs were group-housed in different rooms to prevent cross-vaccination. The HSC piglets, and not LSC piglets, were vaccinated against *Mycoplasma hyopneumoniae*, porcine circovirus type 2, porcine reproductive and respiratory syndrome, *Lawsonia intracellularis*, *Actinobacillus pleuropneumoniae*, and influenza A virus in the first nine weeks of age as specified in van der Meer et al. (2016).

The HSC and LSC pigs were transported separately to another commercial farm where the experiment was conducted (Vlierbos V.O.F., Neerloon, The Netherlands) with half of the pigs at 10 weeks of age and the other half at 11 weeks of age (two batches of 288; LSC: 144, HSC: 144). Upon arrival, pigs were allocated to their pen based on body weight (**BW**) in order to minimize variation in BW between pens (mean BW ± standard error of the mean; LSC batch 1: 17.3 ± 0.06 kg, LSC batch 2: 18.1 ± 0.07 kg, HSC batch 1: 15.9 ± 0.07 kg, HSC batch 2: 17.4 ± 0.07 kg). In total 64 concrete pens were used, each pen had a partly slatted floor and contained nine pigs (0.8 m^2 space per pig). All pens were distributed over eight rooms; four rooms we selected for LSC pigs and four rooms for HSC pigs. Per room each experimental diet was distributed to two pens, resulting in one repetition of treatment combination per room. Distribution of experimental diet per room was done randomly. Per treatment combination there were eight replicates (pens). Each room had separate manure pits and separate negative pressure mechanical ventilation regulation. The temperature in the rooms was set at 24°C at start of the experiment and was decreased to 20°C during the experiment. The LSC rooms were not cleaned after containing a previous batch of commercial finisher pigs that left the facility two days before, and no specific hygiene protocol was applied to these rooms. Starting at five weeks after arrival, fresh manure of another commercial pig farm was spread in the LSC pens every two weeks until end of the experiment to enhance antigenic pressure. The LSC pigs did not receive any preventative medication. In contrast, HSC pigs received a dose of antibiotics (Fenflor; AUV Veterinary Services B.V., Cuijk, the Netherlands; 1 mL/pig, intramuscular at day 1 and 3 of the experiment) after arrival at 10 weeks of age and were placed in four disinfected rooms in a distinct part of the pig facility with a strict hygiene protocol see van der Meer et al. (2016).

Animals were monitored for the complete fattening period, divided in three phases, i.e. starter (0-34 d), grower (35-49 d), and finisher phase (from day 50 until a target average pen BW of 110 kg).

Diets, feeding and analysis. Pens were allocated to a diet with either a low crude protein (**CP**) level (**LP**) or a normal CP level (**NP**), each having either a basal AA-profile or a supplemented AA-profile, resulting in four different diets: LP-AA-B, LP-AA-S, NP-AA-B, NP-AA-S. The apparent ileal digestible (**AID**) Lys/ MJ NE ratio of the diets was reduced in each subsequent phase of the experiment to follow a three phase feeding system. For the NP diet, the lysine (**Lys**)/ MJ NE ratio was set to 95% of the requirement values for boars, as published by NRC (2012), to prevent dietary energy to be limiting for growth performance.

The respective diets contained 0.90 g AID Lys/ MJ net energy (NE) for the starter phase, 0.81 g AID Lys/ MJ NE for the grower phase, and 0.75 g AID Lys/ MJ NE for the finisher phase. The LP diets were created by decreasing the inclusion level of all protein-containing ingredients by 20% in exchange for maize starch and cellulose, resulting in diets with 0.72 g AID Lys/ MJ NE for the starter phase, 0.65 g AID Lys/ MJ NE for the grower phase, and 0.60 g AID Lys/NE for the finisher phase.
The basal AA-profile (AA-B) was designed to cover the AA requirements for body protein deposition (Bikker, 1994; Le Bellego and Noblet, 2002; NRC, 2012) and to cover AA losses associated with basal endogenous proteins in ileal digesta (Jansman et al., 2002; NRC, 2012), AA losses in skin and hair (NRC, 2012), and AA losses related to cell and tissue turnover in the body (Moughan, 1998), as specified in van der Meer et al. (2016). The supplemented AA-profile (AA-S) was derived from the AA-B profile by increasing the Met, Thr, and Trp ratio relative to Lys on a AID basis by 20%. These AA are believed to be increasingly important as building blocks for synthesis of specific proteins, such as acute phase proteins, synthesized in case of immune system activation (Melchior et al., 2004; Le Floc'h et al., 2008; Rakhshandeh et al., 2010; Le Floc'h et al., 2012), and effects on immune processes (Wu, 2009). The composition of the diets is shown in Table 2.5, and the AA-profiles are shown in supplementary Table I. All diets were isocaloric on a NE basis and provided as pellets during the experiment. Per pen one single space feeder and one nipple drinker were present and feed and water were offered ad libitum.
Diets were analyzed for AA composition by acid hydrolysis at 110°C for 23 h and ion-exchange chromatography with post-column derivatization with ninhydrin (ISO, 2005a) and Trp by alkaline hydrolysis at 110°C for 20 h ion-exchange chromatography with fluorescence detection (ISO, 2005b).

Behavioural observations. Pigs were observed at 20 (grower phase) and 23 (finisher phase) weeks of age using behaviour sampling. Frequencies of the behaviours, described in Table 3.1, were recorded during live observations from 8.00-12.20 h and 14.00-16.50 h during two consecutive days. In case of continuous biting for > 30 sec, after 30 sec a new occurrence was scored. Animals in each pen were observed 10 × 10 min, resulting in 100 min of observations per pen per week of observation. Distribution of the 10-min observation blocks over the day and over the two observation days was balanced for experimental treatments. Observers were blind to the dietary treatments and divided over the pens balanced for treatment. All four observers were trained by the same person before behavioural observations.

Damage and wound scores. Tail damage was recorded at 15, 18 and 24 weeks of age as indicator of being tail bitten, using the following scores as described in Ursinus et al. (2014b): 1. No tail damage, 2. Bite marks; thin scratches. The individual bite marks have the size of a pinhead, 3. Small wound; clearly visible lesion with fresh or dried blood on the (top

of the) tail, but the tail retains its entire length, 4. Medium wound; clearly visible lesion with fresh or dried blood on the tail and the tail is partly shortened, 5. Severe wound; lesion with fresh or dried blood, the tail is completely removed. Ear damage was recorded together with the tail damage scoring. Only damage to the backside of the ears was recorded. Scoring was done as follows: 1. No ear damage, 2. Top or bottom lesions; thin scratches, 3. Top and bottom lesions; thin scratches, 4. Severe damage, part of ear is missing. These scores were determined as described by Ursinus et al. (2014b).

Table 3.1. Ethogram

Behaviour	Description
Oral manipulation of group mates	
Tail biting	Nibbling, sucking or chewing the tail of a pen mate
Ear biting	Nibbling, sucking or chewing the ear of a pen mate
Manipulating other	Nibbling, sucking or chewing of another part of the body of a pen mate
Aggression	
Fighting	Ramming or pushing a pen mate with or without biting the other pen mate. Can be either mutual or unilateral
Fighting at feeder	Pushing, head knocking or biting a pen mate at the feeder
Belly nosing	Rubbing the belly of a pen mate with up and down snout movements
Mounting	Standing on hind legs while having front legs on other pig's body
Enrichment object biting	Chewing on an enrichment object provided in the pen. The enrichment object was a chain with a hard plastic pipe fixed at the top of the pen wall.

Cases of ear necrosis were given a score of 5. It is not fully known whether ear necrosis is a result of ear biting. Recent epidemiological studies point to a possible infection route for ear necrosis through biting, however, the spread of ear necrosis in pig herds is difficult to explain solely by opportunistic bacterial infections (Pringle et al., 2009; Karlsson et al., 2013). Damage scores of both ears were recorded, and the average score of both ears was used for further analysis, resulting in one value per pig per recording week for further analysis. Pigs received a binary score for both tail and ear damage: (0) no damage (score1) vs. (1) damage (score >1) per phase. Similarly, pigs received a binary score for having (0) no wound (score < 3 for tails, and score < 4 for ears) vs. (1) a wound (score > 2 for tails, and > 3 for ears) per phase. These scores were averaged per pen and phase and used for further analysis. In case of a score > 4 for tail damage or ear damage, pigs were individually treated with a iodine spray and another wound spray to prevent bacterial infections (MS jodium

bruin and MS protect verband spray, MS Schippers B.V., Bladel, The Netherlands). No pigs were removed from the experiment.

Statistical analysis. Statistical analyses was performed using SAS (SAS 9.3, Institute Inc.). Behavioural frequencies were averaged per pen and phase (grower and finisher) before analysis. Residuals of the response variables were checked for normality, and if needed square root transformed. All variables for behavioural frequencies were analyzed in a Mixed Model that included sanitary condition, dietary CP level, dietary AA profile, batch, phase, sanitary condition × dietary CP level, and sanitary conditions × dietary AA profile as fixed effects. Other interactions were deleted from the model when not significant. The effect of room nested within sanitary conditions was used as a random effect to correct for differences between rooms.

To analyze damage scores, the prevalence of damage was analyzed by expressing damage as a 0-1 variable (0 = no damage, 1 = damage) per pig per phase. The pen-averaged prevalences were analyzed per phase by a Mixed model that included sanitary condition, dietary CP level, dietary AA profile, batch, sanitary condition × dietary CP level, and sanitary conditions × dietary AA profile as fixed effects. Other interactions were deleted from the model when not significant. The effect of room nested within sanitary condition was used as a random effect to correct for differences between rooms. The prevalence of wounds was analyzed by expressing wounds as a 0-1 variable (0 = no wound, 1 = wound) per pig per phase and expressed as prevalence per pen per phase. The pen-averaged wound prevalences were analyzed per phase with the same model as the pen averaged damage prevalences. All values are presented as raw means ± standard error of the mean (SEM).

RESULTS

Behavioural observations. Oral manipulation of group mates occurred more often (4.1 ± 0.4 times per pig per hour) than chewing on enrichment object (1.0 ± 0.2) and aggression (2.4 ± 0.3). Oral manipulation was less directed at tails (0.3 ± 0.1) than ears (1.5 ± 0.2) or body parts other than tails or ears (2.3 ± 0.3). Frequency of belly nosing, was rather low (0.1 ± 0.1), and the same held for mounting (0.4 ± 0.1).

Frequency of total oral manipulation of pen mates (chewing or nibbling tail, ear or other body parts) was higher for LSC than for HSC pigs (4.5 vs. 3.8 times per pig per hour, $P \leq 0.05$, Table 3.2). Ear biting was affected by the interaction between sanitary conditions and dietary AA treatment ($P \leq 0.05$, Fig 3.2). Ear biting was more frequently recorded for LSC pigs fed AA-B diets compared with LSC pigs fed AA-S diets (1.9 vs. 1.5 times per hour), whereas , frequency of ear biting in HSC pigs was similar for pigs fed the AA-B diet and the AA-S diets (1.2 vs. 1.3 times per hour). For tail biting behaviour also an interaction between sanitary conditions and dietary AA level was found ($P \leq 0.05$). Tail biting was more

frequently recorded in HSC pigs fed AA-B diets compared with HSC pigs fed AA-S diets (0.4 ± 0.1 vs. 0.3 ± 0.1 times per hour), whereas dietary AA level did not affect frequency of tail biting in LSC pigs (0.4 ± 0.1 vs. 0.4 ± 0.1 times per hour). Sanitary conditions, dietary AA profile or their interaction did not affect chewing on an enrichment object or aggressive behaviour.

Apart from sanitary conditions and dietary AA profile, also dietary protein level affected the pigs' behaviour. The LP fed pigs showed higher frequencies of total oral manipulation of group mates (4.4 ± 0.4 vs. 3.8 ± 0.4 times per hour), ear biting (1.6 ± 0.2 vs. 1.3 ± 0.32 times per hour), manipulation of other body parts (2.5 ± 0.3 vs. 2.2 ± 0.3 times per hour), enrichment object biting (1.2 ± 0.2 vs. 0.8 ± 0.2 times per hour), total aggression (2.7 ± 0.3 vs. 2.1 ±0.3 times per hour), fighting (1.3 ± 0.2 vs. 0.9 ± 0.2 times per hour), belly nosing (0.2 ± 0.1 vs. 0.1 ± 0.0 times per hour) (All $P \leq 0.05$, Table 3.2, Fig. 3.1), and a tendency for higher frequencies of tail biting than the NP fed pigs (0.4 ± 0.1 vs. 0.3 ± 0.1 times per hour; $0.05 \leq P \leq 0.10$).

Pigs fed with the AA-B diet had higher frequencies of mounting than pigs fed the AA-S diet (0.4 ± 0.1 vs. 0.3 ± 0.1 times per hour, $P \leq 0.05$). Mounting was recorded more for LP than NP fed pigs in LSC (0.5 ± 0.1 vs. 0.4 ± 0.1 times per hour), but less for LP than NP fed pigs in HSC (0.3 ± 0.1 vs. 0.4 ± 0.1 times per hour) (sanitary conditions × dietary protein level interaction, $P \leq 0.05$).

Table 3.2. Frequencies of behaviours collected by behaviour sampling during the grower and finisher phase in pigs kept under low or high sanitary conditions, and provided with diets containing either low or normal protein levels and either a basal or a supplemented amino acid profile

	LSC[2]				HSC[2]									
	LP[3]		NP[3]		LP		NP		*p*-values[5]					
Behaviour[1]	AA-B[4]	AA-S[4]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SC	CP	AA	batch	SC×CP	SC×AA
n	8	8	8	8	8	8	8	8						
Oral manipulation of group mates	5.0 ± 0.4	4.5 ± 0.4	4.3 ± 0.4	4.2 ± 0.5	4.3 ± 0.3	4.0 ± 0.3	3.5 ± 0.4	3.3 ± 0.4	**0.02**	**0.002**	0.23	**0.0004**	0.54	0.89
Tail biting	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.59	0.06	0.23	0.09	0.58	**0.02**
Ear biting	2.0 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.0 ± 0.1	1.2 ± 0.2	**0.03**	**0.02**	0.38	0.95	0.99	**0.03**
Manipulating other	2.6 ± 0.3	2.5 ± 0.3	2.3 ± 0.2	2.4 ± 0.3	2.6 ± 0.2	2.3 ± 0.2	2.1 ± 0.3	1.9 ± 0.2	0.19	0.04	0.47	**<.0001**	0.30	0.41
Chewing enrichment object	1.3 ± 0.2	1.3 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	1.0 ± 0.2	1.2 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.54	**0.002**	0.99	0.48	0.59	0.87
Aggression	2.8 ± 0.3	2.8 ± 0.3	2.3 ± 0.3	2.1 ± 0.2	2.7 ± 0.3	2.3 ± 0.3	2.2 ± 0.3	1.6 ± 0.3	0.37	**0.001**	0.14	0.65	0.95	0.29
Fighting	1.4 ± 0.2	1.2 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.2 ± 0.2	0.7 ± 0.1	0.84	**0.001**	0.17	0.35	0.80	0.22
Feeder fighting	1.4 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	1.0 ± 0.2	0.9 ± 0.2	0.25	0.11	0.41	0.23	0.72	0.74
Belly nosing	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.39	**0.0003**	0.39	0.09	0.79	0.73
Mounting	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.54	0.59	**0.01**	0.16	**0.03**	0.64

[1]Behaviours are expressed in frequencies per pig per hour observed. In case of continuous behaviour a new bout was scored when behaviour was > 30 seconds. [2]LSC = low sanitary conditions, SC = high sanitary conditions. [3]LP = low dietary crude protein level, NP = normal dietary crude protein level. [4]AA-B = basal dietary amino acid profile, AA-S = supplemented dietary amino acid profile containing 20% more Met, Thr, and Trp compared with the basal profile. [5]*P*-values for manipulating other and belly nosing were based on square root transformed data. Significant *P*-values are indicated in bold and for a tendency values are underlined. Means are presented as raw means ± standard error. [6]n = number of pens, a pen contained nine pigs.

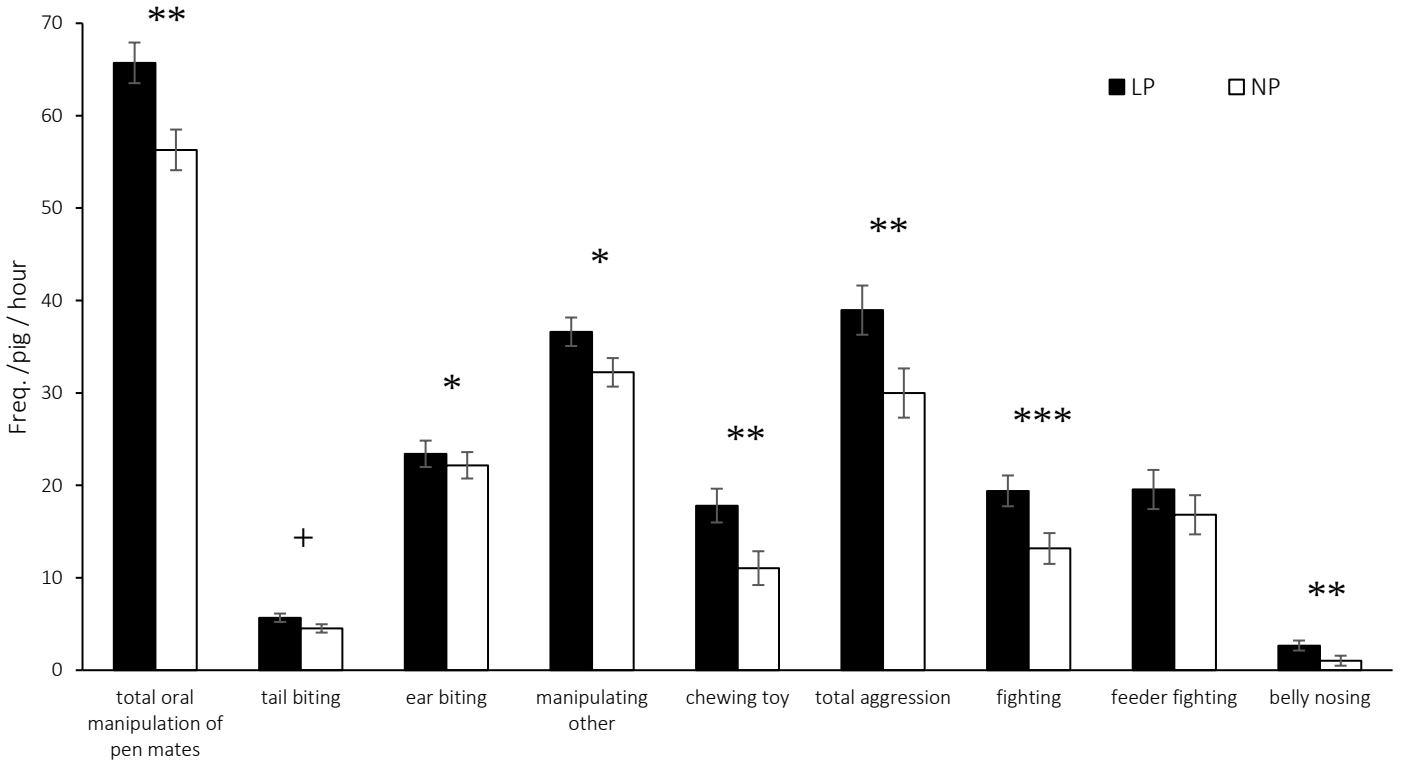

Figure 3.1. Behaviour of pigs during the grower and finisher phase for pigs fed diets with low (**LP**) or normal (**NP**) crude protein level. $^{+}$ $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

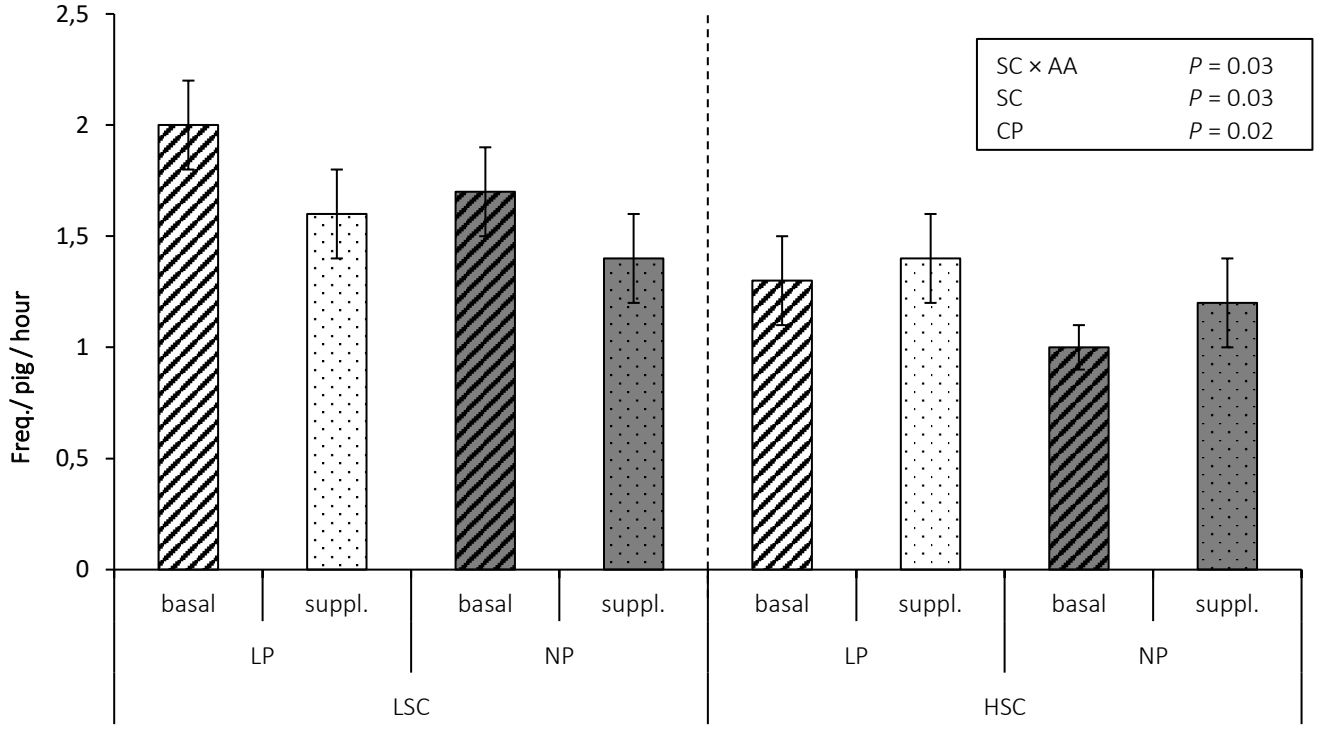

Figure 3.2. Frequency of ear biting in pigs kept under different sanitary conditions and fed a diet with either a low or normal dietary protein level and a basal or supplemented amino acid profile. Ear biting was recorded during live ad libitum sampling for 100 min per pen in total. The bars represent the raw means per treatment group ± standard error of the mean. LSC = low sanitary conditions, HSC = high sanitary conditions, LP = low dietary crude protein level (white bars). NP = normal dietary crude protein level (grey bars), AA-B = basal dietary amino acid profile (striped bars), AA-S = supplemented dietary amino acid profile containing 20% more Met, Thr, and Trp compared with the basal profile (dotted bars), SC = sanitary conditions, AA = amino acid profile.

Tail and ear damage scores. When considering the prevalence of tail damage as a binary score (tail damage vs. no tail damage), almost all of the pigs had tail lesions with a score higher than 1, i.e. at least bite marks, in at least one phase during the experiment. Furthermore, 39% of the pigs had a tail wound (score > 2), in at least one of the phases. These proportions were not affected by sanitary conditions or diet.

When averaging binary tail damage scores per pen and phase, the proportion of pigs with tail damage in LSC pens (0.80 ± 0.03) was higher than that in HSC pens (0.65 ± 0.03) in the starter phase ($P \leq 0.05$; Table 3.3). In the grower phase, the proportion of pigs with tail damage in LSC (0.81 ± 0.04) was lower than that in HSC (0.92 ± 0.02; $P \leq 0.05$, Table 3.3). An interaction for tail damage in the finisher phase was found for sanitary conditions (SC) × CP, $P \leq 0.05$; NP fed pigs in HSC (0.82 ± 0.04) had lower damage scores than LP fed pigs in HSC (0.92 ± 0.02), whereas this score was not different for pigs in LSC (0.91 ± 0.02 vs. 0.92 ± 0.02).

In total, 78% of LSC and 58% of HSC pigs had at least in one phase an ear damage score higher than 1 ($P \leq 0.05$). When averaging binary ear damage scores per pen and phase, the proportion of animals with ear damage was higher in pens fed AA-B diets (0.37 ± 0.04) compared to those fed AA-S diets (0.25 ± 0.03) in the grower phase ($P \leq 0.05$). The proportion of pigs with ear damage in the grower phase tended to be higher for LSC (0.33 ± 0.04) than for HSC pigs (0.28 ± 0.04). For the finisher phase the proportion of ear damage was higher for LSC (0.92 ± 0.02) than for HSC pigs (0.87 ± 0.02; $P \leq 0.05$). The distribution of LSC and HSC pigs over tail and ear damage scores are presented in Supplementary Table II and III, respectively.

When considering the prevalence of tail wounds as a binary score (tail wound vs. no tail wound), in LSC a lower proportion of pigs had tail wounds (0.13 ± 0.02) than in HSC (0.22 ± 0.03) in the grower phase ($P \leq 0.05$; Table 3.4). The LP diet tended to result in a lower proportion of pigs with tail wounds (0.32 ± 0.04) compared with the NP diet (0.38 ± 0.04), in the grower phase ($0.05 \leq P \leq 0.10$). Proportion of pigs with a tail wound tended to be lower for the AA-B diet (0.20 ± 0.03) than for the AA-S diet (0.14 ± 0.02) in the grower phase ($0.05 \leq P \leq 0.10$). In the finisher phase, an interaction was found for SC × CP for proportion of pigs with tail wounds, in LSC a lower proportion of pigs with wounds was found when fed a LP diet (0.17 ± 0.03) instead of a NP diet (0.24 ± 0.03), whereas in HSC a higher proportion of pigs had tail wounds when fed a LP diet (0.22 ± 0.04) instead of a NP diet (0.15 ± 0.04) in the grower phase ($P \leq 0.05$).

When averaging binary ear wound scores (scores > 3 for ear wounds vs. scores < 4) per pen and phase, many pens had a score 0 (no wound). Therefore no statistically test was performed on ear wound scores. The raw means are presented in Table 3.4.

Table 3.3. Proportion of pigs (based on pen averages) with tail damage (score > 1) and ear damage (score > 1) observed for each treatment group: low and high sanitary condition pigs both with diets containing either low or normal protein levels and basal or supplemented amino acid profiles

	LSC[1]				HSC[1]									
	LP[2]		NP[2]		LP		NP		*P*-values[4]					
Damage score[5]	AA-B[3]	AA-S[3]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SC	CP	AA	batch	SC×AA	SC×CP
n[6]	8	8	8	8	8	8	8	8						
Tail														
starter	0.80 ± 0.08	0.82 ± 0.04	0.76 ± 0.04	0.83 ± 0.08	0.64 ± 0.06	0.65 ± 0.06	0.78 ± 0.07	0.54 ± 0.04	**<.0001**	0.98	0.45	**0.002**	0.06	0.70
grower	0.83 ± 0.07	0.75 ± 0.09	0.78 ± 0.08	0.88 ± 0.06	0.95 ± 0.03	0.94 ± 0.02	0.94 ± 0.04	0.85 ± 0.06	**<.0001**	0.76	0.52	0.47	0.37	0.21
finisher	0.92 ± 0.03	0.91 ± 0.04	0.93 ± 0.03	0.91 ± 0.04	0.95 ± 0.03	0.88 ± 0.03	0.81 ± 0.06	0.84 ± 0.04	0.68	0.07	0.54	**<.0001**	0.88	**0.04**
Ear														
starter	0.52 ± 0.05	0.41 ± 0.08	0.30 ± 0.07	0.45 ± 0.06	0.47 ± 0.12	0.36 ± 0.08	0.43 ± 0.06	0.28 ± 0.06	0.50	0.13	0.30	**0.02**	0.17	0.78
grower	0.34 ± 0.12	0.32 ± 0.06	0.37 ± 0.05	0.31 ± 0.07	0.42 ± 0.10	0.20 ± 0.05	0.34 ± 0.08	0.16 ± 0.05	<u>0.10</u>	0.63	**0.02**	0.86	0.11	0.53
finisher	0.53 ± 0.08	0.40 ± 0.06	0.51 ± 0.10	0.45 ± 0.09	0.31 ± 0.12	0.29 ± 0.05	0.32 ± 0.07	0.35 ± 0.12	**<.0001**	0.62	0.32	0.76	0.31	0.90

[1]LSC = low sanitary conditions, HSC = high sanitary conditions. [2]LP = low crude protein level in diet, NP = normal crude protein level in diet. [3]AA-B = basal dietary amino acid profile, AA-S = supplemented dietary amino acid profile containing 20% more Met, Thr, and Trp than the basal profile. [4] Significant *P*-values are indicated in bold and for a tendency values are underlined. Means are presented as raw means ± standard error of the mean. [5]Damage scores were either 0 = no damage or 1= damage and were averaged per pen. [6]n = number of pens, a pen contained nine pigs.

Table 3.4. Proportion of pigs (based on pen averages) with tail wounds (score > 2) and ear wounds (score > 3) observed for each treatment group: low and high sanitary condition pigs both with diets containing either low or normal protein levels and basal or supplemented amino acid profiles

	LSC[1]				HSC[1]									
	LP[2]		NP[2]		LP		NP		*P*-values[4]					
Wound score[5]	AA-B[3]	AA-S[3]	AA-B	AA-S	AA-B	AA-S	AA-B	AA-S	SC	CP	AA	batch	SC×AA	SC×CP
n[6]	8	8	8	8	8	8	8	8						
Tail														
Starter	0.08 ± 0.03	0.07 ± 0.03	0.11 ± 0.04	0.16 ± 0.05	0.07 ± 0.04	0.13 ± 0.05	0.13 ± 0.04	0.10 ± 0.03	0.10	0.17	0.50	0.43	0.99	0.38
Grower	0.15 ± 0.04	0.04 ± 0.02	0.22 ± 0.06	0.10 ± 0.03	0.18 ± 0.06	0.21 ± 0.06	0.27 ± 0.08	0.22 ± 0.05	**0.007**	0.10	0.09	**0.0003**	0.14	0.80
Finisher	0.18 ± 0.03	0.15 ± 0.04	0.25 ± 0.05	0.23 ± 0.06	0.18 ± 0.06	0.26 ± 0.04	0.17 ± 0.06	0.12 ± 0.06	0.82	0.94	0.84	0.77	0.56	**0.04**
Ear														
Starter	0.05 ± 0.03	0.04 ± 0.04	0.00 ± 0	0.06 ± 0.03	0.06 ± 0.03	0.01 ± 0.01	0.00 ± 0	0.00 ± 0						
grower	0.05 ± 0.05	0.00 ± 0	0.00 ± 0	0.05 ± 0.03	0.17 ± 0.08	0.07 ± 0.03	0.00 ± 0	0.00 ± 0						
finisher	0.06 ± 0.06	0.02 ± 0.02	0.00 ± 0	0.08 ± 0.04	0.08 ± 0.04	0.05 ± 0.02	0.00 ± 0	0.00 ± 0						

[1]LSC = low sanitary conditions, HSC = high sanitary conditions. [2]LP = low crude protein level in diet, NP = normal crude protein level in diet. [3]AA-B = basal dietary amino acid profile, AA-S = supplemented dietary amino acid profile containing 20% more Met, Thr, and Trp than the basal profile. [4] Significant *P*-values are indicated in bold and for a tendency values are underlined. Means are presented as raw means ± standard error of the mean. For ear wounds no statistically test was done, as there were too many pens without wounds. [5]Wound scores were either 0 = no wound or 1= wound and were averaged per pen. [6]n = number of pens, a pen contained nine pigs.

