

**Alternatives to in-feed antibiotics  
and their effects on development and stability of intestinal  
microbial community in weaning piglets**

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### I. General introduction and outline

In the contemporary pig industry it is a common practice to wean the piglets from their dams abruptly at age of 21 or 28 days. The piglets are susceptible to many infections at this time, as their immune system is still not mature, and additionally the change from liquid feed (the milk) to a solid diet is associated with anatomical and physiological changes in the gastrointestinal tract (GIT). Thus far, the disorders of the GIT during the weaning period were controlled by introducing sub-therapeutic levels of antibiotics in the feed. However, general concerns regarding the cross-resistance to the antibiotics developed by many pathogens have led to the ban of the in-feed antibiotics in the European Union since 1 January 2006. However, the antibiotics have been further widely used as prophylaxis and they are commonly applied to piglets at or shortly after birth. As this is a common practice, a question arose if any late effects on intestinal microbiota appear that could further bias studies on alternatives to antibiotics. In the **Chapter 1** the late effect of parenterally administered amoxicillin on the intestinal microbiota composition is presented. It was clearly shown that the administration of a long-term, broad spectrum antibiotic to piglets one day after birth can result in changes in the intestinal microbiota composition lasting as long as 39 days. Thus these results confirmed the hypothesis that prophylactic antibiotics can bias dietary studies. Therefore, all further studies presented in this thesis were performed without any application of antibiotics, either prophylactic or therapeutic.

Many alternative approaches have been attempted to replace the in-feed antibiotics since their ban. One of such alternatives displaying interesting properties *in vitro* or *ex vivo* are plant extracts, among them essential oils (EO) have been widely investigated. Before the effects of selected essential oils and other alternatives to antibiotics were tested in trials on pigs (Chapters 7 - 10), several studies were performed to increase the knowledge on pig intestinal microbial composition, its changes with age and its adaptation to new feed components, such as complex carbohydrates or other phytogetic ingredients present in a solid diet. **Chapters 2 to 6** are devoted to these issues. **Chapters 2** and **3** focus on lactobacilli, a bacterial group agreed to be a beneficiary for the host. Applying culture-independent methods based on 16S-rRNA gene approach, allowing studying of bacterial composition of complex ecosystems, diversity of the pig intestinal lactobacilli and the changes within

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the lactobacilli population were investigated. PCR with following denaturing gradient gel electrophoresis (DGGE) together with fluorescent in-situ hybridization (FISH) were applied to study this particular bacterial group. Culture-dependent technique based on carbohydrate fermentation profile was applied to distinguish the main cultivable lactobacilli isolated from porcine intestine and to characterize their functional properties regarding the changes in the feed after weaning. The total bacterial and lactobacilli communities were also the object of the **Chapter 4**.

Despite multiple works on the intestinal bacterial communities, only very scarce knowledge on intestinal yeasts exists. Therefore, **Chapters 5** and **6** are devoted to this rather forgotten microbial group and describe not only the diversity of the yeasts harbouring the porcine intestine, and name its dominating species, but also provide information on for their source and development.

The five chapters mentioned above provide much additional information allowing better understanding of the gastrointestinal milieu in pigs. Further investigations were focused on changes of this milieu. Field-isolates of *Lactobacillus plantarum* showing promising effects *in vitro* were chosen as possible probiotic and fed to weaning pigs (**Chapter 7**). The chapter shows the qualitative changes of the microbial population caused by these lactobacilli and discusses its possible effect on the host.

Another group of alternatives to antibiotics consists of prebiotics and plant bioactive compounds (PBC). The main mode of action of these compounds is believed to be the support of the beneficiary microbiota of the GIT, according to the *in vitro* recordings. However, *in vivo* results very often disagree with the observations collected *in vitro*. Therefore animal studies are important when any potential prebiotic or PBC should be introduced on the market. Here (**Chapter 8**), a mixture of selected EO was fed to weaned pigs in order to investigate its effect on the intestinal microbiota. Furthermore, different sanitary conditions were the additional issue of the studies, an issue often forgotten in studies on effects of dietary regimes on intestinal microbial communities. In the **Chapter 8** the changes in the total intestinal microbial community caused by the investigated EO are presented. The assumed differences between the farms of different sanitary status were confirmed.

