FACULTY OF SCIENCE
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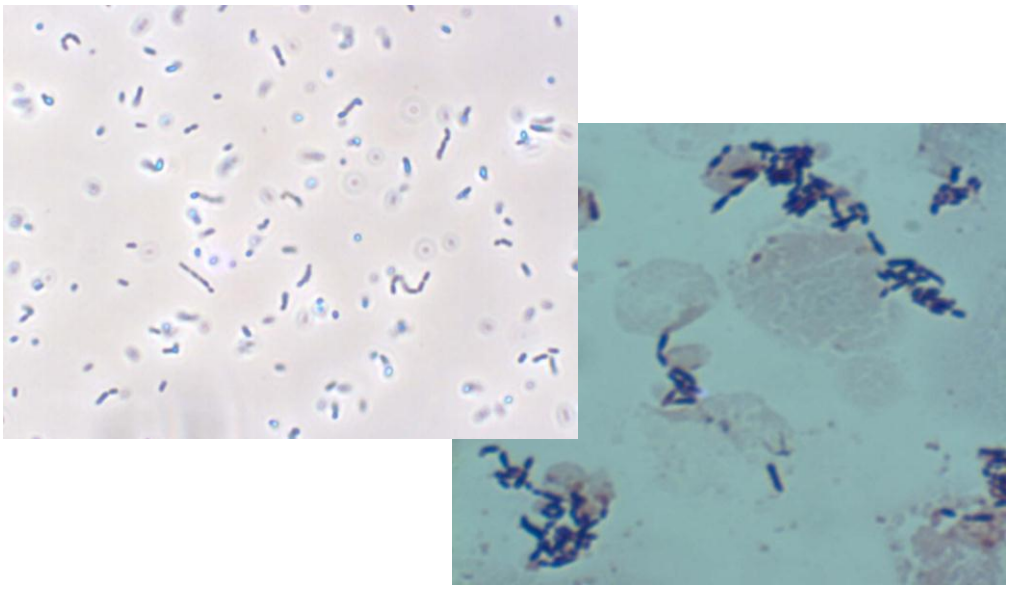
Master's thesis

Rikke Lassen Jensen
zvt384

Feed interventions and skatole deposition

Characterisation of a skatole producing bacterium isolated from the gastrointestinal tract of pigs



Academic advisor: Christian Fink Hansen, Ph.D., Associate Professor, Department of Large Animal Scenic, Copenhagen University

Co Academic adviser: Bent Borg Jensen, Professor, Department of Animal Science, Immunology and Microbiology, Aarhus University

45 ECTS

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Preface

This thesis is the completion of my MSc in Animal Science at the Faculty of Life Science, University of Copenhagen.

I am grateful for the excellent guidance from my supervisor, associate professor Christian Fink Hansen, Department of Large Animal Science, Faculty of Health and Medical Sciences, University of Copenhagen.

The thesis has been carried out in cooperation with Department of Animal Science, Immunology and Microbiology, Aarhus University. I wish to thank the department for the opportunity to conduct my thesis in cooperation with them and in particular my co-academic adviser professor Bent Borg Jensen for inspiration and helpful guidance.

The experiment was part of the Danish collaboration project: "No Cast – Organic pig production in 2014 without castration", supported by GUDP. The collaboration was between University of Aarhus, University of Copenhagen, the Pig Research Centre, the Knowledge Centre for Agriculture and the Centre of Development for Outdoor Livestock Production. I am grateful for the opportunity to get insight in this project. I wish to thank Professor Ole Højberg and PhD Morten Poulsen for answering all my questions. Finally, I wish to thank the laboratory staff Karin Durup, Thomas Rebsdorf, Mona Dinesen and Trine Poulsen for invaluable support and guidance in the laboratory.

Rikke Lassen Jensen

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Abstract

Skatole produced in the large intestine of the pig and the testicular steroid androstenone are the main substances contributing to boar tainted meat from entire male pigs. Boar taint decreases the quality of the meat and is not accepted by consumers. Until now boar taint has been avoided by castrating male pigs. Surgical castration reduces lean meat percentage, growth rate and feed efficiency, and it causes pain to the animal. This constitutes a problem in relation to productivity and welfare. Different attempts on avoiding surgical castration were either not fully effective, not accepted by the market, or they have a long time horizon for implementation. However, when focusing on the effect of feed interventions on boar taint, previous studies have showed a reducing effect through reduced skatole production in the large intestine after a one week application period. Skatole is produced from the microbial fermentation of L-tryptophan in the large intestine. In the literature it is well documented that skatole production in the large intestine is positively correlated with skatole deposition in adipose tissue. Moreover skatole production can be decreased by adding non-digestible and easy fermentable carbohydrates to the feed. However, little is known about skatole producing bacteria from the large intestine of pigs, and how these bacteria can be affected.

This thesis reviews skatole production and metabolism in the pig, and how skatole production can be reduced by affecting the microbial production of skatole in the large intestine. A skatole producing bacterium SK9 K4 was isolated from the gastrointestinal tract of pigs. No such bacterium has previously been described. The bacterium was characterised by 16S RNA sequencing, gram stain, analysis of DNA G-C content, cellular fatty acids composition and DNA hybridisation with closely related bacteria. The fermentation of different carbohydrate sources, the growth pattern, the production of organic acids and the skatole production were studied *in vitro*.

The production of skatole in the large intestine was correlated with skatole deposition in adipose tissue. Skatole production could be reduced when adding a minimum of 20 % raw potato starch or 9 % inulin to the feed. The problem concerning deposition of skatole in adipose tissue seems to be solved through the introduction of feed interventions. However high concentrations of androstenone deposited in adipose tissue remains a challenge. Thus, the feed interventions were not fully effective against boar taint.

SK9 K4 was described as cells being strictly anaerobic, occurred singly or in pairs and were gram positive. It was identical with an Olsenella sp. strain isolated from the rumen, an uncultured Olsenella sp. clone isolated from sludge and an uncultured bacterium colon isolated from the oral cavity. Moreover SK9 K4 was closely related to *Olsenella uli*, *Olsenella profusa*, *Olsenella umbonata* and *Atopobium parvulum*. SK9 K4 and *O. uli* produced skatole from idole-3-acetic acid but not from L-tryptophan. The major fermentation products were lactic acid together with acetic acid and formic acid. SK9 K4 was not able to ferment raw potato starch, inulin and raw corn starch. Thus, when feeding resistant starch or inulin, the growth of skatole producing bacteria might be reduced followed by a reduced ability to produce skatole. The characterisation of a skatole producing bacterium isolated from the gastrointestinal tract of pigs gives the opportunity to further study the bacterium *in vivo*. Studies should be conducted to investigate the effect of a control diet compared to a diet added a non-digestible and easy fermentable carbohydrate on the growth of SK9 K4 in the large intestine of the pig.

Resumé

Skatol produktion i grisens tyktarm og kønshormonet androstenone er de primære stoffer, der bidrager til kød fra hangrise med ornelugt. Ornelugten nedsætter kvaliteten af kødet og er ikke accepteret af forbrugerne. Hidtil er ornelugt undgået ved at kastrere hangrise. Kastration reducerer kødprocenten, tilvæksten og fodereffektivitet og forårsager smerte til dyret. Dette udgør et problem i forhold til produktivitet og velfærd. Forskellige forsøg på at undgå kastration har vist sig ikke at være fuldt ud effektive, blev ikke accepteret af markedet eller havde en lang tidshorisont. Fokuseres der på virkningen af foder interventioner på ornelugt, har tidligere undersøgelser vist en reducerende effekt gennem reduceret skatol produktion i tyktarmen efter en periode på en uge. Skatol er et produkt fra den mikrobielle fermentering af L-tryptophan i tyktarmen. I litteraturen er det veldokumenteret, at skatol produktion i tyktarmen er positivt korreleret med skatol aflejring i fedtvæv. Desuden kan skatol produktion reduceres ved tilsætning af ikke-fordøjelige og let fermenterbare kulhydrater til foderet. Der er dog begrænset viden om skatol-producerende bakterier isoleret fra tyktarmen hos svin, samt hvordan disse bakterier kan påvirkes.

Dette speciale undersøger ved en litteraturgennemgang skatol produktion og metabolisme hos svin og hvordan skatol produktion kan reduceres ved at påvirke den mikrobielle produktion af skatol i tyktarmen. En skatol-producerende bakterie SK9 K4 blev isoleret fra mave-tarmkanalen hos grise. En sådan bakterie er ikke tidligere blevet beskrevet. Bakterien blev karakteriseret ved 16S RNA sekventering, gram farvning, analyse af GC indhold i DNA, den cellulære fedtsyrere sammensætning og DNA hybridisering med nært beslægtede bakterier. Fermentering af forskellige kulhydrat kilder, vækst, produktion af organiske syrer og skatol produktion blev undersøgt *in vitro*.

Produktionen af skatol i tyktarmen var korreleret med skatole aflejring i fedtvæv. Skatol produktionen kunne reduceres ved at tilsætte minimum 20 % rå kartoffel stivelse eller 9 % inulin til foderet. Udfordringen med skatol aflejring i fedtvævet lader til at kunne løses gennem foder interventioner. Dog er aflejring af høje koncentrationer androstenon i fedtvæv fortsat en udfordring. Derfor var de fundende foder interventionerne ikke fuldt ud effektive mod ornelugt.

SK9 K4 blev beskrevet som celler, der er strengt anaerobe, forekom enkeltvis eller parvis og var gram positive. Bakterien var identisk med en Olsenella sp. stamme isoleret fra vommen, en ukultiveret Olsenella sp. klon isoleret fra slam og en ukultiveret bakterie klon isoleret fra mundhulen. Desuden var SK9 K4 nært beslægtet med *Olsenella uli*, *Olsenella profusa*, *Olsenella umbonata* og *Atopobium parvulum*. SK9 K4 og *O. uli* producerede skatol fra idole-3-eddikesyre, men ikke ud fra L-tryptophan. De store fermenteringsprodukter var mælkesyre sammen med eddikesyre og myresyre. SK9 K4 var ikke i stand til at fermentere rå kartoffelstivelse, inulin og rå majsstivelse. Ved fodring med resistent stivelse eller inulin, er det derfor muligt at væksten af skatol producerende bakterier reduceres, efterfulgt af en reduceret skatol produktion. Karakteriseringen af en skatol producerende bakterie isoleret fra grisens tarmsystem giver mulighed for yderligere at studere bakterie *in vivo*. Undersøgelser bør udføres med henblik på at undersøge virkningen af en kontrol diæt sammenlignet med en diæt tilsat ufordøjelige og let fermenterbare kulhydrater på væksten af SK9 K4 i tyktarmen.

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1 Introduction

Meat from entire male pigs is not accepted by consumers due to a decrease in meat quality through a strong off odour, flavour and taste in the heated fat of the meat from some boars (Bonneau et al., 2000; Matthews et al., 2000). This is through the entire thesis referred to as boar taint. Boar taint can be avoided by castrating the pig, which causes that surgical castration without anaesthesia of male piglets is a common practice in most pig-producing countries. Several studies have showed that castration reduces lean meat percentage, growth rate and feed efficiency (Review: Xue et al., 1997). Castration causes pain to the animal during and after the procedure (Hay et al., 2003) which is unacceptable from an ethical point of view. Furthermore, castration is time consuming to perform which costs labour. Altogether, it would be beneficial for the efficiency of the production and animal welfare if castration was not necessary. With the animal welfare concerns and the negative effects on production efficiency, there has become an increasing interest in finding alternatives to surgical castration to control boar taint.

Boar taint is mainly caused by elevated concentrations of androstenone (α-androst-16-en-3-one) (Patterson, 1968) and skatole (3-methyl-indole) (Vold, 1970; Walstra and Maarse, 1970) in adipose tissue(Patterson, 1968)(Patterson, 1968). Androstenone is a steroid produced in the Leydig cells of the testicles at sexual maturity. It originates partly from the same biosynthetic pathways as male sex hormones (Brooks and Pearson, 1986). The Lydig cells are controlled by the neuroendocrine system. A variety of environmental factors such as nutrition, season and social factors leads to an associated change in gonadotrophin releasing hormone (Gn-RH) secretion (Clarke and Pompolo, 2005). Gn-RH regulates secretion of Leutinizing hormone (LH) which stimulates the Lydig cells. The system allows only an overall regulation which means that androstenone and male sex hormones are produced in parallel and are affected by the same factors (Claus et al., 1994; Zamaratskaia et al., 2004a). Skatole is formed during the microbial degradation of L-tryptophan in the large intestine (Jensen et al., 1995a). Degradation of L-tryptophan mainly results in the formation of skatole and indole (figure 1.1). The production of indole occurs in one step and has a minor effect on boar taint. The production of skatole occurs in a two-step process (Jensen et al., 1995a). L-tryptophan is converted into indole-3-acetic acid (IAA) by many different types of microbes. The further metabolism to skatole is performed by only a few specialised bacteria (Yokoyama and Carlson, 1979; Deslandes et al., 2001). In addition Knarreborg et al. (2002) found that indole-3-propionic acid also could be produced from the degradation of L-tryptophan. It is not

known if this compound can be found in adipose tissue or if it contributes with reduced quality of the meat.

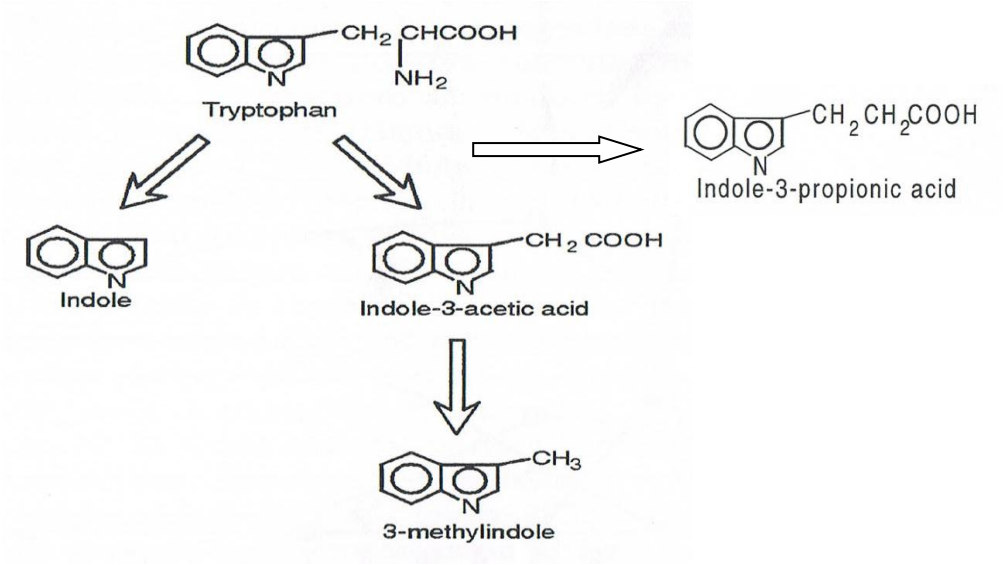


Figure 1.1 Microbial production of 3-methylindole (skatole), indole and indole-3-propionic acid in the large intestine of pigs. The fermentation processes illustrated by the arrows occur in different bacteria that are more or less specialised (Jensen et al., 1995; Jensen and Jensen, 1998).

In Denmark entire male pigs are delivered to the slaughterhouse in Ringsted where skatole and indole concentration in adipose tissue can be analysed. An international study revealed that differences exist between sensory evaluation panels from different countries (Matthews et al., 2000). In Denmark the level of skatole leading to boar taint is estimated to be above 0.250 ppm, and the level of androstenone leading to a boar taint is estimated to be above 1 ppm in adipose tissue (Andersen, 1997).

Skatole production might be reduced through reduced amount of L-tryptophan available for microbial fermentation in the large intestine. An obvious way to reduce the amount of available L-tryptophan is to reduce the tryptophan content in the feed. However, studies conducted to investigate the effect of this showed no effect on skatole production and deposition in adipose tissue (Lundström et al., 1994). Instead a major source of L-tryptophan for skatole production originates from endogenous protein by the turnover of cells from gut mucosa regulated by a balanced rate of mitosis and apoptosis (Claus et al., 1994). The rate of mitosis and apoptosis has been shown to be regulated by the composition of the feed. An increase in apoptosis leads to an increased amount of L-tryptophan for skatole production in the large intestine (Claus et al., 1996; Raab et al., 1998).

Thus, the production of skatole is regulated by the amount of available L-tryptophan, composition of the intestinal microbial population and composition of the feed. Skatole is absorbed from the large intestine into the blood stream and transported to the liver together with androstenone from the Lydig cells. Skatole and androstenone is partially metabolized by the liver and excreted in urine and partly deposited in adipose tissue causing boar taint of the meat (Babol et al., 1998b). It was previously found that there is a relationship between the metabolism of the two compounds. A variable metabolic potential in the liver was suggested to cause the variation of boar taint in pigs, reared under the same environmental and dietary conditions (Babol and Squires, 1999).

Different attempts have been made on finding alternatives to surgical castration. The most pronounced attempts are immunocastration and sexing of seamen for production of only females. Immunocastration is performed with a vaccination against the release of Gn-RH. The vaccine has to be given by two injections at least four weeks apart with the last injection given 4-6 weeks before slaughter. Immunocastration offers advantages regarding the productivity. Until the last injection is given, the pigs are practically still entire male pigs with increased lean meat percentage, growth rate and feed efficiency (Pauly et al., 2009; Fàbrega et al., 2010). Furthermore, it is suggested that immunocastration is associated with welfare improvements by reducing sexual and aggressive behaviour during sexual maturity (Fàbrega et al., 2010) and by avoiding the painful incision with associated increased risk of infections. Potential disadvantages are that the procedure of vaccinating group housed and nearly full grown slaughter pigs can be difficult and time consuming. Furthermore, there is a concern about the impact of the vaccination on food safety (de Roest et al., 2009). In Denmark, immunocastration is not used due to concerns about negative reactions from the international market (Villadsen, 2012, personal massage). Sexing of seamen would provide a method for only producing female pigs. Practical application of sexing of seamen depends on cost-benefit, fertility results, efficiency and ease to use. A method that lives up to these criteria have still not been developed (Maxwell et al., 2004; Johnson et al., 2005; Bathgate et al., 2008).