DISCUSSION

In this study we assessed the influence of sanitary conditions, dietary protein concentration, and dietary AA profile on behaviour of pigs kept under practical conditions. To the best of our knowledge, this is the first study on damaging behaviour in pigs that are experimentally exposed to diverging sanitary conditions and receive different dietary treatments in a commercial farm setting. We demonstrate evidence for a causal relationship between sanitary conditions and damaging behaviour, indicating that biting behaviour, particularly ear biting in pigs is linked to health status in a diet-dependent manner. In addition, low dietary protein levels clearly increased the frequencies of both damaging biting behaviour and aggression. Dietary supplementation of Thr, Met and Trp modified these behavioural responses in low sanitary conditions pigs or low dietary protein fed pigs to some extent.

In our study ear biting and manipulation of body parts other than ears and tails were scored more frequently than tail biting. Given the frequent occurrence of tail wounds, tail docking was applied on the experimental farm. This is still common practice in the Netherlands and many other countries. It is known that ear biting occurs more frequently on farms with docked tails, likely because docked tails are not the most attractive body part to bite in (Goossens et al., 2007). Also in studies on pigs with intact (Camerlink et al., 2015) and half-docked tails (Ursinus et al., 2014b), ear biting and manipulation of other body parts was observed more frequently than tail biting. Oral manipulation of pen mates occurred far more frequent than biting of the enrichment object provided, probably because body parts of conspecifics better meet the requirements of pigs for suitable chewing and rooting materials (Van de Weerd et al., 2003), and more pen mates than enrichment objects were available.
Tail docking, i.e. amputation of the tail or part of it in young pigs, is still done in several EU countries to prevent tail damage at later age (Sutherland et al., 2011). The underlying causes of tail biting are, however, not removed by this practice, and tail wounds remain a problem (Valros and Heinonen, 2015). Our results, with almost all of pigs showing at least bite marks and 39% having a small or large tail wound in one of the production phases, confirm that tail docking is no guarantee for the absence of tail biting. This is line with other studies. For instance, in a study with Irish slaughter pigs, 99% of the studied pigs were tail docked and 72.5% of these pigs had tail lesions at slaughter (Harley et al., 2014). In an observation study with pigs with half-docked tails (Ursinus et al., 2014b) frequency of ear biting (1.7 ± 0.1 per hour) in pigs was higher than tail biting (0.6 ± 0.1 per hour), as was the case in our study. Overall, the percentage of pigs with tail lesions was higher than the percentage of pigs with ear lesions, suggesting that, in spite of its relatively lower frequency, tail biting was more severe than ear damage in our study.

Damaging behaviour and interactions with sanitary conditions and diet. Pigs in LSC showed more ear biting (+39%) compared with HSC pigs. Dietary supplementation with extra Met, Thr, and Trp reduced the frequency of ear biting (-16%) only in LSC pigs. The proportion of pigs with ear damage was higher in LSC than in HSC during the grower (tendency) and finisher phase. Similarly, proportions of pigs with tail damage was higher in LSC during the starter phase, although the opposite was found for proportion of pigs with tail damage or wounds in the grower phase. The LSC pigs had higher haptoglobin concentrations in blood and higher pleuritis scores for lungs at slaughter than HSC pigs (van der Meer et al., 2016), confirming a difference in health status. The impact of LSC on both damaging behaviours and the lesions and wounds they induce suggests a causal relationship between poor health and the occurrence of behavioural problems.

The increased propensity of pigs with a poor health status to start biting their pen mates could be related to increased immune system activation. In line with this, in laying hens the stimulation of immune reactivity, by intra-tracheal exposure to human serum albumin, increased damaging feather pecking (Parmentier et al., 2009), a redirected foraging behaviour showing a strong similarity to tail biting in pigs (Rodenburg and Koene, 2007). Immune stimulation may lead to a change in the animal's nutrient requirements, particularly also for AA. Recent studies have shown indeed that the requirements for Trp (Melchior et al., 2004; Le Floc'h et al., 2008; Rakhshandeh et al., 2010), Met + cysteine, (Rakhshandeh et al., 2010) and Thr (Li et al., 2007; Ren et al., 2014) are increased in case of immune system stimulation. Increased immune system activity in growing animals may redirect nutrients from growth to other processes that require AA. Indeed the LSC pigs in our study showed a lower body weight gain than the HSC pigs (van der Meer et al., 2016). Notably, and unlike in most studies using model agents like lipopolysaccharide (LPS) (Rakhshandeh et al., 2010; de Ridder et al., 2012; Kim et al., 2012), Complete Freund's Adjuvant (CFA) (Le Floc'h et al., 2012; Kampman-van de Hoek et al., 2015), to stimulate the immune system, contrasts in sanitary status in this experiment occurred strictly without pigs showing signs of clinical illness. Pigs with suboptimal health may thus need more AA to support the immune system whilst maintaining growth. Indeed, particularly in the pigs kept under poor sanitary conditions, the diets with supplemented Met, Thr and Trp led to higher gain-to-feed ratios and a higher body weight gain (van der Meer et al., 2016). If particular AA are limiting for optimal body functioning, pigs might intensify their chewing and rooting behaviour to satisfy their nutritional needs, and, under commercial conditions, direct part of their oral behaviours towards pen mates. This is confirmed by the lower frequency of ear biting recorded for AA-S compared with AA-B fed pigs in LSC. The frequency of tail biting and the ear and tail damage scores in LSC pigs were not always reduced by the supplementary AA diet. The former suggests that the link between health status, AA availability in metabolism, and damaging behaviour is complex and possibly influenced by multiple mechanisms.

An imbalance in AA might also influence behaviour by altering brain neurotransmitter metabolism. Several brain neurotransmitters are synthesized from AA (Anderson, 1979; Massey et al., 1998). Threonine is a precursor of brain glycine (Castagné et al., 1993), Met can be used as a donor of methyl groups used for synthesis of many substrates such as choline (Burke et al., 1951; Rodwell, 2003), which is an acetylcholine precursor (Purves et al., 2001), and Trp is a precursor of serotonin (5-hydroxytryptamine; 5-HT; Rodwell, 2003), a neurotransmitter which is known to affect behaviour and emotional state. Aggressive behaviour and stress have been shown to be related to the supply of dietary Trp and/or brain 5-HT in several animal and human studies (Gibbons et al., 1979; Chamberlain et al., 1987; Salomon et al., 1994; Winberg et al., 2001; Lepage et al., 2002), including studies on pigs (Koopmans et al., 2005; Edwards, 2006). Ursinus et al. (2014a) recently found that pigs that perform tail biting behaviour had lowered blood 5-HT levels, and, in line with this, feather pecking chickens also showed altered peripheral (Bolhuis et al., 2009) and central 5-HT (Kops et al., 2013; Kops et al., 2014) metabolism. This could either be due to a direct effect of 5-HT on damaging behaviour demonstrated experimentally for chickens only (van Hierden et al., 2004), or reflect lowered AA availability for neurotransmitter synthesis, either or not caused by a lowered health status.
As Trp competes with other large neutral AA (LNAA) for passage over the blood brain barrier, the Trp: LNAA ratio in blood is a major determinant of brain 5-HT concentration (Wurtman and Fernstrom, 1975). Despite that there were differences in Trp: LNAA ratio between the AA-B and AA-S diets, no clear effects of dietary AA profile on aggression and on some of the damaging behaviours were found. In contrast, supplementation of AA in the AA-S diet resulted in lower recorded frequencies of mounting than for pigs fed the AA-B diet. It is not really clear via which mechanisms dietary AA provision affects mounting behaviour.

Apart from pigs in poor health being more likely to start biting, as indicated also affected by the dietary AA supply, the health-biting relationship (Moinard et al., 2003b; Kritas and Morrison, 2007; Marques et al., 2012) can be fortified by the poor ability of ill pigs to avoid being bitten (Taylor et al., 2010). Finally, it can be speculated that immune activation in wounded pigs (e.g. Heinonen et al., 2010; Munsterhjelm et al., 2013), in turn, increases the probability of these pigs to start biting as well, which consequently leads to an escalation of tail or ear biting in the entire group.

Dietary protein reduction: a risk for damaging behaviour? Almost all damaging and aggressive behaviours recorded were more frequently scored for pigs fed the LP diet, considered to be dietary deficient in essential AA, than for those fed the NP diet, demonstrating a clear and direct effect of dietary protein level on damaging behaviour. In addition, a higher proportion of pigs with tail damage and tail wounds was found in HSC in the finisher phase when pigs were fed a LP diet. In a study of McIntyre and Edwards (2002)

pigs had a higher preference for a wet blood-soaked tail model when fed a low protein diet (98 g CP/kg) compared to their preference when fed a control diet (189 g CP/kg). These results suggest that a diet deficient in essential AA increases preference for blood. However, they used different protein sources in both diets (McIntyre and Edwards, 2002). Therefore an effect of protein source rather than CP level alone cannot be excluded in their study. In our study, however, the same protein sources at a different inclusion level were used in the respective diets. As average daily feed intake was not significantly different between LP an NP diets, we conclude that a the difference in AA uptake lead to more oral manipulation of pen mates and more aggression.

The LP diets might have led to a shortage or imbalance of AA resulting in restless pigs searching for missing nutrients. It might be that some brain neurotransmitters cannot be synthesized from AA derived from dietary protein as was stated by Harper and Peters (1989), simply because insufficient precursors were available. Nutritional imbalance, probably due to an overall shortage in AA in this case, can increase foraging activity and redirected, damaging behaviours.

Apart from the effect on damaging behaviour, an overall shortage of the dietary supply of AA might also be responsible for the increase in aggressive behaviour in pigs fed the LP diets. The LP diets, regardless of the AA profile, were diets with a lower Trp concentration compared with the NP diets. A low Trp concentration in the brain, due to a low concentration of Trp in the diet, might affect brain serotonin metabolism resulting in more aggressive behaviour as found by Martinez-Trejo et al. (2009), or might affect aggressive behaviour via other hormones such as cortisol or noradrenaline as found by Koopmans et al. (2005). Also the effect of AA concentration on blood insulin concentration might have played a role, as blood insulin is known to play a role in Trp uptake in the brain as well (Kritas and Morrison, 2007).

Alternatively, Fraser et al. (1991) suggested that the effect of deficient dietary essential AA level on biting behaviour is indirectly caused by a lower growth rate of these animals, rather than by the dietary composition as such. In support of this, McIntyre and Edwards (2002) found a tendency for a correlation between body weight gain and preference for a blood tail model vs. water tail model. Also Larsen (1983) found that tail biting pigs often were slower growing animals. In contrast, Ursinus et al. (2014b) reported a higher phenotypic and genotypic growth in fanatic tail biters. These authors suggested that fast growing pigs and pigs with a low growth rate due to health problems may have a high metabolic demand for protein synthesis for muscle tissue or immune processes in common, which could explain their increased biting tendencies. A larger variation in size of animals within the same pen has also been reported to negatively influence biting behaviour (Taylor et al., 2010). When calculating the standard error for body weight per pen in our study the standard error was higher for LSC than HSC animals, which means that

variation in weight per pen was higher in LSC animals. When calculating this standard error for body weight with the measured behaviours, no clear correlations were found.
Even if growth rate is related to biting behaviour, more research is needed to determine cause and effect, and to study the impact of a potential shared underlying factor, such as, for instance, an actual status or history of poor health. A low protein diet with deficiencies in essential AA should thus be considered as a risk factor for biting behaviour and aggression in pigs, which can only partly be counteracted by supplementation with specific essential AA, such as Met, Thr and Trp.

In conclusion, this study shows a causal link between sanitary conditions, dietary CP level and damaging behaviours. Both LSC and a reduction of dietary CP level, increase the occurrence of damaging behaviours in pigs and therefore may negatively impact pig welfare. These effects could only partly be counteracted by supplementation with specific essential AA that are known to be increasingly required in case of immune system activation.
Tail and ear biting clearly are multifactorial problems and combating these requires a multidisciplinary effort. This study demonstrates that care should be taken in reducing dietary protein concentrations to improve protein efficiency in pigs, even when maintaining the ratio of the most important essential AA relative to energy. It incurs a risk to increased damaging behaviours, particularly when pigs are kept under poor sanitary conditions.

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SUPPLEMENTAL TABLES

Supplemental table I Ingredient and nutrient composition of the starter diets

	LP[1]		NP[1]	
Item	AA-B[2]	AA-S[2]	AA-B	AA-S
Ingredient, g/kg of feed				
Maize	320.00	320.00	400.00	400.00
Soybean meal	182.02	182.00	227.54	227.54
Barley	160.00	160.00	200.00	200.00
Wheat	45.53	45.53	56.91	56.91
Maize starch	206.79	204.64	40.65	37.90
Sugarcane molasses	20.00	20.00	20.00	20.00
Limestone	13.94	13.94	14.11	14.11
Monocalcium phosphate	9.99	9.99	8.93	8.93
Soybean oil	10.65	10.98	15.98	16.38
Vitamin + mineral mix[3]	5.00	5.00	5.00	5.00
Salt	3.19	3.19	3.83	3.83
L-lysine HCl	1.95	1.94	2.35	2.35
Titanium dioxide	2.50	2.50	2.50	2.50
Sodium bicarbonate	2.58	2.58	1.34	1.34
L-threonine	0.60	1.46	0.63	1.71
L-tryptophan	0.03	0.31	0.00	0.37
DL-methionine	0.23	0.94	0.23	1.13
Cellulose[4]	15.00	15.00	0.00	0.00
Nutrients calculated, g/kg				
NE, MJ/kg[5]	9.80	9.80	9.80	9.80
DM	889.6	889.8	893.2	884.9
Crude protein	138.0	136.0	168.0	167.0
Starch[4]	474.1	472.3	410.0	407.7
Lys[6]	8.60	8.60	10.50	10.50
Thr[6]	5.40	6.20	6.60	7.40
Trp[6]	1.70	1.90	2.00	2.30
Met + Cys[6]	4.30	4.80	5.20	5.90
Ile[6]	5.60	5.60	6.90	6.80
Arg[6]	8.30	8.40	10.50	10.30
Phe[6]	6.60	6.60	8.20	8.10
His[6]	3.40	3.40	4.20	4.20
Leu[6]	10.9	11.00	13.60	13.40
Tyr[6]	4.40	4.50	5.70	5.70
Val[6]	6.40	6.40	7.90	7.70

[1] LP = low CP concentration diet, NP = normal CP concentration diet.

[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile.

[3] Supplied the following per kilopgram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[4] Opticell (Agromed Austri GmbH, Kremsmünster, Austria).

[5] Based on chemical composition, digestibility, and energy value for pigs from the Centraal Veevoeder Bureau livestock feed table (CVB, 2011).

[6] Analyzed values.

Supplemental table II Ingredient and nutrient composition of the grower diets

	LP[1]		NP[1]	
Item	AA-B[2]	AA-S[2]	AA-B	AA-S
Ingredient, g/kg of feed				
Maize	400.00	400.00	500.00	500.00
Soybean meal	138.15	138.15	172.69	172.69
Barley	171.36	171.36	214.19	214.19
Maize starch	199.19	197.24	43.34	40.86
Wheat	20.00	20.00	20.00	20.00
Sugarcane molasses	13.11	13.11	13.33	13.33
Limestone	8.62	8.62	7.67	7.67
Monocalcium phosphate	9.19	9.48	10.20	10.57
Soybean oil	5.00	5.00	5.00	5.00
Vitamin + mineral mix[3]	3.95	3.95	3.61	3.61
Salt	2.45	2.45	2.92	2.92
L-lysine HCl	2.50	2.50	2.50	2.50
Titanium dioxide	1.56	1.56	2.00	2.00
Sodium bicarbonate	3.87	3.87	1.52	1.52
L-threonine	0.72	1.49	0.74	1.72
L-tryptophan	0.21	0.46	0.22	0.55
DL-methionine	0.12	0.76	0.07	0.87
Cellulose[4]	20.00	20.00	0.00	0.00
Nutrients calculated, g/kg				
NE, MJ/kg[5]	9.84	9.84	9.84	9.84
DM	883.7	885.9	882.8	887.7
Crude protein	124.0	124.0	152.0	152.0
Starch[5]	497.1	495.5	448.9	446.9
Lys[6]	8.00	7.90	9.70	10.00
Thr[6]	5.10	5.90	5.90	7.00
Trp[6]	1.56	1.75	1.91	2.16
Met + Cys[6]	3.98	4.52	4.76	5.58
Ile[6]	4.80	4.70	5.90	5.80
Arg[6]	7.20	7.00	9.00	9.00
Phe[6]	5.80	5.70	7.10	7.10
His[6]	3.00	3.00	3.70	3.60
Leu[6]	10.0	9.90	12.3	12.3
Tyr[6]	3.80	3.90	5.00	5.00
Val[6]	5.70	5.80	6.90	6.90

[1] LP = low CP concentration diet, NP = normal CP concentration diet.

[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile.

[3] Supplied the following per kilogram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[4] Opticell (Agromed Austri GmbH, Kremsmünster, Austria).

[5] Based on chemical composition, digestibility, and energy value for pigs from the Centraal Veevoeder Bureau livestock feed table (CVB, 2011).

[6] Analyzed values.

Supplemental table III Ingredient and nutrient composition of the finisher diets

	LP[1]		NP[1]	
Item	AA-B[2]	AA-S[2]	AA-B	AA-S
Ingredient, g/kg of feed				
Maize	360.10	360.10	450.10	450.10
Soybean meal	115.70	115.70	144.60	144.60
Barley	240.00	240.00	300.00	300.00
Maize starch	187.20	185.30	36.40	34.10
Wheat	20.00	20.00	20.00	20.00
Sugarcane molasses	12.20	12.20	12.50	12.50
Limestone	7.40	7.40	6.50	6.50
Monocalcium phosphate	14.00	14.30	13.20	13.60
Soybean oil	5.00	5.00	5.00	5.00
Vitamin + mineral mix[3]	2.30	2.30	2.60	2.60
Salt	2.40	2.40	2.80	2.80
L-lysine HCl	2.50	2.50	2.50	2.50
Titanium dioxide	5.30	5.30	3.00	3.00
Sodium bicarbonate	0.00	0.60	0.00	0.70
L-threonine	0.70	1.40	0.70	1.60
L-tryptophan	0.10	0.40	0.10	0.40
DL-methionine	0.10	0.10	0.00	0.00
Cellulose[4]	25.00	25.00	0.00	0.00
Nutrients calculated, g/kg				
NE, MJ/kg[5]	9.84	9.84	9.84	9.84
DM	885.9	886.5	881.6	887.7
Crude protein	132.0	126.0	151.0	148.0
Starch[5]	541.7	509.4	541.7	540.2
Lys[6]	8.00	7.60	8.90	8.90
Thr[6]	5.30	5.60	5.90	6.50
Trp[6]	1.51	1.62	1.68	1.91
Met + Cys[6]	4.03	4.22	4.68	5.04
Ile[6]	4.90	4.60	5.70	5.60
Arg[6]	7.30	6.80	8.20	8.20
Phe[6]	6.00	5.60	6.80	6.80
His[6]	3.10	2.90	3.50	3.50
Leu[6]	10.10	9.60	11.90	11.30
Tyr[6]	4.00	3.70	4.70	4.70
Val[6]	6.00	5.70	6.60	6.60

[1] LP = low CP concentration diet, NP = normal CP concentration diet.

[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile.

[3] Supplied the following per kilogram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[4] Opticell (Agromed Austri GmbH, Kremsmünster, Austria).

[5] Based on chemical composition, digestibility, and energy value for pigs from the Centraal Veevoeder Bureau livestock feed table (CVB, 2011).

[6] Analyzed values.

Supplemental table IV. Formulated dietary amino acid profiles relative to lysine on apparent ileal digestible level, fed in combination with different dietary protein levels to pigs kept under different sanitary conditions

Amino acid	AA-B profile[1]	AA-S profile[1]
Lysine	100	100
Methionine + cysteine	51	61
Threonine	59	71
Tryptophan	18	22
Arginine	87	87
Histidine	43	43
Isoleucine	53	53
Leucine	101	101
Phenylalanine	54	54
Valine	69	69

[1] AA-B = basal dietary amino acid profile ; AA-S profile = supplemented dietary amino acid profile with 20% extra methionine, threonine, and tryptophan than basal profile.

Supplemental table V. The percentage of pigs in different tail damage score categories from observations taken across all phases for low and high sanitary conditions

Score	LSC	HSC
1	15.6	18.5
2	69.4	64.6
3	14.5	16.5
4	0.5	0.5
5	0.0	0.0

Score 1: No tail damage; 2: Bite marks; 3: Small wound; 4: Medium wound, part of tail missing; and 5: Severe wound, no tail is left. LSC: low sanitary condition pigs, HSC: high sanitary condition pigs.

Supplemental table VI The percentage of pigs in different ear damage score categories from observations taken across all phases for low and high sanitary conditions

Score	LSC	HSC
1	73.4	77.4
2	20.8	15.6
3	1.3	1.4
4	0.1	0.8
5	4.4	4.7

Score 1: No ear damage, 2: Top or bottom lesions, 3: Top and bottom lesions, 4: Severe damage, part of the ear is missing, and 5: ear necrosis. LSC: low sanitary condition pigs, HSC: high sanitary condition pigs.

Chapter 4

Low sanitary conditions increase maintenance energy expenditure and decrease incremental protein efficiency in growing pigs

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ABSTRACT

Background. Requirements for energy as well as for some essential amino acids (**EAA**) are known to be influenced by the nature and extent of immune system stimulation. Most studies on this topic use immune challenge models mimicking clinical conditions, making results of such studies difficult to extrapolate to practical conditions, where subclinical infections prevail. Therefore, a contrast in sanitary conditions was applied to study effects of chronic, low grade immune system activation on nutrient partitioning.

Objective. To quantify the difference in energy requirements for maintenance, and in incremental efficiencies for deposition of dietary energy and protein in the body of clinically healthy pigs kept under low or high sanitary conditions, fed a basal diet either or not supplemented with additional methionine (**Met**), threonine (**Thr**) and tryptophan (**Trp**).

Methods. In a 2 × 2 factorial arrangement, 24 groups of 6 pigs each were allocated to either a low sanitary condition (**LSC**) or high sanitary condition (**HSC**), and were offered two different diets having either a basal or a dietary AA profile supplemented with Met, Thr and Trp. For each group of pigs, complete energy and nitrogen balances were determined during two consecutive weeks, during which feed was available ad libitum or at 70% of ad libitum. Following the second week, the fasting heat production (**FHP**) was determined after a 25 h period.

Results. Low SC conditions increased FHP from 696 to 750 kJ/(kg $BW^{0.6}$. d), regardless AA supplementation. The incremental efficiency of ingested N for retention was reduced in LSC pigs from 73 to 53 %, but incremental efficiencies of DE for fat deposition were unaffected by the experimental treatments.

Conclusions. Chronic, low grade immune system stimulation by imposing low sanitary conditions to pigs increases FHP. Under such conditions, the incremental efficiency of N utilization for body protein deposition is reduced, but the incremental efficiency of absorbed energy for energy or fat deposition are unaffected. Supplementation of Met, Thr and Trp did not affect any of the incremental efficiencies measured.

Keywords: efficiency, pig, energy, sanitary conditions, health, amino acid

INTRODUCTION

For developing concepts in clinical nutrition of animals, changes in protein and energy metabolism are often studied using models, such as *i.m.* or *i.p.* administered lipopolysaccharide (LPS) (e.g. (Rakhshandeh et al., 2010; de Ridder et al., 2012; Kim et al., 2012), or *s.c.* Complete Freund's Adjuvant (CFA) (Melchior et al., 2004; Le Floc'h et al., 2008; Kampman-van de Hoek et al., 2015) provoking clinical symptoms. It is questionable whether results obtained from such models apply to conditions of low grade, chronic immune system stimulation (ISS) as often encountered in animal production systems. Under subclinical health conditions, quantifying alterations in protein/AA and energy requirements are important for developing targeted nutritional strategies.

Clinical ISS has been shown to increase the metabolism and requirements of some essential amino acids (EAA) such as methionine (Met) + cysteine (Rakhshandeh et al., 2010; Litvak et al., 2013), and tryptophan (Trp) (Melchior et al., 2004; Le Floc'h et al., 2008; Le Floc'h et al., 2012) relative to lysine. In a previous study (van der Meer et al., 2016), 20% extra dietary supplementation of Met, threonine (Thr), and Trp relative to lysine (Lys) resulted in increased growth and G:F ratio for pig kept under either low or high sanitary conditions. The beneficial effect, however, was greater for LSC pigs, indicating that low grade, chronic ISS increases the requirements for these AA.

Apart from AA metabolism, energy metabolism is also found to be influenced during ISS (Benson et al., 1993; Humphrey and Klasing, 2004). Responses such as fever and acute phase protein production in response to ISS consume energy (Lochmiller and Deerenberg, 2000; Parmentier et al., 2002). For example, Lochmiller and Deerenburg (2000) described that stimulated macrophages, like other immune cells, use energy to a level comparable to a maximally functioning heart muscle. Immune challenges in pigs often result in decreased average daily gain (ADG) for ISS treatment groups which cannot be completely accounted for by a related decreased average daily feed intake (ADFI), as reported in a meta-analysis by Pastorelli et al. (2012b). They estimated a 7% reduction of feed efficiency for respiratory diseases, 12% for poor housing conditions, and 30% for bacterial infections of the gastro-intestinal tract.

The change in energy efficiency during ISS might be due to an increased energy expenditure for the immune system, reflected in an increase in the resting or fasting heat production (FHP). Furthermore an the priority for fat deposition during ISS can be altered as the consequence of a decreased priority for protein deposition (Lochmiller and Deerenberg, 2000). Alternatively, it may be beneficial for the animal to have increased body fat stores, preparing for increased requirements for energy for the future under such conditions. For example, Parmentier et al. (2002) found an increased fat, but not protein deposition in chicken exposed to a repeated LPS challenge compared with unchallenged chickens.

The effect of ISS on protein and fat deposition is the final result of altered requirements for the use of nutrients for maintenance purposes and of changes in responses of nutrient deposition to incremental intake of protein or energy via the diet. Hence, studying relationships between ISS and nutrient partitioning requires estimates for nutrient requirements for maintenance and values for incremental efficiencies of energy and protein deposition in the body.

In this study, we measured incremental efficiencies for deposition of dietary energy and protein in the body of clinically healthy pigs kept under low and high sanitary conditions, fed a basal diet either or not supplemented with additional MET, Thr, and Trp in ratio to Lys, and measured FHP as a proxy for maintenance energy expenditure. Within-day patterns of heat production and carbohydrate and fat oxidation are reported elsewhere.

MATERIALS AND METHODS

The present study was carried out at the experimental facilities of Wageningen University, the Netherlands. The experimental protocol was approved by the Animal Care and Use Committee of Wageningen University, The Netherlands.

Experimental design. In a 2 × 2 factorial arrangement, 24 groups of 6 pigs each were allocated to either a low (**LSC**) or high sanitary condition (**HSC**), and were offered one of two diets having either a basal (**AA-B**) or supplemented dietary AA profile (**AA-S**). Pigs were used from week 3 till week 12 of age. From week 3 until 8, pigs were adapted to the different sanitary conditions, after which the dietary treatments were introduced. Energy balances studies were performed from week 9 to 12. During week 9, pigs were allowed ad libitum access to their diets. During week 10, pigs were fed twice daily at 70% of the measured feed intake during week 9. During week 12, pigs were allowed restricted access to their diets for 2d, prior to a 25h period of feed deprivation, for the measurement of FHP. These three experimental weeks are referred to as the ad lib feeding period, the restricted feeding period and the fasting period.

Animals and treatments. In total, 144 (Topigs 20 × Danish Duroc; Topigs, Helvoirt, The Netherlands; Danbred International, Herlev, Denmark) newborn female piglets were selected at one week of age in three subsequent batches of 48 pigs on a commercial farm in The Netherlands, and allocated to either the LSC or HSC treatment. Per farrowing room, half of the female piglets were selected for LSC and the other half for HSC treatment. Only HSC piglets received vaccinations during the period of the first nine weeks of age. At one week of age, HSC piglets were vaccinated against Mycoplasma hyopneumoniae (Porcilis M Hyo, MSD Animal Health, Boxmeer, The Netherlands by subcutaneous injection in the neck. Piglets of both LSC and HSC treatments were housed in the same rooms until weaning at 21 ± 0.2 d of age.

At weaning (at 3 weeks of age) LSC and HSC piglets were transported to the research farm in Wageningen (±70 km) and housed in separate rooms (LSC and HSC room). Upon arrival at the research farm, all pigs were weighed and allocated to their pen within sanitary condition (**SC**) treatment, based on BW in order to minimize variation between pens (LSC batch 1: 5.7 kg, LSC batch 2: 5.9 kg, LSC batch 3: 5.9 kg, HSC batch 1: 5.5 kg, HSC batch 2: 5.2 kg HSC batch 3: 5.9 kg). Upon arrival (± 21 d of age) and 2 d thereafter, HSC pigs received an antibiotic injection (Fenflor, AUV, Cuijk, The Netherlands, 1 mL per pig, *i.m.* per time). At the research facility, the vaccination program for HSC pigs was continued: In week 4: Mycoplasma hyopneumoniae, Porcine circovirus type 2, Porcine Reproductive and Respiratory Syndrome (PRRS), and *Lawsonia intracellularis* (Porcilis M Hyo, Porcilis Circo, Porcilis PRRS, all MSD Animal Health, Boxmeer, The Netherlands, and Enterisol Ileitis,

Boehringer Ingelheim, Alkmaar, The Netherlands); at 6 weeks of age against Actinobacillus pleuropneumoniae, and Influenza A virus (Porcilis APP, MSD Animal Health, Boxmeer, The Netherlands, and Gripovac3, Merial, Velserbroek, The Netherlands); and at 8 weeks of age against Actinobacillus pleuropneumoniae, and Influenza A virus (Porcilis APP, MSD Animal Health, Boxmeer, The Netherlands, and Gripovac3, Merial, Velserbroek, The Netherlands) by subcutaneous injection in the neck or in case of Enterisol by oral drench.

Housing. After arrival until 9 weeks of age pigs were housed in identical pens located in either a room in which HSC or LSC conditions were applied. The HSC room was intensively cleaned before arrival of each batch of pigs: once with high pressure washing, and once disinfected with a mixture of hydrogen peroxide and peracetic acid gas (Destbest 400, Frans Veugen Bedrijfshygiene B.V., Nederweert, The Netherlands) with an ecofogger (Coldfogger, Frans Veugen Bedrijfshygiene B.V., Nederweert, The Netherlands) for 2 h. In addition, all people entering the HSC room were obliged to shower, change clothes and were a hairnet and face mask. The manure in HSC pens was removed daily.
The LSC room was not cleaned and no hygiene protocol was applied. A mixture of fresh manure of three commercial pig farms, obtained fresh weekly, was spread weekly in the LSC pens to increase pathogenic pressure. The LSC pigs were exposed to nylon bags containing a mixture of pig manure and straw, ground to pass a 1 mm screen, as a model for dust. The bags were mounted on a chain above the LSC pens, for pigs to play with. The LSC pigs did not receive any medication.
During the measurement period (10 until 13 weeks of age), within each batch, groups of six pigs were housed in one of eight identical, 40-m^3 respiration chambers, described by Heetkamp et al. (2015). Each chamber contained one pen of 1.75 × 2.85 meter with 40% slatted floor. During this period, pre-weighed quantities of fresh manure and dust were introduced weekly in LSC chambers similar to their introduction in the pre-experimental period. The HSC chambers were cleaned and disinfected before start measurement at the ad lib period (week 9 of age), as was done with the rooms for the pre-period. During the measurement period a strict hygiene protocol was adhered to when entering the HSC chambers similar to the one described above. Temperature in the chambers was maintained at 20 °C, relative humidity at 65%, and air velocity at < 0.2 m/s. Pigs were exposed to artificial light from 0730 to 1930 h (420 lux) and to darkness (3.5 lux) during the remainder of the day.

Diets and feeding. From arrival (start week 3) until week 5 (12 d post weaning) all pigs were allowed ad libitum access to a commercial diet containing 164 g/kg crude protein (CP), 18.0 kJ/g gross energy (GE) (Comfort 3, De Heus Animal Nutrition, The Netherlands) and from week 6 until week 9 containing 170 g/kg CP and 17.8 kJ/g GE (Comfort 4, De Heus Animal Nutrition, The Netherlands). From week 9 onwards, pigs were allocated to one of two treatment diets either containing a basal or a supplemented AA-profile. The diets were

formulated to be slightly limiting in Lys (95% of the requirement value for lysine relative to the dietary net energy value (NE), to prevent that the dietary energy concentration could limit the growth performance of the pigs in the study (NRC, 2012).
The AA-B diet was designed as described in van der Meer et al. (2016). Briefly, this was developed by a factorial approach to cover AA requirements for maintenance (skin and hair losses, intestinal endogenous losses, and cell turnover) and whole body protein deposition. The AA-S diet was derived from the AA-B profile by increasing the Met, Thr, and Trp ratio relative to Lys by 20%.
The ingredient and nutrient composition of the experimental diets is shown in Table 1. The diets were isocaloric on an NE basis and contained TiO_2 as indigestible marker for measuring fecal nutrient digestibility. The diets were analyzed for AA composition by acid hydrolysis at 110°C for 23 h and ion-exchange chromatography with post-column derivatization with ninhydrin and Trp by alkaline hydrolysis at 110 °C for 20 h, followed by ion-exchange chromatography and fluorescence detection (ISO, 2005b; ISO 2005c). During the ad libitum feeding period, pigs were fed ad libitum from a single space feeder. During the restricted feeding period, pigs were fed 70% of their ad libitum intake divided over two meals at 0730 h and 1530 h, fed from a through which allowed all 6 pigs to eat at the same time. Per meal pigs had 30 minutes to eat their meal after which the through was closed to prevent contamination of feeding troughs with urine.

Measurements. During the ad libitum, restricted feeding period and fasting period, gas exchange of each group of pigs was measured in the calorimetric chambers in 6-min intervals by measuring exchange of O_2, CO_2, and CH_4, as described by Heetkamp et al. (2015). A CO_2 recovery test with the respiration chambers was performed immediately prior to the start of the experiment, according to procedures described by Heetkamp et al. (2015). In the eight chambers, 99, 96.4, 97.9, 100.7, 98.7, 98.3, 99.2, and 100.5 % of the CO_2 released was recovered. A radar device was mounted above each chamber completely covering the housing area of the pigs to measure physical activity continuously during the ad libitum, restricted, and fasting period. Complete energy and nitrogen (N) balances were determined in for the ad libitum and the restricted period (Supplement tables I to IV). Individual pigs were weighted before and after each balance week. In each balance week, manure was collected quantitatively, weighed, mixed thoroughly and sampled for analysis of GE, DM, ash, and N. Aerial NH_3 was collected from a quantified sample of the outgoing air after trapping in H_2SO_4, and NH_4^+ in water that condensed on the heat exchanger. For LSC pigs, the contribution of the manure and dust introduced into the pens to the energy and nitrogen balances was quantified.
From each group of six pigs, a representative rectal feces sample was obtained in each balance week by sampling each pig once during 3 subsequent days. Fecal samples of individual pigs were pooled by weight per chamber and stored at -20 for later analysis of GE, DM, neutral detergent fibre (NDF), and N and worms (coccidian parasite, Ascaris,

Trichostrongulus/Strongylus, other endoparasites; P217, De Gezondheidsdienst voor Dieren, Deventer, The Netherlands). After the restricted feeding period, pigs had restricted feeding for 2 more days (0.1 kg diet · kg $BW^{-0.6}$· d^{-1}), after which they were fasted for 25 h to measure FHP.

Dissection. From the fasting heat measurement in the fasting heat period pigs were fed ad libitum for 4-5 days until dissection. At the dissection day, three pigs per room were euthanized on one day between 8.30 and 15.00 h to collect blood samples for further analysis and lungs for lesion scores. The order of euthanizing was balanced per room and treatment group. Pigs were restrained and anaesthetized with an *i.m.* injection in the neck with in total 6 mg Zoletil/kg BW (Zoletil, Virbac Laboratories, Carros, France). After sedation they were euthanized by an *i.v.* injection of 24 mg/kg BW of sodium pentobarbital (Euthasol 20%, AST Farma B.V., The Netherlands) in the ear vein and subsequently blooded by incision in the neck. Blood samples were collected during bleeding in two 9-mL tubes per animal were filled: 1 EDTA tube for blood cell counts (Vacuette; Greiner Bio-One, Kremsmünster, Austria) and 1 serum tube (Vacuette) for acute-phase protein and natural antibody (Nab) analysis. Blood samples collected in EDTA tubes were immediately stored on ice and transported to the lab where blood cell counts were performed using a Microcell counter (Sysmex pocH- iV Diff; Toa Medical Electronics Co., Ltd., Kobe, Japan). Blood samples in serum tubes were allowed to clot for 1 h at room temperature, after which serum was collected after centrifugation for 10 min at 5,251 × *g* at room temperature and stored at -80°C pending analysis of haptoglobin (Pig haptoglobin Elisa kit, , catalog number ab205091, Abcam, Cambridge, UK), pig major acute-phase protein (Cusabio Pig-MAP, ELISA, catalog number CSB-E13425p; Cusabio Biotech Co, Ltd., Wuhan, Hubei Province, China), C-reactive protein (CRP; Pig CRP Elise kit, catalog number ab205089, Abcam, Cambridge, UK), and Nab titers against keyhole limpet hemocyanin (KLH) types IgG and IgM were determined as described by de Koning et al. (2015) with the minor modification that a 4-step dilution (40, 160, 640, 2,560 times diluted) of the sera was made instead of a 3-step dilution.

Chemical analysis. Samples of diets were pooled per batch of pigs and analysed for DM, ash, N, GE, Ti, and NDF content (ISO, 1998; ISO, 1999; ISO, 2005a;). Pooled fecal samples were analysed for DM, N, GE, Ti, and NDF content. For determination of DM content, faeces were freeze-dried and feces and diets were ground to pass a 1-mm screen before analysis using a Retsch ZM 100 mill (Retsch GmbH, Haan, Germany). Dry matter content was determined by drying at 103 °C. Crude ash content was determined by combustion at 550 °C . Nitrogen content was determined by kjeldahl in fresh material. Neutral detergent fiber content was analysed according to the method of van Soest et al. (1991), with prior amylase treatment. The GE content was analysed using bomb calorimetry (model C7000 calorimeter; IKA Werke GmbH & Co. KG, Staufen, Germany). Ti was analysed after

hydrolysis with concentrated sulphuric acid in the presence of a copper catalyst at 420 °C and subsequent addition of peroxide (Short et al., 1996; Myers et al., 2004). All analysis were carried out in duplicate.

Calculations. Gross energy intake was calculated as feed intake (supply minus refusals) multiplied by the GE content of the diet. Intake of metabolizable energy (ME) was determined by subtracting energy excretion in manure and CH_4 from the gross energy intake. Heat production (HP) was calculated from consumed volumes of O_2, produced volumes of CO_2, and CH_4, and quantitative urinary N excretion (Brouwer, 1965), and physical activity of the pigs as measured by radar (Gerrits et al., 2015). Energy retention was calculated as the difference between ME intake and HP. Nitrogen retention was calculated as N intake minus N losses in manure, aerial NH_3, and NH_4^+ in water that condensed on the heat exchanger. Protein retained (N retention × 6.25) and energy retained as protein (protein retained × 23.7 kJ/g) were calculated from N retention. Energy retained as fat was calculated from the difference between total energy retained and energy retained as protein in the body. Apparent total tract digestibility for DM, GE, NDF, and N was calculated using TiO_2 as an indigestible marker (Kotb and Luckey, 1972).

Incremental efficiencies and incremental digestion coefficients. From data on parameters related to energy and N utilization and deposition in the body measured in the ad lib and restricted feeding period, incremental energy and nutrient efficiencies were calculated as the difference in a response parameter X divided by the difference in dietary intake between the ad lib and restricted feeding period (Fig 4.1). The incremental efficiency of energy retained in the body was thus calculated as the increase in energy retained (kJ·kg BW-0.6·d-1) per extra unit of GE intake, digestible energy (DE), or ME intake (kJ·kg BW-0.6·d-1). Likewise, the incremental efficiency of N retention was expressed as a percentage of extra units of N retained (g N·kg BW-1·d-1) per extra unit of gross or digestible N intake (g N·kg BW-1·d-1). Analogously, incremental apparent total tract digestion coefficients for all nutrients analysed were calculated as the difference in digestible nutrient intake between the ad libitum and restricted feeding periods, divided by the difference in gross intake of that nutrient between both feeding periods (all in g/kg BW). As incremental efficiencies are dependent on the level of feeding (Labussière et al., 2011), we chose to determine these values using feed intake levels close to the maximum voluntary intake, i.c. using first a period of ad lib feed intake followed by a period of restricted supply, set at 70% of the voluntary feed intake.

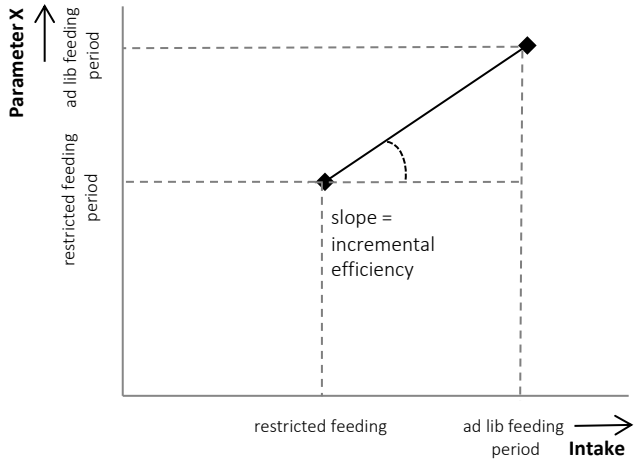

Figure 4.1. Example for calculating incremental efficiency of intake converted to parameter X. The difference in measured parameter X in the ad lib and restricted feeding period divided by the difference in intake between the ad lib and restricted feeding period. The outcome is equal to the slope of the equation between the points in the graph and equals the incremental efficiency.

Fasting heat production and resting metabolic rate. Two days after termination of the restricted feeding period, pigs were fasted in the respiration chambers for measurement of FHP for 25 h i.c. from after the morning meal provided at 0730 until 0900 am on the next day. For the ad libitum and the restricted feeding period, and the FHP measurement separately, HP was partitioned between heat production related to physical activity and resting metabolic rate (**RMR**), using penalised β-spline regression procedures, developed and described by van Klinken et al. (2012). The FHP was calculated as the average RMR during the last 2 h of feed deprivation. By using this method there were three missing values out of 24 observations in total. Parameters related to heat production or energy balance were scaled to $BW^{0.60}$, according to Noblet et al. (1999) Parameters related to digestion or N retention were scaled to BW.

Statistical analysis. Data were analyzed using the GLM procedure of SAS (SAS 9.3; SAS Inst. Inc., Cary, NC) with pen as experimental unit for all parameters. For all data, the normality of the distribution of studentized residuals was assessed by the Shapiro–Wilk statistic. If required, transformation of data was performed to obtain normal distribution of residuals. Sanitary conditions, diet, batch, and their interactions were used as fixed effects. Values are presented as least squares means ± SEM. Effects were considered significant at $P \leq 0.05$ and a trend was defined as $0.05 < P \leq 0.10$.

RESULTS

One pig had a volvulus and died just before entering the respiration chambers. Therefore, one pen had five instead of six animals in the experimental period. No clinical signs of illness were observed during the experiment in the other pigs. All fecal samples were found to be negative for Ascaris, Eimeria spp., Trichostrongylus, and other endoparasites. Five out of 36 LSC pigs (13%) and 8 out of 36 HSC pigs (22%) had a small pneumonia lesion in the lungs at dissection. The remainder of the pigs had no lung lesions.

In the restricted feeding period, pigs were offered 70% of the feed intake of the ad libitum feeding period, supplied in two meals per day. Not all groups consumed the feed supplied within 30 min, leading to a contrast in feed intake between periods slightly exceeding the 30% anticipated, 39% for LSC and 31% for HSC. Due to a technical problem in batch 2 and 3 with the device measuring physical activity in one chamber of the LSC treatment, and one chamber in the HSC treatment, the activity measurements in these chambers were not considered reliable and were therefore discarded.

The results of the energy and nitrogen balances are presented as incremental efficiencies. The energy and nitrogen balances of the groups of pigs during the ad libitum and restricted feeding period are presented in supplemental tables I-IV. To facilitate interpretation of treatment differences in these tables, al parameters were standardized to the same level of feed intake within period, by including GE intake as a covariate in the statistical model.

Performance. Mean BW at weaning was greater for LSC pigs (5.8 ± 0.1 kg) compared with HSC pigs (5.6 ± 0.1 kg; $P \leq 0.01$; Table 4.2). The difference in mean BW at weaning between sanitary conditions (SC) differed between batches (interaction batch SC, $P \leq 0.01$). The difference in BW for LSC and HSC pigs in batch 2 was greater (+0.7 ± 0.1 kg) than for batch 1 (+0.2 ± 0.1 kg) and batch 3 (0.0 ± 0.1 kg). Mean BW at weaning of batch 3 pigs (5.9 ± 0.1 kg) was greater compared with batch 1 and 2 pigs (5.6 ± 0.1 kg; $P \leq 0.01$). A batch effect was still observed for the mean BW at dissection ($P \leq 0.01$). The BW at the dissection day was lower for LSC (28.4 ± 0.8 kg) compared with HSC pigs (38.0 ± 0.8 kg , $P \leq 0.05$).

During the ad libitum feeding period, ADFI, ADG and G:F were on average 9, 22% and 10% higher in HSC pigs compared with LSC pigs for ADFI, ADG, and G:F respectively (all, $P < 0.01$), depending on batch (SC × batch, all $P \leq 0.001$)., Differences between SC for these parameters were in the same direction for ADG (+6, +145, and +245 g/d for batch 1, 2, and 3, respectively), but not for ADFI (-77, +308, and +81 g/d for batch 1, 2, and 3, respectively) and G:F (+0.04, -0.03, and +0.16 g/g for batch 1, 2, and 3, respectively). In the restricted feeding period, pigs were restricted to the level of their intake in the ad libitum feeding period. Hence, treatment effects during the ad lib feeding period for ADFI, ADG and G:F ratio were sustained in the restricted week.

Acute-phase proteins, natural antibodies against keyhole limpet hemocyanin in serum, and blood cell counts. The LSC pigs showed greater (740 µg/mL) haptoglobin and lower (54 µg/mL) CRP concentrations in serum than HSC pigs ($P \leq 0.05$; Table 4.3). The LSC pigs had (0.2×10^{12}/L) a lower RBC count, and a (0.4 mmol/L) lower haemoglobin concentration, and (1%) lower haematocrit value in blood than HSC pigs ($P \leq 0.05$). For mean corpuscular volume (MCV) an interaction was found between SC × batch ($P \leq 0.01$), caused by a positive difference between LSC and HSC pigs in batch 1, but negative differences in batches 2 and 3.

Apparent total tract digestion and incremental ATTD coefficients. The average apparent total tract digestibility (ATTD) of DM, GE and N for the ad libitum and restricted feeding period was lower for LSC than for HSC pigs (-3%, -3%, and -7% for DM, GE and N respectively, all $P \leq 0.0001$). Incremental ATTD coefficients for DM, GE NDF, and N were not affected by SC ($P > 0.05$). The incremental ATTD coefficient for N was affected by batch ($P \leq 0.05$), being 69, 77, and 74 % for batch 1, 2 and 3, respectively. A tendency for SC × diet was found for the ATTD coefficient for N ($0.05 < P \leq 0.10$), the value being higher for the AA-S diet than for the AA-B diet in LSC pigs, while the opposite result was obtained in HSC pigs.

Incremental efficiencies of energy and nitrogen retained as protein or fat and fasting heat production. Incremental responses of heat production to increased GE, and DE, but not ME intake were 7% and 8% points higher for HSC than for LSC pigs, respectively ($P \leq 0.01$). Incremental responses of retained energy to increased GE, DE and ME intake were unaffected by experimental treatments ($P > 0.05$). Incremental responses of protein deposition to increased GE, DE or ME intake, and to increased N or digestible N intake were substantially higher for HSC compared with LSC pigs (all, $P \leq 0.05$). For example, the incremental N efficiency was nearly 20% points higher in HSC (73%) compared with LSC (53%) pigs. Conversely, the incremental response in fat deposition to increased ME intake, but not to increased GE or DE intake, was higher in LSC compared to HSC pigs ($P \leq 0.05$). Surprisingly, none of the incremental efficiencies were affected by dietary AA supplementation.
Fasting heat production was (55 kJ · $BW^{0.6}$ · day^{-1}) greater in LSC than HSC pigs ($P \leq 0.05$).

Table 4.1. Ingredient and nutrient composition of the experimental diets.

Item	AA-B[1]	AA-S[1]
Ingredient, g/kg of feed		
Wheat	308.6	308.6
Maize	200.0	200.0
Barley	200.0	200.0
Soybean meal	184.5	184.5
Maize starch	25.8	23.4
Sugarcane molasses	20.0	20.0
Limestone	14.7	14.7
Monocalcium phosphate	9.0	9.0
Soybean oil	19.2	19.2
Vitamin + mineral mix[2]	5.0	5.0
Salt	3.5	3.5
L-lysine HCl	3.4	3.4
Titanium dioxide	2.5	2.5
Sodium bicarbonate	2.1	2.1
L-threonine	1.0	2.1
L-tryptophan	0.0	0.4
DL-methionine	0.4	1.3
L-Valine	0.3	0.3
Analyzed nutrients composition, g/kg		
NE, MJ/kg[3]	9.8	9.8
DM	889.6	889.8
Crude protein[4]	166	167
Starch	474	472
Lys[4]	9.8	10.1
Thr[4]	6.6	7.8
Trp[4]	2.2	2.6
Met + Cys[4]	5.2	6.2
Ile[4]	8.2	8.3
Arg[4]	11.8	12.2
Phe[4]	9.4	9.9
His[4]	5.5	5.3
Leu[4]	15.0	15.3
Tyr[4]	6.2	6.2
Val[4]	9.6	9.8

[1] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile.

[2] Supplied the following per kilogram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[3] Based on chemical composition, digestibility, and energy values for pigs from CVB (2011).

Table 4.2. Performance data of pigs kept under low or high sanitary conditions and fed diets differing in AA composition

	LSC[1]		HSC[1]			*P*-values[5]					
Item	AA-B[2]	AA-S[2]	AA-B	AA-S	SEM[4]	SC	diet	batch	SC×diet	SC×batch	diet×batch
n[3]	6	6	6	6							
BW week 1, kg	2.4	2.5	2.2	2.4	0.1	0.22	0.16	0.25	0.61	0.28	0.35
BW weaning (± 21 d), kg	5.8	5.8	5.5	5.6	0.1	**0.001**	0.82	**0.01**	0.80	**0.004**	0.81
BW start ad lib feeding, kg	17.8	19.0	24.2	24.5	0.5	**<.0001**	0.15	**<.0001**	0.42	0.21	0.42
BW start restricted feeding, kg	21.6	23.4	29.3	29.5	0.6	**<.0001**	0.13	**<.0001**	0.20	0.83	0.74
ADFI, g											
ad lib feeding period	1136	1200	1292	1252	32	**0.006**	0.72	**0.001**	0.13	**0.001**	0.46
restricted feeding period	743	797	973	923	29	**<.0001**	0.95	0.17	0.10	**0.02**	0.55
ADG, g											
ad lib feeding period	545	636	737	709	26	**0.0002**	0.25	0.83	**0.04**	**0.008**	0.55
restricted feeding period	299	332	405	367	13	**<.0001**	0.88	**0.03**	**0.02**	**<.0001**	0.14
G:F, g/g											
ad lib feeding period	0.48	0.54	0.57	0.56	0.02	**0.007**	0.19	**0.0004**	0.10	**0.001**	0.87
restricted feeding period	0.40	0.42	0.42	0.39	0.01	0.84	0.78	**0.02**	0.14	**0.0001**	0.28

[1] LSC= Low sanitary conditions, HSC= High sanitary conditions. [2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile.[3] n = number of pens, a pen contained 6 pigs. [4] SEM= pooled standard error of the mean, means are presented as least square means. [5]Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$.

Table 4.3. Blood parameters of pig kept under low or high sanitary conditions and fed diets differing in AA composition

	LSC[1]		HSC[1]			*P*-values[5]					
Item	AA-B[2]	AA-S[2]	AA-B	AA-S	SEM[4]	SC	diet	batch	SC×diet	SC×batch	diet×batch
n[3]	6	6	6	6							
pigMAP, µg/mL	783	826	855	816	62	0.62	0.97	**0.02**	0.52	0.47	0.50
Haptoglobin, µg/mL	1884	1502	1156	750	326	**0.04**	0.09	0.06	0.71	0.52	0.45
CRP, µg/mL	170	167	251	194	23	**0.03**	0.22	0.80	0.25	0.67	0.90
IgG_KLH	4.5	4.7	4.7	4.0	0.3	0.21	0.35	0.99	0.07	0.74	0.54
IgM_KLH	4.8	5.1	4.9	4.9	0.2	0.78	0.33	0.73	0.38	0.23	0.49
WBC, 10^9 /L	12.6	13.9	13.7	13.3	0.7	0.76	0.57	0.16	0.24	0.73	0.62
RBC, 10^{12}/L	5.7	5.7	5.8	6.0	0.1	**0.02**	0.14	0.07	0.25	0.12	0.77
Hb, mmol/L	6.5	6.4	6.8	6.8	0.1	**0.002**	0.80	**0.003**	0.29	0.42	0.11
Ht, %	32.0	31.6	32.4	33.2	0.4	**0.01**	0.61	**0.01**	0.12	0.50	0.34
MCV, 10^{-15}/L	56.2	55.4	56.3	55.4	0.4	0.94	0.07	**0.003**	0.94	**0.003**	0.15
PTL, 10^9/L	405	409	420	427	21	0.46	0.80	0.61	0.94	0.34	0.23

[1] LSC = Low sanitary conditions, HSC = High sanitary conditions. [2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile. [3] n = number of pens, a pen contained 6 pigs and 3 pigs were sampled for blood. [4] SEM = pooled standard error of the mean, means are presented as least square means. [5] Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$.

Table 4.4 Apparent total tract digestibilities averaged over an ad libitum and restricted feeding period for pigs kept under low (**LSC**) or high (**HSC**) sanitary conditions and fed diets differing in AA composition

	LSC		HSC			*P*-values[4]					
Item	AA-B[1]	AA-S[1]	AA-B	AA-S	SEM[3]	SC	diet	Week	batch	SC×diet	SC×batch
n[2]	6	6	6	6							
DM	79.9	79.9	83.1	83.0	0.3	**<.0001**	0.96	0.68	0.24	0.74	0.46
GE	80.1	80.6	83.5	83.6	0.4	**<.0001**	0.46	0.38	0.24	0.67	0.41
NDF	39.2	41.5	41.5	41.5	1.5	0.15	0.96	0.75	0.29	0.94	0.07
N	69.9	70.9	77.7	77.3	0.6	**<.0001**	0.66	0.53	0.14	0.27	0.80

[1] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile [2] n = number of pens, a pen contained 6 pigs. [3] SEM = pooled standard error of the mean, means are presented as least square means. [4] Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$.

Table 4.5. Incremental apparent total tract digestibility (**ATTD**) coefficients in pigs allowed ad libitum or 70% of ad libitum access to diets differing in AA composition, and kept under low (**LSC**) or high (**HSC**) sanitary conditions

		LSC		HSC			*P*-values[4]			
Increment in intake of:	Response parameter:	AA-B[1]	AA-S[1]	AA-B	AA-S	SEM[3]	SC	AA	batch	SC×AA
	n[2]	6	6	6	6					
DM, g·kg BW·d^{-1}	ATTD DM, g·kg BW·d^{-1}	82	84	84	82	2	0.86	0.84	0.13	0.26
GE, g·kg BW·d^{-1}	ATTD GE, g·kg BW·d^{-1}	80	82	82	80	2	0.96	0.84	0.17	0.32
NDF, g·kg BW·d^{-1}	ATTD NDF, g·kg BW·d^{-1}	42	45	35	34	7	0.22	0.85	0.70	0.76
N, g·kg BW·d^{-1}	ATTD N, g·kg BW·d^{-1}	70	74	77	72	2	0.21	0.89	**0.03**	0.10

[1] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile. [2]n = number of pens, a pen contained 6 pigs. [3] SEM = pooled standard error of the mean, means are presented as least square means. [4] Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$.

Table 4.6. Incremental responses of heat production, activity related heat, and body retention to increases in dietary energy and N intake in pigs, of pigs kept under low (**LSC**) or high (**HSC**) sanitary conditions and fed diets differing in AA composition

		LSC[1]		HSC[1]			*P*-values[5]			
Increment in intake of:	Response parameter	AA-B[2]	AA-S[2]	AA-B	AA-S	SEM[4]	SC	diet	batch[6]	SC×diet
	n[3]	6	6	6	6					
GE , kJ·kg $BW^{-0.6}$·d^{-1}	Heat production, kJ·kg $BW^{-0.6}$·d^{-1}	0.23	0.22	0.30	0.28	0.02	**0.0005**	0.39	0.13	0.63
GE , kJ·kg $BW^{-0.6}$·d^{-1}	Activity heat, kJ·kg $BW^{-0.6}$·d^{-1}	0.47	-0.05	-1.47	1.25	1.0	0.74	0.28	0.21	0.12
GE , kJ·kg $BW^{-0.6}$·d^{-1}	Energy retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.51	0.52	0.55	0.59	0.05	0.36	0.64	0.95	0.81
GE , kJ·kg $BW^{-0.6}$·d^{-1}	Protein retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.12	0.13	0.19	0.17	0.02	**0.004**	0.85	0.64	0.54
GE , kJ·kg $BW^{-0.6}$·d^{-2}	Fat retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.39	0.39	0.36	0.41	0.04	0.95	0.49	0.88	0.58
DE , kJ·kg $BW^{-0.6}$·d^{-1}	Heat production, kJ·kg $BW^{-0.6}$·d^{-1}	0.28	0.27	0.36	0.35	0.02	**0.0003**	0.43	0.08	0.89
DE , kJ·kg $BW^{-0.6}$·d^{-1}	Activity heat, kJ·kg $BW^{-0.6}$·d^{-1}	0.64	0.0	-1.69	1.51	1.2	0.74	0.29	0.19	0.12
DE , kJ·kg $BW^{-0.6}$·d^{-1}	Energy retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.65	0.64	0.67	0.74	0.07	0.39	0.70	0.98	0.60
DE , kJ·kg $BW^{-0.6}$·d^{-1}	Protein retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.16	0.16	0.23	0.22	0.02	**0.005**	0.87	0.52	0.78
DE , kJ·kg $BW^{-0.6}$·d^{-1}	Fat retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.49	0.48	0.44	0.52	0.05	0.95	0.57	0.97	043
ME , kJ·kg $BW^{-0.6}$·d^{-1}	Heat production, kJ·kg $BW^{-0.6}$·d^{-1}	0.32	0.30	0.35	0.33	0.03	0.37	0.56	0.48	0.86
ME , kJ·kg $BW^{-0.6}$·d^{-1}	Activity heat, kJ·kg $BW^{-0.6}$·d^{-1}	0.47	-0.25	-1.92	1.33	1.3	0.69	0.29	0.20	0.15
ME , kJ·kg $BW^{-0.6}$·d^{-1}	Energy retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.68	0.70	0.65	0.67	0.03	0.37	0.56	0.48	0.86
ME , kJ·kg $BW^{-0.6}$·d^{-1}	Protein retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.17	0.17	0.22	0.20	0.01	**0.02**	0.43	0.68	0.42
ME , kJ·kg $BW^{-0.6}$·d^{-1}	Fat retention, kJ·kg $BW^{-0.6}$·d^{-1}	0.51	0.53	0.43	0.47	0.03	**0.04**	0.32	0.31	0.58
GE , kJ·kg $BW^{-0.6}$·d^{-1}	ME intake, kJ·kg $BW^{-0.6}$·d^{-1}	0.74	0.74	0.85	0.87	0.05	**0.03**	0.8	0.66	0.91
DE , kJ·kg $BW^{-0.6}$·d^{-1}	ME intake, kJ·kg $BW^{-0.6}$·d^{-1}	0.93	0.90	1.04	1.09	0.07	**0.05**	0.85	0.73	0.56
N , g·kg BW^{-1}·d^{-1}	N retention, g·kg BW^{-1}·d^{-1}	0.51	0.55	0.75	0.71	0.06	**0.003**	0.99	0.60	0.52
Digested N[7], g·kg BW^{-1}·d^{-1}	N retention, g·kg BW^{-1}·d^{-1}	0.75	0.75	0.98	0.98	0.08	**0.007**	0.98	0.34	0.99
	FHP[8], kJ·kg $BW^{0.6}$·d^{-1}	753	747	708	683	25	**0.04**	0.54	0.13	0.69

[1] LSC = Low sanitary conditions, HSC = High sanitary conditions.[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile.[3] n = number of pens, a pen contained 6 pigs. [4] SEM = pooled standard error of the mean, means are presented as least square means.[5] Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$.[6] There were no interactions with batch.[7] Apparent total tract digested N-intake. [8] FHP = fasting heat production.