Production of entire male pigs that are not affected by boar taint has some exclusive advantages if the tainted pigs can be detected and sorted out at the slaughter line, and research can identify how boar taint can be avoided without castrating the pig. Research has been done on genetic selection against boar taint (Gregersen et al., 2012), economical optimal weight at slaughter (Babol et al., 2004b; Aluwé et al., 2011), environmental conditions having an effect on degree of slurring, feeding strategy and feed composition. Additionally improved methods for efficient boar taint

detection are under development. Genetic selection has a long time horizon and cannot work as a solution against boar taint at the moment. Economical optimal weight at slaughter will be a decreased weight to avoid androstenone production followed by sexual maturity (Zamaratskaia et al., 2005; Chen et al., 2007). This will have a negative influence on the economic value of the pig. Furthermore, the method is not fully effective due to the variation in time of sexual maturity (Andersson et al., 1999). Environmental factors enhancing a clean environment with little or no faeces deposition in the pen has a decreasing effect on boar taint (Hansen et al., 1994) but is not fully effective against boar taint. Several papers have been published concerning the effect of different feed interventions on boar taint. The effect is primarily due to reduced skatole production and absorption in the intestines and has a small influence on androstenone. Most often tested feedstuffs are raw potato starch with a high content of resistant starch (Xu et al., 2002; Losel and Claus, 2005; Loesel et al., 2006; Chen et al., 2007; Hansen et al., 2008; Li et al., 2009; Øverland et al., 2011) and chicory root being effective due to a high content of inulin (Hansen et al., 2006; Byrne et al., 2008; Hansen et al., 2008). The interventions are often tested for different time periods before slaughter and are evaluated against one or several of the following factors: skatole, indole, pH, volatile fatty acid and ATP in intestinal content and skatole and androstenone concentration in blood plasma and back fat. Some of the studies include a sensory evaluation of meat quality to find a connection between sensory and chemical evaluations. None of the tested interventions have been fully effective against boar taint.

Thus, the different attempts on avoiding surgical castration are either not fully effective, not accepted by the market or have a long time horizon before they can be implemented. Research on the development of feed interventions might lead to a more effective method for avoiding boar tainted meat from entire male pigs and can be implemented within a short period of time. Studies on how feed interventions, that have showed an effect on boar taint, are affecting the environment in the intestines might provide an opportunity to evaluate alternative feed interventions with improved effect on boar taint. Skatole is the main substance, involved in boar taint, that are affected when focusing on the effect of feed interventions (Lundström et al., 1988; Hansen et al., 2006; Byrne et al., 2008; Øverland et al., 2011). Thus, this thesis will focus mainly on the production and metabolism of skatole in entire male pigs.

1.1 Objective

The objective of the current thesis is to investigate how skatole production in the gastrointestinal tract can be affected, and how the level of skatole production affects skatole absorption into the portal vein, metabolism in the liver and deposition in adipose tissue. Moreover, it was investigated how the microbial production of skatole in the gastrointestinal tract can be decreased through different feed interventions.

1.2 Hypothesis

This thesis hypothesises that skatole deposition in adipose tissue can be reduced through feed interventions having a reducing effect on skatole production in the large intestine.

1.3 Structure of the thesis

The thesis consists of two main parts. The first part is a literature review consisting of two chapters. The first chapter examines the skatole metabolism in the pig through four main steps which is the production in the large intestine, the absorption into the bloodstream, the metabolism in the liver and the deposition in adipose tissue. The second chapter examines feed related factors affecting skatole production and deposition. The literature review functions as background research for the second part which consists of a microbiological study performed *in vitro.* The study was presented as a scientific paper within the thesis addressed for the International Journal of Systematic an Evolutionary Microbiology. In the study a bacterium isolated from the gastrointestinal tract of pigs was characterised. Moreover the bacterium and closely related bacteria were studied for their ability to produce skatole from L-tryptophan or indole-3-acetic acid. Following the paper is a general discussion of the results from both parts of the thesis and perspectives for further investigation. The paper contains a separate reference list whereas references used in the literature review, general discussion and perspectives are listed in the end of the thesis.

2 Skatole metabolism in the pig

Skatole is produced by microbial fermentation of L-tryptophan in the large intestine and is absorbed into the blood through the intestinal wall. Skatole is then transported to the liver through the portal vein where it is either metabolised and excreted with urine or deposited into adipose tissue. All the steps more or less influence the level of skatole deposition in adipose tissue. The aim of the following chapter is to investigate how skatole production in the gastrointestinal tract can be affected and how the level of skatole production affects absorption into the portal vein, metabolism in the liver and deposition in adipose tissue. The following four parts of this chapter review the skatole production in the large intestine, the absorption into the portal vein, the metabolism in the liver and the deposition in adipose tissue.

2.1 Production

Skatole is produced by microbial fermentation of L-tryptophan in the large intestine. Moreover, indole and small amounts of indole-3-propionic acid (IPA) can be produced from the fermentation of L-tryptophan (figure 2.1) (Agergaard at al., 1998 Knarreborg et al., 2002).

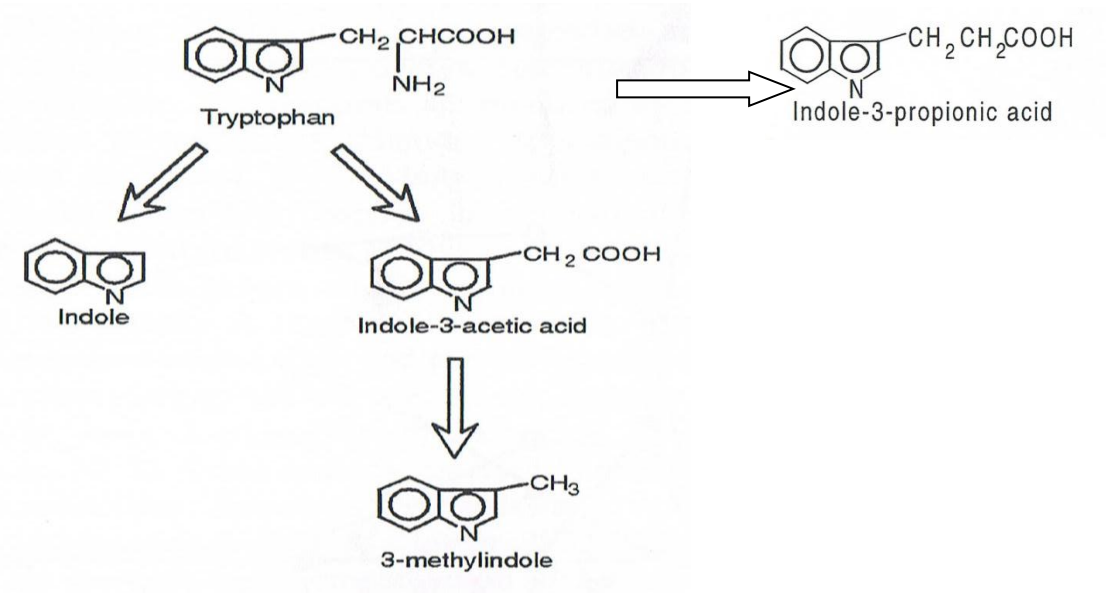


Figure 2.1 Microbial production of 3-methylindole (skatole), indole and indole-3-propionic acid in the large intestine of pigs. The fermentation processes illustrated by the arrows occur in different bacteria that are more or less specialised (Jensen et al., 1995; Jensen and Jensen, 1998).

Agergaard at al. (1998) studied the effect of L-tryptophan infusion into the large intestine on skatole, indole and IPA production for a period of 12 hours. Two groups of five pigs were fed either a low fibre diet based on barley, wheat and soya-bean meal or a high fibre dietary diet added sugar beet pulp and corrugated for energy content. When pigs were fed the low fibre diet, it was estimated that 69 %, 7 % and 26 % of infused L-tryptophan was converted into indole, IPA and skatole respectively. When pigs were fed the high fibre diet, it was estimated that 35 %, 16 % and 6 % of the infused L-tryptophan were converted into indole, IPA and skatole respectively. Thus, on the low fibre diet approximately all the infused L-tryptophan was fermented by microbes whereas on the high fibre diet around half of the infused L-tryptophan was fermented. This indicates that there is a reduced requirement for proteolytic activity of microbes when fed a high fibre diet. This was probably due to an increased amount of carbohydrates from sugar beet pulp, reaching the large intestine, available for fermentation. According to Agergaard et al. (1998) and Knarreborg et al. (2002) skatole, indole and indol-3-acetc acid (IAA) decrease when feeding a high fibre diet compared to a low fibre diet. IPA shows a reverse pattern. The concentration of IPA increases when feed a high fibre diet compared to a low fibre diet. The concentration of IPA was highest in the proximal part of the colon where the primary production of IPA probably occurs. Throughout the rest of the colon, the concentration of IPA decreases properly due to the absorption of IPA from the colon to the blood. The role of IPA in relation to boar taint needs to be investigated.

Studies have analysed the site of skatole production by analysing skatole concentration in samples from different parts of the gastrointestinal tract (Jensen et al., 1995b; Knarreborg et al., 2002). The studies revealed that skatole production was to a large extend affected by the availability of carbohydrates through the gastrointestinal tract. The microorganisms obtained energy from the fermentation of available substrate in form of adenosine 5'triphosphate (ATP) for growth and maintenance. When carbohydrates were available, the microbes in turn produced volatile fatty acids (VFA), which was absorbed into the blood and used for energy

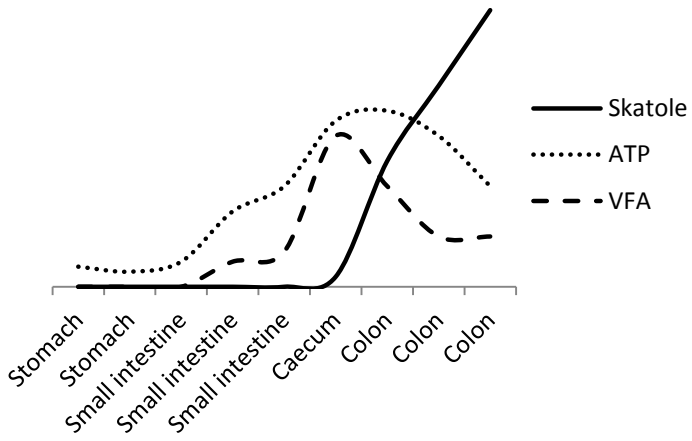


Figure 2.2 Realtionship between production of ATP, VFA and skatole in various regions of the gastrointestinal tract (Jensen and Jensen, 1998)

by the host. Thus, ATP and VFA concentration in the caecum and colon reflected the level of microbial activity when fermenting carbohydrates (figure 2.2). VFA and ATP decreased when available carbohydrates were limited, and microbes started to draw their energy for maintenance from protein fermentation and in turn produced compounds like the indolic compounds indole and indole-3-acetic acid (IAA) (figure 2.2) (Jensen et al., 1995b; Smith and Macfarlane, 1996). Moreover, when carbohydrates were available for bacterial fermentation in the large intestine, bacteria were growing and available amino acids, including L-tryptophan, were incorporated into bacterial biomass. Less L-tryptophan would then be available for microbial fermentation and skatole production. This indicated that skatole production increases when the amount of available carbohydrates becomes a limiting factor as the intestinal content passes through the large intestine (Jensen and Jensen, 1998 Knarreborg et al., 2002). Thus, skatole is primarily produced in the distal part of the colon.

Agergaard at al. (1998) studied the effect of tryptophan availability *in vivo* by the infusion of tryptophan into the caecum of entire male pigs. The level of L-tryptophan used in the experiment cannot be compared with levels naturally occurring in the colon. However, the high amount of L-tryptophan makes it possible to register changes that would be too small to detect during normal physiological conditions. The *in vivo* study concluded that increased amounts of tryptophan in the colon result in the production of more indole at the expense of skatole. Furthermore, the amount of IAA became detectable which during normal conditions is often not possible. This indicates an accumulation of IAA at these L-tryptophan levels (Agergaard at al., 1998). Thus, the production of skatole from IAA is somehow more limited than the production of IAA and indole from L-tryptophan. However, the production of indole and skatole do increase after an L-tryptophan infusion which indicates an unexploited microbial potential to ferment L-tryptophan and produce skatole during physiological conditions.

Jensen at al. (1995a) studied the kinetics of indole and skatole formation *in vitro* by incubating pig faecal slurries either with no addition or with addition of IAA or L-tryptophan. The three reactions, occurring when L-tryptophan is metabolised, can be fitted to hyperbolic kinetics where the output is inversely proportional to its input. The effect of substrate concentration on the production of skatole, IAA and indole can be described by the Michaelis-Menten equation (Eq. 2.1).

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \text{Eq. 2.1}$$

V = Reaction rate

[S] = Substrate concentration

V_{max} = maximum reaction rate achieved by the system

K_m = substrate concentration at ½V_{max}

Eq. 2.1 and constants are illustrated in figure 2.3.

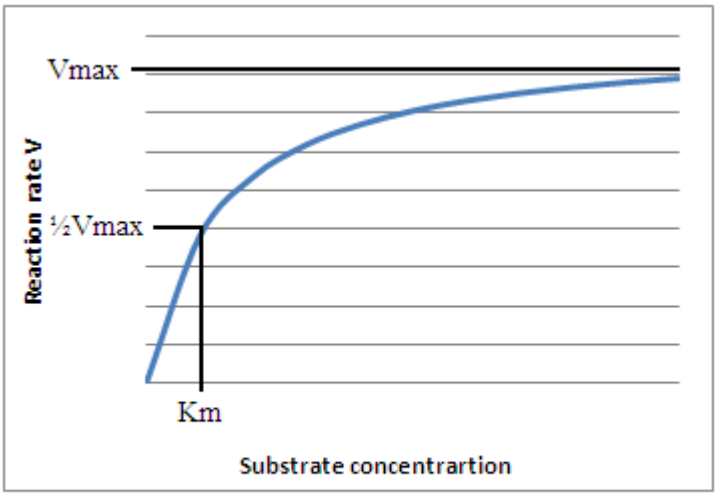


Figure 2.3 Effect of substarte concentration on reaction rate. Micaelis-Menten equation (Eq. 2.1)

The two Michaelis-Menten constants K_m and V_{max} were calculated for the three reactions; L-tryptophan to indole or IAA and IAA to skatole (table 2.1). The calculations were done on the basis of several *in vitro* studies simulating the metabolism of L-tryptophan in the pig. Faecal samples for the *in vitro* study was taken from at least eight pigs feed the same diet (Jensen et al., 1995a).

Table 2.1 Estimated values of Michaelis-Menten constants for the metabolism of L-tryptophan to indole, IAA and skatole after infusion of L-tryptophan (Jensen et al., 1995a).

	K_m (µM)	V_{max} (µmol (kg faeces)$^{-1}$ h^{-1})
L-tryp. → Indole	34	415
L-tryp. → IAA	52	580
IAA → Skatole	136	450

K_m for the skatole production was 4 and 2.5 times as high as K_m for the indole and IAA production respectively. This means that an equally higher concentration of substrate was needed to achieve half of the maximum production rate. The maximum production rate for the production of skatole was only slightly faster than the production of indole and a bit slower than the production of IAA. This means that the maximum reaction rate for the production of skatole from IAA can reach the level of the two other reactions, but more substrate is needed for this to happen. Thus, the production of skatole was sensitive to substrate concentrations; hence it was sensitive to the concentration of IAA produced from L-tryptophan. Agergaard et al. (1998) showed in an *in vivo* study that indole and IAA had a rapid increase in formation rate reaching a maximum around 700

and 150 µmol h^{-1} within one hour after infusion. In contrast, the skatole formation rate increased until four hours after L-tryptophan infusion and reached only a maximum of nearly 80 µmol h^{-1} (Agergaard et al., 1998). Thus, the conversion of IAA into skatole seems like a natural barrier for skatole production in the large intestine. In conclusion two factors have an effect on the level of skatole production. 1) Concentration of substrate available for skatole production. 2) The activity of bacteria responsible for the decarboxylation of IAA into skatole.

2.2 Absorption

Skatole is absorbed through the intestinal wall into the portal vein. Studies analysed skatole absorption by taking samples from the portal vein and peripheral vein and analysed the samples for skatole concentration (Knarreborg et al., 2002; Zamaratskaia et al., 2005). Samples from the peripheral vein estimated the background level of skatole in the blood before the absorption of skatole from the large intestine. Samples from the portal vein estimated the amount of skatole added to the background level after absorption from the large intestine. Analysis of blood plasma has the advantage that samples can be taken several times during a trail without killing the animal. Samples from the peripheral vein can be taken through a catheter (Claus et al., 2003). Samples from the portal vein needs to be taken through a simple catheter operated into the portal vein (Hooda et al., 2009). The absorption rate can be estimated according to equation 2.2 (Rerat et al., 1987).

$$q = [C_p - C_j] \times D \qquad \text{Eq. 2.2}$$

q = quantity absorbed per minute

C_p = concentration in portal vein

C_j = concentration in jugular vein

D = Blood flow rate in the portal vein

Knarreborg et al. (2002) conducted a feeding trial, studying the effect of diets with high or low content of non-starch polysaccharide (NSP) where blood samples were taken from the jugular vein and portal vein for a period of 12 hours after feeding. Samples from 11 different segments of the gastrointestinal tract were taken after killing the animal. The samples were analysed for skatole content. The results showed a significant reduced skatole production in the distal part of the colon when feeding high NSP compared with a low NSP diet. This was followed by a corresponding reduced absorption rate. Thus, there were a correlation between production and absorption of skatole when calculated for the individual animal (Knarreborg et al., 2002). Correlations calculated between animals showed no clear results which might be due to genetic differences between

animals (Claus et al., 1993; Babol et al., 2004a). Thus, the level of skatole production in the large intestine will affect the amount of absorbed skatole.