DISCUSSION

The main objective of the present experiment was to quantify the difference in energy requirements for maintenance, and in incremental efficiencies for energy and protein deposition of clinically healthy pigs, kept under low or high sanitary conditions and fed a diet with or without extra supplemented Met, Thr and Trp. The measured FHP was used as a proxy for the energy requirement for maintenance. Incremental efficiencies for energy and N retention were derived from nutrient digestibility and retention values obtained with animals fed ad libitum or at a level of 70% of ad libitum intake.

Methodological issues. There were several batch effects and interactions found for SC × batch in this study, which means that the treatment effects varied among batches. The batch effect was a composite effect of the batch of pigs, originating from the same farm but taken at a different point in time, the effect of batches of manure and dust introduced into the pens, and the effect of time. As housing conditions were well standardized, we assume that the batch effect is dominated by differences between batches of pigs and batches of manure. It should be noted that the direction of most observed effects, however, was not affected by batch, and that none of the incremental efficiencies were affected by batch. Variation in responses between experiments and between batches of animals in the same experiment are commonly observed when imposing either challenges with live micro-organisms and multifactorial challenges as used in the present study. The observed variation among batches illustrates the importance to carefully consider variation among batches rather than reducing experimental variation by the use of a single batch, as often done in studies applying similar models (Williams et al., 1997; Le Floc'h et al., 2009; Pastorelli et al., 2012a).

In our study, the contrast in feed intake, allowing the determination of incremental nutrient efficiencies, was created within each group of pigs. The advantages of this approach are that the effect of feed intake is estimated using the same animals and that it enables the incremental efficiencies to be estimated at levels of feed intake close to the maximum voluntary feed intake level. The disadvantage is that the restricted period inevitably follows the ad libitum period, and that it is conducted later in time. Effects of the latter are minimized by standardizing the experimental housing conditions (hygiene management, temperature, relative humidity, air velocity). To account for differences in BW between weeks, we expressed energy metabolism parameters relative to $BW^{0.60}$ (Noblet et al. 1999). In addition, we found our conclusions unaltered when expressing the results relative to the traditionally used $BW^{0.75}$.

Contrast in sanitary conditions affecting pig performance and the immune system. The contrast in SC in the present study was created by a combination of factors; vaccinations

against specific pathogens, a preventive antibiotic treatment, application of a hygiene protocol, and weekly introduction of a mixture of manure and dust in pens. The slightly higher BW at weaning for LSC pigs likely results from the Mycoplasma vaccination that HSC pigs received in the period just before weaning (Thacker et al., 2000). After weaning until the end of the experiment LSC pigs had substantially lower BW and BW gain than HSC pigs. At dissection day, the difference in BW for LSC and HSC pigs was 10 kg. The difference in weight gain during the experiment was caused both by a reduction in ADFI and G:F. In addition, LSC pigs had a greater haptoglobin concentration in blood compared with HSC pigs. Taken together, the lower BW, lower ADFI, lower ADG, greater haptoglobin levels in LSC pigs, and the absence of clinical signs of illness in both SC treatment groups indicate a contrast in subclinical health between LSC and HSC pigs slightly more severe than a previous study using a similar model of ISS (van der Meer et al., 2016).

Because pigMAP, haptoglobin and CRP are all positive acute phase proteins, the blood concentrations of these acute phase proteins were expected to be greater for LSC pigs than for HSC pigs. The results did, however, not meet the expectations, as only the haptoglobin concentration was significantly greater in LSC pigs and CRP concentrations were even lower. The absence of an effect of subclinical ISS on pigMAP concentration, was also found in van der Meer et al. (2016) and suggests a less sensitive response for pigMAP compared with haptoglobin as found in a number of other studies as well (Heegaard et al., 1998; Kampman-van de Hoek et al., 2016; Saco et al., 2016). We have no explanation for the unexpected lower CRP level in blood in LSC compared with HSC pigs. Haptoglobin and CRP have different functions and may therefore respond differently to low grade inflammation. Chen et al. (2003) who determined haptoglobin and CRP concentrations in pig blood at different farms, found also increased haptoglobin concentrations in subclinical ill pigs, but no differences in CRP concentrations. Based on their observations it was concluded that haptoglobin is a better indicator of inflammatory reactions in pig herds than serum CRP.

Natural antibodies levels against KLH as measure of the response to low grade immune stimulation were not significantly different, which was in contrast with higher values found for LSC compared with HSC pigs in a previous experiment (van der Meer et al., 2016). In that study the effect was most pronounced shortly after the transfer of the piglets to the novel sanitary conditions. In the current study, KLH specific natural antibodies were measured only 10 weeks after the time point at which the piglets were exposed to the different sanitary conditions. The increased infectious pressure that likely occurs at low sanitary conditions might be the stimulus that causes a short term increase in NAb levels. These increased NAb levels may gradually disappear on the long term. The period of measurement might therefore have contributed to the lack of effect on the antibody response against KLH in the current experiment. Furthermore, the SC to create a contrast in ISS were different between the two experiments. For example manure was introduced more often and was a mixture of manure from more farms compared with the first experiment, introduction of dust in the pens was not applied in the previous experiment,

pigs were housed in groups of 6 instead of 9, and pigs were housed in experimental facilities compared with a commercial pig farm in the previous experiment. We cannot exclude that these differences in sanitary conditions are responsible for the contradicting observations.

Dietary amino acid supplementation. The basal AA profile was designed based on a factorial estimate of the requirements for protein deposition, basal endogenous losses, cell turnover, and skin and hair losses, slightly increasing Met and Trp (Knowles et al., 1998; Jansman et al., 2010). The rationale for this choice was that requirement estimates originating from dose response studies will always include the requirements for meeting increased needs associated with ISS under the conditions of that study. Supplementing Met, Thr and Trp was based on studies in which increased requirements for these AA were demonstrated, mostly using clinical models (Melchior et al., 2004; Le Floc'h et al., 2008; Litvak et al., 2013; Rakhshandeh et al., 2014) and the level of supplementation was arbitrarily set to 20%. In a previous study we showed that performance of LSC pigs was increased by a similar supplementation strategy with Met, Thr and Trp. In the present study, however, pig performance was not improved by supplementation of these AA.
A reason for the lack of effect might be that pigs in the current study were exposed to the contrast in SC at younger age (weaning age; 21 d of age) than in the previous study (10 weeks of age; van der Meer et al., 2016), while the measurements in both studies started at the same age (9-10 weeks of age). The effects of the extra supplemented AA in the previous study was mainly expressed in the starter phase (week 10 until 15 of age) in HSC pigs, and may result from compensatory growth performance when introduced to the clean environment after a period of reduced growth in the pre-experimental period as also observed by Kampman-van de Hoek et al. (2016). In our previous study, the reduced growth in the pre-experimental period was related to the administration of a number of vaccinations in this period (van der Meer et al., 2016). In the current study such an effect might not have occurred as the period of measurements started 6 weeks later. We find, in contrast to other published information using clinical models of ISS (Melchior et al., 2004; Le Floc'h et al., 2008; Rakhshandeh et al., 2014; Kampman-van de Hoek et al., 2015), no effects of AA supplementation on performance, and on incremental efficiency of N utilization. Also our findings do not corroborate the effects of SC on Trp as was found by Le Floc'h et al. (2009).

Use of dietary energy and protein. We expected G:F ratio to be reduced in case of ISS due to the LSC treatment, comparable to results from other ISS studies (Williams et al., 1997; Le Floc'h et al., 2006). We found a higher FHP for LSC compared with HSC pigs which reflects a lower energetic efficiency for LSC compared with HSC pigs due to an increased energy expenditure for maintenance purposes, of which e.g. functioning of the immune system is

part of. The extra energy required by LSC pigs compared with HSC pigs was estimated at 55 kJ · kg $BW^{0.6}$·d^{-1}, when considering a HSC pig as reference.

The incremental efficiencies for ATTD of DM, GE, NDF, and N were not influenced by the different treatments, however, the average ATTD of DM, GE and N were reduced for LSC pigs compared with HSC pigs with 3, 3 and 7% respectively for the ad libitum and restricted period together. A reduced ATTD of N and DM for LSC pigs was in line with van der Meer et al. (2016) and Kampman-van de Hoek (2016) where 1% and 4% reduced ATTD N values and 1% and 1% reduced ATTD DM values were reported for LSC pigs compared with HSC pigs. The reduced ATTD found for LSC pigs might be due to intestinal infections, intestinal damage, or an increased digesta passage rate (Sandberg et al., 2006; Pastorelli et al., 2012b).

The incremental efficiencies for energy retention were not affected by the different treatments, however a substantial reduction in the utilization of dietary N for N retention was found for LSC compared with HSC pigs. The reduced incremental efficiency for N retention of LSC pigs was due to a difference in the gross efficiency of retained N of 71% and 79% in the ad libitum feeding period, while the gross efficiency for retained N in the restricted feeding period was similar (71%) for LSC and HSC pigs. The difference found for the incremental efficiency of retained N between LSC and HSC might be due to a reduced maximal rate of protein deposition for LSC pigs compared with HSC pigs, an increased rate of AA oxidation due to increased protein turnover for LSC pigs, or an imbalance of AA available for protein retention in the body in LSC pigs. The latter, however, is unlikely when considering the lack of effect of, dietary supplementation of Met, Thr, and Trp, unless another essential AA was limiting protein deposition in LSC pigs or the dietary protein supply in general was limiting.

The incremental efficiency of ingested energy for fat retention was greater for LSC pigs than HSC pigs when calculated for ME intake retained as fat, however, this effect was absent when calculated for GE or DE intake retained as fat. The significant effect found for the incremental efficiency of ME intake retained as fat was likely related to a higher urinary energy excretion in LSC pigs compared with HSC pigs, coinciding with an increased urinary N output. Hence it is unlikely that incremental efficiencies of DE for fat retention were affected by SC.

In conclusion, a chronic, low grade stimulation of the immune system by imposing low sanitary conditions to pigs increases FHP by 8% and reduces apparent total tract nutrient digestibility, particularly of protein. Under such conditions, the incremental efficiency of digestion is unaffected, but the incremental N utilization for body protein deposition is reduced by 20% points. The incremental efficiency of digestible energy for fat deposition is unaffected by sanitary conditions. Dietary supplementation of Met, Thr and Trp did not affect incremental efficiencies for energy and protein deposition.

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SUPPLEMENTAL TABLES

Supplemental table I. Least square means of energy balance parameters for ad libitum fed pigs kept under low or high sanitary conditions and fed diets containing basal or supplemented amino acid profiles. All parameters except gross energy (**GE**) intake were adjusted to a similar level of feed intake by including GE intake as a covariate.

	LSC[1]		HSC[1]			*P*-values[4]						
Item, g/kg BW$^{0.60}$/day	**AA-B[2]**	**AA-S[2]**	**AA-B**	**AA-S**	**SEM[3]**	**SC**	**diet**	**batch**	**SC×diet**	**SC×batch**	**diet×batch**	**GE intake[6]**
BW, kg	17.8	19.0	24.2	24.5	0.6	**<.0001**	0.15	**<.0001**	0.42	0.21	0.42	-
GE intake	2843	3007	2872	2843	56	0.246	0.24	**0.0008**	0.10	**<.0001**	0.06	-
ME intake[5]	2285	2329	2449	2439	36	**0.002**	0.64	0.44	0.49	0.23	0.15	**0.002**
ME:GE ratio, %	79	81	85	84	1	**0.003**	0.68	0.52	0.44	0.22	0.18	0.30
Methane production	3.8	4.8	9.2	10.5	1.1	**0.0002**	0.28	0.26	0.89	0.96	0.80	0.26
Heat production	1373	1368	1429	1401	20	**0.04**	0.43	**0.05**	0.58	0.24	0.61	0.10
Energy retention total	912	961	1020	1039	47	0.07	0.48	0.13	0.77	0.36	0.49	**0.05**
Energy retention protein	358	376	440	421	11	**<.0001**	0.97	0.28	0.15	0.42	0.71	**0.02**
Energy retention fat	554	585	580	618	41	0.48	0.42	0.13	0.94	0.35	0.50	0.10

Supplemental table II. Least square means of energy balance parameters restricted fed pigs kept under low or high sanitary conditions and fed diets containing basal or supplemented amino acid profiles. All parameters except gross energy (**GE**) intake were adjusted to a similar level of feed intake by including GE intake as a covariate.

	LSC[1]		HSC[1]			*P*-values[4]						
Item, g/kg BW$^{0.60}$/day	**AA-B[2]**	**AA-S[2]**	**AA-B**	**AA-S**	**SEM[3]**	**SC**	**diet**	**batch**	**SC×diet**	**SC×batch**	**diet×batch**	**GE intake[6]**
BW, kg	21.6	23.4	29.3	29.5	0.6	<.0001	0.13	<.0001	0.20	0.83	0.74	-
GE intake	1819	1934	2035	1933	49	**0.05**	0.89	**0.007**	**0.04**	**0.001**	0.24	-
ME intake[5]	1592	1601	1636	1615	11	**0.03**	0.57	0.74	0.25	0.06	0.41	**<.0001**
ME:GE ratio, %	82	83	85	84	1	**0.04**	0.65	0.75	0.26	0.09	0.33	0.52
Methane production	4.6	5.2	10.3	10.3	0.8	**<.0001**	0.61	0.62	0.74	0.67	0.46	0.71
Heat production	1162	1159	1153	1142	17	0.49	0.64	**0.008**	0.83	0.77	0.97	**0.02**
Energy retention total	430	442	483	473	21	0.08	0.94	**0.01**	0.64	0.41	0.72	**<.0001**
Energy retention protein	244	249	261	254	6	0.11	0.82	0.08	0.39	0.21	0.06	**<.0001**
Energy retention fat	185	193	222	219	18	0.13	0.88	**0.02**	0.79	0.62	0.98	**0.0002**

[1] LSC = Low sanitary conditions, HSC = High sanitary conditions.[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile.[3] SEM = pooled standard error of the mean, means are presented as least square means.[4] Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$. [5] ME = metabolizable energy, [6] This value represents the significance of the covariable GE-intake.

Supplemental table III. Least square means of nitrogen (**N**) balance parameters for ad libitum fed pigs kept under low or high sanitary conditions and fed diets containing basal or supplemented amino acid profiles. All parameters were adjusted to a similar level of feed intake by including gross energy (**GE**) intake as a covariate.

	LSC[1]		HSC[1]			*P*-values[4]						
Item	AA-B[2]	AA-S[2]	AA-B	AA-S	SEM[3]	SC	diet	batch	SC×diet	SC×batch	diet×batch	GE intake[5]
BW, kg	17.8	19.0	24.2	24.5	0.6	**<.0001**	0.15	**<.0001**	0.42	0.21	0.42	-
N intake	1.42	1.38	1.27	1.24	0.01	**<.0001**	**0.02**	**<.0001**	0.55	0.96	0.26	**<.0001**
N retention	0.73	0.75	0.80	0.76	0.02	0.09	0.81	0.02	0.21	0.38	0.53	**0.05**
N retention/N intake	0.51	0.55	0.63	0.61	0.02	**0.0001**	0.69	0.42	0.14	0.46	0.66	0.93

Supplemental table IV. Least square means of nitrogen (**N**) balance parameters for restricted fed pigs kept under low or high sanitary conditions and fed diets containing basal or supplemented amino acid profiles. All parameters were adjusted to a similar level of feed intake by including gross energy (**GE**) intake as a covariate.

	LSC[1]		HSC[1]			*P*-values[4]						
Item	AA-B[2]	AA-S[2]	AA-B	AA-S	SEM[3]	SC	diet	batch	SC×diet	SC×batch	diet×batch	GE intake[5]
BW, kg	21.6	23.4	29.3	29.5	0.6	<.0001	0.13	<.0001	0.20	0.83	0.74	-
N intake	0.89	0.86	0.81	0.79	0.00	**<.0001**	**0.002**	**<.0001**	0.93	0.50	0.06	**<.0001**
N retention	0.46	0.47	0.45	0.44	0.01	0.15	0.64	**0.006**	0.43	0.19	0.17	**0.0007**
N retention/N intake	0.52	0.54	0.56	0.55	0.01	0.11	0.48	0.09	0.35	0.26	**0.02**	0.15

[1] LSC = Low sanitary conditions, HSC = High sanitary conditions.[2] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared to the profile in the basal profile.[3] SEM = pooled standard error of the mean, means are presented as least square means.[4] Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$. [5] GE = gross energy, this value represents the significance of the covariable GE-intake.

Chapter 5

Diurnal patterns of heat production and oxidation of carbohydrate and fat in pigs kept under different sanitary conditions

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ABSTRACT

In Chapter 4 we showed that low grade, chronic inflammation, imposed on pigs by keeping them under low sanitary conditions (LSC) increased fasting heat production (FHP) by 8% and reduced incremental utilization of dietary protein by 20% points compared with high sanitary conditions (HSC). Dietary supplementation of methionine (Met), threonine (Thr), and tryptophan (Trp) affected neither the incremental utilization of dietary protein nor any of the energy balance traits. By studying diurnal patterns of heat production and substrate oxidation rates we aimed to gain insight in the underlying mechanisms for increased energy expenditure and reduced marginal protein efficiency in LSC pigs fed diets with either a basal amino acid profile or supplemented with Met, Thr and Trp. In a 2 × 2 factorial arrangement, 24 groups of 6 pigs each were allocated to either LSC or HSC, and were offered one of two different diets having either a basal or supplemented dietary AA profile. For each group of pigs, heat production and physical activity was measured in 6 min intervals by indirect calorimetry during two consecutive weeks, during which feed was available ad libitum or at 70% of ad libitum. The LSC pigs had reduced rates of body weight gain, feed intake, gain:feed ratio and higher serum haptoglobin concentrations than HSC pigs, indicating that the contrast in sanitary conditions was reflected in physiological and immunological parameters. Supplementation of Met, Thr and Trp did not affect any of the traits measured. When compared with HSC pigs, LSC pigs reduced their energy expenditure on physical activity and increased their resting metabolic rate in the restricted feeding period compared with HSC pigs, especially when lights were on. This confirms our previous observation that FHP was increased at LSC. Within day patterns of net fat and carbohydrate oxidation were not affected by sanitary conditions. It is speculated that the reduction of the incremental utilization of dietary protein under LSC is not restricted to particular periods within the day.

Keywords. heat production, pig, energy expenditure, sanitary conditions, health, amino acid.

Implications. The results of this study indicate that sanitary conditions of pigs cause variation in energy utilization and expenditure over the day. The results help to understand the effect of sanitary conditions on dietary requirements of pigs. Providing diets that match the requirements of the pig on the correct moment of the day, might help to increase the efficiency of pig production and to reduce the environmental pressure of the pig industry.

INTRODUCTION

Sanitary conditions (SC), have been shown to affect energy expenditure for fasting heat production (FHP) and incremental efficiency for protein retention in pigs (In Chapter 4 we showed that low grade, chronic inflammation, imposed on pigs by keeping them under low sanitary conditions (LSC) increased fasting heat production (FHP) by 8% and reduced incremental utilization of dietary protein by 20% points. Dietary supplementation of methionine (Met), threonine (Thr), and tryptophan (Trp) affected neither the incremental utilization of dietary protein nor any of the energy balance traits measured in Chapter 4. In the study described in Chapter 4, the low grade, chronic inflammation was apparent from reduced performance and increased serum haptoglobin concentrations. Whereas immune system stimulation in the clinical range is known to increase heat production (Lochmiller and Deerenberg, 2000, Parmentier et al., 2002), little is known about the metabolic or immunological processes underlying the increased FHP and reduced incremental protein utilization at LSC. As energy is also needed to deposit fat or protein in the body, and the requirements for specific amino acids (AA), Met, Thr and Trp are also known to be affected by immune system stimulation (Le Floc'h et al., 2008, Kampman-van de Hoek et al., 2016, van der Meer et al., 2016), an interaction between energy and AA metabolism affected by immune stimulation was expected, but was found to be absent in Chapter 4. Detailed analysis of within day patterns of heat production, activity related or resting, and net rates of substrate oxidation will allow to track the observed effects of SC and (absence of) effects of AA supplementation (Chapter 4) to particular periods within the day. Treatment differences in heat production or net rates of substrate oxidation directly following a meal would typically indicate altered metabolism of ingested nutrients, indicating substantial contribution of visceral tissues.

To this end, we explored the diurnal patterns of energy expenditure by partitioning total heat production (Htot) into heat production related to physical activity (Hact), and resting metabolic rate (RMR) of pigs kept under different sanitary conditions fed diets with or without extra supplemented Met, Thr, and Trp. In addition, the diurnal patterns of the respiratory quotient (RQ) and net rates of carbohydrate and fat oxidation of these pigs were also evaluated.

MATERIALS AND METHODS

In a 2 × 2 factorial arrangement, 24 groups of six pigs were allocated to either LSC or HSC, and were offered two different diets having either a basal (AA-B) or supplemented dietary AA profile (AA-S). Pigs were followed from week 3 until week 12 of age. Pigs were used from week 3 till week 12 of age. From week 3 until 8, pigs were adapted to the different sanitary conditions, after which the dietary treatments were introduced. Energy balances

studies were performed from week 9 to 12. During week 9, pigs were allowed ad libitum access to their diets. During week 10, pigs were fed twice daily at 70% of the measured feed intake during week 9. During week 12, pigs were allowed restricted access to their diets for 2 d, prior to a 25h period of feed deprivation, for the measurement of FHP. These three experimental weeks are referred to as the ad lib feeding period, the restricted feeding period and the fasting period.

Animals and treatments. In total, 144 (Topigs 20 × Danish Duroc; Topigs, Helvoirt, The Netherlands; Danbred International, Herlev, Denmark) newborn female piglets were selected at one week of age in three subsequent batches of 48 pigs on a commercial farm in The Netherlands, and allocated to either the LSC or HSC treatment as explained in Chapter 4.

In brief, piglets were selected at the same farrowing rooms and HSC piglets received six vaccinations (against Mycoplasma hyopneumoniae, procine circovirus type 2, porcine reproductive and respiratory disease, Lawsonia intercellularis, action bacillus pleuropneumoniae, and influenza A virus) during the period of the first nine weeks of age. Piglets of both LSC and HSC treatments were housed in the same rooms at the commercial farm until weaning at 21 ± 0.2 d of age. At weaning (start pre-period) LSC and HSC piglets were transported to the research farm in Wageningen and housed in separate rooms (LSC and HSC room) until nine weeks of age (end adaptation period).

The HSC room was intensively cleaned and disinfected before arrival of the pigs. In addition, a strict hygiene protocol was adhered to when entering the HSC rooms. The LSC room was not cleaned and no hygiene protocol was applied. A mixture of manure of three commercial pig farms, obtained fresh weekly, was spread weekly in the LSC pens to increase microbial pressure. In addition, the LSC pigs were exposed to nylon bags containing a mixture of pig manure and straw, ground to pass a 1 mm screen, as a model for dust. The bags were mounted on a chain above the LSC pens, for pigs to play with.

Upon arrival at the research farm, all pigs were individually weighed and, allocated to their pen within sanitary condition treatment, based on BW in order to minimize variation between pens (LSC batch 1: 5.7 kg, LSC batch 2: 5.9 kg, LSC batch 3: 5.9 kg, HSC batch 1: 5.5 kg, HSC batch 2: 5.2 kg HSC batch 3: 5.9 kg). At arrival day (± 21 d of age) and 2 d thereafter, HSC pigs received an antibiotic injection (Fenflor, AUV, Cuijk, The Netherlands, 1 mL per pig, sub muscular per time).

Housing experimental period. During the experimental period (9 until 12 weeks of age), groups of six pigs were housed in one of eight identical, 40 m^3 indirect calorimetric chambers, described by Heetkamp et al. (2015), containing a pen of 1.75 × 2.85 m with 40% slatted floor. During this period, pre-weighed quantities of fresh manure and dust were introduced weekly in LSC chambers similar to their introduction in the pre-experimental period. The HSC chambers were cleaned and disinfected before start measurement at week

10, as was done with the rooms in the pre-period. During the experiment period a strict hygiene protocol was adhered to when entering the HSC chambers as was done in the pre-period. Temperature in the chambers was maintained at 20 °C, relative humidity at 65%, and air velocity at < 0.2 m/s. Pigs were exposed to artificial light from 0730 to 1930 h (420 lux) and to darkness (3.5 lux) during the remainder of the day.

Diets and feeding. From arrival (start week 3) until week 5 (12 d post weaning) all pigs were allowed ad libitum access to a commercial diet containing 164 g/kg CP, 18.0 kJ/g GE (Comfort 3, De Heus Animal Nutrition, The Netherlands) and from week 6 until week 9 containing 170 g/kg CP and 17.8 kJ/g gross energy (GE) (Comfort 4, De Heus Animal Nutrition, The Netherlands). From week 9 onwards, pigs were allocated to one of two treatment diets either containing a basal or a supplemented AA-profile. The diets were formulated to be slightly limiting in lysine (95% of the requirement value for lysine relative to the dietary net energy value (NE, NRC, 2012), to prevent that the dietary energy concentration could limit the growth performance of the pigs in the study.
The basal AA-profile (AA-B) was designed as described in van der Meer et al. (2016). Briefly, this was developed by a factorial approach to cover AA requirements for maintenance (skin and hair losses, intestinal endogenous losses, and cell turnover) and whole body protein deposition. The supplemented AA-profile (AA-S) was derived from the AA-B profile by increasing the Met, Thr, and Trp ratio relative to Lys by 20%.
The ingredient and nutrient composition of the experimental diets is shown in supplemental Table I. The diets were isocaloric on an NE basis and contained TiO_2 as indigestible marker for measuring fecal nutrient digestibility. The diets were analyzed for AA composition by acid hydrolysis at 110°C for 23 h and ion-exchange chromatography with post-column derivatization with ninhydrin (ISO, 2005a) and Trp by alkaline hydrolysis at 110 °C for 20 h, followed by ion-exchange chromatography and fluorescence detection (ISO, 2005b). During the ad libitum feeding period, pigs were fed ad libitum from a single space feeder. During the restricted feeding period, pigs were fed 70% of their ad libitum intake divided over two meals at 0730 h and 1530 h, fed from a through which allowed all 6 pigs to eat at the same time. Per meal pigs had 30 minutes to eat their meal after the trough was closed, to prevent contamination of the trough with urine.

Feeding strategy during experimental period. During the experimental period (week 10 until 12 of age) pigs were fed ad libitum for one week (week 10 of age) and restricted in the second week (week 11 of age). Feed was provided ad libitum in a single feeder during the ad libitum week and caretakers visited the chambers at 7:30 am and 3:30 pm. In the restricted week, pigs were fed 70% of the ad libitum intake in two meals provided in a trough at 7:30 am and 3:30 pm. Water was available ad libitum in both weeks. In week 12 of age, the pigs were fed four more restricted meals and subsequently they were starved

for 25 h for FHP measurement. After FHP measurement the pigs were fed ad libitum until the end of the experiment.

Measurements in the experimental period. During the ad libitum, restricted feeding period and fasting period, gas exchange of each group of pigs was measured in the calorimetric chambers in 6-min intervals by measuring exchange of O_2, CO_2, and CH_4, as described by Heetkamp et al. (2015). A CO_2 recovery test with the respiration chambers was performed immediately prior to the start of the experiment, according to procedures described by Heetkamp et al. (2015). In the eight chambers, 99, 96.4, 97.9, 100.7, 98.7, 98.3, 99.2, and 100.5 % of the released CO_2 was recovered. A radar device was mounted above each chamber completely covering the housing area of the pigs to measure physical activity in 6-min intervals during the three experimental weeks. Changes in the frequency of the reflected radar waves due to movement of the animals (Doppler effect) were converted into electrical pulses and pulses were recorded. Complete energy and nitrogen (N) balances were determined in for the ad lib and the restricted period. Individual pigs were weighted before and after each balance week. In each balance week, manure was collected quantitatively, weighed, mixed thoroughly and sampled for analysis of GE, DM, ash, and N. Aerial NH_3 was collected from a quantified sample of the outgoing air after trapping in H_2SO_4, and NH_4^+ in water that condensed on the heat exchanger. For LSC pigs, the contribution of the extra administered manure and dust to the energy and nitrogen balances was quantified.

The Htot was partitioned between Hact and RMR, using a penalised β-spline regression procedure, developed and described by van Klinken et al. (2012). The FHP was estimated as the average RMR during the last 2 h of feed deprivation. The thermic effect of feeding (TEF) in the restricted week, was calculated by subtracting the average RMR in the restricted week by the FHP. Short TEF (sTEF) was calculated by subtracting the average RMR of the restricted by the lowest RMR value, and long TEF (lTEF) was calculated by subtracting the lowest RMR value in the restricted week by FHP. The RQ was calculated as the ratio between O_2 consumption and CO_2 production. The carbohydrate and fat oxidation (in g) were calculated from gas exchange (in L), as described by Brouwer (1958), ignoring the contribution of methane production and protein oxidation (van den Borne *et al.*, 2015):

$$\textit{Carbohydrate oxidation in g} = -2.965 \times VO_2 + 4.170 \times VCO_2$$

$$\textit{Fat oxidation in g} = 1.718 \times VO_2 - 1.718 \times VCO_2,$$

where VO_2 = volume O_2 consumption and VCO_2 = volume CO_2 produced

Dissection. From the fasting heat measurement in the fasting heat period pigs were fed ad libitum for 4-5 days until dissection. At the dissection day, three pigs per room were euthanized on one day between 8.30 and 15.00 h to collect lungs for lesion scores. The order of euthanizing was balanced per room and treatment group. Pigs were restrained and anaesthetized with an *i.m.* injection in the neck with in total 6 mg Zoletil/kg BW (Zoletil, Virbac Laboratories, Carros, France). After sedation they were euthanized by an *i.v.* injection of 24 mg/kg BW of sodium pentobarbital (Euthasol 20%, AST Farma B.V., The Netherlands) in the ear vein and subsequently blooded by incision in the neck.

Statistical analysis. The experimental unit for all measurements was the group of 6 pigs, i.e. one pen of six pigs in each chamber. Effects of sanitary conditions, AA profile, batch, and their interactions on the hourly means of Htot, Hact, RMR, RQ, carbohydrate oxidation and fat oxidation were assessed by analysis of variance using the GLM procedure in SAS (v. 9.3, SAS Institute Inc., Cary, NC, USA). Non significant interactions with batch were deleted from the model. The partitioning of heat in Htot, Hact and RMR in the restricted feeding period was analysed with the same model as was used for the hourly means. In addition, GE intake was included in the model for the partitioning of heat in the ad libitum and restricted feeding period as a covariate to correct for feed intake. *P*-values were considered significant when $P \leq 0.05$, and considered as tendency when $0.05 < P \leq 0.10$. Results are presented as least square means ± SEM.