Agergaard et al. (1998) studied skatole absorption rates by taking samples from the portal vein one hour before and for a 12 hours period after tryptophan infusion. The concentration of indole in the portal vein blood increased rapidly during the first three hours after infusion into a maximum concentration around 500 ng/ml corresponding to an absorption rate around 150 µmol/h. Compared to indole, the absorption of skatole was 2.5 times slower reaching a maximum concentration around 200 ng/ml six hours after tryptophan infusion corresponding to an absorption rate around 60 µmol/h (Agergaard at al., 1998). The delay in skatole absorption corresponds to the delayed increase in skatole production observed in the large intestine and corresponds to the reaction scheme where indole is produced directly from tryptophan, whereas skatole is produced indirectly from tryptophan through IAA. The slow skatole absorption rate indicates a natural barrier for the transport of skatole from the intestine to the vascular system. In addition the correlation between skatole production in the large intestine and absorption indicates that a decrease in skatole production will result in reduced skatole absorption.

2.3 Metabolism in the liver

Skatole is metabolised in the liver into a variety of different metabolites to convert small lipophilic molecules into large more water soluble compounds which can be excreted with urine. Studies analysed skatole metabolism in the liver by taking samples from the liver after killing the animal and analyse the activity of enzymes involved in the metabolism of skatole. In addition, urine samples were analysed for metabolites from skatole metabolism in the liver. However, many different metabolites originate from the metabolism of skatole (Babol et al., 1998a), which makes it difficult to use the method to identify the level of skatole metabolism in the liver. Agergaard and Laue (1998) studied the level of skatole metabolism on four pigs by measuring the absorption of skatole to the portal vein, the hepatic inflow of skatole and the hepatic outflow estimated as skatole concentration in blood plasma from the jugular vein. Blood samples were taken every half hour for a five hour period. Liver extraction percentage of skatole varied from 43 % to 96 % indicating a marked variation in the ability of individual pigs to extract skatole from the blood (Agergaard and Laue, 1998).

Using ^{14}C-skatole, four different metabolites from the metabolism of skatole were detected in plasma, and more than fifteen metabolites were detected in urine. The major metabolites found were

6-sulfatoxyskatole (MII) and 3-hydroxy-3-methyloxindole (MIII). High concentrations of a conjugate of MII was primarily found in pigs that were able to rapidly metabolise skatole and excrete it from the body, whereas high MIII were related to slow clearance of skatole (Babol et al., 1998a Bæk et al., 1998, pp. 111-112). It has been demonstrated that the potential of the liver to metabolise skatole and increase skatole clearance from the blood can greatly exceed what is found during physiological conditions (Agergaard and Laue, 1998). The potential of the liver to metabolise skatole depends on the expression of enzymes catalysing the metabolic processes. Studies have identified aldehyd oxidase (Diaz and Squires, 2000a) and cytochromes of a bigger enzymatic complex called P450 to play a major role in this metabolism. Especially cytochrome CYP2E1, CYP2A6, CYP2A19 and CYP2C49 of the P450 enzymatic complex were identified as key regulators of skatole metabolism (Babol et al., 1998a; Diaz and Squires, 2000b; Wiercinska et al., 2012). Babol et al. (1998) found that high levels of CYP2E1 were only found in plasma of pigs that were able to rapidly clear skatole from their body. Doran et al. (2002) found a negative correlation between skatole deposition in adipose tissue and CYP2E1 activity in the liver. Thus, a high activity of CYP2E1 was associated with a high metabolism of skatole in the liver and less skatole deposition in adipose tissue. This indicates that CYP2E1 might be a key regulator having a direct or indirect ability to catalyse the metabolism of skatole. Thus, CYP2E1 expression is used by many studies as an indicator of the level of skatole metabolism in the liver.

The expression of key liver enzymes is affected by a variety of direct and indirect factors, of which some of them are not yet known, and some of them have been studied with no clear conclusions. A clear age and weight related decrease in activities of CYP2E1 in entire male pigs has been found around the onset of puberty (Whittington et al., 2004; Zamaratskaia et al., 2012). Skatole is usually not accumulated in gilts and barrows in an increased amount. Thus, increased skatole levels in entire male pigs at the onset of puberty could be due to regulation of skatole metabolism by testicular steroids (Zamaratskaia et al., 2005). Correlation has been found between elevated levels of testicular steroids and reduced metabolism of skatole followed by increased skatole deposition in adipose tissue. This was leading to the conclusion that some testicular steroids have a reducing effect on skatole metabolism (Babol et al., 1998b; Babol et al., 1999; Doran et al., 2002; Zamaratskaia et al., 2004b). The underlying mechanism responsible for the increased skatole level is unknown. Thus, it is uncertain if there is a direct effect through inhibition of the liver metabolism or if there is an indirect effect for example through repressing the expression involved in skatole metabolism. Doran et al (2002) concluded from an *in vitro* study that: *"CYP2E1 is induced by*

skatole and this induction is blocked by androstenone". This connection explains the increase in skatole deposition at the onset of puberty, where elevated amounts of androstenone are produced in the testicles. The conclusion contains two statements. 1) CYP2E1 is induced by skatole 2) The induction is blocked by androstenone. The first statement was not in agreement with later studies where no correlation between low levels of skatole and reduced CYP2E1 enzyme activity were found (Zamaratskaia et al., 2005b; Rasmussen et al., 2011a). The second statement was discussed in later studies where a possible association between testicular steroids and skatole has been investigated. The association has been investigated by two different *in vivo* methods. 1) To compare enzyme activity between entire male pigs producing testicular steroids and castrated males and female pigs producing no testicular steroids. 2) By increasing testicular steroid level artificially through stimulation with human chorionic gonadotropin (hCG) (Chen et al., 2006). Studies testing the association by the first method (Whittington et al., 2004; Brunius et al., 2012) and the second method (Zamaratskaia et al., 2008) and by an *in vitro* method (Rasmussen et al., 2011b) all agreed that the induction of CYP2E1 is blocked by androstenone. However, it was also realised that other testicular steroids than androstenone may be due in part of the decrease in the expression of CYP2E1 and other enzymes involved in the metabolism of skatole. For example there has been found a correlation between free oestrone and skatole in adipose tissue. This indicated that oestrone might be involved in the regulation of skatole level as well (Zamaratskaia et al., 2005a). None of the studies were able to finally conclude which testicular steroids were involved and to what degree they were involved in the changes. Studies were also found to disagree with the conclusion that CYP2E1 is blocked by androstenone or other testicular steroids. One study found no change in the enzyme activity of CYP2E1 after hCG administration (Zamaratskaia et al., 2006). Another study aimed to investigate the effect of different concentrations of testicular steroids on enzyme activities of different enzymes belonging to the P450 enzyme complex. The results showed that direct inhibition by the testicular steroids was not the primary cause of reduced enzyme activities in entire male pigs compared to castrated pigs (Zamaratskaia et al., 2009). Thus, it is likely that testicular steroids have an effect on the activity of enzymes involved in the metabolism of skatole. This effect is most likely an indirect affect, affected by yet unknown factors.

Studies indicated that some feed ingredients might have an effect on skatole deposition not only through a decrease in skatole production in the intestines. It was suggested that feeding sugar beet fibre (Whittington et al., 2004) and dried chicory root (Rasmussen et al., 2011c) increased the expression of hepatic enzymes involved in skatole metabolism incl. CYP2E1. This was expected to

increase the skatole clearance in the liver. The underlying mechanisms of this increased expression are still unknown. Thus, there are still many aspects of skatole metabolism in the liver that is unknown, and studies made on the subject so far have realised that the process is very complex. Important to realise was that, when skatole production was low, the potential of the liver to metabolise skatole was found to be less important for the level of skatole deposition (Whittington et al., 2004; Zamaratskaia et al., 2005b; Zamaratskaia et al., 2006).

2.4 Deposition

Skatole, which is not being metabolised in the liver, is transported with the vascular system to the peripheral tissue. Due to the lipophillic properties of skatole, it is deposited in adipose tissue. Studies analysed skatole deposition in the pig by analysing skatole concentration in samples from adipose tissue (Zamaratskaia et al., 2005; Pauly et al., 2008). Samples from adipose tissue have the disadvantage that it can only be taken once after killing the animal. Samples could be taken by a biopsy, but there is a high risk of not getting a representative sample. It is likely that there is a correlation between skatole concentration in blood plasma and in adipose tissue, which has been found within groups of slaughter pigs slaughtered at 115 kg live weight (Zamaratskaia et al., 2005). If there is a correlation, skatole deposition could be estimated from samples of peripheral vein blood which can be taken several times from each animal through the experimental period.

Deposition of skatole into adipose tissue is where skatole contributes to boar taint of the meat. Thus, it is the main site of interest when the effect of an intervention is measured. It has not been well established how fast the clearance rate of skatole in adipose tissue is. Studies aimed to identify the optimal application time for a feed intervention which are directly affected by the time needed for clearance of skatole from adipose tissue. Studies have revealed that 25 % lupine, 25 % chicory root or 30 % raw potato starch added to the feed, fed to entire male pigs, decreased skatole concentration in adipose tissue after an application period of seven days. (Claus et al., 1994; Hansen et al., 2006; Hansen et al., 2008; Pauly et al., 2008; Pauly et al., 2010). No study on the effect of feed interventions has tested shorter application periods. Hansen et al (1997) found a reducing effect of treating pigs with antibiotics on skatole concentration in adipose tissue. Application periods of 3 and 7 days were tested and only the application period of 7 days had a significant reducing effect on skatole concentration in adipose tissue (Hansen et al., 1997). This supports that a period of 7 days are needed for the clearance of skatole from adipose tissue.

2.5 Summary

L-tryptophan can be converted by microbes in the large intestine of the pig into indole-3-acetic acid (IAA), indole and indole-3-propionic acid (IPA). IAA can further be converted into skatole. The production of skatole in the colon can be affected by the amount of carbohydrate and L-tryptophan reaching the colon. Estimates of the conversion of infused L-tryptophan to the large intestine indicate a reduced need for proteolytic activity by microbes when pigs where fed a high fibre diet compared to a low fibre diet. This was expected to be a result of increased amounts of carbohydrates reaching the large intestine, being available for fermentation, when pigs were fed a high fibre diet. Moreover, a high fibre diet reduced the concentration of IAA and indole in the large intestine but increased the concentration of IPA. This indicates a need for further investigation of the role of IPA in relation to boar taint.

The three reactions occurring when L-tryptophan was converted into indole or IAA and IAA into skatole can be described by Michaelis-Menten equation. The constants describing the three equations were estimated. The constants reveal that more substrate was needed for the conversion of IAA into skatole in order to obtain a reaction rate as fast as the reaction rate for the conversion of L-tryptophan into indole or IAA. Due to this limitation, reaction rates measured for the conversion of IAA into skatole are often much slower compared to reaction rates for the conversion of L-tryptophan into indole or IAA. Thus, the conversion of IAA into skatole seems like a natural barrier for skatole production in the large intestine. This can either be affected by affecting the microbes responsible for the conversion of IAA into skatole or by reducing the availability of substrate available for the production of skatole.

Skatole is absorbed through the intestinal wall into veins ending up in the portal vein. The absorption rate is correlated with the production rate in the colon when calculated within individual animals. Thus, a decrease in skatole production in the large intestine will also decrease the absorption of skatole.

Skatole is transported with the portal vein to the liver. In the liver skatole are metabolised primarily into 6-sulfatoxyskatole (MII) or 3-hydroxy-3-methyloxindole (MIII) which can be excreted with urine. MII was found in high concentrations in urine only from pigs that were able to rapidly metabolise skatole, whereas high concentrations of MIII were only found in pigs with low clearance of skatole. The potential of the liver to metabolise skatole was shown to greatly exceed what was observed during physiological conditions. The ability of the liver to metabolise skatole was affected by the expression of enzymes catalysing the metabolism. This was primarily aldehyd oxidase and

enzymes of the bigger enzymatic complex P450. The activity of these enzymes was affected by a verity of factors, of which most of them are unknown. Studies found an effect of age and weight which might correspond to the hormonal changes occurring in the pig at the onset of puberty. Testicular steroids were in some studies found to have a major decreasing effect on the activity of enzymes involved in the metabolism of skatole. Other studies disagreed and found a smaller effect indicating that the effect of testicular steroids is only indirect. Thus, many aspects of skatole metabolism are unknown due to the complexity of the system. However, one study did conclude that when skatole production and absorption was low, the potential of the liver to metabolise skatole was less important for the level of skatole deposition.

Skatole, which is not metabolised in the liver, is deposited in adipose tissue. It is not well known, how fast the transition time is for this deposition. Feeding trails revealed that an application period of seven days of a skatole reducing feed intervention before slaughter is sufficient to observe a significant decrease in skatole concentration in adipose tissue.

Common for all steps of skatole metabolism, which were investigated, is that they are affected by the level of skatole production in the large intestine. Thus, reduced skatole production in the large intestine has an effect through all the metabolic processes of skatole. Additionally skatole production in the large intestine seems to be naturally limited by specialised bacteria performing the conversion of IAA into skatole. Moreover, it seems to be limited when the availability of substrate needed for skatole production is reduced.

3. Feed related factors affecting skatole production in the gastrointestinal tract

Fermentation of L-tryptophan into indole or skatole in the large intestine of the pig is affected by feed composition which affects the relationship between available L-tryptophan and carbohydrates entering the large intestine and the composition of the microbial population in the large intestine. If a feed related factor should have a potential to influence skatole production in the large intestine of the pig, it should have the ability to pass undigested through the stomach and small intestine. The following chapter is a review on feed related factors having this ability and their effect on skatole production in the large intestine.

Several feed interventions with reducing; increasing or no effect on skatole production has been studied.

Protein: Since skatole are produced from L-tryptophan, it is easy to believe that reducing the protein content of the diet would lead to decreased skatole production (Lundström et al., 1994). However, proteins are digested and absorbed in the small intestine and rarely enter the large intestine. Primarily endogenous protein reaches the large intestine for microbial fermentation and skatole production (Claus et al., 1994).

Brewers' yeast slurry had an increasing effect on skatole production. It is suggested that this is due to a low digestibility of yeast cells in the small intestine leading to increased amounts of yeast protein entering the large intestine for protein fermentation. Yeast slurry can be included in experimental diets to ensure a greater skatole production, making it easier to observe a potential reducing effect of a feed intervention on skatole production and deposition (Jensen et al., 1995b).

Antibiotics: Antibiotic growth promoters showed a reducing effect on skatole production through reduced microbial activity in the gastrointestinal tract (Hawe et al., 1992; Hansen et al., 1997). In January 2006, the use of antibiotics as growth promoters was prohibited in EU due to the concern of bacterial resistance to antibiotics.

Organic acids: Organic acids, such as benzoic acid, showed some of the same growth promoting properties as antibiotics. Thus, organic acids might also have the same reducing effect on skatole production as the antibiotics had shown. Øverland et al. (2008) studied the effect of adding 0.85 % formic acid, benzoic acid or sorbic acid to the feed fed to entire male pigs and found no significant reducing effect on skatole production (table 3.1). However, this might be due to a low skatole concentration of 130 ng/g found in adipose tissue of pigs from the control group making it difficult to detect a significant difference. When entire male pigs where fed diets added formic acid, benzoic acid or sorbic acid, skatole concentration in adipose tissue dropped from 130 ng/g to 30, 20 and 20

ng/g respectively (Øverland et al., 2008). Thus, even though the reducing effect of adding organic acids was not significant, it might have an effect in a large scale experiment including more animals.

Liquid feeding: Liquid feeding was suggested to affect boar taint based on results from the entire male pig data base of the Federation of Danish Pig Producers and Slaughterhouses. These results indicated that entire male pigs from herds using liquid feed had a lower skatole level in adipose tissue than entire male pigs from herds using dry feed (Kjeldsen and Udesen, 1998). Jensen et al. (1998) and Hansen et al. (2000) showed a significant effect on the microbiological environment in the gut but found inconsistent effects on skatole production, absorption and deposition in adipose tissue, mostly indicating no or little effect (Jensen at al., 1998 Hansen et al., 2000). This indicates that liquid feeding only have a small potential of decreasing skatole production alone. However, it might have a potential to enhance the effect of diets showing an effect on boar taint. At the time of writing, this has not been studied.

Fasting: Ambrosen (1993) found a reducing effect on fasting entire male pigs for 12 hours before slaughter on skatole concentration in adipose tissue. Further investigation is needed to identify who fasting has an effect and if fasting will contribute with an effect in addition to a feed intervention.

Thus, many different feed related factors have shown the potential of affecting the site of skatole production in the large intestine. However, source and amount of carbohydrate added to the feed with the ability to enter the large intestine undigested showed the most pronounced effect on skatole production in the large intestine of the pig. The following part of this chapter contains a review on the effect of different sources and amounts of carbohydrates on skatole production.