RESULTS

General. One LSC pig fed the AA-S diet in the adaptation period, had a volvulus and died just before entering the respiration chambers. Therefore, one pen had five instead of six animals in the experimental period. Throughout the experiment, no clinical signs of illness were observed. All fecal samples were negative for Ascaris, coccidia, Trichostrongylus, and other endoparasites. Five out of 36 LSC pigs (13%) and 8 out of 36 HSC pigs (22%) had a small pneumonia lesion in the lungs at dissection, the remainder of the pigs had no lung lesions.

In the restricted feeding period, pigs were offered 70% of the feed intake of the ad libitum feeding period, supplied in two meals per day. Not all groups consumed the feed supplied within 30 min, leading to a contrast in feed intake between periods slightly exceeding the 30% anticipated, 39% for LSC and 31% for HSC. Due to a technical problem in batch 2 and 3 with the activity device in one chamber of LSC in and one chamber in HSC, the activity measurements of these chambers were not reliable and had to be discarded.

Ad libitum feeding period. During the ad libitium feeding period, Htot peaked twice daily. This variation in Htot was almost completely accounted for by variation in Hact, resulting in

only minor within-day variation in RMR (Fig 5.1). There were no significant effects of treatments at any point in time on RMR, and only few on Hact and Htot.

An interaction for sanitary conditions (SC) × AA was found for Htot at 8:00 am ($P \leq 0.05$), the LSC pigs fed the AA-B diet had higher Htot than the LSC pigs fed the AA-S diet, whereas the HSC pigs had a similar Htot independent of the diet. The HSC pigs had higher Htot at 0:00, 4:00, 8:00 am, and 1:00 pm than LSC pigs ($P \leq 0.05$). The pigs fed the AA-B diet had higher Htot at 7:00 pm.

At 2:00, and 3:00 pm a tendency for an interaction between SC × AA was found for Hact ($0.05 < P \leq 0.10$), the Hact for LSC pigs fed the AA-B diet was lower than for LSC pigs fed the AA-S diet, whereas for HSC pigs fed the AA-B diet the Hact was higher compared with HSC pigs fed the AA-S diet.

The RQ exceeded unit 1 during the whole day (Fig 5.2). Positive values were found for carbohydrate oxidation and negative values for fat oxidation, indicating substantial synthesis of fatty acids *de novo.*

Restricted feeding period. During the restricted feeding period, Htot peaked twice daily. This variation in Htot was partly accounted for by variation in Hact, and partly by within-day variation in RMR (Fig 5.3). There were only a few significant effects of treatments at points in time on Htot and Hact, and RMR values were higher for LSC pigs from 8:00 am until 1:00 pm and from 3:00 pm until 7:00 pm (Fig 5.3C). An interaction for SC × AA was found for Htot at 6:00 and 7:00 pm ($P \leq 0.05$), the LSC pigs fed the AA-B diet had higher Htot production than the LSC pigs fed the AA-S diet, whereas the HSC pigs had a similar Htot production independent of the diet. The HSC pigs had higher Htot production at 0:00, 1:00, 4:00 am, and 6:00, 8:00, 9:00, and 10:00 pm than LSC pigs ($P \leq 0.05$) and tended to have higher Htot production at 2:00, and 7:00 am than LSC pigs ($0.05 < P \leq 0.10$). At 8:00 pm an interaction SC × AA was found for Hact ($0.05 < P \leq 0.10$). The Hact was lower for LSC pigs at 9:00 and 11:00 am ($P \leq 0.05$).

The RQ in the restricted feeding period values were below 1 between 12:00 and 7:00 am and between 2:00 and 3:00 pm. Positive values were found for the net rate of carbohydrate oxidation and negative values for fat oxidation were only found between 8:00 am to1:00 pm, and 4 pm to 1 am, indicating synthesis of fatty acids de novo only after consumption of the meals (Fig 5.4).

Partitioning of heat in the restricted week. Overall, no treatment effects were found on parameter of heat partitioning, except for a SC tendency for RMR. The LSC pigs tended to have higher RMR values than the HSC pigs in the restricted feeding period ($P = 0.05$, Table 5.1). This tendency corresponded with a tendency for a higher estimate of FHP in LSC pigs ($P = 0.06$).

FIGURE CAPTIONS

Figure 5.1 Heat partitioning of pigs kept under different sanitary conditions (**SC**) and fed a diet (ad libitum) with or without extra supplemented amino acids (**AA**). Symbols are least square means of 6 groups of pigs, adjusted to equal GE intake among treatments. **A.** Total heat production (**Htot**), **B.** physical activity related heat (**Hact**), **C.** resting metabolic rate (**RMR**). The dashed lines represent low sanitary condition pigs and the closed lines high sanitary condition pigs. The circles represent pigs fed a basal AA profile diet and the triangles represent pigs fed a supplemented AA profile. * represent $P \leq 0.05$, + represents $0.05 < P \leq 0.10$, and – represents $P > 0.10$.

Figure 5.2 Respiration quotient (**RQ**) and net rates of carbohydrate and fat oxidation of pigs kept under different sanitary conditions (**SC**) and fed a diet (ad libitum) with or without extra supplemented amino acids (**AA**). **A.** RQ, **B.** carbohydrate oxidation, **C.** fat oxidation. The dashed lines represent low sanitary condition pigs and the closed lines high sanitary condition pigs. The circles represent pigs fed a basal AA profile diet and the triangles represent pigs fed a supplemented AA profile. * represent $P \leq 0.05$, + represents $0.05 < P \leq 0.10$, and – represents $P > 0.10$.

Figure 5.3 Heat partitioning of pigs kept under different sanitary conditions (**SC**) and fed a diet (restricted 70% of ad libitum intake) with or without extra supplemented amino acids (AA). Symbols are least square means of 6 groups of pigs, adjusted to equal GE intake among treatments. **A.** Total heat production (**Htot**), **B.** physical activity related heat (**Hact**), **C.** resting metabolic rate (**RMR**). The arrows represent the moments of meal feeding. The dashed lines represent low sanitary condition pigs and the closed lines high sanitary condition pigs. The circles represent pigs fed a basal AA profile diet and the triangles represent pigs fed a supplemented AA profile. * represent $P \leq 0.05$, + represents $0.05 < P \leq 0.10$, and – represents $P > 0.10$.

Figure 5.4 Respiration quotient (**RQ**) and net rates of carbohydrate and fat oxidation of pigs kept under different sanitary conditions (**SC**) and fed a diet (restricted 70% of ad libitum intake) with or without extra supplemented amino acids (**AA**). **A.** RQ, **B.** carbohydrate oxidation, **C.** fat oxidation. The arrows represent the moments of meal feeding. The dashed lines represent low sanitary condition pigs and the closed lines high sanitary condition pigs. The circles represent pigs fed a basal AA profile diet and the triangles represent pigs fed a supplemented AA profile. * represent $P \leq 0.05$, + represents $0.05 < P \leq 0.10$, and – represents $P > 0.10$.

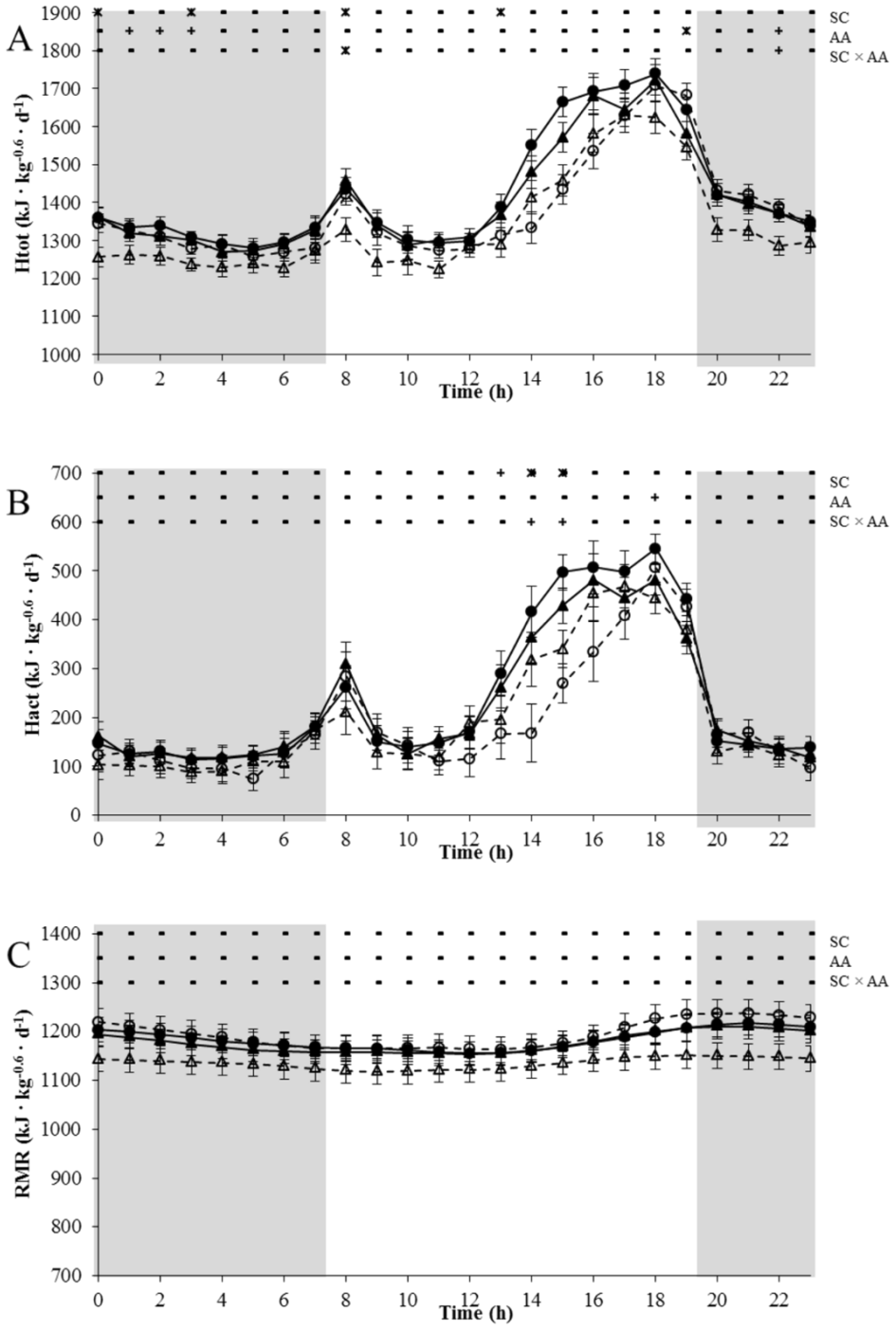
A
B
C
SC
AA
SC × AA
Htot (kJ · kg^-0.6 · d^-1)
Hact (kJ · kg^-0.6 · d^-1)
RMR (kJ · kg^-0.6 · d^-1)
Time (h)

Figure 5.1

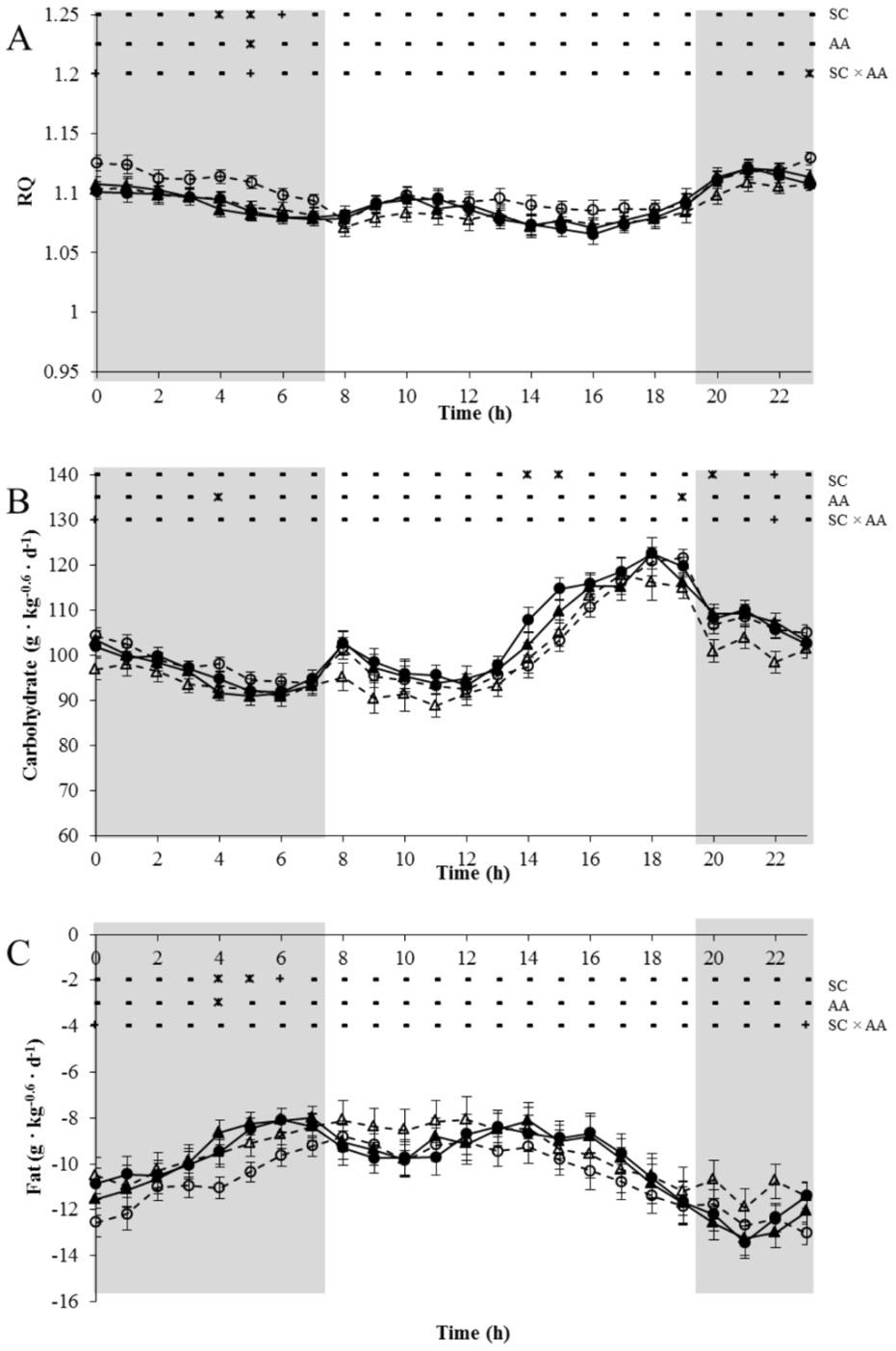
A
RQ
Time (h)
SC
AA
SC × AA
B
Carbohydrate (g · kg-0.6 · d-1)
Time (h)
SC
AA
SC × AA
C
Fat (g · kg-0.6 · d-1)
Time (h)
SC
AA
SC × AA

Figure 5.2

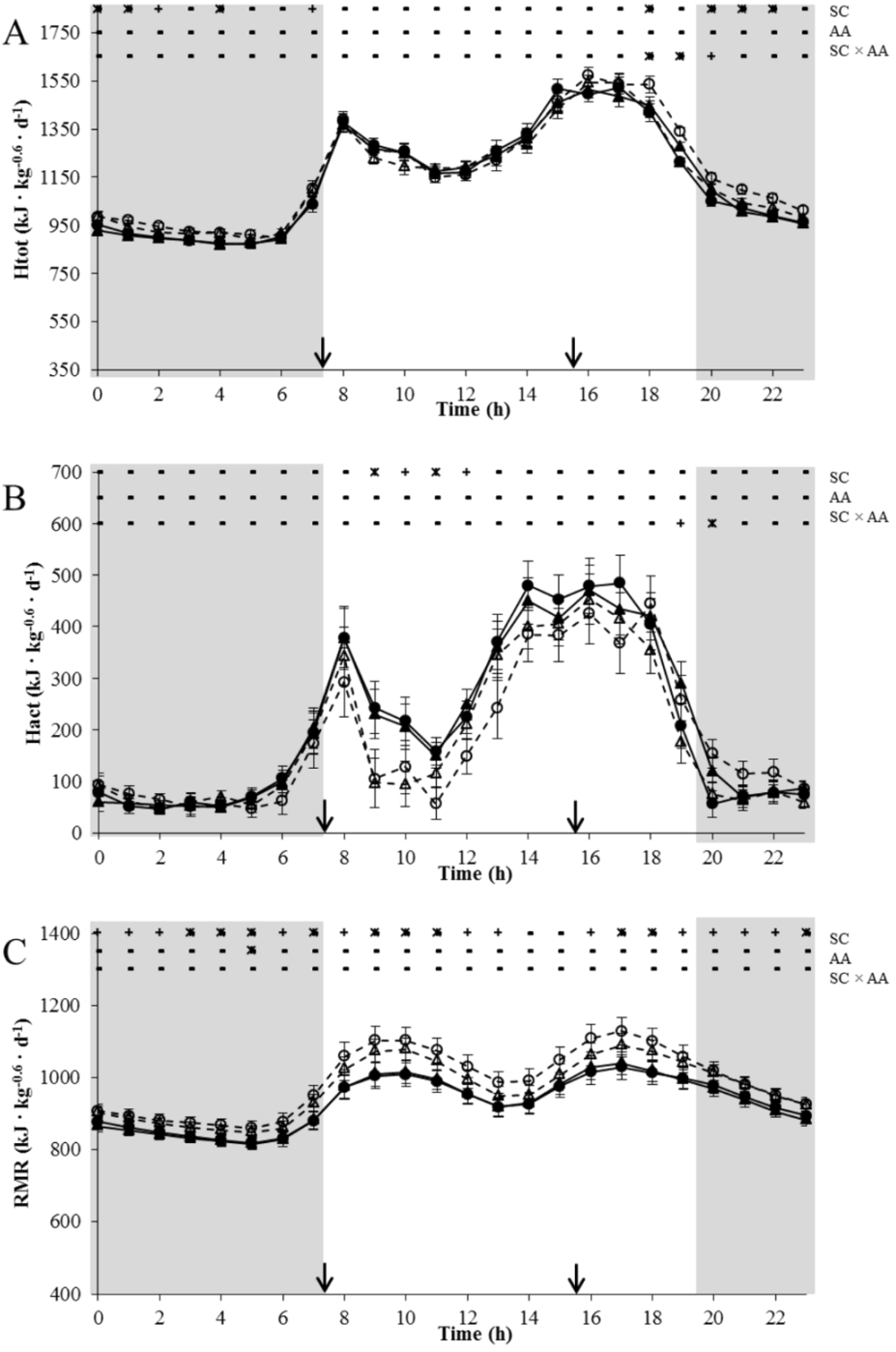
A
Htot (kJ · kg-0.6 · d-1)
1750
1550
1350
1150
950
750
550
350
0 2 4 6 8 10 12 14 16 18 20 22
Time (h)
SC
AA
SC × AA
B
Hact (kJ · kg-0.6 · d-1)
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22
Time (h)
SC
AA
SC × AA
C
RMR (kJ · kg-0.6 · d-1)
1400
1200
1000
800
600
400
0 2 4 6 8 10 12 14 16 18 20 22
Time (h)
SC
AA
SC × AA

Figure 5.3

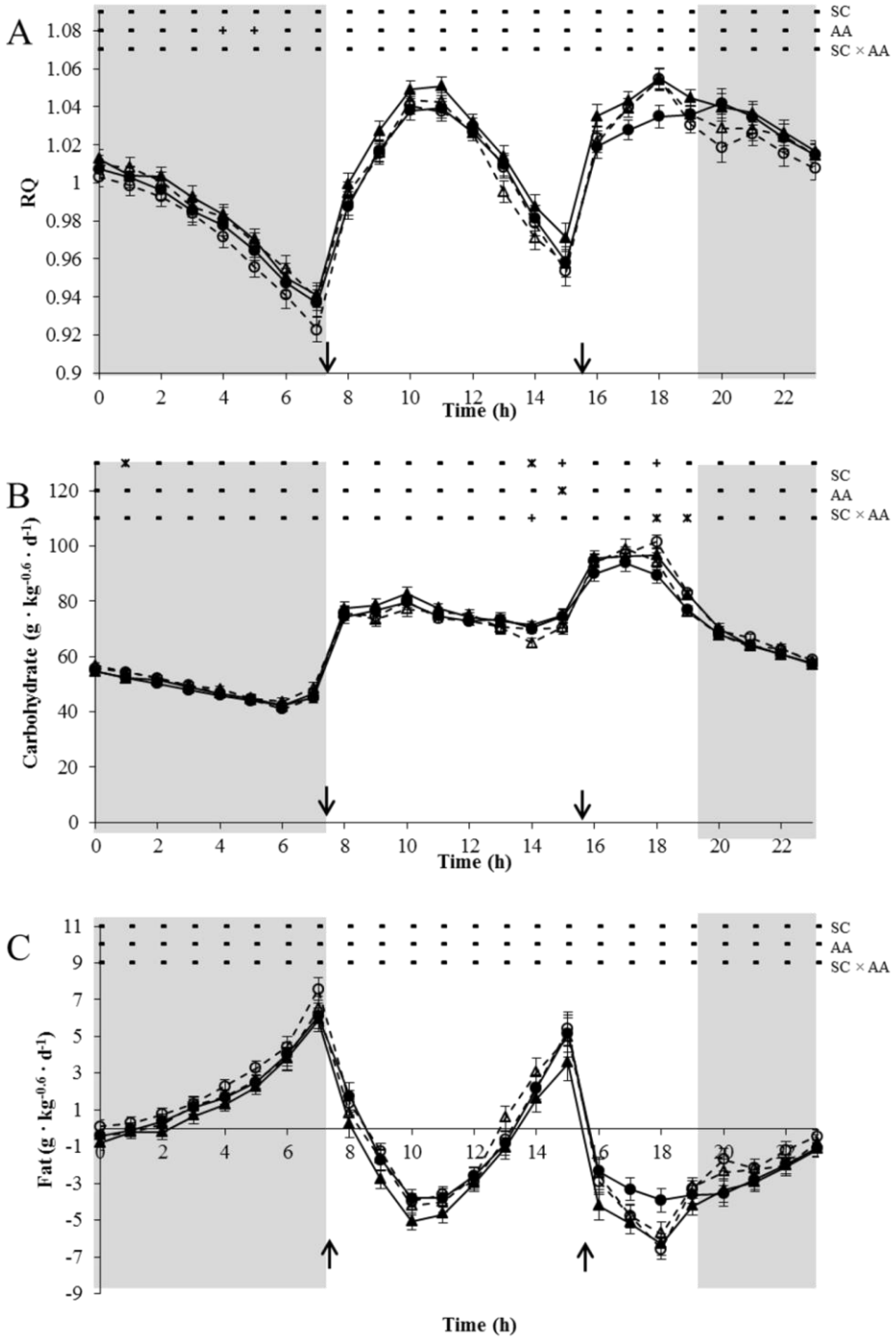
A
RQ
Time (h)
SC
AA
SC × AA
B
Carbohydrate (g · kg-0.6 · d-1)
Time (h)
SC
AA
SC × AA
C
Fat (g · kg-0.6 · d-1)
Time (h)
SC
AA
SC × AA

Figure 5.4

DISCUSSION

We explored the diurnal patterns of heat production, RQ, and net rates of carbohydrate and fat oxidation of LSC and HSC pigs fed a diet with or without extra supplemented AA with the objective to gain insight in underlying mechanisms of increased energy expenditure and reduced incremental efficiency of protein retention for LSC pigs. This was determined by measurement of heat production, RQ, carbohydrate and fat oxidation in 6 min intervals and averaging these values per hour per day per ad libitum and restricted feeding period. Feed intakes differed among treatments during the ad libitum and restricted feeding periods (Chapter 4). For the objective of this study, we adjusted all parameters to the same GE intake to ease interpretation of the effects of SC and AA supplementation.

Contrast in sanitary conditions. The contrast in SC was created by a combination of vaccination, antibiotic treatment, hygiene, disinfecting, and introducing manure and dust in pens. The LSC pigs had a lower rate of BW gain, feed intake, gain:feed ratio (Chapter 4), and higher haptoglobin concentrations in serum than HSC pigs, which is in accordance with a previous study (van der Meer et al., 2016). In addition, no clinical signs of illness were observed during the experiment. Overall, these results indicate that a contrast in SC is reflected by the physiology of the pigs, and illustrate the chronic, low grade inflammation that was induced, which is detailed in Chapter 4.

The LSC pigs had an 8% increased FHP, 20% points reduced incremental efficiency for protein deposition, compared with HSC pigs. The incremental efficiency for fat deposition remained unaffected (Chapter 4). Supplementation of the basic AA profile with 20% Met, Thr and Trp did not affect any of the parameters measured.

The difference in FHP seems to be present during the whole day, as RMR values in the diurnal pattern in the restricted feeding period were higher or tended to be higher for LSC pigs compared with HSC pigs for almost all hours. The difference between LSC and HSC pigs was close to 50 kJ · kg $^{-0.6}$ · d^{-1}, which is similar to the difference in FHP (+55 kJ · kg $^{-0.6}$ · d^{-1}) found between the LSC and HSC pigs as described in Chapter 4. It is likely that the difference found in FHP, was causing the difference in RMR between the SC treatment groups in the restricted feeding period. The maximal difference in RMR of LSC and HSC pigs in the restricted week however was higher than 50 kJ · kg $^{-0.6}$ · d^{-1}, namely 80 kJ · kg $^{-0.6}$ · d^{-1} at max. at 9:00 and 10:00 am. This greater difference was especially present during the peak moments in RMR over the day. Assuming that FHP stays constant over the day, the difference in RMR higher than the difference in FHP should be due to a difference in the TEF post meal between LSC and HSC pigs, but this could not be detected significantly. Interestingly, the difference in RMR between SC was not present during the ad libitum period, during which RMR values were 300 kJ higher than during the restricted period.

Physical activity in ad libitum and restricted fed pigs. The increase in energy expenditure for physical activity with increasing feed intake was not affected by the experimental treatments (Chapter3). Nonetheless, differences were detected for physical activity during the ad libitum and restricted periods, and treatment effects were present during the restricted period. During the restricted, but not during the ad libitum feeding period, activity of LSC pigs was lower after the morning meal and increasing in the beginning of the night compared with the HSC pigs.

The stable RQ found in the ad libitum week (Fig 5.2A) suggests that the ad libitum fed animals ate several times during the 24 h period. In a study of Souza da Silva et al. (2014) was reported that group housed control pigs, that were fed ad libitum, had 17.5 meals per day and spend 81.5 min eating per 24 h period, supporting that ad libitum fed animals eat several times per day. As the ad libitum pigs in our experiment did not eat only during the moment of high Hact, the two peaks found for Hact in the ad libitum fed pigs cannot be explained by feeding activity alone. The two similar Hact peaks for the restricted fed animals might be largely affected by meal related behaviour though. In the ad libitum week caretakers visited the chambers at 7.30 am and 3.30 pm for taking rectal faecal samples, perhaps these events affected the activity pattern of the ad libitum fed pigs.

At 2:00 and 3.00 pm in the ad libitum week and at 9.00 and 11.00 am in the restricted week the Hact was lower for LSC pigs than for HSC pigs. In the ad libitum feeding period, we also observed a lower carbohydrate oxidation for LSC pigs at 2:00 and 3:00 pm than for HSC pigs. It might be that LSC pigs in the ad libitum feeding period were less active and therefore used less energy for activity, leading to lower carbohydrate oxidation for LSC pigs than for HSC pigs. In the restricted feeding period no effects on carbohydrate oxidation were found at 9.00 and 11.00 am. For RMR there were differences found at these moments in the 24 period, with higher RMR for LSC pigs than for HSC pigs.

It might be that the differences found for Hact in LSC and HSC pigs was related to differences in satiety between LSC and HSC pigs. It is known that in pigs, activity patterns reflect differences in satiety (Brouns et al., 1994, Beattie and O'Connellt, 2002, de Leeuw et al., 2008). Lower Hact values might reflect higher satiety in LSC pigs than in HSC pigs. When comparing the feed intake per kg $BW^{0.6}$ of the ad libitum and restricted feeding period for both SC groups, we see a larger reduction in intake between the two periods for LSC than for HSC pigs. The LSC pigs had more difficulty with consuming 70% of their ad libitum intake in two meals of 30 min. than HSC pigs, even though the absolute reduction in g/d was less big for LSC pigs. More feed refusals for the LSC pigs in the restricted feeding period led to a stronger reduction in feed intake between the two periods for the LSC compared with the HSC pigs. Taking this into consideration, the reduction in energy expenditure for physical activity following the morning meal likely reflects increased satiety of these pigs, suggesting that feeding patterns over the day may be affected by SC.

The RQ for the oxidation of carbohydrates, fats and proteins is approximately 1, 0.7 and 0.8, respectively, slightly depending on the type of substrate within each class. An RQ exceeding 1 indicates *de novo* fatty acid synthesis from carbohydrates (Kuhla *et al.*, 2015, van den Borne et al., 2015). The RQ value for the ad libitum feeding period was continuously exceeding 1, reflecting fat deposition from carbohydrates, and corresponds with the constant negative fat oxidation found during the day. The diurnal pattern for the RQ of pigs in the restricted feeding period was comparable to restricted fed pigs with a diet containing pregelatinized starch in a study of Gerrits et al. (2012) and to restricted fed pigs with a pregelatinized or a native potato starch in a study of Bolhuis et al. (2008). Hence, the diurnal pattern found for the restricted feeding period seems to be typical for restricted fed pigs. Diurnal patterns for carbohydrate and fat oxidation were unaffected by SC. Hence, there are no indications that differences in the incremental efficiency of dietary protein for protein deposition can be linked to particular differences in fat or carbohydrate oxidation at particular times of the day. It can be concluded from the present study that LSC pigs reduced their energy expenditure on physical activity and increased their resting metabolic rate in the restricted feeding period compared with HSC pigs, especially when lights were on. Therefore the diurnal energy expenditure pattern of LSC and HSC pigs can be considered as different. Within day patterns of net fat and carbohydrate oxidation were not affected by sanitary conditions. Therefore It is speculated that the reduction of the incremental utilization of dietary protein under low sanitary conditions is not restricted to particular periods within the day.

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Table 5.1 Partitioning of heat in pigs kept under different sanitary conditions and fed a diet with or without extra AA at 70% of ad libitum intake and during a 25 h period of fasting[1]

	LSC[2]		HSC			*P*-values[6]			
Item (kJ·BW$^{-0.6}$·d^{-1})	AA-B[3]	AA-S	AA-B	AA-S	SEM[5]	SC	AA	batch[7]	SC×AA
n[4]	6	3	6	4					
Htot	1168	1134	1149	1135	15	0.16	0.59	**0.002**	0.58
Hact	177	204	179	206	26	0.32	0.95	0.42	0.99
RMR	990	930	970	929	23	0.05	0.66	0.26	0.70
TEF	250	228	237	248	20	0.78	0.83	0.74	0.41
sTEF	165	139	155	161	18	0.58	0.72	0.82	0.40
lTEF	85	89	82	87	17	0.77	0.87	0.98	0.99
FHP	741	702	733	680	23	0.06	0.52	0.09	0.77

Htot = total heat production; Hact = physical activity related heat; RMR = resting metabolic rate; TEF = thermic effect of feeding; sTEF = short thermic effect of feeding; lTEF = long thermic effect of feeding; FHP = fasting heat production; GE = gross energy.[1]Results are predicted means, adjusted to the same GE intake by including observed GE intakes per group as covariate in the model. [2] LSC = Low sanitary conditions, HSC= High sanitary conditions. [3] AA-B = basal amino acid profile, AA-S= supplemented amino acid profile 20% more Met, Thr, and Trp compared with basal. [4] n = number of pens, a pen contained 6 pigs. [5] SEM = pooled standard error of the mean, means are presented as least square means. [6] Considered significant when $P \leq 0.05$, considered as tendency when $0.05 < P \leq 0.10$. [7]No interactions with batch were found.

SUPPLEMENTAL TABLE

Supplemental table I. Ingredients and nutrient composition of the treatment diets

Item	AA-B[1]	AA-S[1]
Ingredient, g/kg of feed		
Wheat	308.6	308.6
Maize	200.0	200.0
Barley	200.0	200.0
Soybean meal	184.5	184.5
Maize starch	25.8	23.4
Sugarcane molasses	20.0	20.0
Limestone	14.7	14.7
Monocalcium phosphate	9.0	9.0
Soybean oil	19.2	19.2
Vitamin + mineral mix[2]	5.0	5.0
Salt	3.5	3.5
L-lysine HCl	3.4	3.4
Titanium dioxide	2.5	2.5
Sodium bicarbonate	2.1	2.1
L-threonine	1.0	2.1
L-tryptophan	0.0	0.4
DL-methionine	0.4	1.3
L-Valine	0.3	0.3
Nutrients calculated, g/kg		
NE, MJ/kg[3]	9.8	9.8
DM	889.6	889.8
Crude protein[4]	166.0	167.0
Starch	474.1	472.3
Lys[4]	9.8	10.1
Thr[4]	6.6	7.8
Trp[4]	2.2	2.6
Met + Cys[4]	5.2	6.2
Ile[4]	8.2	8.3
Arg[4]	11.8	12.2
Phe[4]	9.4	9.9
His[4]	5.5	5.3
Leu[4]	15.0	15.3
Tyr[4]	6.2	6.2
Val[4]	9.6	9.8

[1] AA-B = basal dietary AA profile, AA-S= supplemented dietary AA profile containing 20% more Met, Thr, and Trp compared with the basal profile.

[2] Supplied the following per kilogram of diet: 3.0 mg riboflavin, 20 mg niacine, 20 mg d-pantothenic acid, 10 mg choline chloride, 0.015 mg cyanocobalamin, 40 mg dl-α-tocopheryl acetate, 1.5 mg menadione, 6,000 IU retinyl acetate, 1,200 IU cholecalciferol, 0.2 mg folic acid, 1.0 mg thiamin, 1.0 mg pyridoxine HCl, 50 mg manganese oxide, 267 mg iron $SO_4 \cdot H_2O$, 60 mg copper $SO_4 \cdot 5H_2O$, 140 mg zinc $SO_4 \cdot H_2O$, 0.44 mg disodium selenium trioxide,1.0 mg potassium iodate.

[3] Based on chemical composition, digestibility, and energy value for pigs from the Centraal Veevoeder Bureau livestock feed table (CVB, 2011).

[4] Analyzed values.

Chapter 6

General discussion

INTRODUCTION

It is economically and environmentally important to match the nutrient supply to the nutrient requirements in pig production. Until now, the effects of different sanitary conditions (SC) on energy and nutrient requirements are not implemented in recommendations for nutrient requirement values (ARC, 1981; Whittemore et al., 2003; Santioga Rostagno et al., 2011; NRC, 2012; CVB, 2016). The nutrient requirement data in these tables are based on studies in with pigs are kept under conditions that can be regarded as close to optimal. Changes in nutrient requirements caused by differences in sanitary conditions are poorly documented. As in pig industry hygienic conditions are variable it is of major importance to determine the effects of low sanitary conditions (LSC) on amino acids (AA) and energy requirements in growing pigs. Pigs under LSC have an increased risk of (subclinical) infections, resulting consequently in a more chronic stimulation of their immune system. Immune system stimulation is known to influence energy and AA metabolism. However, most studies in pigs evaluating the relationship between immune system stimulation and nutrient requirements use specific experimental challenge models. Whereas such models have the obvious advantage of reproducibility and allow mechanistic insight in the effects of stimulating specific parts of the immune system, these models often are in the range of clinical illness and results from such models may therefore be difficult to translate to practical situations.

Therefore the objective of this thesis was to study the effects of low and high sanitary conditions (HSC) on AA and energy metabolism in pigs. In this chapter, the different experiments presented in this thesis are discussed with emphasis on the different models used in comparison with other challenge studies. Additional data are presented in text boxes. Methodological aspects related to measurements on AA and energy metabolism are discussed and an overall summary of effects for LSC pigs compared with HSC pigs and consequences for nutrient requirements is given with implication of the results for current pig feed industry. General conclusions are provided at the end of this chapter.

CHALLENGE STUDIES TO STIMULATE THE IMMUNE SYSTEM

To implement effects of SC on energy and nutrient requirements for growing pigs in feed advise tables, data on the effects of SC on energy and nutrient metabolism are needed. Sanitary conditions are known to influence the energy and nutrient requirements via the immune system, and therefore it is interesting to collect data of pigs with a contrast in immune stimulation. To study the effects of immune stimulation due to LSC, a model is needed that mimics chronic immune stimulation, on a subclinical level, and allows to study the impact on nutrient requirements. In literature several immune stimulating models have been described and several porcine immune stimulation models used in relation to nutrition or metabolism are listed in Table 6.1.

Table 6.1 Overview of models used in pigs experiments to stimulate the immune system in relation with nutrition

challenge	dose	estimated BW[1]	response	fever	duration	ΔADFI[2]	ΔADG[2]	ΔG:F ratio[2]	references
Lipopolysaccharide (LPS)									
LPS	repeated increasing i.v. dose, every 48 h for 7 d	21-25 kg	haptoglobin↑, fibrinogen↑	yes	7 days, from start to end	-6%	.	.	Rakhshandeh et al. (2010)
LPS	repeated i.v. dose, every 48 h for 7 d; initial dose 20 µg/kg BW, +15% per dose	20-25 kg	haptoglobin↑, albumin↓, fibrinogen↑, white blood cell count↑	yes	7 days, from start to end	.	.	.	de Ridder et al. (2012)
LPS	repeated i.v. dose, every Monday and Thursday for 6 weeks;, initial dose 30 µg/kg BW, +15% per dose	53-60 kg	haptoglobin↑, albumin↓, neutrophils↑	yes	6 weeks, from start to end	.	.	.	Kim et al. (2012)
LPS	repeated i.v. dose, d1 every 48h until day 7, initial dose 50 µg /kg BW, +12% each dose and the last one +15%	18-22 kg	haptoglobin↑, albumin↓, fibrinogen↑	yes	7 days, from start to end, ± 12h of anorexia per injection	.	.	.	Litvak et al. (2013)
LPS	Repeated i.v. dose, d1, 3, 5, 7, 9;initial dose 30 µg/kg BW, +12% per dose	64-68 kg	.	yes	10 days, from start to end	-25%	-67%	-55%	Campos et al. (2014)
LPS	single i.v. dose, 2 µg/kg BW	12-16 kg	TNF-α↑, IFN-γ↑	yes	± 6 hours	.	.	.	Clouard et al. (2015)
LPS	single i.v. dose, 25 µg/kg BW		TNF-α↑, IL-1b↑, IL-6↑, haptoglobin↑, WBC↑	.	± 6 hours	.	.	.	Williams et al. (2009)

[1] BW = body weight, [2] Δ = the difference between challenged and unchallenged pigs, ADFI = average daily feed intake, ADG average daily gain, G:F = gain to feed. [3] RBC = red blood cells.

challenge	dose	estimated BW[1]	response	fever	duration	ΔADFI[2]	ΔADG[2]	ΔG:F ratio[2]	references
Complete Freund's adjuvant (CFA)									
CFA	*i.v.* 3 mL per pig	11-16 kg	haptoglobin↑, respiratory rythm↑	Yes	7 days, from start to end, clinical sign seem to decrease after 2 days	.	no effect	.	Le Floc'h et al. (2008)
CFA	*i.v.* 3 mL per pig	11-15 kg	haptoglobin↑, respiratory rythm↑	Yes	10 days, from start to end, clinical sign seem to decrease after 2 days	-50% immediately after	no effect	.	Melchior et al. (2004)
CFA	*i.v.* 0.2 mL/kg BW	28-35 kg	haptoglobin↑, CRP↑, PigMAP↑	.	9 days, from start to end	.	.	.	Kampman-van de Hoek et al. (2015)
CFA	*i.v.* 0.2 mL/kg BW	30-40 kg	haptoglobin↑, CRP↑, PigMAP↑ respiratory rythm ↑	Yes	9 days, from start to end	-30% immediately after, no effect from 2 d onwards	-10%	.	Kampman-van de Hoek et al. (2011)
Turpetine oil (TO)									
TO	*s.c.* 1.0 mL/kg BW every 72h	24-27 kg	IL-1β ↑, RBC[3] ↓, skin ulcers↑	Yes	10 days, from start to end	±-50%	.	.	Rakhshandeh and de Lange (2012)
TO	*s.c.* 0.3 mL/kg BW	20-25 kg	haptoglobin↑, CRP↑, PigMAP↑	.	± 14 days	.	.	.	Carpintero et al. (2005)
TO	*s.c.* 0.3 mL/kg BW	18-22 kg	haptoglobin↑, CRP↑, PigMAP↑	Yes	± 15 days	.	.	.	Heegaard et al. (2011)
TO	*s.c* 0.3 mL/kg BW	30-40 kg	haptoglobin↑, CRP↑, PigMAP↑	Yes	9 days, from start to end	-17% immediately after, no effect from 2 d onwards	no effect	.	Kampman-van de Hoek et al. (2011)

[1] BW = body weight, [2] Δ = the difference between challenged and unchallenged pigs, ADFI = average daily feed intake, ADG average daily gain, G:F = gain to feed. [3] RBC = red blood cells.

challenge	dose	estimated BW[1]	response	fever	duration	ΔADFI[2]	ΔADG[2]	ΔG:F ratio[2]	references
A. pleuropneumoniae									
A. pleu	Serotype 5b, biotype 1	28-35 kg	haptoglobin ↑,CRP ↑, MAP ↑	yes	± 15 days	.	.	.	Heegaard et al. (1998)
A. pleu	inhalation with serotype 4 isolate	15-20 kg	haptoglobin ↑,CRP ↑, PigMAP ↑	yes	± 20 days	loss of appetite direct after	.	.	Heegaard et al. (2011)
A. pleu	intranasal with serotype 5b, isolate L20	15-20 kg	CRP ↑, IL-6 ↑	.	.	.	.	.	Skovgaard et al. (2009)
A. pleu	serotype 5b, biotype 1	28-35 kg	PigMAP ↑, Apo A-1 ↓	.	± 12-15 days	.	.	.	Carpintero et al. (2005)
E. coli (ETEC)									
ETEC	5 ml of E. coli K88+ dose of 10^{10} CFU/ml per piglet	5-10-kg	diarrhoea↑, mortality↑	.	2 days, from start to end	.	.	.	Marquardt et al. (2006)
ETEC	oral challenge of 2×10^{9} CFU comprised of two strains of F18 ETEC	6-10 kg	IL-6↑, IL-8↑, TNF-α↑, neutrophils↑, diarrhea↑, villus length↓, mucosal mast cells↑	.	4 days, from start to end	.	-30%	.	Mclamb et al. (2013)
ETEC	orally dose of 10 ml PBS containing 1 × 10^{8} CFU of ETEC K88 2 subsequent days	8-10 kg	diarrhoea↑, gene expression IL-10, and IL12a↑	.	.	-35%	-60%	-30%	Heim et al. (2014)
S. suis									
S.suis	serotype 2, strain 93	30-35 kg	PigMAP↑, ApoA-1↓	.	± 12-15 days	.	.	.	Carpintero et al. (2005)
S.suis	s.c. serotype 2, ribotype I isolate, 1 mL (1010 CFU)	10-15 kg	haptoglobin↑, CRP↑, PigMAP↑	yes	± 20 days	loss of appetite at 1st day p challenge	.	.	Heegaard et al. (2011))

[1] BW = body weight, [2] Δ = the difference between challenged and unchallenged pigs, ADFI = average daily feed intake, ADG average daily gain, G:F = gain to feed.

The effects on the immune system and/or pig performance are listed per model in Table 6.1. All described models stimulated the innate immune system of the pig, which was expected. This means that cytokines produced by innate immune cells and acute phase proteins in the blood, and rectal temperature increase significantly in response to the challenge. The adaptive immune system was not evaluated in these studies, which does not mean that this part of the immune system was not influenced by these challenges.

Administration of a single *i.v.* dose of lipopolysaccharide (LPS) leads to a short sickness response of about 6 hours (Williams et al., 2009; Clouard et al., 2015). Injection with LPS, typically leads to an increase in proinflammatory cytokines (IL-6, TNF-α, IFN-γ), body temperature, acute phase proteins (haptoglobin, CRP, pigMAP, fibrinogen) and the number of white blood cells (WBC). Attention should be paid to the effects of LPS injection between individual pigs, as the sickness response for some pigs might be very severe and even may cause death (Williams et al., 2009). By repeating the administration of LPS every two days the immune system can be stimulated for a longer period than 6 hours (for example for 7-10 days, (Rakhshandeh et al., 2010; de Ridder et al., 2012; Kim et al., 2012; Litvak et al., 2013; Campos et al., 2014). Pigs develop, however, rapidly tolerance to LPS. To maintain a similar immune stimulating effect, an increased dosage of 12% or 15% should be given at each subsequent injection (Table 6.1). Campos et al. (2014) and Rakshandeh et al. (2010) reported a reducing effect on ADFI when pigs were repeatedly challenged with LPS. Campos et al. (2014) also found a negative effect on ADG and G:F ratio, suggesting that the lower ADG found for challenged pigs was not only due to the reduced feed intake but also partly due to a different efficiency. In contrast, Pastorelli et al. (2012b) showed in a meta-analysis based on 12 studies with LPS challenged pigs, that a reduction in ADG after this challenge was associated with a reduction in feed intake rather than related to changes in feed efficiency.

Another model to stimulate the immune system of pigs is *i.v.* injection with Complete Freund's Adjuvant (CFA) (Melchior et al., 2004; Le Floc'h et al., 2008; Kampman-van de Hoek et al., 2011). Complete Freund's Adjuvant is a mineral oil containing 1 mg of dead *Mycobacterium tuberculosis* cells per mL. As CFA contains dead bacteria this challenge is not infectious to other animals in the same room or pen. After injection with CFA pigs will develop a chronic lung inflammation, and acute phase proteins like haptoglobin, pigMAP, and CRP increase. Pigs will become lethargic, and the respiratory rhythm of the pig is accelerated. The clinical signs of illness are reported to decrease after 2 days post challenge (Melchior et al., 2004; Le Floc'h et al., 2008) and after dissection lung lesions can be found, which were found to be the most severe between 7 and 14 days post injection (Edwards and Slauson, 1983). Severity of the response to CFA challenge can be variable between individual animals. Kampman-van de Hoek et al. (2015) reported that eight out of 16 pigs responded clinically more severe than expected based on the response in a previous pilot study with the same challenge.

Turpentine oil (TO), an oil from a coniferious tree (*Pinus spp*) is *s.c.* injected and is used in pigs and also other animal as a model agent, such as LPS and CFA, to stimulate the immune system. Similar to LPS and CFA, TO leads to increased acute phase protein concentrations in blood and fever after injection, most likely due to cytokine production (IL-6, TNF-α, and IL-1β). Kampman-van de Hoek et al. (2016) found a greater within animal variation and a lower magnitude of response for acute phase proteins of pigs challenged with TO compared with CFA. In a study of Rahkshandeh and de Lange (2012) an extreme reduction in feed intake (50%) and severe skin ulcerations were found in repeatedly TO injected pigs, which indicates that TO as a model leads to severe responses.
Next to the non-pathogenic agents such as LPS, CFA and TO, there are models in which pigs are exposed to live pathogenic bacteria, such as *Actinobacillus pleuropneumoniae*, *Escherichia coli,* and *Streptococcus suis* bacteria to stimulate the immune system. These bacteria are orally dosed or intranasal administered to the pig. *E. coli* is used for inducing an intestinal infection leading to diarrhoea, *A. pleuropneumoniae* to induce a lung inflammation, and *S. suis* challenge might lead to clinical signs of illness, such as lameness and meningitis. The effects of these three experimental models using living pathogenic bacteria can be managed by using a standard dosage of bacterial cells to infect the pigs. It is difficult to estimate how long the immune system remains stimulated after the described bacterial challenges, as in many studies pigs are dissected before responses were back to baseline again. Based on the references listed in Table 6.1 the effects of a challenge with *E. coli* last at least several days (± 4 days; McLamb et al., 2013) and a challenge with *A. pleuropneumoniae* and *S. suis* can result in a stimulated immune system up to 20 days (Heegaard et al., 2011).

In general, models with agents such as LPS, CFA, and TO and living pathogens are suitable for studies evaluating nutritional costs of disease or mechanistic relationships between immune stimulation and nutrition. These models already lead to valuable information about the effects of immune stimulation on AA requirements in pigs, and showed for example that requirements for tryptophan, and methionine were increased for immune stimulated pigs. The results of studies using such challenge models, however, are difficult to translate to an on farm situation. The models using live pathogens are associated with variable responses, high mortality rate and extreme loss of BW, depending on the nature and pathogenicity of the micro-organism (Wichterman et al., 1980). When using living pathogens in an immune stimulation model, it should be taken into account that special housing conditions should be available to prevent spreading of the pathogen to unchallenged animals. Models with non-pathogenic agents such as LPS, CFA and TO are sterile models which can be more easily controlled and can induce more reproducible responses. Administering these agents in a high concentration single dose, however, might lead to severe clinical responses and it is not unusual that some of the challenged pigs die (Williams et al., 2009). Reduction of the concentration per dose gives less severe responses,

however shortens the recovery time to several hours and repeated or continuous administration of LPS leads to tolerance.

The natural challenges for a growing pig on a commercial farm are mostly less extreme and more low-grade (often subclinical instead of clinical) than the immune stimulation models presented in Table 6.1. Some examples of factors stimulating the innate immune system of growing pigs on commercial farms are the presence of pathogenic bacteria, viruses, and parasites that can spread via faeces, dust, feed, water, or low hygiene conditions in general. A sub-optimal climate (too cold or warm, air flow too high or low), high animal density and behavioural problems (biting behaviour, aggressive behaviour) may aggravate this by increasing transmission of pathogens, causing stress and irritated tissue.

Pigs on farm often have not to deal with one single immune stimulating factor, such as LPS or an infection with *E. coli,* but with a combination of the earlier mentioned factors. To measure the effects of these commercial farm factors on energy and AA requirements of pigs, these commercial farm conditions should be mimicked with a model in an experimental setting or the measurements should be done on a farm having these conditions. The following criteria were set for an experimental model to study energy and AA metabolism of growing pigs affected by sanitary conditions:

- The experimental conditions should lead to a contrast in innate immune stimulation, however clinical signs of disease should be absent.
- The contrast in innate immune stimulation, should be created in a group of pigs with the same origin and background
- Interaction with pen mates in group housing should be possible by using the model

For the experiments described in Chapter 2 and 4 an experimental model based on these criteria was used. In both experiments pigs with clinical signs of illness were removed from these studies (n = 1 and n = 0 for Chapter 2 and 4, respectively). The ADG of LSC pigs was lower, and LSC pigs had higher serum haptoglobin levels than HSC pigs. In addition, LSC pigs of Chapter 2, had greater KLH-IgG titers than HSC pigs. This indicates that in the absence of clinical signs of illness a contrast in immune effector functions was found between LSC and HSC pigs. In addition, after slaughter more lung lesions were observed in LSC than in HSC pigs, which is a sign of increased subclinical infections for LSC pigs.

Kampman-van de Hoek et al. (2016) selected pigs at a low and high health status farm based on a contrast in serological monitoring program for the presence of antibodies against several pathogens and moved these pigs to an experimental farm for measurements. Upon arrival at the experimental farm, all pigs were housed on metabolism cages. Low health status pigs were housed in a clean but not disinfected area, and were not treated with antibiotics, while high health pigs were housed in a cleaned and disinfected area including HEPA filters, and received an antibiotic treatment. High health pig personnel

had to follow a strict hygiene protocol. Despite the contrast in conditions for low and high health pigs, low health pigs showed a greater ADG, more efficient N digestion, and greater N retention during the experiment than high health pigs (Kampman-van de Hoek et al., 2016), which was explained as possible compensatory effect due to improved health during the experimental period for low health status pigs compared with the period on the farm of origin before the experimental period. The study of Kampman-Van de Hoek et al. (2016) shows that it is difficult to maintain the health status of pigs of a farm of origin in an experimental setting, particularly when housed in metabolism cages. When a contrast in health status is imposed to a group of pigs with the same original health status and are obtained from the same farm, as done in Chapter 2 and 4, low sanitary status could be maintained more easily.

For the studies described in Chapter 2 and 4 several batch effects were found for performance and immunological parameters but not for parameters related to the incremental efficiency of nutrient utilization, which means that the response of pig batches varied among treatments. The batch effects might be caused by a combination of effect of the batch of pigs, originating from the same farm per experiment but obtained at a different time point, the effect of batches of manure and dust introduced to the pens, and the effect of time. As the housing conditions during the experiments were standardised, it is assumed that the batch effects were due to pig batch and manure and dust batch. Most of the effects found for batch were not changing the direction of the observed effects. Reducing experimental variation by the use of a single batch, as done by others using similar models (Williams et al., 1997; Le Floc'h et al., 2009; Pastorelli et al., 2012a), should be reconsidered as the observed variation among batches of Chapter 2 and 4 indicate the importance of having more than a single batch in studies using a chronic low grade immune stimulation approach.

VARIATION IN CONDITIONS FOR DIFFERENT EXPERIMENTAL MODELS

In literature several studies have been described that used a similar chronic low-grade immune system stimulation model (Williams et al., 1997; Lee et al., 2005; Le Floc'h et al., 2006; Le Floc'h et al., 2009; Pastorelli et al., 2012a; Jayaraman et al., 2016). Each study uses a combination of factors to create a contrast in immune system stimulation and was compared with the studies described in Chapter 2 and 4 (Table 6.2). It is interesting to see whether these models, by using a different combination of factors, result in a similar effect as in our studies.

Haptoglobin concentration. When comparing the haptoglobin concentration in the blood of pigs from these eight comparable studies (Fig 6.1), haptoglobin concentration was greater

for LSC pigs compared with HSC pigs in four out of six studies that measured this parameter. Jayaraman et al. (2016) found a tendency for higher haptoglobin concentration for LSC compared with HSC pigs. The haptoglobin values for Jayaraman et al. (2016) were in a higher range than in the other studies. Perhaps this was due to more severe conditions such as a high aerial ammonia concentration for both LSC and HSC pigs compared with the other studies. Pastorelli et al. (2012a) did not find a significant difference for average haptoglobin concentration between LSC and HSC pigs over the complete experimental period. Pastorelli et al. (2012a) found a tendency for greater haptoglobin values in LSC compared with HSC pigs in a period before the pigs were transferred from the weaner to the grower unit, which was an event meant as stressor in both LSC and HSC groups. Perhaps transfer from weaner to grower unit stimulated the immune system of both LSC and HSC pigs resulting in similar haptoglobin values in blood.

Table 6.2 Overview of factors used to create a contrast in immune stimulation for six comparable studies with pigs

Nr.	Reference	n	Housing	m^2/ pig	Weaning age	Initial age contrast	Duration of experiment
1	experiment 1 (Chapter 2)	612	Group	0.8	24 d	70 d	16 wk
2	experiment 2 (Chapter 4)	144	Group	0.8	21 d	21 d	8 wk
3	le Floc' h et al., 2006	40	Individual	2.3	28 d	28 d	±6.5 wk
4	le Floc' h et al., 2009	80	Individual	.	28 d	28 d	±6 wk
5	Pastorelli et al., 2012	20	Individual	0.5	28 d	28 d	±6 wk
6	Williams et al., 1997	30	Individual	0.4	12 vs. 19 d	12 vs. 19 d	± 7 wk
7	Jayaraman et al., 2016	180	Group	.	21 d	21 d	2 wk
8	Lee et al., 2005	272	group/individual	0.54	21 d	21 d	5 wk

	low sanitary condition	high sanitary condition
vaccination program		1,2, (6)[a]
clean and disinfected rooms		1,2,3,4,5,6,7,8
preventive antibiotics		1,2,3,4,5
deworming		1
introduction of non-experimental pigs	3,4,6	
spreading manure	1,2,7	
spreading dust	2	
hygiene protocol personnel		1,2
recycling air in room	8	

[a] The number between brackets means that these pigs were the offspring of vaccinated sows instead of vaccinated experimental pigs.

Vaccination as part of the model. Pastorelli et al. (2012a) vaccinated both LSC and HSC pigs against Influenza, as controlled immunological challenge to compare the responses of both LSC and HSC pigs. The LSC pigs tended to have higher serum antibodies against influenza virus than HSC pigs, which suggest that LSC stimulated the antigen-specific humoral response.

The studies described in Chapter 2 and 4 included a vaccination program as part of the sanitary condition model. It is debatable whether vaccination contributes to HSC. It is generally known that vaccination leads to initial innate immune activation as vaccines contain a weak or dead pathogenic microorganism. Vaccination might therefore cause loss of appetite and fever and/or adverse behaviour for a short period as side effect (Maes et al., 1999; Thacker et al., 2000). The vaccination program used in the studies described in Chapter 2 and 4 was not meant to challenge HSC pigs but to protect HSC pigs against disease. The vaccination program included administration of six vaccines during the first nine weeks of age. In the first experiment (Chapter 2) HSC pigs were 1 kg lighter than LSC pigs at 10 weeks of age (after a complete vaccination program) and in the second experiment (Chapter 4) HSC pigs were 0.3 kg lighter at weaning (± 21 days of age, after one vaccination) than LSC pigs of the same age. In both experiments this effect was in the opposite direction during the experimental period. The differences in BW at the beginning of the experiments might be due to a negative effect immediately after vaccination on feed intake and growth. These negative effects on performance disappeared, however, within 2 weeks. It can however not be excluded that vaccination of the HSC pigs reduced unintentionally the immunological contrast between LSC and HSC pigs. Inclusion of a vaccination programme as a factor in the experimental model in this sense can be controversial. In the experiment described in Chapter 2, however, the LSC pigs were tested positive for porcine circo virus type 2 (PCV2), a virus HSC pigs were vaccinated against. It is likely that PCV2 vaccination protected the HSC pigs and consequently maintained a contrast in immune stimulation between LSC and HSC pigs. In this sense vaccination as part of an experimental model for high sanitary conditions can be beneficial. Vaccination of the experimental pigs should therefore be considered based on the risks for disease during the experiment. When taking this into account vaccination of the HSC pigs in the experiment described in Chapter 4 was probably not needed as this experiment was conducted under (clean) experimental facilities with a low risk for pig diseases.

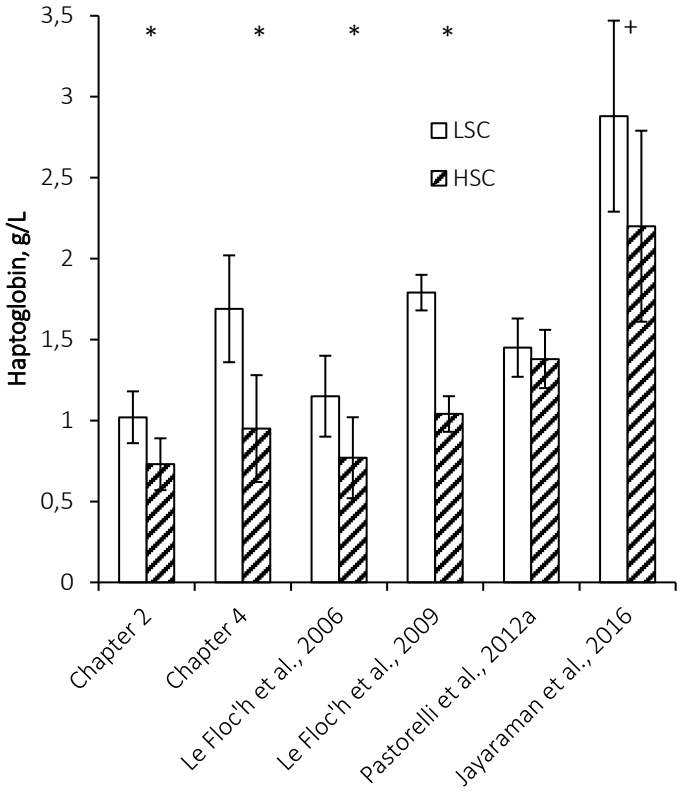

Figure 6.1. Comparison of haptoglobin concentration in blood of low (**LSC**) and high (**HSC**) sanitary condition pigs for several references using a similar model to stimulate the immune system (Le Floc'h et al., 2006; Le Floc'h et al., 2009; Pastorelli et al., 2012a; Jayaraman et al., 2016). * $P \leq 0.05$, + $0.05 < P \leq 0.10$.