3.1 Carbohydrates

Studies have shown that the amount and source of carbohydrates in the diet can have an effect on skatole production. Carbohydrates in general are a very diverse group of compounds which from a chemical approach can be classified according to their size measured as degree of polymerisation (DP). Sugars: DP 1-2, Oligosaccharides: DP 3-9 and Polysaccharides: DP $\geq$ 10. Furthermore, they can be classified according to starch and non-starch polysaccharides (NSP) and the linkage between the compounds. The linkage and the interrelation between the carbohydrate compounds have an impact on where the carbohydrates are digested, and how they influence the physiology of the gastrointestinal tract. Almost all α-linked carbohydrates can be broken down to monosaccharaides by endogenous enzymes in the small intestine. In contrast, compounds linked together with β-

linkage have to be fermented by microbes primarily located in the caecum and the colon (Cummings and Stephen, 2007; Englyst et al., 2007). Digestible carbohydrates are defined as carbohydrates which can be broken down by endogenous enzymes in the small intestine. Starch and sugars belong to this group and do never or rarely reach the large intestine in pigs with a fully developed digestive tract (Knudsen et al., 2012). Thus, it is not expected that digestible carbohydrates will have an effect on the fermentation processes in the large intestine. In contrast, non-digestible carbohydrates (NDC) cannot be broken down by endogenous enzymes, and most of it will reach the large intestine undigested. NDC consist of three main categories of carbohydrates: 1) Non-starch polysaccarides (NSP). NSP contributes to the fibre fraction of the diet. 2) Oligosaccharides. 3) Starch resistant to enzymatic breakdown referred to as resistant starch (RS). RS are glucans linked together as branched chained polysaccharides with β-linkage. The structure of the branched chains forms a crystalline protecting layer. When heated in water, the crystal layer is broken down, a process called gelatinisation. The starch then becomes a gel no longer resistant to enzymatic breakdown. Thus, this type of starch needs to be in its raw form to be resistant to enzymatic breakdown (Cummings and Stephen, 2007; Englyst et al., 2007). NDC can interact with the digestive processes along the entire gastrointestinal tract and have an influence on the structure and function of the gut. NDC reaching the large intestine are available for microbial fermentation and have an effect on the microbiological community (Knudsen et al., 2012). Thus, it is expected that non-digestible carbohydrates might have a pronounced impact on skatole production. The theoretical line of actions is illustrated in figure 3.1.

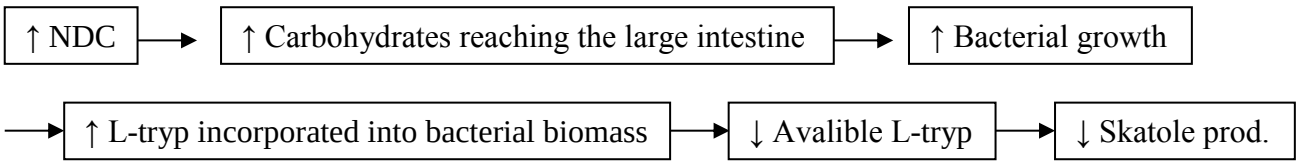


Figure 3.1 Theoretic line of action when increasing the amount of non-digestible carbohydrates in the diet. NDC: Non-digestible carbohydrates; L-tryp: L-tryptophan (Louis et al., 2007; Knudsen et al., 2012).

NDC with the most pronounced effects found in literature were adding inulin from chicory root, raw potato starch or dietary fibre in the form of sugar beet pulp, wheat bran, soybean hulls or lupine. An overview of results from feed experiments testing the effect of different feed interventions is listed in table 3.1. The following three parts of this chapter review the effect of these feed interventions.

Table 3.1 Results from studies testing the effect of inulin from chicory root, raw potato starch, the dietary fibres sugar beet pulp, wheat bran, soybean hulls and lupine and the organic acids formic acid, benzoic acid and sorbic acid. Results are presented as "results from control group" / "result from experimental group".

				Skatole				Andro-stenone	
Product	Inulin %	N	App. period days	Colon content µg/g	Faeces µg/g	Plasma µg/l	Adipose tissue ng/g	Adipose tissue µg/g	Reference
4 % DA	2.1	11	7	4.6/7.4	13.0/15.6		37/55	1.8/2.7	Vhile et al., 2012
8 % DA	4.2	10	7	4.6/1.8	13.0/7.6		37/15	1.8/2.0	Vhile et al., 2012
12 % DA	6.3	11	7	4.6/0.5	13.0/4.7		37/10	1.8/1.6	Vhile et al., 2012
3 % DC	1.8	30	14				237/129		Zammerini at al., 2012
6 % DC	3.6	30	14				237/124		Zammerini at al., 2012
9 % DC	5.4	30	14				**237/47***		Zammerini at al., 2012
9 % DC	6.3	11	7	4.6/1.3	13.0/9.7		37/17	1.8/1.3	Vhile et al., 2012
10 % DC	4.0	24	8-15			2.43/1.95	160/140		Hansen et al., 2008
15 % DC	9.0	50	14	**26.3/6.0***			**110/40***	1.14/1.08	Maribo et al., 2010
25 % DC		32	7			**3.49/0.11***			Hansen et al., 2006
3 % PI	3.0	11	31	36.2/33.2			40/60	1.59/1.99	Øverland et al., 2011
5 % PI	5.0	18	28-42				110/90	0.29/0.36	Aluwé et al., 2009
5 % PI	5.0	6	14		183/79.2$^{(*)}$				Rideout et al., 2004
6 % PI	6.0	12	31	36.2/15.9			40/20	1.59/1.87	Øverland et al., 2011
9 % PI	9.0	12	31	**36.2/5.4***			**40/10**$^{(*)}$	1.59/2.64	Øverland et al., 2011
14 % PI	14.0	32	42			**3.49/0.68***			Hansen et al., 2006
10 % RPS		18	28-42				110/130	0.29/0.25	Aluwé et al., 2009
20 % RPS		10	16	**37.6/4.7***			**170/20***	3.08/1.72	Øverland et al., 2011
20 % RPS		8▪	14-21	134/76			**159/67***		Lösel and Claus, 2005
30 % RPS		20	nd	**39.8/nd***		**0.77/0.42***			Lösel et al., 2006
30 % RPS		12	7			**20.0/7.0**$^{(*)}$	**850/220***	1.7/2.0	Pauly et al., 2008
30 % RPS		12	7				**140/60***	0.9/1.1	Pauly et al., 2010
30 % RPS		8▪	14-21	**134/8.6***			**159/26***		Lösel and Claus, 2005
40 % RPS		7▪	14-21	**134/4.8***			**159/20***		Lösel and Claus, 2005
58 % RPS		12	19		**99/1.87***	**1.62/0.19***	**236/nd***		Claus et al., 2003
10 % SBP		6	30	**33.4/13.1***	**45.9/19.8**$^{(*)}$	**3.5/1.3***			Knarreborg et al., 2002
15 % SBP		15	nd				65/37		Van Oeckel et al., 1998
20 % SBP		7	nd	**26.5/8.1***			**150/100***		Jensen et al., 1995
20 % WB		7	nd				150/200		Jensen et al., 1995
30 % WB		15	nd				65/126		Van Oeckel et al., 1998
15 % SBH		15	nd				65/55		Van Oeckel et al., 1998
10 % lupine		18	28-42				110/60	**0.29/0.15***	Aluwé et al., 2009
25 % lupine		24	8-15			**2.43/0.43***	**160/40***		Hansen et al., 2008
0.85% formic acid		10	~78			**1.76/0.18***	130/30	1.63/0.84	Øverland et al., 2008
0.85% benzoic acid		9	~78			**1.76/0.15***	130/20	1.63/2.34	Øverland et al., 2008
0.85% sorbic acid		9	~78			1.76/1.90	130/20	1.63/1.38	Øverland et al., 2008

n: number of pigs feed on the experimental diet
▪male pigs together with female gilts
DC: Dried Chicory root
DA: Dried Artichoke
PI: Pure inulin
RPS: raw potato starch
SBP: sugar beet pulp
WB: Wheat bran
SBH: Soybean hulls
* significant decrease compared to control
(*) tendency for decrease compared to control
nd: not detected, below detection limit or not defined

3.1.1 Dietary fibre sources

Dietary fibre is characterised as non-starch polysaccharides (NSP) and reach the large intestine undigested making it available for microbial fermentation (Cummings and Stephen, 2007). A definition on dietary fibre has not been very well defined, but the basic definition is that they are of fibrous, coarse or structural nature (Englyst et al., 2007). Moreover, NSPs are the most diverse group of carbohydrates with different properties which affect the gastrointestinal tract in different ways (Knudsen et al., 2012). The level of skatole production depends on sufficient availability of fermentable carbohydrates throughout the entire large intestine to supply gut bacteria with energy (Jensen et al., 1995b). Thus, an important property in relation to their ability to affect microbial skatole production is the fermentability of NSP. Li Cai Yan et al. (2009) studied the effect of three different NSP sources differing in fermentability on their effect on skatole production tested *in vivo*. Sugar beet pulp was used as an easy fermentable NSP source. Ray grass hay and alfalfa hay were used as less fermentable NSP sources. Skatole production increased when adding ray grass hay and alfalfa hay and decreased when adding sugar beet pulp to faecal slurry. (Li CaiYan et al., 2009). Thus, sugar beet pulp contributed with more carbohydrates available for microbial fermentation compared to the two hay sources (figure 3.2).

Moreover, it is proposed that dietary fibre contributes with an increased endogenous loss from the gut epithelial cells increasing the amount of protein hence L-tryptophan reaching the large intestine. More L-tryptophan will then be available for microbial fermentation and skatole production (Low, 1989) (figure 3.2).

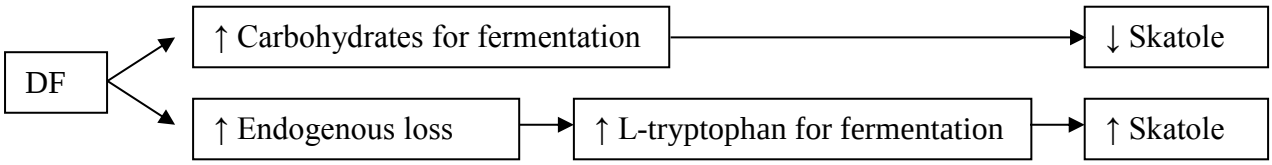


Figure 3.2 Theoretical line of action when adding Dietary fibre (DF) to the feed fed to entire male pigs. (Low, 1989; Jensen et al., 1995).

When studying the effect of different sources of NSP on skatole production, high inclusion rates were necessary to observe a reducing effect on skatole production and deposition. The inclusion of 25 % lupine had a decreasing effect on skatole (Hansen et al., 2008) whereas 10 % inclusion showed no effect on skatole deposition in adipose tissue (Aluwé et al., 2009).

Another proposed effect of NSP on skatole deposition is decreased absorption of skatole due to gut content with more structure. The inclusion of 10 % sugar beet pulp did not show significant differences in skatole production in the large intestine but was found to decrease skatole absorption into portal vain blood (Knarreborg et al., 2002). Thus, NSP had a limited potential to reduce skatole deposition in adipose tissue depending on amount and fermentability of the NSP source.

3.1.2 Raw potato starch

Raw potato starch is a resistant starch with a high content of amylopectin which cannot be digested by the endogenous enzyme amylase, excreted in the small intestine. Thus, it passes undigested through the small intestine into the large intestine where it contributes with carbohydrates for microbial fermentation. In contrast, starches with a high content of amylose will be digested in the small intestine by amylase (Cummings and Stephen, 2007). Raw potato starch is believed to have an effect on skatole production by affecting the level of apoptosis of gut epithelial cells regulating the availability of L-tryptophan.

The gut is characterised by a high turnover rate of gut mucosa. The daily loss of cells by apoptosis is compensated by mitotic division of steam cells. The process is regulated by endocrine and paracrine processes which balance the degree of apoptosis and mitosis (Ramachandran et al., 2000). Apoptosis contributes with endogenous protein for digestion. In the small intestine, the proteins are digested by proteases and absorbed through the intestinal wall. In the large intestine, the protein is accumulated into microbial biomass or fermented by microbes. Thus, the level of apoptosis has an effect on availability of protein hence L-tryptophan in the large intestine.

Several studies showed that the level of apoptosis can be degreased by the volatile fatty acid butyrate produced during the fermentation of carbohydrates in the large intestine (Hass et al., 1997; Luciano et al., 2002; Claus et al., 2003; Mentschel and Claus, 2003). Butyrate plays a specific role because it is easily metabolised by β-oxidation in the mitochondria. Thus, it is estimated to provide 60-70 % of the total energy demand for colonocytes which are the cells lining the surface of the gut (Luciano et al., 2002). This might be related to the findings of an interaction between butyrate level and apoptosis. *In vitro* and *in vivo* studies have identified contradicting effects of butyrate on apoptosis. *In vitro* showed that butyrate stimulates apoptosis (Heerdt et al., 1997). In contrast, *in vivo* studies showed that the absence of butyrate stimulates apoptosis (Luciano et al., 2002; Claus et al., 2003; Mentschel and Claus, 2003). In general results from *in vivo* studies are most reliable since they reveal what happens in the animal and take the underlying mechanisms of the apoptotic pathways occurring in the animal into account (Heerdt et al., 1997).

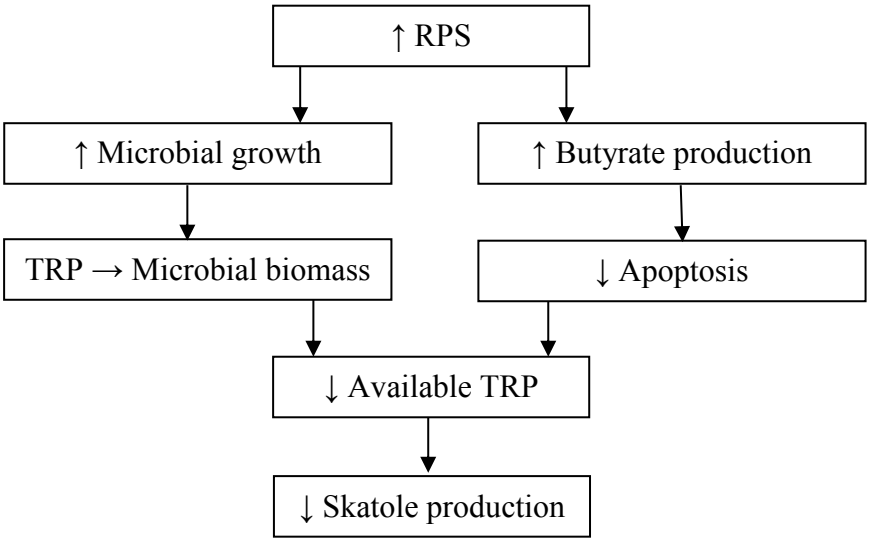


Figure ?? Theoretical line of action when adding raw potato starch (RPS) to feed fed to entire male pigs . TRP: L-tryptophan (Mentschel and Claus, 2003; Heerdt et al., 1997).

Martin et al. (1998) compared volatile fatty acid production in the caecum, proximal and distal colon from pigs fed a diet containing 65 g/kg raw potato starch and 57 g/kg maize starch respectively. The diets were corrugated for energy content with pregelatinised potato starch. Raw potato starch contained 23 % amylose and 77 % amylopectin. Maize contained 70 % amylose and only 30 % amylopectine. The study showed that raw potato starch favoured butyrate formation in the caecum and proximal colon compared to maize starch (Martin et al., 1998). Following this study, the effect of raw potato starch on butyrate formation, skatole production and accumulation of skatole in adipose tissue was further investigated (Mentschel and Claus, 2003; Losel and Claus, 2005; Loesel et al., 2006; Aluwé et al., 2009; Pauly et al., 2010). Table 3.1 shows an overview of results testing the effect of raw potato starch on skatole production. Mentschel and Claus (2003) found an increase in butyrate formation in the colon when adding more than 60 % raw potato starch. Losel and Claus (2005) found a significant dose dependent reducing effect of adding 20 %, 30 % and 40 % raw potato starch on skatole concentration in the distal part of the colon and adipose tissue. Pauly et al (2010) also found a reducing effect of adding 30 % raw potato starch on skatole deposition in adipose tissue. Loesel et al (2006) found significant flavour improvements of the meat from entire male pigs, assessed by a trained test panel, when adding 30 % raw potato starch to an experimental diet. Aluwé et al (2009) failed to detect a reduction in skatole when only adding 10 % raw potato starch. All diets were corrugated to a control diet for similar energy content. Thus,

several studies agreed to observe a reducing effect on skatole production and deposition when at least 20 % raw potato starch were added to the feed.

Further studies were conducted to investigate the effect of adding butyrate directly to the feed. To avoid an immediate digestion of butyrate, it had to be coated. Fat coated butyrate ensured a gradually digestion of butyrate through the small intestine. Butyrate became available for digestion, as the fat was digested by lipases (Claus et al., 2003; Claus et al., 2007; Øverland et al., 2008). Thus, fat coated butyrate never got to enter the large intestine. Øverland et al. (2008) and Lacorn et al. (2010) investigated the effect of coating butyrate to the inulin. Inulin is a non-digestible carbohydrate, and it was expected that the inulin coating would not be removed before entering the colon where inulin would be fermented by microbes. However, it was already removed in ileum where there was a limited but sufficient microbiological activity to remove inulin (Øverland et al., 2008; Lacorn et al., 2010). Future studies must reveal, if a more resistant inulin coating or increased amounts of butyrate fed to the pig, is sufficient to carry butyrate to the colon. Before further efforts are made on finding out, how butyrate added to the feed can reach the large intestine, it could be beneficial to find out if butyrate alone even has an effect on skatole production. Infusion of butyrate directly into the large intestine could reveal this the same way infusion of L-tryptophan to the large intestine has revealed how L-tryptophan affects skatole production (Agergaard et al., 1998). It is possible that the effect of butyrate alone is limited when the effect of undigested starch reaching the large intestine are removed.

3.1.3 Inulin from Chicory root

Inulin is a linear polysaccharide consisting of fructose units and a terminal glucose unit (Louis et al., 2007). In structure, it is very similar to frugtooligosaccarides (FOS), which consists of short chains of fructose unites, while inulin consists of medium or long chains. Inulin is the storage carbohydrate in chicory root and artichokes and has been detected in smaller amounts in wheat, rye, asparagus, onion, garlic and leeks. The molecules in inulin and FOS are linked together by β-linkage, and therefore they are not susceptible to endogenous enzyme breakdown (Knudsen et al., 2012). Several studies showed that FOS has prebiotic properties, and it is assumed that inulin has the same properties. The aim of supplementing the feed with prebiotics is beneficial management of the gut microbes. Skatole production is affected by the composition of the microbial population in the gut. Thus, prebiotics might have an effect on skatole production through an effect on skatole producing microbes. Gibson and Roberfroid (1995) wrote a review on the concept of prebiotics. In the review they formulated a definition of a prebiotic.

"A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995).

This definition turned out not to be specific enough leading the attribution of prebiotic properties to many food components that were in fact not prebiotic at all. Gibson et al (2004) established criteria for classifying feed ingredients as a prebiotic

1. *Resists gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption*
2. *Is fermented by intestinal microflora*
3. *Stimulates selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing.*

(Gibson et al., 2004)

To be able to classify a feed ingredient as prebiotic, scientific demonstrations of the three properties need to be demonstrated. The first criterion can be tested by adding the ingredient to acidic conditions occurring in the stomach and enzymatic conditions from saliva, pancreas and the small intestine. The second criterion can be tested by adding mixed faecal bacteria populations to the ingredient under investigation in an environment imitating the gastrointestinal tract. The third criterion is more difficult to determine. Kolida and Gibson (2007) listed examples of in vivo and in vitro studies on the prebiotic effect of different tested carbohydrates. In these studies, the third requirement is often consistent with the growth of bacteria seen as health promoting with special attention to bacteria from the genera lactobacilli or bifidobacteria together with maintained low levels of *Escherichia coli* and *Clostridium perfringens.*

Gibson et al. (2004) reviewed different oligo- and polysaccharides claimed to be prebiotics according to the three criteria. Frugtooligosaccharides (FOS) was the only carbohydrate that without doubt fulfilled all the criteria. Some of the studies leading to the classification of FOS as a prebiotic were performed on humans. Due to the physiological and anatomical similarity of the digestive tract of slaughter pigs and humans, the results are accepted as applicable for pigs (Miller and Ullrey, 1987). Furthermore, there has not been found negligible contradictions between the conclusions of studies on the effect of FOS on humans and pigs (Kolida and Gibson, 2007; Molbak et al., 2007).

Several studies on adding inulin from chicory root and artichoke to feed fed to entire male pigs showed a decreasing effect on skatole production in colon and deposition in adipose tissue (Hansen

et al., 1994; Xu et al., 2002; Rideout et al., 2004; Hansen et al., 2006; Jensen and Hansen, 2006; Byrne et al., 2008; Maribo et al., 2010 Øverland et al., 2011; Vhile et al., 2012; Zammerini et al., 2012). Table 3.1 shows an overview of results testing the effect of raw potato starch on skatole production. Aluwé et al. (2009) found no effect of including 5 % inulin on skatole concentration in adipose tissue even though Rideout et al (2004) observed a reduction of skatole concentration in faecal content when adding 5 % inulin to the feed. Øverland et al. (2011) investigated the effect of including 3, 6 and 9 % inulin to the feed fed to entire male pigs for 31 days before slaughter. 3 and 6 % inclusion showed no effect compared to a control diet without inulin. Adding 9 % inulin significantly reduced skatole concentration in intestinal content in colon descendens and rectum where most skatole is expected to be produced. No significant difference was observed when measuring skatole concentration in adipose tissue (Øverland et al., 2011). However, skatole concentration in adipose tissue of the control group was only 40 ng/g and decreased to 10 ng/g in the group given 9 % inulin in the feed. A low skatole concentration in the control group makes it difficult to observe a significant effect of adding inulin, but a decreasing tendency was observed. Jensen and Hansen (2006) and Hansen (2006) found that including 25 % chicory root reduced skatole in adipose tissue and improved eating quality. Dried chicory root from similar experiments contained around 46 % inulin. An inclusion of 25 % chicory root might then correspond to around 11 % inulin. Thus, reduced amounts of chicory root were expected to have an effect on skatole concentration in adipose tissue. However Hansen et al (2008) failed to detect an effect of adding 10 % chicory root due to an inulin content of chicory root of around 36 % corresponding to an inulin content of 3.6 %. Thus, the minimum amount of chicory root in the diet to obtain a reduction of skatole production and deposition depends on the inulin content. Moreover, there was an effect according to application periods. Zammerini et al (2012) found an effect of including 5.4 % inulin for a period of two weeks. Hansen et al (2006) and Hansen et al (2008) found an effect of 9 % inulin after an application period of one week.

It is believable that the reducing effect of inulin on skatole production is associated with its prebiotic properties. Furthermore, several studies showed that inulin increased bifidobacteria, suppressed *Escherichia coli* and *Clostridium perfringens*. No studies showed a significant effect on lactobacilli (Kolida and Gibson, 2007; Molbak et al., 2007). Knowledge about bacteria species that can produce skatole is necessary to identify how prebiotics affect skatole producing bacteria in the large intestine. Maybe it enhanced growth of microbes followed by incorporation of tryptophan into microbial biomass and decreased fermentation of tryptophan. Maybe it has an additional effect

through suppressing skatole producing bacteria by the growth of bacteria inducing gut health enhanced by prebiotics.

Rasmussen et al. (2011) found that adding 10 % chicory root to the feed increased the expression of cytochrome P450 enzymes, which is enzymes involved in the metabolism of skatole in the liver (Rasmussen et al., 2011c). Thus, another reducing effect of chicory root on skatole deposition might be that it increases the skatole clearance rate from blood through increased skatole metabolism in the liver.

3.2 Summary

The non-digestible carbohydrates, raw potato starch (RPS) and inulin from chicory root, showed most pronounced results on reduced skatole production. Reducing effects on skatole production were observed when adding minimum 20 % RPS, 5.4 % inulin two weeks before slaughter or 9 % inulin one week before slaughter. Optimal application period for RPS needs to be further investigated. RPS had an effect on cell turnover of gut mucosa which affects the availability of L-tryptophan for fermentation by microbes. Inulin had prebiotic properties which affects the microbial population in the caecum and colon. Thus, it is assumed that the prebiotic properties are related to the skatole reducing property of inulin. Knowledge about and identification of skatole producing bacteria are necessary to identify, whether this assumption is correct. By then it can be investigated how skatole producing bacteria react to a prebiotic like inulin, and how they are affected by availability of L-tryptophan and the presence of other species of bacteria. Moreover, it was found that chicory root increased the expression of liver enzymes involved in skatole metabolism in the liver. Thus, it was suggested that chicory root increased the clearance rate of skatole from the blood. All feed interventions with a reducing effect on skatole production in the large intestine had in common that they were non-digestible carbohydrates. Thus, most of the carbohydrates reached the site of skatole production in the large intestine. Moreover, it was easy fermentable carbohydrates contributing to increased bacterial growth and incorporation of L-tryptophan into bacterial biomass. Thus, less L-tryptophan was fermented leading to reduced skatole production.

4 Conclusion and introduction to paper

The previous chapters consisted of a literature review on skatole metabolism in the pig through production in the large intestine, absorption into the vascular system, metabolism in the liver and deposition in adipose tissue. The level of absorption, liver metabolism and deposition were all affected by the level of skatole production in the large intestine. Skatole production in the large intestine seemed to be naturally limited by specialised bacteria responsible for the conversion of indol-3-acetic acid (IAA) into skatole and concentration of substrate available for skatole production. Moreover, feed interventions affecting skatole production and deposition in adipose tissue were reviewed. Investigated feed interventions with the most pronounced reducing effect on boar taint were adding a minimum of 20 % raw potato starch or 9 % inulin to the feed with an application period for one week or 5.4 % inulin with an application period of two weeks. The following chapter contains a conclusion on the literature review and an introduction to the experimental part of the thesis.

4.1 Conclusion

Skatole is produced from bacterial fermentation of L-tryptophan primarily in the large intestine in the pig. A low skatole production in the large intestine was correlated with a low deposition of skatole in adipose tissue.

Skatole is produced in the large intestine, absorbed into the blood and transported through the portal vein into the liver. In the liver, skatole is metabolised and excreted with urine. If skatole, reaching the liver, exceeds the capacity of the liver to metabolise skatole, then skatole are deposited in adipose tissue. Production, absorption and metabolism in the liver all have an impact on skatole deposited in adipose tissue. Thus, factors affecting these processes can have an effect on skatole deposition.

Production: microbial fermentation of L-tryptophan in the large intestine of pigs results in the production of indole, indol-3-propionic acid (IPA) and indole-3-acetic acid (IAA). IAA is further converted into skatole. A high protein supply into the large intestine tends to increase skatole production. A high supply of fermentable carbohydrates reaching the large intestine tends to lower skatole production. If the carbohydrate supply is high, the bacterial biomass grows faster, and available protein, including L-tryptophan, is incorporated into bacterial biomass and will not be available for skatole production. Providing more carbohydrate into the large intestine therefore indirectly reduces skatole production. IPA seemed to increase when providing more carbohydrate into the gastrointestinal tract. The effect of IPA on boar taint needs to be investigated further.

Absorption: the production of skatole was correlated with the absorption when calculated within individual animals. Thus, the level of skatole absorbed is increased if the level of skatole produced in the large intestine is increased.

Metabolism in the liver: in the liver, skatole is metabolised into many metabolites. Two major metabolites 6-sulfatoxyskatole (MII) and 3-hydroxy-3-methyloxindole (MIII) were associated with the metabolism. MII and MIII were found in high concentrations in urine from pigs with rapid and slow clearance of skatole respectively. Metabolism was found to be catalysed by aldehyd oxidase and a bigger enzyme complex called P450. The activity of these enzymes was believed to be affected by a variety of indirect and direct factors, of which much is still unknown. Skatole concentrations increased at the onset of puberty, indicating that testicular steroids had an effect on skatole metabolism in the pig. Testicular steroids were by some studies found to have a major effect on the expression of some enzymes involved in skatole metabolism in the pig. Other studies found only a small effect of testicular steroids indicating an indirect effect. Thus, the potential of the liver can be affected, but factors affecting metabolism in the liver are highly complex. However, when the production level of skatole is low, the potential of the liver to metabolise skatole was found to be less important in relation to level of skatole deposition.

Deposition: Skatole, which is not metabolised in the liver, is deposited in adipose tissue. It is not fully known how fast the transition time is. Feeding trials have revealed that an application period for a skatole reducing feed intervention of one week before slaughter can reduce skatole deposited in adipose tissue significantly.

Common for all steps of skatole metabolism, which have been investigated, was that they were all affected by the level of skatole production in the large intestine. Thus, reduced skatole production in the large intestine has an effect through all the metabolic processes of skatole in the pig and will lead to reduced skatole deposition in adipose tissue.

Providing more carbohydrate to the large intestine indirectly reduces skatole production. Thus, feeding entire male pigs with non-digestible and easily fermentable carbohydrates can reduce skatole production in the large intestine and contribute to reduced skatole deposition in adipose tissue. Inulin from chicory root and artichoke, raw potato starch (RPS) and dietary fibre from sugar beet pulp and lupine have shown most pronounced reducing effect on skatole production in the large intestine and deposition in adipose tissue. In addition to the effect of non-digestible carbohydrates itself, RPS and inulin were believed to have an additional reducing effect on skatole

production. RPS had an effect on the availability of L-tryptophan for fermentation through an effect on cell turnover of gut mucosa. Bacterial fermentation of RPS increased the production of the volatile fatty acid butyrate. Butyrate showed a decreasing effect on apoptosis of gut mucosa cells, which was followed by a decrease in available L-tryptophan in the lumen of the large intestine. Thus, RPS had an indirect effect through decreased amounts of available L-tryptophan for bacterial fermentation and skatole production. Inulin might have an effect through its prebiotic properties having a positive effect on the growth and activity of bacteria inducing gut health. It has not been investigated how prebiotics affect skatole producing bacteria. However, the prebiotic properties of inulin, having an impact on gut micro flora are suggested to induce the reducing effect on skatole production when feeding inulin.

In conclusion, the effort was to reduce skatole deposition in adipose tissue. All steps from production of skatole in the large intestine to deposition in adipose tissue were directly affected by the level of skatole production in the large intestine. Non-digestible and easy fermentable carbohydrates reduced skatole deposition in adipose tissue. However, none of the tested feed interventions were fully affective against the production of skatole.

Little is known about bacteria capable of performing the decarboxylation of indol-3-acetic acid to produce skatole. Further studies needs to be conducted to identify bacterial species responsible for the conversion of IAA into skatole in the large intestine of pigs. Knowledge about skatole producing bacteria from the large intestine of pigs could reveal how skatole production can be affected and reduced further.

4.2 Introduction to paper

The conclusions of the literature review were the background for the experimental part presented as a paper in chapter 5. The objective of the experimental part was to characterise a skatole producing bacteria isolated from the gastrointestinal tract of pigs. No previous studies have identified bacteria from the gastrointestinal tract of pigs with the property of producing skatole. The characterisation of bacteria with this property might contribute with additional knowledge about skatole production in the gastrointestinal tract of pigs. The characterisation involved sequencing of 16S rRNA, gram staining, analysis of cellular fatty acids composition, analysis of DNA base composition and a DNA-DNA hybridisation to closely related bacteria. Fermentation of different carbohydrate sources, growth pattern, production of organic acids and skatole production were studied *in vitro*.

4.3 Hypotheses of the experimental work

The experimental part of this thesis hypothesises that a new bacterial strain, isolated from the gastrointestinal tract of pigs, is capable of producing skatole from indol-3-acetic acid. Characteristics of the bacteria will contribute with knowledge to understand skatole production in the large intestine of entire male pigs.

5 Paper

This chapter contains the scientific paper presenting the results of the experimental part of the thesis

Characterisation of a skatole producing bacterium isolated from the gastrointestinal tract of pigs.

Rikke Lassen Jensen

Department of Large Animal Science, University of Copenhagen,
Grønnegårdsvej 2, DK-1870 Frederiksberg C, Denmark

ABSTRACT: The objective of the experiment was to characterise a skatole producing bacterium isolated from the gastrointestinal tract of pigs temporarily named SK9 K4. A phylogenetic analysis based on 16S rRNA sequences revealed that SK9 K4 is closely related to *Olsenella uli* (sequence similarity of 94 %), *Olsenella profusa* (sequence similarity of 94.3 %), *Olsenella umbonata* (sequence similarity of 94.5 %) and *Atopobium parvulum* (sequence similarity of 91.7 %). The characterisation of the bacteria included 16S RNA sequencing, gram stain, analysis of DNA G-C content, cellular fatty acids composition and DNA hybridisation with *Olsenella uli*, *Olsenella prufosa*, *Olsenella umbonata* and *Atopobium parvulum*. Fermentation of different carbohydrate sources, growth pattern, production of organic acids and skatole production from L-tryptophan or indol-3-acetic acid (IAA) were studied. SK9 K4 was described as cells being strictly anaerobic and occurring singly or in pairs. Best suited media for growth was Colon and modified PYG media. The 16S rRNA sequence was 99 % identical with an Olsenella sp. strain isolated from the rumen, an uncultured Olsenella sp. clone isolated from sludge and an uncultured bacterium colon isolated from the oral cavity. Cells were gram positive. Major cellular fatty acid content was $C_{14:0}$ and $C_{18:1}$. The DNA G+C content was 62.1 mol %. The DNA-DNA hybridisation revealed that SK9 K4 does not belong to *Olsenella umbonata* while results from the hybridisation with *Olsenella profusa* and *Atopobium parvulum* could not be used. SK9 K4 had 69 % DNA-DNA similarity to *Olsenella uli* from which it could not be decided whether SK9 K4 is a new bacterial species or belongs to *Olsenella uli*. Glucose, lactose, sucrose, maltose, mannose, rhamnose and fructose were fermented. Cellobiose and raw potato starch might be fermented to a small degree. Salicin, xylose, arabinose, mannitole, raffinose, sorbitol, trehalose, ribose, inulin and raw corn starch were not fermented. The major fermentation products were lactic acid together with acetic acid and formic acid. SK9 K4 and *Olsenella uli* produced skatole from idole-3-acetic acid but not from L-tryptophan. The 16S RNA sequence and the description of the bacteria place it in the Actinobacteria class. From the DNA G+C content it was concluded that SK9 K4 belongs to the Olsenella genera. From the DNA-DNA hybridisation it could not be decided whether SK9 K4 is a new bacterial species or belongs to *Olsenella uli*. However a sequence dissimilarity of 6 % on 16S RNA between SK9 and *O.uli*, difference in major cellular fatty acid content and variations in the ability to ferment different carbohydrates sources indicate that SK9 K4 is a new bacterial species. However, more biochemical test is necessary to conclude that.

Keywords: Boar taint, skatole, pig, lactic acid bacteria, rRNA, Atopobium, Olsenella

1. Introduction

Skatole, produced in the large intestine of monogastric animals, (Vold, 1970; Walstra and Maarse, 1970) and the testicular steroid androstenone (Patterson, 1968), are the main substances associated with an off odour and off taste of meat from entire male pigs when cooked. This is referred to as boar taint. Boar taint decreases the quality of the meat and is not accepted by consumers (Bonneau et al., 2000; Matthews et al., 2000).

Until now, boar taint has been avoided by castrating male pigs, which eliminated the production of androstenone and decreased the deposition of skatole into adipose tissue. Surgical castration reduces lean meat percentage, growth rate and feed efficiency (Review: Xue et al., 1997) and causes

pain to the animal during the procedure (Hay et al., 2003). Due to the negative effect on productivity and the animal welfare concerns, there has been an increasing interest in finding alternatives to castration.

Studies have found that adding non digestible carbohydrates to the feed had the potential to decrease boar taint through decreased skatole production in the large intestine (Losel and Claus, 2005; Hansen et al., 2006; Jensen and Hansen, 2006; Loesel et al., 2006; Øverland et al., 2011). Feed interventions with the most pronounced decreasing effect on skatole deposition in adipose tissue had an immediate effect and only had to be implemented around one week before slaughter (Hansen et al., 2006; Hansen et al., 2008). However, none of the studied feed interventions has been found to be sufficient to ensure all entire male pigs against skatole deposition in adipose tissue contributing to boar taint. Knowledge about the origin of skatole, and how it is produced, is necessary to improve interventions that reduce the production of skatole.