Performance and sanitary condition model. When comparing performance data of the previously mentioned eight studies the results for average daily gain (**ADG**) give a similar picture (Fig 6.2). The difference in ADG between LSC and HSC treatments was significantly different for all studies, with HSC pigs having the highest ADG. The proportion of the difference in ADG, when expressing the difference between LSC and HSC as a percentage of HSC was variable between the studies (Fig 6.3). The lowest difference (6% reduction in ADG for LSC compared with HSC) was found in the experiments described in Chapter 2 and for a study of Le Floc'h et al. (2006). The highest difference was found in the experiment described in Chapter 4 (26% difference in ADG). In all studies, except for Jayaraman et al. (2016), the difference in ADG was due to both a difference in average daily feed intake (**ADFI**) and gain to feed (**G:F**) ratio for the LSC and HSC treatment groups. In a meta-analysis of Pastorelli et al. (2012b) the reduction in ADG was reported for different immune challenged pigs compared with control pigs. For poor housing conditions, a combination of studies with poor hygiene conditions, exposure to extreme temperatures, and limiting

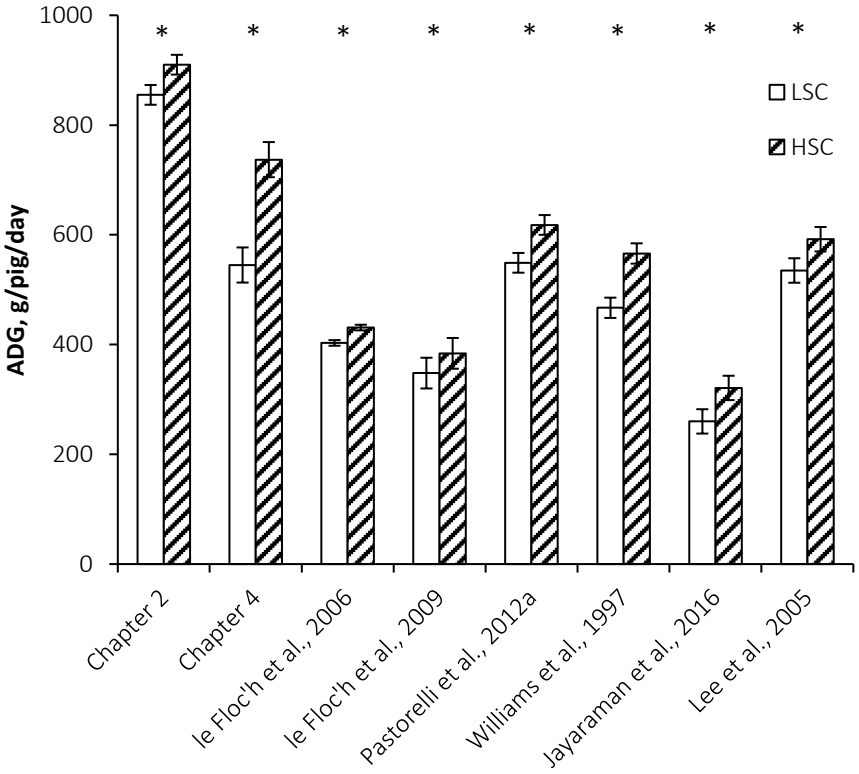

Figure 6.2. Average daily gain (**ADG**) of low (**LSC**) and high (**HSC**) sanitary condition pigs for several references using a similar model to stimulate the immune system (Williams et al., 1997; Lee et al., 2005; Le Floc'h et al., 2006; Le Floc'h et al., 2009; Pastorelli et al., 2012a; Jayaraman et al., 2016). * $P \leq 0.05$.

space allowance, a reduction of 15% in ADG was reported with 4.1% due to a reduction in ADFI and 12.2% due to reduced efficiency or G:F ratio. Despite that all the eight studies (Fig 6.3) have a contrast in hygiene conditions the results of these studies are not completely in line with Pastorelli et al. (2012b). All studies represented in in Fig 6.3 differed in more factors than only a contrast in hygiene conditions, other factors were different which might explain the difference in results with the previous mentioned meta-analysis. The difference in ADG was highest for the study described in Chapter 4, Williams et al. (1997), and Jayaraman et al. (2016). In these studies piglets were weaned at 21 d of age or younger (Table 6.1) and the contrast in sanitary conditions was created from weaning onwards for a duration of 2 to 8 weeks. The other studies except for Lee et al. (2005) used pigs weaned at 24 d (Chapter 2) or 28 d of age (Le Floc'h et al., 2006; Le Floc'h et al., 2009; Pastorelli et al., 2012a) and the contrast in SC was created from weaning onwards or later in life (Chapter 2, initial age 70 d), and the experimental period lasted for 40 d to 16 weeks. Pastorelli et al. (2012b) found that the difference in ADG decreased with increasing initial age and

increased duration of the experimental period for models containing poor housing conditions and digestive bacterial infections which is in line with the findings in Fig 6.3.

The difference in initial age and duration of the study might explain the difference in ADG contrast between the treatments of the studies described in Chapter 2 and 4, however, the experimental models used in both studies were not completely similar, which might have affected ADG as well. In the study of Chapter 4 a manure mixture of 3 growing pigs farms was spread weekly in the LSC pens, whereas in the study of Chapter 2 manure of one sow farm was spread every 2 weeks in the LSC pens from 5 weeks in experimental period onwards. In addition, dust (ground pig manure and straw) was introduced in the study in Chapter 4, which might have increased the contrast in SC compared with the study in Chapter 2. Despite the differences in spreading manure or dust, the composition of manure is also variable in time, which lowers the repeatability of such studies.

Effect of aerial ammonia on performance. When comparing the studies in which aerial ammonia concentrations were reported there is variability in results (Table 6.3). Ammonia is known to be irritating for respiratory tissues and eyes and will influence consequently respiratory function (Urbain et al., 1994; Groot Koerkamp et al., 1998). The threshold for ammonia was set on 20 ppm measured in the mean dwelling zone of animals by CIGR (CIGR, 1984). It is unclear where the value of the CIGR is based on. In a study of Groot Koerkamp et al. (1998) was described that the labour inspectorate in several countries in Europe has set the threshold limit for an 8 h working day on 25 ppm ammonia. In a study of Jones et al. (1999) was reported that weaner pigs showed averse to ammonia concentrations of more than 20 ppm in a choice study. In this perspective ammonia concentration was more severe in the study of Jayaraman et al. (2016) compared with the study in Chapter 2 and Lee et al. (2005). Drummond et al. (1980) found a negative effect of high concentrations of aerial ammonia (50 ppm) on pig growth (-12% growth). This negative effect of high ammonia concentrations on growth might partly explain the greater difference in ADG between LSC and HSC pigs in the study of Jayaraman et al. (2016) compared with the study in Chapter 2 and Lee et al. (2005).

Behaviour and sanitary conditions. The relation between sanitary conditions and behaviour of pigs is not often made. In the experiment described in Chapter 3, LSC pigs were more frequently biting in ears of pen mates than HSC pigs. The mechanism behind the relationship between LSC and redirected behaviour is not completely understood yet. Redirected behaviour might be a result of an increased drive for foraging behaviour as a consequence of shortages of nutrients caused by immune stimulation. In a study of Pastorelli et al. (2012a) individual housed LSC and HSC pigs were submitted to a diet change from a starter to a weaner diet over a 3 day period (12 to 14 days post weaning). The HSC pigs did not react on a change in diet, whereas the LSC pigs increased their exploration

behaviour towards the trough, which included sniffing, licking, touching and chewing on the trough. The absence in the behavioural effect of HSC pigs was explained as a lower reactivity for new situations associated with a more stable environment as described by Greenberg (2003) and the redirected behaviour of LSC pigs was considered as a typical signal of maladjustment as found by Wood-Gush and Vestergaard (1989). Despite that, a link between immune system stimulation due to LSC and a possibly consequential shortage in nutrients and increased redirected behaviour was not made in the study of Pastorelli et al. (2012a), behavioural measurements showed that sanitary conditions and behaviour were related. Behaviour of pigs as an indicator for wellbeing, can be considered as an important parameter for immune stimulation studies. The measurement of pig behaviour in studies about sanitary conditions should therefore be considered more often.

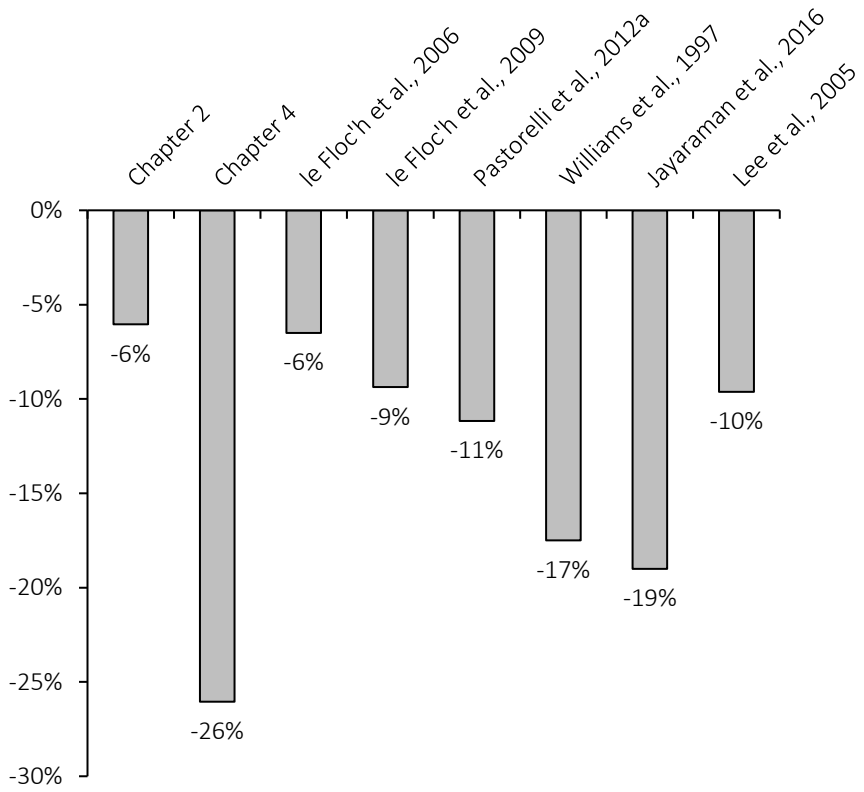

Figure 6.3. Difference in average daily gain (**ADG**) of low (**LSC**) and high (**HSC**) sanitary condition pigs for several references using a similar model to stimulate the immune system (Williams et al., 1997; Le Floc'h et al., 2006; Le Floc'h et al., 2009; Pastorelli et al., 2012a; Jayaraman et al., 2016). The difference is expressed as a percentage relative to HSC.

Table 6.3 Ammonia concentration for low and high sanitary condition pigs for several references using a similar model to stimulate the immune system

	Estimated BW	low sanitary condition	high sanitary condition	*P*-value
Chapter 2[a]	25-110 kg	17.1 ± 1.8 ppm	13.8 ± 1.3 ppm	0.14
Lee et al., 2005	6-20 kg	13.0 ± 0.7 ppm	6.0 ± 0.5 ppm	0.001
Jayaraman et al., 2016	7-12 kg	26.7 ± 1.0 ppm	18.2 ± 1.1 ppm	≤ 0.001

[a] unpublished data measured weekly per room from 17 until 22 weeks of age

Immune system stimulation. It is difficult to estimate the severity of the stimulation of the innate immune system in pig studies. Most of the studies about immune stimulation in relation to nutrition and health report haptoglobin values, some other acute phase proteins and body temperature, however, often not many more parameters are presented (Melchior et al., 2004; Le Floc'h et al., 2009; Rakhshandeh et al., 2010; Pastorelli et al., 2012a). Acute phase proteins are relevant parameters as it is known that these proteins are relatively stable in comparison to e.g. cytokines, however, more parameters are needed to gain insight in the physiological/metabolic processes in the pig under different sanitary conditions. Analysis of natural antibodies in blood can for instance be such an additional parameter. Natural antibodies are defined as antigen-specific antibodies that are present in the absence of intentional immunization with specific antigens (Star et al., 2007). Because natural antibodies play a role in the first line of defence against pathogens (Ochsenbein and Zinkernagel, 2000), an increase in natural antibody concentration might be an adaptive response of pigs to the higher infection pressure for instance in the case of LSC. The experiment described in Chapter 4 showed higher KLH specific natural antibody levels in LSC than in HSC pigs and this suggests together with the results presented in Textbox 6.1, that there was a contrast in immune system stimulation between LSC and HSC pigs. Measurement of white blood cell concentrations and blood cell differentiation might give extra insight, in the stimulation of the immune system. For clinical infection studies this might work as the results can be compared with reference values for clinically healthy animals. Results for blood cell counts as described in Chapter 2 and 4, however, were more difficult to interpret and gave variable results. Partly the problems with interpreting these blood cell counts, were due to the fact that these values were within the reference values for clinically healthy pigs.

The pathogenic pressure on the immune system can be measured by testing the presence of antigens against specific pathogens in the blood or the saliva of pigs. A method to analyse the presence of saliva was applied in the experiment described in Chapter 2. Collecting saliva of pigs, is rather easy when using chewing ropes, and the samples can subsequently be analysed for the presence of pathogenic bacteria, viruses and parasites with PCR or culture techniques. Saliva collection is not stressful for the pigs, so it will not influence other parameters measured in the experiment. Moreover, it is possible and easy to sample a pen of pigs with one chewing rope to get a representative saliva sample. The collected saliva samples in the study described in Chapter 2 were only used for qualitative

testing of the presence of antibodies against specific pathogens. Other available analytical methods allow to quantify antibody concentrations in saliva samples. Collection and analysis of saliva can therefore be of interest in studies evaluating the effects of sanitary conditions.

The pig performance and acute phase protein information reported in several studies about sanitary conditions, is too limited to conclude whether the innate immune system was stimulated differently in LSC than in HSC pigs. Additional measurements of natural antibodies, and antibodies against specific pathogens can give additional insight in the infectious pressure, however, further research is needed develop a method or to evaluate what combination of sanitary condition parameters are needed to get more insight into the actual level of innate immune responses.

It is interesting to investigate whether robustness of pigs is affected by different sanitary conditions. To this end, we studied within the setting of the experiment described in Chapters 2 and 3, the effect of sanitary conditions on the sickness response of pigs, provoked by a low, *i.v.* dose of LPS. This test is described in Textbox 6.1. From the results of this study, there are no indications that the sickness response of pigs is altered by sanitary conditions.

Textbox 6.1. Effects of RGG and LPS challenge in pigs kept under low or high sanitary conditions

Aim. To determine whether sanitary conditions (**SC**) affect the sickness response and antibody levels of pigs *i.v.* challenged with rabbit gamma globulin (**RGG**) and lipopolysaccharide (**LPS**).

Materials and methods. In Chapter 2, 612 pigs (9 per pen) were subjected to one of 8 experimental treatments in a 2 × 2 × 2 factorial arrangement. Pigs were kept under low (**LSC**) or high (**HSC**) sanitary conditions and offered ad libitum access to either a normal CP concentration diet or low CP concentration diet, each of which containing a basal AA profile or a supplemented AA profile. Pigs were followed for a complete fattening period and slaughtered at a targeted pen weight of 110 kg.
In total, 68 pigs (40 kg BW) of Chapter 2 (one per pen) were *i.v.* challenged with LPS (*Escherichia coli* LPS, serotype O111:B4; Sigma Aldrich, Zwijndrecht, The Netherlands, product code L4391) and RGG (ICN Biomedicals, Inc. code:824551). LPS, an endotoxin of *E. coli,* was used to induce a sickness response (Clouard et al. 2015), and RGG protein was used to induce a specific humoral immune response. The challenge solution was prepared the day prior to the injection by dissolving 1 mg RGG and 80 µg LPS in 1 ml sterile physiological saline and kept at 4 °C. The average BW of the challenged animals was 40 kg at the day of challenge, resulting in a challenge dose of about 2 µg of LPS/kg BW, similar to the dosage used by Clouard et al. (2015) and a challenge dose of 0.024 mg RGG /kg BW. One pig per pen was assigned to an i.v. injection with the challenge solution in the ear vein. The challenge pigs were restrained, the ear was cleaned with a gauze drained in alcohol and the vein at the ear base was clamped down by gently pressing it. The solution was injected using 0.5 ml syringes with 29 gauge needles.
Rectal temperature of the challenged pigs was measured just before the challenge and 1.5, 3.5 and 6.5 h after challenge. In order to assess sickness behaviour, a human approach test (**HAT**) was performed at 1, 3 and 6 h after the challenge. Although this test is normally used to assess fear and anxiety in pigs, in the present study, high latency in this test was considered indicative of sickness behaviour (Clouard et al., 2016). During the test, a person entered each pen and walked to a fixed position in the centre. The latency of the challenged pig to approach, or seek physical contact with the person was recorded, with a maximum of 120 sec.
Blood was sampled from the jugular vein of the challenged pigs at 5 and 10 days post challenge and blood serum samples were analysed for antibody concentrations (both IgG and IgM against each LPS, RGG and keyhole limpet hemocyanin (**KLH**) by ELISA. Antibody titers were determined as described by de Koning et al. (2015) with the minor modification that a 4-step dilution (40, 160, 640, 2,560 times diluted) of the sera was made instead of a 3-step dilution. Rectal temperature at the 3 time points following the challenge, and antibody titers at day 5 and 10 post-challenge were analysed by the proc MIXED procedure of SAS 9.3 (SAS 9.3; SAS Inst. Inc., Cary, NC) with SC, dietary protein level, dietary AA-profile, batch, and their interactions as fixed effects.

Results. The rectal temperature increased after a LPS/RGG injection for all challenged pigs and was almost back to baseline at 6.5 h post challenge (Fig 6.4). This response in rectal temperature was similar to the response of pigs in a study of Clouard et al. (2015) and shows a clear response on the LPS challenge for both treatments.

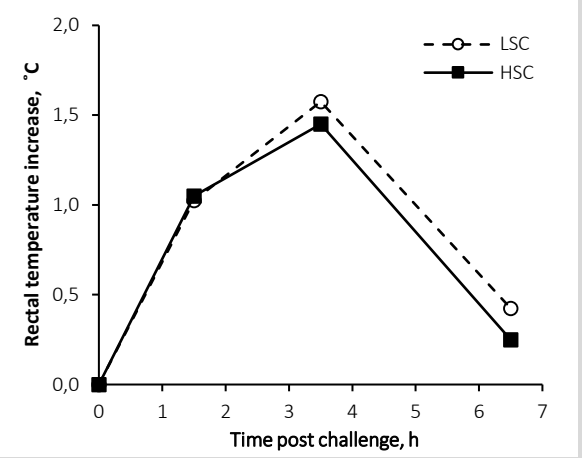

Figure 6.4 Rectal temperature response of low (**LSC**) and high (**HSC**) sanitary condition pigs challenged with LPS and RGG. * $P \leq 0.05$.

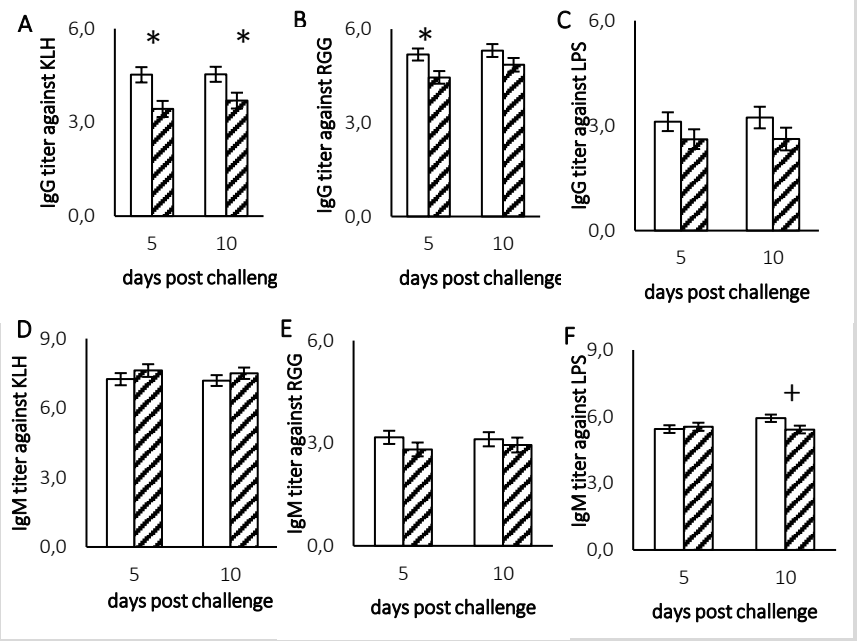

Figure 6.5. Antibody titers (type IgG and IgM) against keyhole limpet hymocyanin (**KLH**), rabbit gamma globulin (**RGG**), and lipopolysaccharide (**LPS**) in pigs measured at 5 and 10 days post *i.v.* challenge with LPS and RGG. * $P \leq 0.05$, + $0.05 < P \leq 0.10$. The open bars represent low sanitary condition pigs and the striped bars high sanitary condition pigs.

Rectal temperature increase in relation to the rectal temperature before challenge did not differ between LSC and HSC pigs (P = 0.58). Latency time in the HAT did not differ between LSC pigs and HSC pigs (P = 0.19).

Type IgG antibody titers against KLH, RGG were higher in LSC pigs compared with HSC pigs ($P \leq 0.05$) at 5 days post challenge, whereas LPS specific IgG titers, although numerically higher, were not significantly different between pigs of different sanitary conditions (Fig 6.5). In unchallenged pigs, the KLH specific IgG titers were also higher for LSC pigs compared with HSC pigs ($P \leq 0.05$; Chapter 2). It might therefore be that the SC effect in IgG against KLH, and maybe also against LPS and RGG, in challenged pigs is due to the contrast in sanitary conditions rather than to the difference in response to the combined LPS and RGG challenge. Unfortunately no blood sampling was done (to avoid stress due to blood sampling) before the LPS, RGG challenge to distinguish between effects due to this challenge or SC effects.

Conclusion. Low and high sanitary condition pigs do not respond differently to a HAT test and have very likely also no different rectal temperature, or antibody response after a combined i.v. RGG and LPS challenge. The effects found for antibody response are probably due to the SC effect rather than to the LPS RGG challenge effect.

TECHNIQUES TO MEAUSURE AMINO ACID AND ENERGY METABOLISM

In several studies, effects of sanitary conditions or acute immune stimulation on AA metabolism were studied by using a titration method between pigs (Williams et al., 1997; de Ridder et al., 2012; Kim et al., 2012; Kahindi et al., 2014; Jayaraman et al., 2015) or within individual pigs (Kampman-van de Hoek et al., 2013). In most of the studies using a titration method between pigs, different treatment groups with pigs were offered treatment diets with a contrast in dietary AA concentration developed by supplementing the AA in free form to a diet that is limiting in the AA of interest. For example, Kahindi et al. (2014) created a difference in lysine concentration in the treatment diets by addition of increasing levels of free lysine to the diet in exchange for maize starch. In a study of Kampman-van de Hoek et al. (2013) a within-animal titration approach was used in individual meal-fed pigs. The dietary Lys concentration was stepwise increased or decreased with a step length of 3 days, resulting in a minimum experimental time period of 21 days as 7 different Lys concentrations were evaluated. The advantage of this titration approach is that it results in the estimation of a requirement value for an individual animal. Nevertheless, a minimum time period of 21 days was required in the study of Kampman-van de Hoek. (2013), and standardization of effects of ISS over a prolonged period of time remains difficult, particularly when housing the pigs on metabolism cages.

In general, the titration method is an easy and accurate method to quantify the effect of sanitary conditions on the AA requirement of pigs. Nevertheless, by using the dose-response method the focus is only on a single supplemented AA that is assumed to be limiting.

Using multiple AA in an alternative approach, Kampman-van de Hoek et al. (2016) measured whole body fluxes of 9 essential AA simultaneously. This flux, referred to as the irreversible loss rate from the plasma AA pool, was measured in pigs assumed to be in a steady state (i.e. hourly fed) following an *i.v.* bolus of a mixture of U-^{13}C-labelled essential AA, after which the disappearance of ^{13}C-labeled AA from the plasma pool was measured for 60 min. When assuming that the measurement is in physiological steady state, differences in ILR between treatments (sanitary conditions) and between individual AA are assumed to reflect differences and changes in AA requirements and their metabolism. Unfortunately, data on ILR of AA do not discriminate between use in oxidative metabolism or protein synthesis (Kampman-van de Hoek et al., 2016). In addition, individual housing and frequent blood sampling is required.

The factorial method used in the experiments described in Chapter 2 and 4 was not meant to determine quantitative AA requirements of the pigs, but to determine whether the requirements of LSC and HSC pigs were different. The method used allowed to study the effect of a diet with a basal AA profile compared with a supplemented AA profile containing extra Met, Thr, and Trp in ratio to Lys compared with the basal diet in group housed pigs. By using this method it was possible to study the effect of supplementing more than one AA at the same time. In Chapter 2, the supplemented diet lead to greater ADG and G:F ratio than the basal diet in both LSC and HSC pigs. The increase in G:F was greater in LSC pigs, indicating that supplementing Met, Thr and Trp were more beneficial for LSC pigs than HSC pigs. With the method used it is not possible to distinguish between the effects of the individual AA supplemented, so it remains unclear if one of the three AA or a combination of AA was responsible for the observed effects. Therefore, results obtained with this approach do not lead to knowledge on quantitative changes in requirements of the specific AA, but these results show whether requirements for the combination of the supplemented AA were affected. This method can therefore be very useful to determine if there is a contrast in AA requirements for different groups of pigs. To determine the exact AA requirements the dose response approach might be useful.

Methods to measure energy metabolism. In Chapter 4 the effect of sanitary conditions on energy metabolism was measured by indirect calorimetry. By using this method the exchange of O_2, CO_2, and CH_4 per group of 6 pigs in climate respiration chambers was measured in 6 minutes intervals as described by Heetkamp et al. (2015). Heat production per chamber was calculated from the measured consumed volumes of O_2, produced volumes of CO_2, and CH_4. This calculation was done by using an equation (Brouwer, 1958) with coefficients based on the stoichiometry of the complete oxidation of carbohydrates,

fat and protein. The indirect calorimetry method is known to be an accurate method, however, attention should be paid in situations where an animals is pushed with a diet, infusate or when using indirect calorimetry to estimate short term variation in heat production (Gerrits et al., 2015), as it is assumed that O_2 and CO_2 pools within the body do not change within a measurement period. The indirect calorimetry method has the advantage that repeated measurements can be performed in group housed animals.

Another method to measure energy metabolism in pigs is the slaughter technique (Kim et al., 2012). Despite that this technique is accurate and simple, it is laborious and does not allow repetitive measurements within one animal. In a study of Kim et al. (2012) the slaughter technique was used to quantify the effects of immune system stimulation on AA metabolism, for an experimental period of 40 d. When using the slaughter technique for such a long experimental period, this means that the contrast in sanitary conditions should be standardized for at least 40 d. It will be a challenge to create a standardized contrast in extent of innate immune system activation over such a period. Therefore the indirect calorimetry method was preferred in Chapter 4. In the study described in this Chapter fasting heat production (FHP) was measured as estimate for energy requirement for maintenance. This was done in pigs fasted over a period of 25 h. To determine whether 25 h of fasting was enough to reach a plateau in resting metabolic rate (RMR) a part of the pigs were fasted for a period of 32 h (Textbox 6.2). The results in this textbox indicate that the RMR was not stable after 25 h of fasting yet, however, the conclusion that LSC pigs had greater FHP compared with HSC pigs was the same for 25 h and 32 h fasted pigs. From FHP, the energy requirement for maintenance (MEm) can be estimated by using the method of Labussière et al. (2011) as illustrated in Textbox 6.3. The MEm was 7% greater for LSC pigs compared with HSC pigs according to this method.

Textbox 6.2 Twenty-five vs. thirty two hours measurement for determining fasting heat production in pigs

In Chapter 4, fasting heat production (**FHP**) was measured as the resting metabolic rate (**RMR**) until 25 h after the start of the fasting period, in 24 groups with 6 pigs each. In addition, FHP of 16 of these groups was also measured until 32 h after initiation of fasting to determine whether 25 h of fasting was sufficient to reach a plateau in energy expenditure as measure for their RMR.

Materials and methods. In a 2 × 2 factorial arrangement, 16 groups of pigs housed in calorimetric chambers, were allocated to either a low sanitary condition (**LSC**) or a high sanitary condition (**HSC**), and were offered either a diet with a basal AA profile (**AA-B**) or a diet with a supplemented AA profile (**AA-S**) as described in Chapter 4. At 12 weeks of age pigs were deprived from feed after the morning meal at 0730 am until 0330 pm the next day. Gas exchange was measured in the calorimetric chambers in 6-min intervals by measuring exchange of O_2, CO_2, and CH_4, as described by Heetkamp et al. (2015). The FHP was measured as described in Chapter 4.

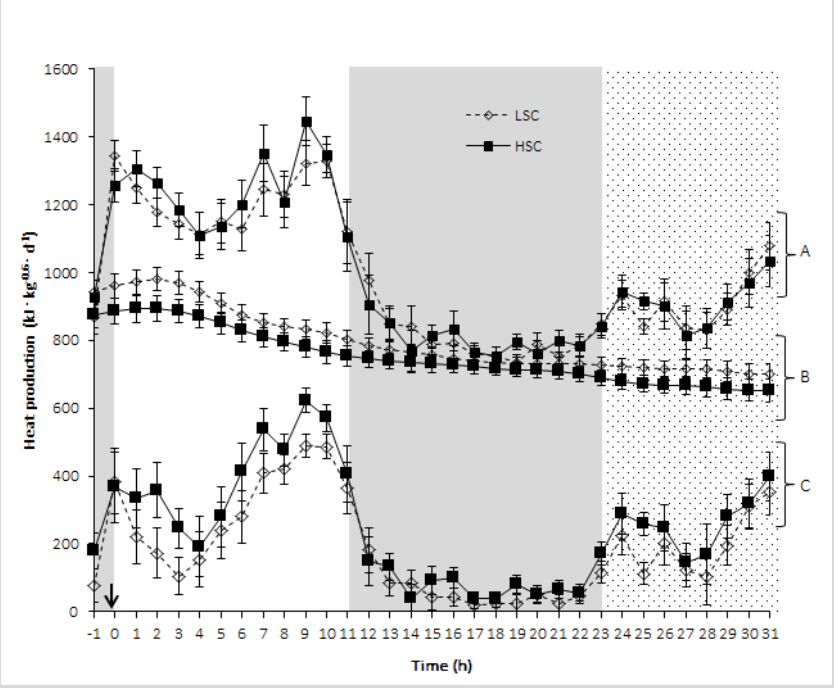

Figure 6.6. Partitioned heat production of pigs kept under low (**LSC**) and high (**HSC**) sanitary conditions and fed a diet with (**AA-S**) or without (**AA-B**) extra AA. **A.** represents the total heat production, **B.** represents resting metabolic rate, **C.** represent heat related to physical activity. The arrow indicates the moment of the last meal. The grey area represents dark time and the white area light time. The dotted area represents the measurement of energy expenditure after 25 h fasting. Results are presented as least square mean ± SEM.

Results. The RMR of the LSC pigs and the HSC pigs fed the AA-B diet was higher 25 h (LSC-AA-B: 698, LSC-AA-S: 743, HSC-AA-B:690 kJ·BW$^{-0.6}$·d^{-1}) than 32 h after the last meal (LSC-AA-B: 668, LSC-AA-S: 733, HSC-AA-B: 648 kJ·BW$^{-0.6}$·d^{-1}, Fig 6.6). The RMR of HSC pigs fed the AA-S diet did not differ using either a 25h or a 32 h period of feed deprivation.