Skatole is formed in the large intestine of monogastric animals and in the rumen of ruminants from the anaerobic fermentation of L-tryptophan (Vold, 1970; Walstra and Maarse, 1970). In the large intestine, the source of L-tryptophan is primarily material released from the gut mucosa by apoptosis (Claus et al., 1994). *In vitro* experiments identified two important steps in skatole formation occurring in both the rumen and the large intestine: The conversion of L-tryptophan into indole-3-acitic acid (IAA) and the decarboxylation of IAA to skatole (Yokoyama and Carlson, 1974; Jensen et al., 1995a).

A verity of bacteria can produce acetic acid compounds like IAA (Chung et al., 1975; Yokoyama and Carlson, 1979; Downes et al., 2001), but only a few bacteria can perform the decarboxylation of IAA and produce skatole. There are few references in literature concerning bacteria that produces skatole. Fellers and Clough (1925) and Spray (1948) identified two species from the Clostridia genera *Clostridia scatologenes* (Fellers and Clough, 1925) and *Clostridia nauseum* (Spray, 1948), isolated from the bovine rumen. The strains were able to decarboxylate IAA into skatole and *C.scatologenes* was in addition able to produce skatole directly from L-tryptophan (Jensen et al., 1995a). Bacteria from the Lactobacillus genera were found to produce skatole from IAA (Yokoyama et al., 1977; Yokoyama and Carlson, 1981). One has been characterised as *Lactobacillus helveticus* and were isolated from cheese (Kowalewska et al., 1985). Attwood et al (2006) studied skatole production of six strains of bacterium isolated form the rumen of sheep and dairy cows. Two of them were identified as *Clostridia sporogenes* and one as *Clostridia aminophilum*. The last three isolates were not closely related to any described species, but belong to the genera of Megasphvera, Prevotella and Actinomyces. *C. sporogenes* were able to produce skatole from IAA in the presence and absence of glucose. *C.aminophilum* and bacteria from the Actinomyces genera produced skatole from IAA and directly from L-tryptophan in the absence of glucose, but produced no skatole in the presence of glucose. Bacteria from the Megasphvera and Prevotella genera produced skatole from IAA as a minor product (Attwood et al., 2006). At the time of writing, there was found no bacteria isolated from the gastrointestinal tract of pigs capable of producing skatole either directly from L-tryptophan or from IAA and little is known about conditions that favour or reduce their growth. During repeated subcultivation of the highest dilution in which skatole could be detected, SK9 K4 was isolated from the gastrointestinal tract of pigs.

The objective of this study was to characterise the bacterial strain SK9 K4 isolated from the gastrointestinal tract of pigs. Moreover SK9 K4 and closely related bacteria from the Olsenella and Atopobium genera were studied *in vitro* for their ability to produce skatole from L-tryptophan and indole-3-acetic acid.

2. Materials and methods

The study was conducted as an *in vitro* study. SK9 K4 was compared to closely related type strains from the genera *Olsenella* and *Atopobium* listed in table 1. The sequences for the *Olsenella* and *Atopobium* genera and SK9 K4 have been deposited in GenBank under the accession numbers listed in table 1.

The characterisation of SK9 K4 included.

- PCR amplification and direct sequencing of the 16S rRNA gene
- Gram stain
- Analysis of DNA base composition
- Analysis of cellular fatty acid composition
- DNA-DNA hybridisation
- Analysis of bacterial growth by measuring OD using light spectroscopy and pH
- Analysis of skatole production using HPLC
- Analysis of volatile fatty acid (VFA) production using GC
- Study of fermentation of different carbohydrate sources
- Study of bacterial growth and production of skatole and VFA

Bacterial growth was tested in seven different anaerobe media. Lysogeny Broth, LB (Merck 10285), Reinforced Clostridial Medium, RCM (Merck 05411), Nutrient Broth, NB (Merck 05443), Colon broth (Holdeman et al., 1977), De Man Rogosa and Sharpe, MRS (Merck 10661) (De Man et al., 1960), Peptone-Yeast extract with Glucose, PYG (Holdeman et al., 1977) and modified Peptone-Yeast extract with Glucose (PYG-mod) (Holdeman et al., 1977; DSMZ, 2009).

All material used to handle the bacteria and media used for bacterial growth were autoclaved for 15 min at temperature 121 ˚C and pressure 200 kPa.

Table 1. Strains included in the analysis

Species	Collection no.	16S rRNA GenBank accession no.	References
SK9 K4		JX905358	This paper
Olsenella uli	DSM 7084^T	AF292373	Dewhirst et al (2001)
Olsenella profuse	DSM 13989^T	AF292374	Dewhirst et al (2001)
Olsenella umbonata	DSM 22620^T	FN178463	Kraatz et al. (2011)
Atopobium parvulum	DSM 20469^T	X67150	Olsen et al. (1991) Collins and Wallbanks (1992)

2.1 PCR amplification and 16S rRNA sequencing

SK9 K4, *Olsenella uli Olsenella profusa* and *Atopobium parvulum* were included in the DNA extraction and 16S rRNA PCR. In addition *Escherichia coli* and a lactic acid bacterium were used as positive control. The method for DNA extraction and PCR was known to be effective on the control bacteria.

DNA was extracted from cells using Maxwell® 16 DNA purification kit (Promega Corporation, Madison, Wisconsin, USA).

16S rRNA PCR was performed on extraction with a Duocycler Thermal cycler (VWR international, USA) using *Escherichia coli* consensus primers (Leser et al., 2002) with modifications to fit lactic acid bacteria. Forward primer 1 lb (*Escherichia coli* positions 8-28; 5´-AGR GTT TGA TYM TGG CTC AG-3´) and reverse primer 6 lb (1510-1492 5´-GGY TAC CTT GTT ACG ACT T-3´). The following steps were used for amplification: Denaturation at 92 ˚C for 45 sec, annealing at 57 ˚C for

60 sec and elongation at 72 ˚C for 60 sec. The amplification was performed 30 times. The cycle was ended at 72 ˚C for 10 min and stored at 10 ˚C.
The 16S rRNA PCR product was purified with QIAquick® PCR purification Kit (Qiagen GmbH, Hilden, Germany). The product was visualised by electrophoresis in a 1 % agorose gel. DNA was stained with one drop of ethidium bromide and viewed under long wavelength UV light.
The purity and sample concentration of the product was measured on Spectrophotometer ND-1000 NanoDrop® (NanoDrop Technologies, Inc., Wilmington, Delaware, USA). The purity was measured as the ratio of sample absorbed at wavelength 260 and 280 nm. A ratio of ~2.0 was accepted as "pure" for RNA sequencing. Sample concentration was measured in ng/µl based on absorbance at 260 nm.
Sequencing was performed on SK9 K4. It was performed commercially by DNA Technology A/S (Aarhus C, Denmark) with 1 lb forward primer, 2 reverse primer (position 518-501 5´- GTA TTA CCG CGG CTG CTG-3´), 4 forward primer (position 905-924 5´-AAA CTC AAA GGA ATT GAC GG-3´) and 5 lb reverse primer (position 1054-1035 5´-ACG AGC TGA CGA CRR CCA TG) as starting primers. Nucleotide sequences were imported into the bioinformatic software Geneious (Drummond et al., 2011). Primer sequences were trimmed off, and sequences were aligned according to their overlapping regions using the integrated MUSCLE alignment tool (Edgar, 2004). Finally, a consensus sequence was re-constructed from the sequences. The consensus sequence was compared to existing sequence data through a nucleotide megablast-based comparison with the nr database at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).
A phylogenetic tree was constructed using the neighbour-joining method (Saitou an Nei, 1987).

2.2 DNA-DNA hybridisation
Bacteria were crown in a modified PYG medium for a period of 2-5 days. Bacterial biomass was separated from the media by centrifugation at 20.000 G for 10 min. at 4 ˚C. The supernatant were removed and the bacteria frozen at -80 ˚C. The hybridisation was carried out by the Identification Service of the DSMZ, Braunschweig, Germany. For the hybridisation, 3 g of wet biomass from all strains included in the hybridisation were needed. The strains included in the hybridisation are listed in table 1.
The hybridisation was performed as a spectroscopic DNA-DNA hybridisation. Cells were disrupted by using a Constant Systems TS 0.75 KW (IUL Instruments, Germany) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA-DNA hybridization was carried out as described by De Ley *et al*. (1970) under consideration of the modifications described by Huss *et al*. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

2.3 Analysis of DNA base composition
SK9 K4 was grown as for the hybridisation. The analysis was carried out by the Identification Service of the DSMZ, Braunschweig, Germany. For the analysis 2 g wet biomass was required. Cells were distributed and DNA was purified using the same method as described for the DNA-DNA hybridisation. The G+C content of DNA was estimated by a HPLC method (Mesbah et al., 1989).

2.4 Analysis of cellular fatty acid content
Bacteria were grown in modified PYG media and sent for analysis as an active growing culture. Fatty acid analyses were carried out by the Identification Service of the DSMZ, Braunschweig, Germany. The method used was described by Kuykendall et al. (1988) and Miller et al. (1982).

2.5 L-tryptophan and indole-3-acetic acid solutions

L-tryptophan or IAA was added when incubating the bacteria to test, whether the bacteria were capable of producing skatole directly from L-tryptophan or from IAA. Two hundred fifty mg IAA was dissolved in 50 ml ethanol and 250 mg, L-tryptophan was dissolved in 50 ml water. The solutions were added to a 100 ml incubation bottle through a Sterile Syringe Filter (VWR international, USA). Both solutions were stored at – 18 ˚C. The IAA solution was protected from light due to light sensitivity of IAA. The solutions were added to a media in an amount corresponding to a 100 times dilution to make up a final concentration of 50 mg/l.

2.6 Analysis of bacterial growth by measuring optical density (OD) using light spectroscopy and pH

During bacterial growth, the bacterial biomass increased. Light with a wavelength of 550 nm was sent through the bacterial medium. The amount of scattered light increased with increasing bacterial growth. OD was measured on UV- 3100PC Spectrophotometer (VWR International Europe bvba, Leuven, Belgium) using 2.5 ml disposable cuvettes. Moreover, during bacterial growth organic acids were produced. The production of lactic acids lowered pH. Thus, during bacterial growth a decrease in pH was observed. MeterLab® PHM201 portable pH meter (Radiometer analytical, Lyon, France) was used to measure pH.

2.7 Skatole content analysed using High Performance Liquid Chromatography (HPLC)

Skatole content in the bacterial culture media was analysed by High Performance Liquid chromatography as described by (Knarreborg et al., 2002). HPLC was performed on a HP 1200 series HPLC system (Agilent Technologies, Inc., Wilmington, Delaware, USA).

2.8 Organic acid content analysed using Gas Chromatography (GC)

The content of volatile fatty acids and lactic acid were analysed by Gas Chromatography (GC) as described by (Jensen et al., 1995b) with modifications described by (Canibe et al., 2007). Volatile fatty acids included in the test were formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptaonic acid, sorbic acid, benzoic acid, succinic acid and hippuric acid. GC was performed on a HP 6890 series GC system (Agilent Technologies, Inc., Wilmington, Delaware, USA).

2.9 Fermentation of different carbohydrate sources

PYG and modified PYG media were made without glucose; thus, it was called PY and PY-mod media. Ten % solutions of different carbohydrate sources were made and autoclaved (table 4). Raw potato starch and raw corn starch were not autoclaved. The reason for this was that high temperatures in the autoclave would gelatinise the starches, which changes the original structure of the starch. Due to the insolubility of the starches, they could not be sterilised through a sterile filter. Instead two empty 50 ml bottles were autoclaved, and the starches were added each into one bottle. Sterile water was added to make up a 10 % solution. The solutions are expected to be sterile, but contaminations might occur. One ml of each carbohydrate was added 9 ml PY media respectively and 9 ml PY-mod media respectively to make up a 1 % final concentration. SK9 K4 was added into each carbohydrate media solution and incubated at 37 ˚C. Six days after incubation, samples were taken for OD and pH measurements *(2.6)* and VFA analysis on the GC *(2.8)*. Fermentation tests in PY media was made once with no repetitions. Fermentation test in PY-mod media were made with three replications. Observation of growth was characterised as increased OD from 0.2 to above 0.4 and decreased pH from around 7 to below 6.

2.10 Bacterial growth and production of skatole and VFA

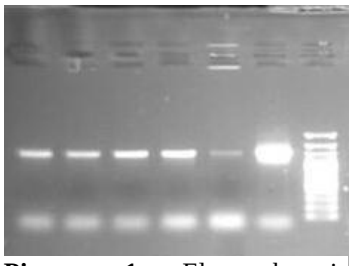
Experimental designs were conducted to investigate the ability of SK9 K4, *O. uli, O. prufosa, O. umbonata* and *A. parvulum* to produce skatole and VFA. SK9 K4, *O. profuse* and *A. parvulum* were tested in duplicate in colon media and once in MRS media. *O. uli* was tested in colon media during five repetitions. *O. umbonata* was tested in dublicate in colon and PYG-mod. Samples were taken five days after incubation. Moreover, SK9 K4 and *O.uli* were tested for a period of 15 days to investigate growth pattern together with skatole and VFA production. Samples were taken twice day 1 and 2 after incubation and once day 3, 4, 8, 9 and 15 after incubation in 50 ml colon media. From each test one sample was included from pure media. The samples were analysed for OD and pH *(2.6)*, skatole on HPLC *(2.7)* and organic acids on GC *(2.8)*.

3. Results and Discussion

All bacteria grew in MRS, Colon and PYG-mod. SK9 K4 was the only bacteria with reliable growth in regular PYG media. Thus, these four media were used during the study. Glucose could be excluded from PYG media and PYG-mod media and be replaced with other carbohydrate sources.

3.1 16S RNA sequencing

The DNA extraction and 16S PCR was successfully visualised for SK9 K4, *Olsenella Uli, Atopobium parvulum, Olsenella profusa* and two controls *E. coli* and a lactic acid bacterium by electrophoresis in a 1 % agorose gel (picture 1).



Picture 1. Electrophoresis performed in a 1 % agorose gel. DNA stained with one drop of ethidium bromide and viewed under long wavelength UV light. From left to right bond: *SK9 K4, Olsenella Uli, Atopobium parvulum, Olsenella profusa*, two controls and the ladda.

SK9 K4 was the only bacterium handed in for 16S rRNA sequencing. The sequencing resulted in a consensus sequence of 1500 bp which was stored in Genbank under the accession number JX905358. The 16s rRNA sequencing placed the bacteria in a phylogeny showed in figure 1. The sequence was 99 % similar to Olsenella sp. isolated from the rumen (GeneBank accession no.: GU045476), Olsenella sp. uncultured clone isolated from sludge (GeneBank accession no.: DQ168838) and an uncultured bacteria clone isolated from the oral cavity (GeneBank accession no.:FJ982998). SK9 K4 was closely related to *Olsenella uli* (sequence similarity of 94 %), *Olsenella profusa* (sequence similarity of 94.3 %), *Olsenella umbonata* (sequence similarity of 94.5 %) and *Atopobium parvulum* (sequence similarity of 91.7 %) (see table 1 for GenBank accession no.).

The genera *Olsenella* (Dewhirst et al., 2001) and *Atopobium* (Olsen et al., 1991; Collins and Wallbanks, 1992) both belong to a group of lactic acid producing bacteria. The group belongs to the class *Actinobacteria* from the family *Coriobacteriaceae. Actinobacteria* are defined as Gram-positive, non-motile, non-spore-forming rods or cocci. They occur singly, in pairs or in short chains and are strictly anaerobic. They have a high G+C content in the DNA base composition. The major fermentation product from glucose is lactic acid together with acetic acid and formic acid. Olsenella and Atopobium can be differentiated by G+C content of the DNA. Olsenella has a DNA G+C content between 63-64 mol%. Atopobium has a DNA G+C content between 35-46 mol% (Collins and Wallbanks, 1992; Dewhirst et al., 2001). According to Dewhirst et al. (2001), the human oral cavity is the main habitat and bovine rumen a likely habitat of *Olsenella*. However, molecular genetic studies have also reported the detection of *Olsenella*-related clones in the gastrointestinal tract of pigs (Leser et al., 2002; Dowd et al., 2008). Bacteria from the Atopobium genera were isolated from the human oral cavity but were also reported to pose one of the predominant groups of lactic acid producing bacteria in the gastrointestinal tract of pigs (Collado and Sanz, 2007).