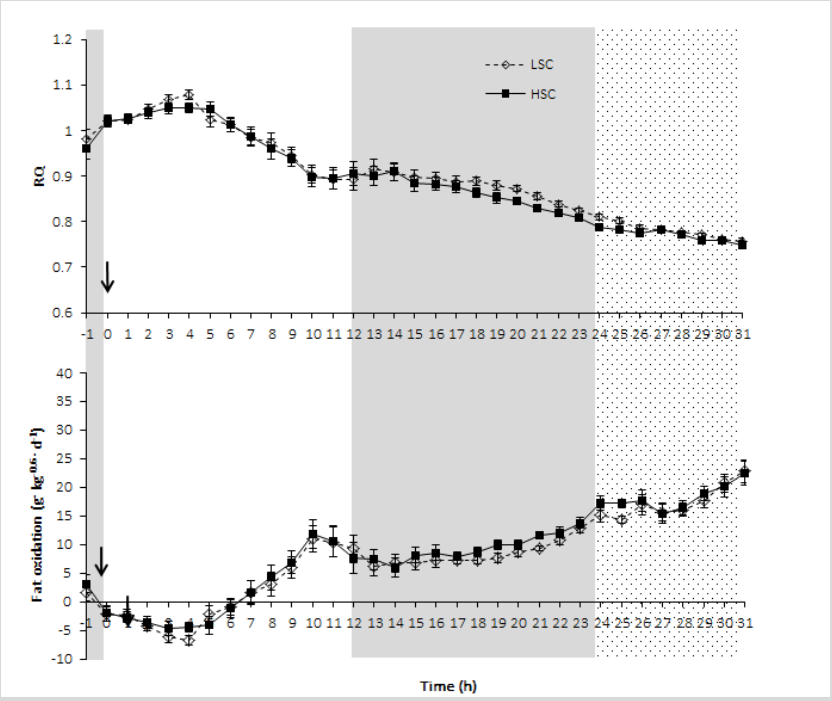

Figure 6.7 Respiration coefficient (**RQ**) and fat oxidation of pigs kept under low (**LSC**) and high (**HSC**) sanitary conditions and fed a diet with (**AA-S**) or without (**AA-B**) extra AA. The arrow indicates the moment of the last meal. The grey area represents dark time and the white area light time. The dotted area represents the extra measurement after 25 hours of fasting. Results are presented as least square mean ± SEM.

These results indicate that RMR, which is needed to estimate the energy was still decreasing after 25 h of fasting. The respiration coefficient (**RQ**) was higher at 25 h (0.78 kJ · kg $^{-0.6}$ · d^{-1}) of fasting compared with 32 h (0.75 kJ · kg $^{-0.6}$ · d^{-1}) of fasting (Fig 6.7), indicating that fat oxidation still increased after 25 h of fasting. Fat oxidation was lower at 25 h of fasting compared with 32 h of fasting, probably partly due to higher physical activity after 32 h compared with 25 h of fasting (Fig 6.7). The FHP was still greater for LSC pigs compared with HSC pigs at 32 h after initiation of fasting ($P \leq 0.05$), as was found for 25 h after initiation of fasting (Chapter 4).

Conclusion. The length of the measurement period for estimating FHP influenced RMR, RQ and fat oxidation. A 32 h fasting measurement gave a lower RMR, a lower RQ value, and a higher fat oxidation than a period of 25 h of fasting.

Textbox 6.3. Energy requirements for maintenance for low and high sanitary condition pigs

Aim. To estimate energy requirements for maintenance for low sanitary conditions (**LSC**) and high sanitary conditions (**HSC**) pigs by using data on fasting heat production (**FHP**).

Materials and methods. In a 2 × 2 factorial arrangement, 24 groups of 6 pigs housed in calorimetric chambers, were allocated to either a LSC or a HSC, and were offered either a diet with a basal AA profile (**AA-B**) or a diet with a supplemented AA profile (**AA-S**) as described in Chapter 4. Heat production of these groups was calculated over an ad libitum and a restricted feeding period, and fasting heat production was measured for the same groups (Chapter 4). Feed intake in ad libitum and restricted feeding periods was registered. Metabolizable energy for maintenance (**MEm**) was calculated by using relationships between heat production (**HP**) or FHP and ME intake by adapting a method used by Labussière et al. (2011). A relationship between ME intake for the restricted and ad lib period was plotted (line A) and for the restricted and FHP and ad libitum and FHP two other lines were plotted (lines B, Fig 6.8). The intercept between line A and the dashed line representing HP corrected for activity = ME, gives the conventional MEm. The MEm according to the method of Labussierre et al. (2011) is represented by the intersection between lines B and HPcorrected = ME.

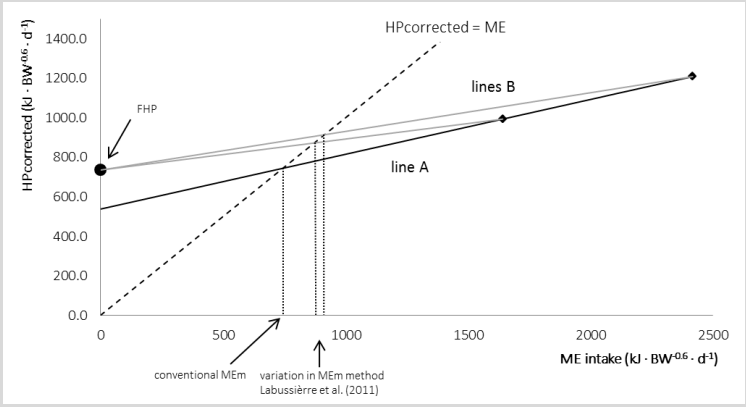

Figure 6.8. Relationships between heat production corrected for activity (**HPcorrected**), fasting heat production (**FHP**) and ME intake for a restricted and ad libitum feeding period for calculating ME for maintenenance (**MEm**). This figure is based on data of a single experimental group of pigs.

Results. The MEm according to the conventional method was 808 kJ · $BW^{-0.6}$ · d^{-1} for LSC and 617 kJ · $BW^{-0.6}$ · d^{-1} for HSC (P = 0.13). This means on average an 30% higher MEm for LSC compared with HSC pigs according to this method, which does not take feeding level into account. MEm, however, is known to depend on feeding level. The MEm calculated by using lines B varied from 885 to 930 kJ · $BW^{-0.6}$ · d^{-1} for LSC pigs and from 818 to 875 kJ · $BW^{-0.6}$ · d^{-1} for HSC pigs. This means on average a 7% higher MEm for LSC compared with HSC pigs according to this method (P = 0.05). This estimate is preferred, as the conventional method requires extrapolation too far outside the measured range.

Conclusion. Consistent with the difference in FHP observed, the MEm for LSC pigs was 7% higher than the MEm for HSC pigs.

NUTRIENT REQUIREMENTS OF LOW SANITARY CONDITION PIGS

To translate the findings of the studies in the present thesis to possible feeding strategies under varying SC, the main findings are summarized (Table 6.4). This summary of results only focusses on data obtained in ad libitum fed pigs and excluded data of pigs fed low protein diets (Chapter 2 and 3).

Table 6.4 Summary of parameters affected for ad libitum fed LSC compared with HSC pigs in Chapter 2 and 4

Item[a]	Difference compared with HSC pigs[b]
ADFI	-6%
ADG	-12%
G:F ratio	-5%
N-digestibility	-6%
Fasting heat production	+8%
Maintenance energy (Textbox 6.3)	+7%
Incremental efficiency nitrogen	-20%
Haptoglobin concentration serum	+69%
Ear biting behaviour	+30%

[a] ADFI = average daily feed intake, ADG = average daily gain, G:F ratio = gain to feed ratio, N = nitrogen, FHP = fasting heat production. [b] Data are based on pigs fed ad libitum with adequate dietary crude protein level.

Based on the summary of parameters affected by SC (Table 6.4), the impact of SC on energy and AA requirements was estimated for a standard pig of 25 kg BW (Table 6.5). Changes in energy requirements for maintenance, N digestibility and incremental efficiency of N for deposition in the body for a LSC compared with a HSC pig were taken into account. For this comparison, differences in ADFI observed in Chapter 2 and 4 (75 and 110 g/d, respectively) between LSC and HSC pigs are not taken into account, and ADFI of LSC pigs was set equal to the ADFI of HSC pigs. A 10 weeks old HSC pig of 25 kg, with an ADFI of 1256 g $\cdot$ pig^{-1} $\cdot$ d^{-1} was taken as reference. The reference diet contained 10.25 g Lys/kg, an 13.4 kJ of ME/g diet, and a dietary CP concentration of 165 g/kg. For the first 950 g of ADFI/d, nutrient digestibility, efficiencies for protein and energy deposition in the body observed for the restricted fed pigs were assumed (Chapter 4), and for the feed intake above this level (i.e. 306 g/d), the incremental energy and N efficiencies were assumed as presented in Chapter 4. Other reference data were based on results presented in this thesis.

Table 6.5 Calculation of the effects of low sanitary conditions (**LSC**) on Lys and ME requirements, by using a high sanitary condition (**HSC**) pig of 25 kg BW as a reference

Item	HSC	LSC
Reference data		
BW[1], kg	25	25
ADFI[2] for ad libitum period, g/d	1256	1256
ADFI for restricted period, g/d	950	950
Dietary ME[3] content, kJ/g	13.4	13.4
Dietary CP[4] content, g/kg	165	165
Dietary Lys[5] content, g/kg	10.3	10.3
MEm[6], kJ/$BW^{0.6}$	847	908
ATTD[7] coefficient for N[8]	0.80	0.75
Incremental ATTD coefficient for N	0.73	0.73
Feed needed for MEm, g/d	436	467
Feed left above maintenance, g/d	820	789
ME intake, MJ/d	16.8	16.8
ATTD protein intake (ad lib), g/d	166	156
ATTD protein intake (restricted), g/d	125	118
Extra ATTD protein intake (ad lib - restricted), g/d	37	37
Protein deposition for restricted intake, g/d	88	84
Incremental protein deposition (ad lib - restricted), g/d	36	28
Total protein deposition in the body, g/d	124	111
Lys intake, g/d	12.9	12.9
ATTD Lys intake (ad lib), g/d	10.1	9.6
Total Lys deposition, g/d	8.8	7.9
Incremental Lys deposition (ad lib - restricted), g/d	2.6	2.0
Lys/ME ratio and increased energy and Lys requirements for LSC pigs		
Lys/ME ratio, g/MJ	0.76	0.76
Increased ME requirement to compensate for increased MEm, MJ/d		0.42
Increased Lys requirement to compensate for reduced ATTD, g/d		0.45
Increased Lys requirement to compensate for reduced incremental efficiency, g/d		0.60
Lys/ME ratio needed for total extra requirements, g ATTD Lys/MJ		2.49

[1]BW = body weight, [2] ADFI = average daily feed intake, [3] ME = metabolizable energy, [4] CP = crude protein, [5] Lys = lysine, [6] MEm = metabolizable energy for maintenance, [7] ATTD = apparent total tract digestibility, [8] N = nitrogen.

From these calculations it appeared that the LSC pig needed 0.42 extra MJ ME/d to compensate for increased MEm, 0.45 g extra ATTD Lys/d to compensate reduced total tract N digestibility, and 0.60 g extra ATTD Lys/d to compensate for reduced incremental N efficiency for LSC compared with HSC pigs. Overall, this means that the Lys / ME ratio of the extra feed required to maintain performance is 2.49 g ATTD Lys/MJ ME, which is higher than the ratio of the diet that was offered to the HSC pigs (0.76 g ATTD Lys/MJ ME). The results described in the different Chapters in this thesis show that energy and AA requirements are both increased for LSC compared with HSC pigs, however, this calculation shows that the increase in AA requirement is proportionally more important than the increase in energy requirement.
The increase in energy for maintenance and the reduction of incremental protein efficiency would have to be accounted for in increased requirement values of all AA per unit of energy. When expressing the total extra requirement for Lys (0.45 + 0.60 g/d, Table 6.5) as a percentage of the ATTD Lys intake for HSC pigs, a 10% increase in Lys would be needed to compensate for reduced ATTD and reduced incremental N efficiency.
Taking the reduction in ADFI that we observed in chapter 2 and 4 into account implies that the increased energy and AA requirements for LSC pigs calculated should be concentrated in a smaller quantity of feed. For a reduction in ADFI of 6% (Table 6.4) this would mean that the ME content of the diet would have to increase to 14.2 kJ of ME/g diet, without considering the increased requirement for MEm as described in Table 6.5. The former could be implemented in practical diet formulation by increasing the dietary fat content. This would only be feasible if the feed intake capacity of LSC pigs is not further reduced by an increased dietary energy concentration. Little is known about the background of satiety regulation in LSC pigs, but our observation that LSC pigs have increased feed refusals upon a 30% feed restriction (Chapter 4) illustrate that LSC pigs tend to eat in smaller meals.

When translating the results of these studies into practise, it is an interesting debate whether the decreased efficiency of nutrient utilization in LSC pigs should be reflected either in the evaluation of feed ingredients or in altered nutrient requirements values. As the effects of SC on ATTD of nutrients appeared independent of the level of feeding, it would appear that effects on nutrient digestibility could be represented in lowering the concentrations of SID AA in feed ingredients as compared to current values (CVB, 2011; NRC, 2012). It would require more information on the effects of LSC on nutrient digestibility, particularly considering low and high digestible feed ingredients.
Regarding the low incremental efficiency of utilization of dietary protein in LSC pigs, it is questionable whether it is cost-effective to completely satisfy the increased AA requirements. As LSC pigs have a reduced incremental efficiency of dietary N for protein retention in the body, increases in dietary AA might lead to a relatively low increase in body protein deposition. It is known that the total efficiency of the use of protein for depositing protein in pigs reduces when protein is fed in excess. The reduced incremental efficiency

found might be related to a lower maximal rate of protein deposition of LSC pigs compared with HSC pigs, a increased AA oxidation for LSC compared with HSC pigs, or an imbalance in AA. In addition, an excess of dietary protein in the gastro intestinal tract might lead to an increase in fermentation of undigested protein and in risk for growth of intestinal pathogens (Prohászka and Baron, 1980; Nollet et al., 1999).

In general, the results presented in the chapters of the present thesis indicate that LSC and HSC pigs have increased requirements for energy and AA. In proportion, the increase in AA requirements seem more important than the increase in energy requirements for LSC pigs. The different requirements found for LSC and HSC pigs were especially present in early life. It might be beneficial to reduce nutrient digestibility values of feed ingredients for young LSC pigs, or to select highly digestible ingredients for diets of these pigs, and to increase the concentrations of AA in the diet for LSC pigs to a certain extent. Results obtained in this thesis help to understand the nutrient requirements of LSC compared with HSC pigs and might be used for implementation in models predicting growth performance of pigs and for targeted feeding strategies for LSC pigs. To further implement the results obtained in LSC and HSC pigs in more exact energy and AA recommendations for diet formulation in practise, additional studies are needed for validation.

CONCLUSIONS

In two large-scale experiments we studied the effect of a chronic, low grade inflammation, by imposing low sanitary conditions, on energy and AA metabolism in growing pigs.. The results provide a scientific basis for developing targeted nutritional strategies for pigs kept under various conditions. In brief, the following conclusions were drawn from this thesis:

- Using low and high sanitary conditions to create a contrast in chronic low grade immune stimulation as described in the present thesis leads to:
    - A 12% reduced body weight gain, a 6% reduced ADFI and a 5% reduced G:F ratio in LSC compared with HSC pigs.
    - An increased occurrence of damaging behaviour for LSC compared with HSC pigs.
- The observed variation in pig performance and innate immune responses among batches and between studies in the current thesis indicates the importance of having more than a single batch in studies using our sanitary condition approach.
- Extra dietary supplementation of Met, Thr, and Trp was beneficial for the growth performance in HSC pigs and in particular for LSC pigs in the first study, however these effects were not observed in the second study.
- Reduction of dietary protein increases the occurrence of damaging behaviours in pigs.
- The apparent total N digestibility of LSC pigs was 6% lower compared with HSC pigs.
- Based on measured rates of fasting heat production, the energy requirement for maintenance is estimated to be 7% greater in LSC than in HSC pigs.
- The incremental efficiency for N intake retained as body protein was 53% for LSC pigs and 73% for HSC pigs.

Overall, the results of this thesis indicate that both energy and AA requirements are greater in LSC pigs compared with HSC pigs. It is questionable, however, whether it is nutrient and cost effective and biologically possible to satisfy these increased nutrient requirements in LSC pigs, as the incremental efficiency of N for retained protein is low, and ADFI is reduced for LSC pigs compared with HSC pigs. The present thesis demonstrates that care should be taken in reducing dietary protein concentrations to improve protein efficiency in pigs, as it incurs a risk to increased damaging behaviours, particularly when pigs are kept under LSC.

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List of abbreviations

AA	amino acids
ADG	average daily gain
ADFI	average daily feed intake
AID	apparent ileal digestibility
APP	acute phase proteins
BW	body weight
CFA	Complete Freund's Adjuvant
CP	crude protein
CRP	C-reactive protein
Cys	cysteine
DE	digestible energy
ECTS	European Credit Transfer System
ETEC	E. coli
FHP	fasting heat production
GE	gross energy
G:F	gain to feed
Hact	physical activity related heat
HP	heat production
HSC	high sanitary condition
Htot	total heat production
i.m.	intramuscular
i.v.	intravenous
IL	interleukin
ILR	irreversible loss rate
ISS	immune system stimulation
KLH	keyhole limpet hymocyanin
LP	low protein
LPS	lipopolysaccharide
LSC	low sanitary condition
Lys	lysine
MCV	mean cell volume
ME	metabolizable energy
Met	methionine
N	nitrogen
Nab	natural antibody
NDF	neutral detergent fibre
NE	net energy
NP	normal protein
PCV2	porcine circovirus type 2
Pdmax	maximal protein deposition
Pig-MAP	pig major acute-phase protein
PTL	platelets
RBC	red blood cells

PRRSV	porcine reproductive and respiratory syndrome virus
RMR	resting metabolic rate
SC	sanitary conditions
s.c.	subcutaneous
SEM	standard error of the mean
SID	standardized ileal digestibility
S. suis	Streptococcus suis
TEF	thermic effect of feeding
Thr	threonine
Ti	titanium
TID	true ileal digestibility
TNF	tumour necrosis factor
TO	turpentine oil
Trp	tryptophan
WBC	white blood cells

SUMMARY

It is economically and environmentally important to match the nutrient supply to the nutrient requirements in pig production. Until now, the effects of different sanitary conditions on energy and nutrient requirements are not implemented in recommendations for nutrient composition of pig diets. The current nutrient requirement data are based on studies with pigs in experimental settings, which can be regarded as rather optimal. Changes in nutrient requirements caused by differences in sanitary conditions are poorly documented. As in the pig production sector farm conditions are variable it is of major importance to determine the effects of low sanitary conditions (LSC) on requirements for amino acids and energy in growing pigs. Pigs under LSC have an increased risk of clinical and subclinical infections, resulting in a chronic stimulation of their immune system. Immune system stimulation is known to influence energy and amino acid metabolism. However, most studies in pigs evaluating the relationship between immune system stimulation and nutrient requirements often use specific experimental challenge models. Whereas such models have the obvious advantage of reproducibility and allow mechanistic insight in the effects of stimulating specific parts of the immune system, these models often induce clinical illness, rather than subclinical infections. Results obtained with such models may therefore be difficult to translate to practical situations. Therefore the objective of the present thesis was to study the effect of low and high sanitary conditions (HSC) on amino acids and energy metabolism in pigs. Also interactions between the immune system, nutrient metabolism and damaging behaviour of pigs were considered in this thesis.

The experiment described in Chapter 2 was designed to study the effect of different dietary crude protein levels and extra amino acid supplementation on the growth performance of pigs kept under different sanitary conditions. In a 2×2×2 factorial arrangement, 68 groups of 9 pigs were allocated to either LSC or HSC, and were offered ad libitum access to two different diets, a normal crude protein concentration diet or a low crude protein concentration diet, each having either a basal dietary amino acid profile or supplemented dietary amino acid profile containing 20% more methionine, threonine, and tryptophan compared with the basal profile. The pigs were followed from 10 weeks of age until slaughter. Haptoglobin concentrations in serum and IgG antibody titers against keyhole limpet heamocyanin, collected in the starter, grower, and finisher phases, and pleuritis scores at slaughter were greater for LSC pigs compared with HSC pigs, illustrating that sanitary conditions affected health conditions. The average daily gain and gain to feed ratio were greater for HSC pigs compared with LSC pigs. A 20% increase in dietary supplementation of methionine, threonine, and tryptophan relative to lysine increased gain to feed ratio more in LSC than in HSC pigs. The results therefore illustrated that dietary

requirements for methionine. threonine, and tryptophan were greater for LSC compared with HSC pigs.

In **Chapter 3** the damaging behaviour of 576 pigs from the experiment in Chapter 2 was evaluated. At 15, 18, and 24 weeks of age, prevalence of tail and ear damage, and of tail and ear wounds was scored. At 20 and 23 weeks of age, frequencies of biting behaviour and aggression were scored by behaviour sampling. The prevalence of ear damage during the finisher phase and the frequency of ear biting were increased in LSC compared with HSC pigs. The frequency of ear biting was increased in low protein fed pigs compared with normal protein fed pigs. The supplemented AA profile reduced ear biting only in LSC pigs. The prevalence of tail wounds was lower for pigs in LSC than for pigs in HSC in the grower phase. Regardless of dietary amino acid profile or sanitary status, pigs fed low protein diets showed more ear biting, tail biting, belly nosing, other oral manipulation directed at pen mates, and aggression than pigs fed normal protein diets, with no effect on ear or tail damage. In conclusion, both LSC and a reduction of dietary protein increased the occurrence of damaging behaviours in pigs and therefore may negatively impact pig welfare.

The experiment of **Chapter 4** was designed to quantify the difference in energy requirements for maintenance, and in incremental efficiencies for deposition of dietary energy and protein in the body of clinically healthy pigs kept under LSC or HSC, fed a basal diet either or not supplemented with additional methionine, threonine and tryptophan.

In a 2 × 2 factorial arrangement, 24 groups of 6 pigs each were allocated to either a LSC or HSC, and were offered two different diets having either a basal or a dietary amino acid profile supplemented with methionine, threonine, and tryptophan. For each group of pigs, complete energy and nitrogen balances were determined during two consecutive weeks, during which feed was available ad libitum or at 70% of ad libitum. Fasting heat production was determined over a 25 h period of fasting after a period of restricted feeding. Low sanitary conditions increased fasting heat production from 696 to 750 kJ/(kg $BW^{0.6}$. d), regardless of the dietary amino acid supplementation. The incremental efficiency of ingested nitrogen for retention in the body was reduced in LSC pigs from 73 to 53%, but incremental efficiencies of digestible energy intake for fat deposition in the body were unaffected by the experimental treatments. These findings showed that the effects of continuous immune stimulation by introducing LSC, was affecting energy and nutrient efficiencies of pigs both at maintenance level and at a feeding level close to ad libitum intake.

In **Chapter 5** diurnal patterns for heat production, respiratory quotient, and carbohydrate and fat oxidation of the pigs studied in the experiment of Chapter 4 were evaluated to get more insight in the mechanisms behind the effects found in Chapter 4. The LSC pigs had reduced activity compared with HSC and a higher resting metabolic rate during the period of restricted feeding, especially during the light parts of the day. Therefore the diurnal energy expenditure pattern of LSC and HSC pigs can be considered as different. Fat and

carbohydrate oxidation patterns were not different for LSC and HSC pigs, indicating that protein and fat deposition during the day was similar for LSC and HSC pigs.

Overall, the results of this thesis indicate that both energy and AA requirements are greater in LSC pigs compared with HSC pigs. It is questionable, however, whether it is nutrient and cost effective and biologically possible to satisfy these increased nutrient requirements in LSC pigs, as the incremental efficiency of N for retained protein is low, and ADFI is reduced for LSC pigs compared with HSC pigs. The present thesis demonstrates that care should be taken in reducing dietary protein concentrations to improve protein efficiency in pigs, as it incurs a risk to increased damaging behaviours, particularly when pigs are kept under LSC.

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Yvonne

About the author

Curriculum vitae
List of publications
Training and supervision plan

Curriculum vitae

Yvonne van der Meer was born in 's-Hertogenbosch, the Netherlands, on the 4th of November 1986. She graduated from secondary education in 2005 at d' Oultremontcollege location Drunen, the Netherlands. Thereafter, she studied Animal husbandry at HAS University in 's-Hertogenbosch where she obtained her BSc. In 2010, Yvonne started her study Animal Sciences at Wageningen University and she obtained her MSc degree in 2012. Yvonne, did her major thesis in Animal Nutrition, studying the effects of dietary chenodeoxycholic acid in weaned piglets. She did her minor thesis in Adaptation Physiology studying the influence of feeding artificial milk to young piglets. In 2012, Yvonne started her PhD at De Heus Animal Nutrition B.V. and the Animal Nutrition Group of Wageningen University & Research. In her PhD, she studied nutrition of pigs kept under low and high sanitary conditions. This research was part of Feed4Foodure and Breed&Feed4Food. The results of her PhD project are presented in this thesis. After concluding her PhD, Yvonne will resume as a nutritionist at De Heus Animal Nutrition B.V.

List of publications

Peer reviewed scientific publications

van der Meer, Y., W. J. J. Gerrits, M. van den Bosch, J. J. Holst, M. Moreto, W. A. Buurman, and T. A. T. G. van Kempen. 2012. Chenodeoxycholic acid reduces intestinal permeability in newly weaned piglets. J Anim Sci, 90: 302-304.

van der Meer, Y., A. Lammers, A. J. M. Jansman, M. M. J. A. Rijnen, W. H. Hendriks, and W. J. J. Gerrits. 2016. Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation. J Anim Sci 94: 4704-4719.

van der Meer, Y., W. J. J. Gerrits, A. J. M. Jansman, B. Kemp, and J. E. Bolhuis. 2017. A link between damaging behaviour in pigs, sanitary conditions, and dietary protein and amino acid supply. Accepted in PLOS one.

van der Meer, Y., A. J. M. Jansman, and W. J. J. Gerrits. Low sanitary conditions increase maintenance energy expenditure and decrease incremental protein efficiency in growing pigs. To be submitted to Journal of Nutrition.

van der Meer, Y., A. J. M. Jansman, A. Lammers, and W. J. J. Gerrits. Diurnal patterns of heat production and oxidation of carbohydrate and fat in pigs kept under different sanitary conditions. To be submitted to Animal.

Conference and symposia proceedings

van der Meer, Y., W. J. J. Gerrits, M. van den Bosch, J. J. Holst, W. Kulik, and T. A. T. G. van Kempen. 2012. Chenodeoxycholic acid improves intestinal permeability in piglets. Proceedings of the 12th Digestive Physiology of Pigs, 29 May - 1 June, Keystone, Colorado.

van der Meer, Y., W. J. J. Gerrits, M. van den Bosch, M. Moretó, W. A. Buurman, and T. A. T. G. van Kempen. 2012. Comparison of three intestinal permeability methods. Proceedings of the 12th Digestive Physiology of Pigs, 29 May - 1 June, Keystone, Colorado.

van der Meer, Y., A. J. M. Jansman, M. M. J. A. Rijnen, W. H. Hendriks, and W. J. J. Gerrits. 2015. Amino acid requirements of growing-fattening pigs affected by sanitary status. Proceedings of the WIAS Science Day, 5 February 2015, Wageningen, The Netherlands.

van der Meer, Y., A. J. M. Jansman, M. M. J. A. Rijnen, W. H. Hendriks, and W. J. J. Gerrits W. 2015. Amino acid requirements differ for fattening boars housed in low or high sanitary conditions. Proceedings of the 13th Digestive Physiology of Pigs, 19-21 May, 2015, Kliczków, Poland.

van der Meer, Y., W. J. J. Gerrits, A. J. M. Jansman, B. Kemp, and J.E. Bolhuis. 2016. Tail and ear biting behaviour in pigs affected by sanitary conditions, dietary amino acid profile, and crude protein level. Proceedings of the WIAS Science Day, 4 February 2016, Wageningen, The Netherlands.

van der Meer, Y., W. J. J. Gerrits, B. Kemp, and J. E. Bolhuis. 2016. Effects of dietary protein and amino acid supply on damaging behaviours in pigs kept under diverging sanitary conditions. Proceedings of the 50th Congress of the International Society for Applied Ethology 12-15th July, 2016, Edinburgh, United Kingdom.

van der Meer, Y., W. J. J. Gerrits, A. J. M. Jansman, B. Kemp, and A. Lammers. 2016. Does a reduction in dietary protein affect immune status of pigs kept under different sanitary conditions? Proceedings of the 5th International Symposium on Energy and Protein Metabolism and Nutrition, 12-15 September, 2016, Krakow, Poland.

Other Publications

van der Meer, Y., W. J. J. Gerrits, and A. J. M Jansman. 2015. Het effect van aminozuuraanbod en -samenstelling van het voer op zoötechnische prestaties van beren gehuisvest onder verschillende sanitaire condities. Vertrouwelijk rapport 445, Wageningen UR Livestock Research, The Netherlands.

Training and supervision plan[1]

Basic package (3 ECTS[2])

WIAS Introduction course	2013
Course on philosophy of science and/or ethics	2013

Scientific exposure (13 ECTS)

International conferences

ADSA-ASAS Joint Annual Meeting Indianapolis, US	2013
13th Digestive Physiology of Pigs, Kliczków, Poland	2015
5th International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland	2016

Seminars and workshops

WIAS Science Day, Wageningen, the Netherlands	2013-2016
International Pig Meeting De Heus	2013-2016
14th GUT Day Symposium, Leuven, Belgium	2012
Animal Nutrition and Research forum	2013
Denkaday, Voorthuizen, the Netherlands	2013
Aspects of sow and piglets performance seminar, Wageningen, the Netherlands	2013
	2013
WIAS Fiber seminar, Wageningen, the Netherlands	2014
WIAS Nutrition, health and welfare of calves seminar, Wageningen, the Netherlands	2014
Schothorst Feed Research symposium: Future dynamics in sustainable animal nutrition, Nijkerk, the Netherlands	2014
Nutrient requirements in relation to animal health symposium, Wageningen, the Netherlands	2014

Presentations

Amino acid requirements of growing-fattening pigs affected by sanitary status., WIAS Science Day, Wageningen, the Netherlands, poster presentation	2015

[1] Completed in the fulfilment of the requirements for the education certificate of the Graduate School Wageningen Institute of Animal Sciences (WIAS).

[2] One ECTS equals a study load of 28 hours.

Amino acid requirements differ for fattening boars housed in low or high sanitary conditions. Digestive Physiology of Pigs, Kliczków, Poland, oral presentation	2015
Tail and ear biting behaviour in pigs affected by sanitary conditions, dietary amino acid profile, and crude protein level. WIAS Science Day, Wageningen, the Netherlands, oral presentation	2016
Amino acid requirements of growing/finishing pigs kept under different sanitary conditions. Wageningen Academy Course Quality of protein in animal diets, Wageningen, the Netherlands, oral presentation	2016
Does a reduction in dietary protein affect immune status of pigs kept under different sanitary conditions? 5th International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland, oral presentation	2016

In-depth studies (11 ECTS)

Varkensvoeding in de praktijk, Wageningen Academy, Sterksel/Wageningen, the Netherlands	2012
Applied molecular microbiology MSc course, Wageningen, the Netherlands	2012
Animal health and immunology discussion group, Wageningen, the Netherlands	2015
Quality of protein in animal diets, Wageningen Academy, the Netherlands	2015
Statistics for the life sciences	2016
Indirect calorimetry course, University of Krakow, Poland	2016

Professional skills support courses (4 ECTS)

Supervising MSc thesis work	2013
Techniques for writing and presenting a paper	2013
Scientific writing	2015
Last stretch of the PhD program	2016

Research skills Training (6 ECTS)

Preparing own PhD research proposal	2012

Didactic skill training (16 ECTS)

Supervising practical's

Supervision research master cluster	2014
Supervision practical Principles of Animal Nutrition	2015

Supervising theses

Supervising MSc thesis (8x)	2013-2017

Management skills training (2 ECTS)

WIAS science day committee member	2013

Education and training total	**52 ECTS**

Colophon

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