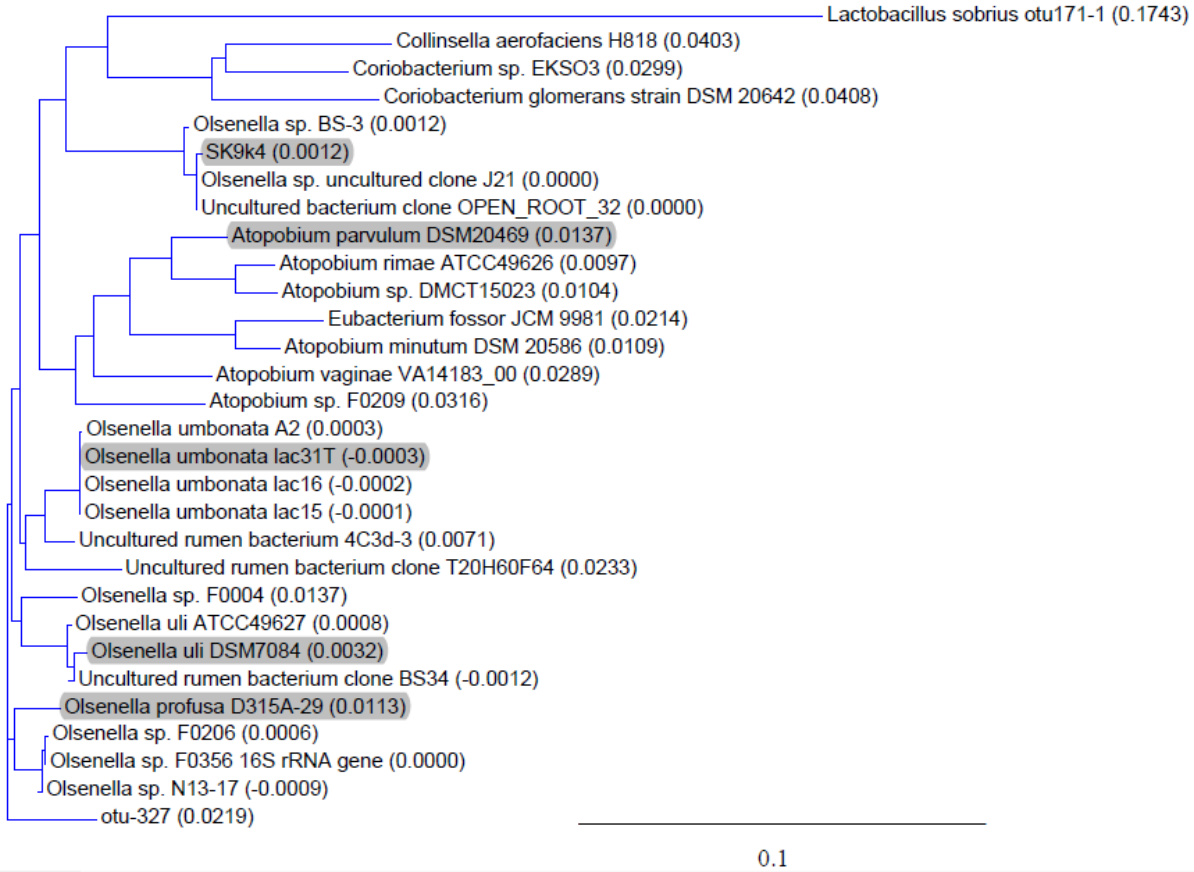
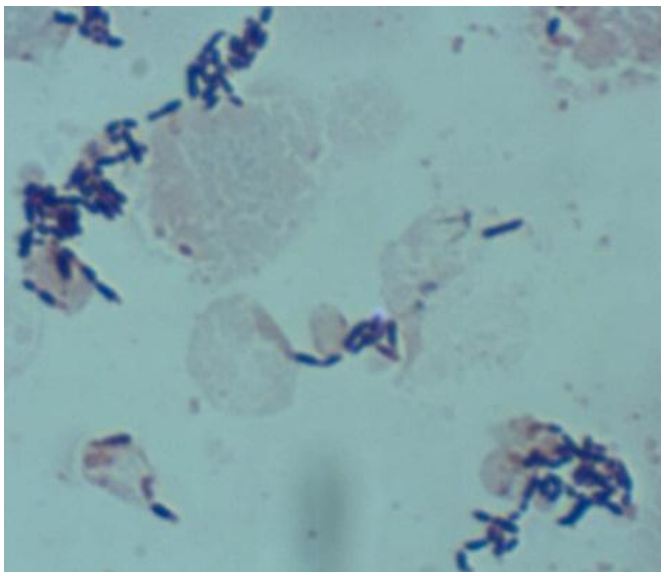


Figure 1. Pylogenetic tree based on 16S rRNA gene sequences from members of *Coriobacteriaceae* family, using *Lactobacillus sobrius* as out-group. The tree was built using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) and analysing pairwise distances of the aligned sequences by the Clustal W algorithm (Thompson et al., 1994). Distances between two sequences are determined by the lengths of the horizontal lines connecting them; the inserted bar indicates 10% dissimilarity (0.1). Grey highlights are bacteria included in this study.

3.2 Gram stain

All bacteria involved in this study were tested gram positive. For the bacteria from the Olsenella and Atopobium genera, the results are in agreement with earlier studies (Olsen et al., 1991; Dewhirst et al., 2001; Kraatz et al., 2011). Picture 2 shows a dark violet, gram positive gram stain of SK9 K4.



Picture 2. Gram positive gram stain of SK9 K4. Picture taken through a microscope with a 100×objetive lens

3.3 Cellular fatty acid content and DNA G+C content

Major cellular fatty acids in the five bacterial spices listed in table 2 are either $C_{14:0,}$ $C_{18:0}$ or $C_{18:1.}$ SK9 K4 had a high content of both $C_{14:0,}$ and $C_{18:1}$ which constituted 34.52 % and 26.54 % of the total cellular fatty acids respectively. Kraatz et al. (2011) and Olsen et al. (1991) disagreed on the major cellular fatty acid content in *O. uli,* as Kraatz et al. (2011) reported the major cellular fatty acid to be C18:0 while Olsen et al,. (1991) reported it to be C18:0 (table2). However, none of them found that $C_{14:0}$ were a major cellular fatty acid which indicates a difference between SK9 K4 and *O. uli.*

The analysis of SK9 K4 indicated a DNA G+C content of 62.1 mol %. Previous it was determined that the distinction between the Olsenella and Atopobium genera are based on high or low DNA G+C content. High was defined as 63-64 mol % and low was defined as 35-46 mol % (Collins and Wallbanks, 1992; Dewhirst et al., 2001). Since the DNA G+C content of SK9 K4 are only 1 mol % unit below the definition for high DNA G+C content, it is suggested that SK9 K4 belongs to the Olsenella genera.

Table 2 Major cellular fatty acid(s) and DNA G+C content of SK9 K4, *Olsenella uli, Olsenella profusa, Olsenella umbonata and Atopobium parvulum*

	SK9 K4	*Olsenella Uli*	*Olsenella profusa*	*Olsenella umbonata*	*Atopobium parvulum*
Major cellular fatty acid(s)	$C_{14:0}$ $C_{18:1}$	$C_{18:0}$* $C_{18:1}$†	$C_{14:0}$*	$C_{14:0}$* $C_{18:0}$*	$C_{18:1}$†
DNA G+C content (mol %)	62	64■	64■	ND	39†

*: Kraatz et al. (2011); †: Olsen et al (1991); ■: Dewhirst et al. (2001)
ND: not determined

3.4 DNA-DNA hybridisation

The threshold value for the definition of a new bacterial species is below 70 % DNA-DNA similarity defined by the ad hoc committee (Wayne et al., 1987). In table 3 results from the DNA-DNA hybridisation are presented. SK9 K4 and *Olsenella uli* had a 69.1 % DNA-DNA similarity. DNA-DNA similarity values can be reproduced in a range of 10 % which means that values around 70 % have to be regarded critical. Thus, further physiological and chemotaxonomical data are needed to determine whether SK9 K4 is a new bacterial species or belongs to the species *Olsenella uli*. The results clearly indicate that SK9 K4 does not belong to the species *Olsenella umbonata.* The 98.2 % similarity of *Olsenella profusa* and *Atopobium parvulum* indicates that one or both samples were contaminated with each other.

Table 3 DNA-DNA hybridisation

	SK9 K4	*Olsenella uli*	*Olsenella profusa*	*Olsenalla umbonata*	*Atopobium parvulum*
Olsenella uli	69.1	1			
Olsenella profusa	28.3	22.4	1		
Olsenalla umbonata	27.2	23.0	26.0	1	
Atopobium parvulum	19.6	16.1	98.2	27.7	1

3.5 Fermentation of different carbohydrate sources

The results from the fermentation test are listed in table 4. Bacterial growth of SK9 K4 in the presence of the added carbohydrate was defined as an increase in optical density (OD) above 0.40 and a decrease in pH below 6. The carbohydrates highlighted with grey tone in table 4 are the ones defined as being carbohydrates fermented by SK9 K4. Raw potato starch and cellobiose do not fall into the definition. However, a slower fermentation might occur due to a minor production of formic-, acetic- and lactic acid found in samples added into this carbohydrate sources.

Table 4 Results from fermentation of different carbohydrate sources by SK9 K4. Results are an average of four measurements. Raw corn starch was not soluble in water and contributed to an increase in optical density (OD) which was not caused by bacterial growth.

Fermentation	pH	OD	Formic acid*	Acetic acid*	Lactic acid*
No addition	7.5	0.190	2.3	2.4	2.1
Glucose	4.0	0.742	14.0	8.3	68.3
Lactose	4.1	0.676	3.2	1.6	70.5
Sucrose	4.3	0.600	3.3	2.3	63.6
Maltose	4.6	0.580	8.8	5.1	48.6
Salicin	7.3	0.200	-0.1	-0.6	1.8
Xylose	7.3	0.186	-0.4	-0.2	0.0
Arabinose	7.3	0.190	-0.9	-0.3	0.0
Cellobiose	7.4	0.244	2.2	1.1	1.3
Mannose	4.6	0.593	9.2	5.6	65.6
Mannitol	7.4	0.176	-0.9	-0.8	0.1
Raffinose	7.4	0.211	0.3	0.5	0.7
Sorbitol	7.4	0.185	-0.7	-0.3	0.1
Rhamnose	5.5	0.434	16.6	19.7	2.6
Trehalose	7.4	0.184	-0.3	-0.4	0.1
Fructose	4.7	0.594	15.8	8.9	40.9
Ribose	7.1	0.245	1.4	1.5	0.2
Inulin	7.4	0.201	0.3	0.1	0.8
Raw potato starch	6.9	0.236	4.4	4.3	1.4
Raw corn starch	7.3	0.677	-1.0	-1.0	-0.4

* Values are in µmol/l and are the difference between concentration with and without carbohydrates added. It reflects the amount produced by SK9 K4 without the amount occurring in the media before incubation. The variance between samples explains way negative values occur.

The results were compared to results from fermentation test performed earlier on bacteria from the Olsenella and Atopobium genera (Olsen et al., 1991; Collins and Wallbanks, 1992; Dewhirst et al., 2001; Kraatz et al., 2011) in table 5. *O. profuse* and *A. parvulum* differentiated from SK9 K4, *O. uli* and *O. umbonata* by being able to ferment almost all carbohydrate sources that has been tested. SK9 K4, *O. uli* and *O. umbonata* were more selective. When comparing them, they were all capable of fermenting glucose, sucrose, maltose, mannose and probably also fructose (*O. umbonata* were not tested for fermentation of fructose). SK9 K4 was the only bacteria of the three selective bacteria capable of fermenting lactose and rhamnose.

Table 5 Fermentation of different carbohydrate sources by SK9 K4 together with results from fermentation test performed earlier on the Olsenella and Atopobium genera (Olsen et al., 1991; Collins and Wallbanks, 1992; Dewhirst et al., 2001; Kraatz et al., 2011).

Fermentation	SK9 K4	*Olsenella uli**†■	*Olsenella profusa**■	*Olsenella umbonata**	*Atopobium parvulum*†
Glucose	+	+	+	+	+
Lactose	+	-	+	-	+
Sucrose	+	-/+	+	+	+
Maltose	+	-/+	+	+	+
Salicin	-	-/+	+	-	+
Xylose	-	-	+	-	ND
Arabinose	-	-	+	-	-
Cellobiose	(-)	-	+	-	+
Mannose	+	-/+	+	+	+
Mannitol	-	-	+	ND	ND
Raffinose	-	-	+	-	ND
Sorbitol	-	-	+	-	ND
Rhamnose	+	-	+	-	ND
Trehalose	-	-	+	+	+
Fructose	+	+	ND	ND	+
Ribose	-	-	ND	ND	-
Inulin	-	-	ND	ND	+
Raw potato starch	(-)	ND	ND	ND	ND
Raw corn starch	-	ND	ND	ND	ND

*: Kraatz et al. (2011); †: Olsen et al (1991); ■: Dewhirst et al. (2001)

\+ The bacterium was able to grow in the presence of the added carbohydrate. Definition of SK9 K4 growth: OD increased above 0.40, pH decreased below 6 (table 4)

\- No bacterial growth was observed

(-) Do not line within the limits of the definition of SK9 K4 growth but seems to have the potential for slow growth

-/+ Previous studies disagree

ND Not determined

3.6 Bacterial VFA and skatole production and growth of SK9 K4

None of the bacteria tested in this study were able to produce skatole directly from L-tryptophan. When adding indole-3-acetic acid to the incubation, SK9 K4 and *Olsenella uli* produced skatole. All bacteria included in the study primarily metabolised glucose to lactic acid and to a smaller amount acetic acid and formic acid (table 6).

Table 6 Skatole and VFA production. SK9 K4, *O. uli*, *O. prufosa*, *O. umbonata* and *A. parvulum* incubated in colon media added with 50 mg/l L-tryptophan or indole-3-acetic acid (IAA). Incubated in duplicate.

	SK9 K4	*Olsenella uli*	*Olsenella prufosa*	*Olsenella umbonata*	*Atopobium parvulum*
Skatole production					
From L-tryptophan	-	-	-	-	-
From IAA	+	+	-	-	-
Metabolic end products	f, L, a	f, L, a	f, L, a	f, L, a	f, L, a

+ Skatole are produced

- Skatole are not produced

a, acetic acid; L, lactic acid; f, formic acid. Capital letters indicate major products.

Growth pattern of SK9 K4 and its production of skatole and organic acids are illustrated in figure 2. The optical density starts to increase day one after incubation and reaches its maximum around 0.4 after four days. This indicates that growth of the bacterium reaches its maximum at this point. The drop in pH starts at day two after incubation and continues until it reaches a minimum around pH 6 eight days after incubation. This indicated that metabolism of glucose into volatile fatty acids and lactic acid primarily occurs at this time.

SK9 K4 converted indole-3-acetic acid (IAA) into skatole. When no IAA was added to the incubation, no increase in skatole concentration was observed. When IAA was added to the incubation, skatole concentration started to increase day 3 after incubation and reached a maximum concentration around 17 mg/l 8 days after incubation. After reaching the maximum, skatole concentration decreased and reached a concentration of 6 mg/l 15 days after incubation where the last samples were taken. A similar development was seen for *O. uli*. Skatole concentration started to increase four days after incubation and reached a maximum concentration around 21 mg/l eight days after incubation. After reaching maximum, skatole concentration decreased and reached a skatole concentration of 6 mg/l 15 days after incubation where the last samples were taken (results not shown).

Compared to the growth pattern of SK9 K4, skatole production initiates in the late exponential phase of the growth curve where the bacterial density reached its maximum. It is assumed that the growth of SK9 K4 takes off due to a reduction in glucose available for bacterial growth. Since SK9 K4 does not produce skatole before the late exponential phase, skatole production might be inhibited by the presence of glucose. Attwood at al. (2006) demonstrated that some skatole producing bacteria isolated from the rumen did not produce skatole in the presence of glucose.

Skatole concentration dropped after 8 days of incubation which remains unexplained.

Lactic acid was the major volatile fatty acid reaching a maximum concentration of 38 mmol/l around 8 days after incubation. Formic acid and acetic acid were produced in smaller amounts. Formic acid reached maximum concentrations of 7 mmol/l 8 days after incubation when IAA was added. Acetic acid reached a concentration of 11 mmol/l and might still be increasing after 15 days of incubation. Formic acid seems to be negatively affected by the production of skatole from indole-3-acetic acid. When no IAA was added to the incubation, formic acid reaches a maximum

concentration of 7 mmol/l. When IAA was added the maximum concentration of formic acid only reaches 0.9 mmol/l.
As for skatole production there seems to be a shift after 8 days of incubation where formic acid and lactic acid reach a maximum concentration and stayed at a constant level. Acetic acid continues to increase after 8 days of incubation from 8 to 11 mmol/l.

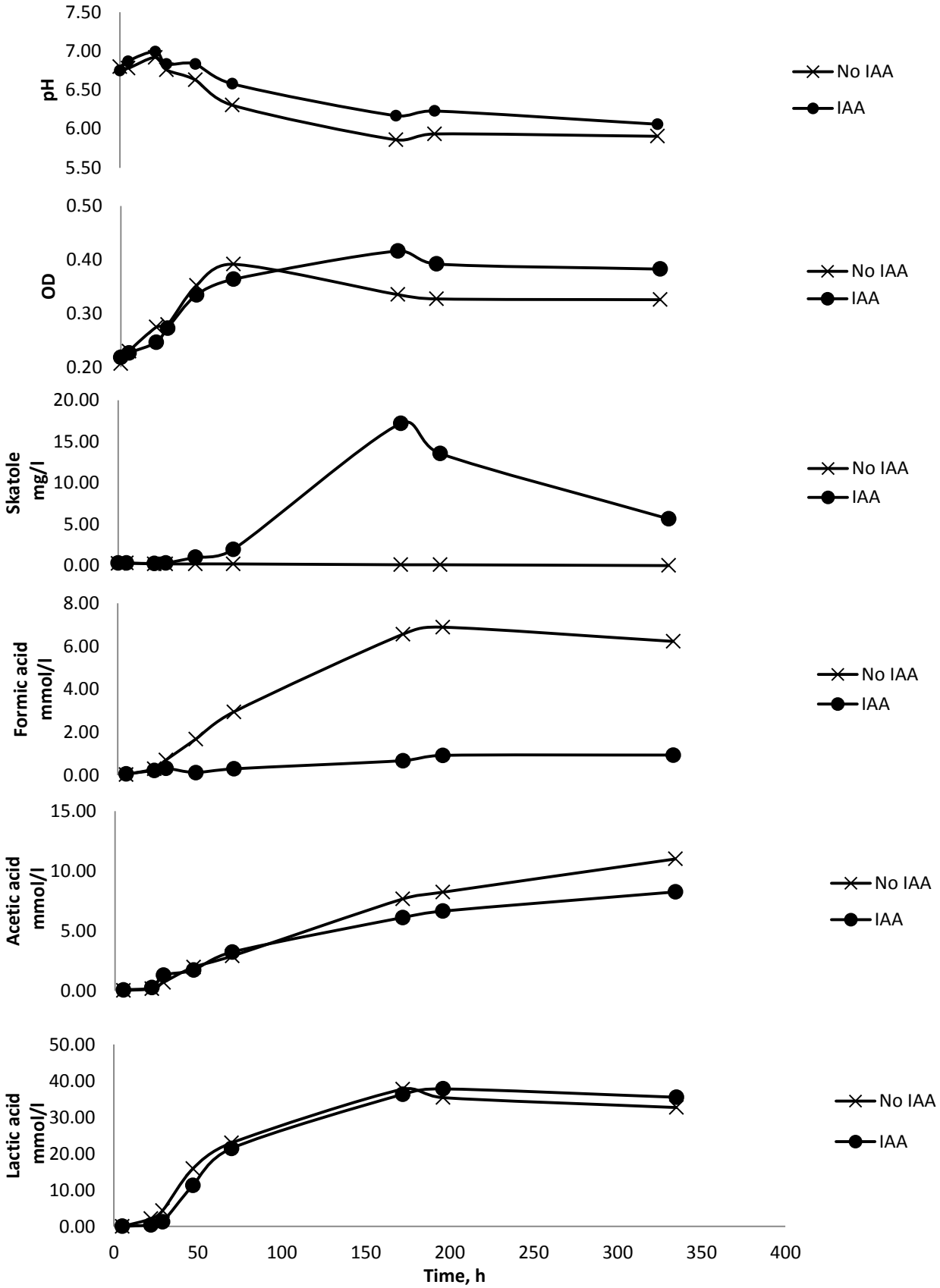


Figure 2. Development in pH, optic density (OD) and production of skatole, formic acid, acetic acid and lactic acid over time after incubation of SK9 K4 with or without adding 50 mg/l indole 3-acetic acid to 50 ml colon media. Incubated in dublicat.
No IAA: no indol-3-acetic acid added to the incubation
IAA: indol-3-acetic acid added to the incubation

5. Conclusion

The description of SK9 K4 was based on one strain isolated from the gastrointestinal tract of pigs. Cells are strictly anaerobic and occur singly or in pairs. Cells can grow in colon, MRS, RCM, PYG and modified PYG media. Best suited media for growth is colon and modified PYG media. Sequencing of 16S RNA showed that the closest related strains were *Olsenella uli* (sequence similarity of 94 %), *Olsenella profusa* (sequence similarity of 94.3 %), *Olsenella umbonata* (sequence similarity of 94.5 %) and *Atopobium parvulum* (sequence similarity of 91.7 %). Cells are gram positive. Major cellular fatty acid content was $C_{14:0}$ and $C_{18:1}$. The DNA G+C content was 62.1 mol %. The DNA-DNA hybridisation revealed that SK9 K4 does not belong to *Olsenella umbonata* while results from the hybridisation with *Olsenella profusa* and *Atopobium parvulum* could not be used. However differences in physiological characteristics indicate that SK9 does not belong to either *O. profusa* or *A. parvulum.* SK9 K4 had 69 % DNA-DNA similarity to *Olsenella uli* from which it could not be decided whether SK9 K4 is a new bacterial species or belongs to *Olsenella uli*.
Glucose, lactose, sucrose, maltose, mannose, rhamnose and fructose are fermented. Cellobiose and raw potato starch might be fermented to a small degree. Salicin, xylose, arabinose, mannitole, raffinose, sorbitol, trehalose, ribose, inulin and raw corn starch are not fermented. The major fermentation products are lactic acid together acetic acid and formic acid. SK9 K4 produced skatole from idole-3-acetic acid but not from L-tryptophan.

The 16S RNA sequence and the description of the bacteria place it in the Actinobacteria class. From the DNA G+C content it was concluded that SK9 K4 belongs to the Olsenella genera. From the DNA-DNA hybridisation it could not be decided whether SK9 K4 is a new bacterial species or belongs to *Olsenella uli*. However a sequence dissimilarity of 6 % on 16S RNA sequence between SK9 and *O.uli* together with differences in major cellular fatty acid content and small variations in the ability to ferment different carbohydrates sources indicates that SK9 K4 is a new bacterial species. However, more biochemical test is necessary to conclude that.

Acknowledgements
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6. General discussion and perspectives

The aim of this thesis was to investigate how boar taint can be decreased by affecting the microbial production of skatole in the large intestine through different feed interventions. Previous chapters contain a review on skatole production and metabolism in the pig and identify sensitive sites of skatole production and metabolism. Moreover, it was described how different feed interventions can decrease skatole production by having different effects on the microbiological environment. In the experimental part, a skatole producing bacteria isolated from the gastrointestinal tract of pigs was identified and characterised. Properties of the bacteria and closely related bacteria to produce skatole and ferment different sources of carbohydrates were studied. In the following chapter the results from the experimental part are related to the literature review and the hypothesis. Finally, perspectives in relation to further research on the microbial production of skatole in the large intestine are presented.

In the literature review it was found that the bacterial conversion of indol-3-acetic acid into skatole in the large intestine might be a sensitive site of skatole production in the pig. Moreover, the level of skatole production in the large intestine was positively correlated with the level found in adipose tissue. Knowledge about how skatole is produced in the large intestine was suggested to contribute with knowledge about how to reduce skatole production and deposition. In the experimental work it was found that SK9 K4 isolated from the gastrointestinal tract of pigs and the type strain of *Olsenella uli* produced skatole from indole-3-acetic acid (IAA). *Olsenella uli* was originally isolated from the oral cavity of humans, but *Olsenella*-related clones have been detected in the gastrointestinal tract of pigs (Leser et al., 2002; Dowd et al., 2008). This indicates that the hind gut of the pig could be a natural habitat for *Olsenella uli.* However, this is not certain. SK9 K4 and *O.uli* were both characterised as gram positive bacteria with high DNA G-C content (Dewhirst et al., 2001). In a previous study, Cook et al (2007) analysed the microbial population of swine lagoon slurry from different treatments having different effects on skatole production. It was found that a group of low G-C content gram positive bacteria increased whit increasing skatole concentration in the swine lagoon slurry (Cook et al., 2007). Due to the increased growth of this group of bacteria, it was concluded that gram positive bacteria with a low DNA G-C content might be responsible for skatole production. However skatole producing bacteria characterised during the present experimental work belong to a group of gram positive bacteria with high DNA G-C content. This gives a small indication that the growth of skatole producing bacteria might not be affected by the level of skatole production.

SK9 K4 and *O. uli* were able to ferment a few of the carbohydrates included in this study and previous studies on *O. uli* (Olsen et al., 1991; Dewhirst et al., 2001; Kraatz et al., 2011). None of them were able to ferment inulin. In addition, SK9 K4 was not able to ferment raw potato starch which has not been tested on *O. uli.* In the literature review, inulin and raw potato starch were found to be non-digestible carbohydrates with the most pronounced reducing effect on skatole production in the large intestine of the pig (Mentschel and Claus, 2003; Losel and Claus, 2005; Hansen et al., 2006; Jensen and Hansen, 2006; Loesel et al., 2006; Øverland et al., 2011). The pronounced effect of raw potato starch on reduced skatole production was suggested to be due to an increased butyrate production by bacteria capable of fermenting raw potato starch. Butyrate triggers a decrease in apoptosis of gut epithelial cells. Decreased apoptosis results in less L-tryptophan available for fermentation. In the experimental study, SK9 K4 was found not to be able to ferment raw potato starch. Thus, an additional effect of feeding raw potato starch might be that growth of SK9 K4 is suppressed by the growth of bacteria capable of fermenting raw potato starch.

The pronounced effect of inulin was suggested to be due to its prebiotic properties (Gibson et al., 2004). By prebiotic properties, the growth of health promoting bacteria in the large intestine is enhanced. Health promoting bacteria are often referred to as bifidobacteria. The bifidobacteria genera are closely related to the Olsenella genera, both belonging to the class *Actinobacteria* from the family *Coriobacteriaceae* (Kraatz et al., 2011). Due to the close relation to bifidobacteria, the growth of SK9 K4 and *O.uli* might also be enhanced by inulin. The fermentation test revealed that SK9 K4 and *O.uli* were not able to ferment inulin. However, they were able to ferment fructose. Inulin is a linear polysaccharide consisting of fructose units and a terminal glucose unit (Louis et al., 2007). Thus, SK9 K4 and *O.uli* might be able to take part of inulin fermentation in the large intestine and in this way be enhanced when feeding pigs with inulin in the diet. If this is the case, reduced skatole production is dependent of sufficient inulin available for bacterial fermentation throughout the entire large intestine. If not, increased amount of skatole producing bacteria will be available for skatole production when no more carbohydrate are available, and an increase in skatole production would be expected. However, feeding trails, studying the effect of feeding small amounts of inulin below 9 % added to the feed, did not show an increase in skatole production. Instead no changes between control and experimental group were observed (Hansen et al., 2008; Øverland et al., 2011). Thus, it is more likely that inulin either reduces the growth of skatole producing bacteria or does not have an effect on the growth of skatole producing bacteria.

A change in the composition and amount of organic acids produced in the large intestine also indicates a change in the microbial composition when more fermentable carbohydrates enter the large intestine. Previous *in vivo* studies have tested the effect of adding different sources of non-digestible and easy fermentable carbohydrates to the feed fed to entire male pigs on the production of organic acids. The amount and composition of major organic acids were analysed in different segments of the gastrointestinal tract. They all had a reducing effect on skatole production. Jensen et al. (1995) added 20 % sugar beet pulp, Maribo at al. (2010) added 15 % chicory root and Øverland et al. (2011) added 9 % inulin. The total amount of volatile fatty acids did not differ significantly between diets. However, when fed a diet added sugar beet pulp, there tended to be a higher total concentration of volatile fatty acids in the colon (Jensen et al., 1995b). When feed were added 15 % chicory root, the concentration of acetic acid increased significantly in the caecum and colon, while the concentration of butyric acid decreased significantly in the colon (Maribo et al., 2010). A decrease in acetic acid was not found when adding 9 % inulin, but a significant drop in butyric acid was found (Øverland et al., 2011). In contrast raw potato starch had an increasing effect on butyrate production in the large intestine as reviewed in chapter 3.1.2. Thus, no study has showed a clear relation between the level of skatole production and changes in amount and composition of organic acids. Results from the experimental work on the analysis of major metabolic end products indicate that the production of skatole had a reducing effect on formic acid production. However formic acid does not constitute a major part of organic acids found in the large intestine; thus, the concentration of formic acid has not been discussed in previous *in vivo* studies. It is expected that the a reduced formic acid production by skatole producing bacteria has a small effect on the microbiological environment in the large intestine.

The effect of different feed interventions on the composition of microbes and growth of skatole producing bacteria needs to be investigated further. The characterisation of SK9 K4 as a skatole producing bacteria isolated from the gastrointestinal tract of pigs makes it possible to study this bacterium *in vivo.* Studies should be conducted to follow the development of SK9 K4 in the large intestine when feeding a control diet compared to a diet added non-digestible and easy fermentable carbohydrates. Knowledge, regarding how skatole producing bacteria are affected by different interventions, could contribute to a better understanding of how to affect these bacteria. This might lead to the development of improved interventions reducing skatole production in the large intestine.

When comparing the pattern of bacterial growth with the pattern for skatole production, then skatole starts to be produced in the late exponential phase of the bacterial growth pattern. Thus, skatole production initiates when bacterial growth has ended. This could be the phase where no more glucose are available for bacterial growth. Thus, skatole production might be blocked by the presence of glucose. Attwood et al. (2006) studied properties of skatole producing bacteria isolated from the rumen. It was found that some of the bacteria were only able to produce skatole in media without glucose, and skatole production seemed to be blocked by the presence of glucose. In the literature review it was found that a high protein supply tended to increase skatole production (Agergaard et al. 1998), whereas a high supply of non-digestible and easy fermentable carbohydrates in the diet tended to lower the skatole production (Knarreborg et al., 2002). This was explained by a fast bacterial growth when carbohydrate supply was high, which was followed by incorporating protein including L-tryptophan into bacterial biomass. Less L-tryptophan would then be available for skatole production. An additional effect of providing more carbohydrate into the large intestine might be that the presence of glucose or maybe carbohydrates in general has a blocking effect on skatole production.

The problem concerning deposition of skatole in adipose tissue seems to be solved through the introduction of feed interventions. However, high concentrations of androstenone deposited in adipose tissue remain a challenge, and it is unlikely that this challenge can be solved through feed interventions. Androstenone contributes to boar taint as well as skatole does. In Denmark, the level of androstenone leading to boar tainted meat is above 1 µg/g in adipose tissue (Andersen, 1997). Several studies have detected androstenone concentrations above this level with no correlation to the level of skatole production and deposition in adipose tissue (Pauly et al., 2008; Aluwé et al., 2009 Maribo er al., 2010; Pauly et al., 2010; Øverland et al., 2011; Vhile et al., 2012). Thus, when the production of skatole is reduced in entire male pigs, it has no effect on androstenone production or deposition in adipose tissue. When castrating male pigs, the production of androstenone is blocked, and this has a reducing effect on skatole deposited in adipose tissue. The reason for this was found in the liver where androstenone was found to block the expression of cytochrome P450 enzymes involved in skatole metabolism in the liver (Whittington et al., 2004; Zamaratskaia et al., 2008; Rasmussen et al., 2011b; Brunius et al., 2012). Androstenone is a testicular steroid and starts to be produced at the onset of puberty (Claus et al., 1994; Zamaratskaia et al., 2004a). Thus, to avoid high concentrations of androstenone in adipose tissue, entire male pigs must be slaughtered before sexual maturity. When low levels of androstenone are correlated with low levels of skatole in

adipose tissue, it might be questionable if the use of skatole reducing feed interventions is necessary when slaughtering entire male pigs before sexual maturity. However, this is not certain and must be investigated further. A variety of environmental factors such as nutrition, season and social factors (Clarke and Pompolo, 2005) together with age and weight (Babol et al., 2004b; Aluwé et al., 2011) are believed to have an effect on time of sexual maturity. Further research needs to identify optimal environmental factors during growth and optimal age and weight at slaughter.

6.1 Conclusion

From the literature reviewed it is concluded that skatole deposition in adipose tissue can be reduced by feed interventions having a reducing effect on skatole production in the large intestine. Skatole production in the large intestine was correlated with skatole deposition in adipose tissue; thus, a reduction in skatole production was followed by a reduction in skatole deposition. A high protein supply into the large intestine tended to increase the skatole production, whereas a high supply of non-digestible and easy fermentable carbohydrates tended to reduce skatole production. A significant reduction of skatole production has been observed when adding a minimum of 20 % raw potato starch, 5 % inulin with an application period of two weeks and 9 % inulin with an application period of one week.

From the experimental part it is concluded that a bacterium from the gastrointestinal tract of pigs can produce skatole from indole-3-acetic acid. Skatole production tended to be blocked by the presence of glucose. The bacterium confirms that skatole is produced by a microbial conversion of indol-3-acetic acid into skatole.

The problem concerning deposition of skatole in adipose tissue seems to be solved through the introduction of feed interventions. However high concentrations of androstenone deposited in adipose tissue remains a challenge thus, the feed interventions were not fully effective against boar taint.

6.2 Perspectives

The production of entire male pigs in commercial pig productions would increase welfare for male piglets and productivity of entire male slaughter pigs. In order to produce entire male pigs, knowledge on how to prevent the development of boar taint is needed. Skatole and androstenone are the main compounds contributing to boar taint. Skatole production can be reduced by adding a minimum of 20 % raw potato starch, 5 % inulin two weeks before slaughter or 9 % inulin one week

before slaughter added to the feed fed to entire male pigs. However, none of the interventions had an effect on androstenone deposition in adipose tissue; thus, the interventions were not fully affective against boar taint. Further research needs to identify how androstenone deposition in adipose tissue can be affected. Until now there has been a challenge in developing feed interventions with a reducing effect on androstenone deposition. However Jen and Squires (2011) found an effect of non-nutritive sorbent materials as intestinal binding agents on androstenone deposition in adipose tissue. Further work is needed to confirm these effects.

In the experimental part of this thesis a skatole producing bacteria was described. The identification of a skatole producing bacterium isolated from the gastrointestinal tract of pigs makes it possible to investigate how skatole production by this bacterium can be reduced or blocked. However further investigation of the properties of the bacterium needs to be identified.

Development of SK9 K4 in the gastrointestinal tract: In vivo studies should be conducted, to investigate how the growth of the bacterium is affected by feed interventions, which have showed a reducing effect on skatole production. A study could be conducted by designing a primer pair encoding for the 16S rRNA sequence of SK9 K4 from the present study. If a primer pair can be successfully designed to specific encode for SK9 K4 the quantity of SK9 K4 in intestinal content could be quantified using Q-PCR (quantitative PCR). This study might be able to conclude if the growth of SK9 K4 is affected when using feed interventions with a reducing effect on skatole production.

Moreover SK9 K4 could be infused into the gastrointestinal tract using the method described by Agergaard et al (1998). In this study L-tryptophan was infused directly into the large intestine and the effect on skatole, indole and indol-3-acetic acid (IAA) production was analysed. When infusing SK9 K4 directly into the large intestine the effect on skatole, indole and IAA production could be analysed by the same method. Furthermore an analysis on the effect of indole-propionic acid (IPA) could be included to further investigate the effect of this compound found by Knarreborg et al. (2002).

Investigate other bacterial species capable of producing skatole: Closely related bacteria and other bacteria isolated from the gastrointestinal tract of pigs should be investigated for their ability to produce skatole. This can be done by using the same method as described in the paper included in this thesis. Moreover bacteria closely related to SK9 K4 which are not capable of producing skatole should be compared to skatole producing bacteria. This might identify special properties that can help identify skatole producing bacteria.

Effect of carbohydrates on SK9 K4: From the experimental work it was suggested that the bacterium was only able to produce skatole in the absence of glucose. This was also found to be an important factor influencing skatole production by skatole producing bacteria isolated from the rumen (Attwood et al., 2006). The blocking effect of glucose or carbohydrates in general on skatole production needs to be investigated further. This could be investigated by using the method described by Attwood et al. (2006). Furthermore it could be studied *in vitro* by adding different carbohydrates sources to intestinal content from the large intestine and observe the effect on skatole production.

Physiological properties: Basic *in vitro* designs should be conducted to investigate the physiological properties of SK9 K4. This could for example aim to investigate the effect of pH, antibiotics, different carbohydrate sources and the interrelation of SK9 K4 with other microbes occurring in the large intestine of pigs.

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