The infections of pigs with *Salmonella* Typhimurium are widely distributed among pig farms. Many EO possess anti-microbial activity *in vitro*. Thymol, the active component of thyme oil present in e.g. thyme or oregano, was shown to kill *S. Typhimurium in vitro*. However, contradictory *in vivo* results were also recorded. As



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the amounts of thymol tested *in vitro* and *in vivo* differed significantly, the proper dose of thymol that would act *in vivo* was to be established. Thus, a well controlled study was performed to investigate the *in vivo* effects of high dose of free thymol on intestinal microbiota of control and pigs challenged with *S. Typhimurium* (**Chapter 9**). Polysaccharides of plant origin are believed to activate the hindgut microbiota fermentation and thus help to stabilize its balance towards the lactic acid bacteria. Especially inulin, the fructooligosaccharide present in chicory has found much interest as prebiotic. However, again discrepancies exist depending on the dietary level or size of the molecules, and leaving necessity to perform further studies. Intestinal microbiota composition was investigated mostly by cultivation of main bacterial groups; therefore more detailed qualitative study applying cultivation-independent approach was designed (**Chapter 10**).

Marine algae have been shown to consist of large amounts of non-digestible polysaccharides. Alginates are extracts from the brown seaweed and are proposed to act as prebiotic when fed to animals or humans. As very limited data on these products exist, sodium alginate was investigated for its prebiotic properties in comparison to inulin, and the study is presented in **Chapter 10**.

In the **General discussion** the results presented in the thesis are discussed and an outline for future work is presented.

The questions that the thesis aimed to answer can be summarized as follows:

- Do parenteral antibiotics affect the gastrointestinal microbiota in piglets, when the prophylactic administration is performed at birth?
- What are the physiological changes in the lactobacilli community of the porcine intestine around weaning? Which species are affected?
- Are the particular species of the gastrointestinal lactobacilli able to adapt to ferment different dietary carbohydrates introduced after weaning?
- What is the kinetics of the changes of other dominating bacteria caused by weaning?
- What is the diversity of porcine intestinal yeasts and are there correlations to bacteria?

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- Does *Lactobacillus plantarum* affect the intestinal microbiota? If yes, then would the changes allow considering it as a possible probiotic? What would be the best time-point to administer it to the weaning piglets?
- Can the *in-vitro* observation on antimicrobial activity of essential oils be confirmed *in-vivo*? Do the essential oils affect the microbial activity with concomitant changes in the intestinal chemical composition?
- Do selected oligosaccharides have an effect on the porcine intestinal microbial activity and composition?
- Do sanitary conditions affect the outcome of the dietary studies?

## **II. Late effects of antibiotics on gastrointestinal microbiota**

## Chapter 1

### ***Parenteral long-term amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration***

Janczyk P., Pieper R., Souffrant W.B., Bimczok D., Rotthkötter H.-J., Smidt H..

The ISME Journal (2007), 1 (2), pp. 180-183

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## **Zusammenfassung**

In der Studie, die im Kapitel 1 vorgestellt wird, wurde der Effekt von einem langfristig wirkenden Antibiotikum auf die Zusammensetzung der intestinalen Mikrobiota untersucht. Die Ferkel der Versuchsgruppe erhielten als Prophylaxe am ersten Lebenstag das Antibiotikum Amoxicillin (15 mg/kg) intramuskulär, während die Kontrollferkel kein Antibiotikum bekamen. Am 39. Lebenstag, elf Tage nach dem Absetzen, wurde die intestinale Mikrobiota mittels der denaturierenden Gradientengelelektrophorese (DGGE) der in Polymerase-Kettreaktion (PCR) amplifizierten Fragmente des 16S rRNA-Gens untersucht. Die Diversität der DGGE-Fingerabdrücke war in der Versuchsgruppe im Vergleich zur Kontrolle stark reduziert ( $0.8 \pm 0.19$  vs.  $1.03 \pm 0.17$ ,  $P=0.012$ ). Die Bande, die anhand der Sequenz *Roseburia faecalis* zugeordnet werden kann, war in der Versuchsgruppe geringer ausgeprägt, während *Shigella* spp., *Escherichia coli* und *Salmonella enterica* serovar Typhi stärker präsent waren. *Lactobacillus sobrius* wurde nur in der Kontrollgruppe gefunden. Das zeigt, dass der prophylaktische Effekt des Antibiotikums durch eine negative dauerhafte Veränderung in der intestinalen Mikrobiota überschattet werden kann.

**III. Porcine gastrointestinal microbial community around weaning  
– its composition and development**

***III.I Intestinal bacteria with emphasis on lactobacilli***

## Chapter 2

### ***Changes in the diversity of pig ileal lactobacilli around weaning determined by means of 16S rRNA-gene amplification and denaturing gradient gel electrophoresis.***

Janczyk P., Pieper R., Smidt H., Souffrant W.B.

FEMS Microbiology Ecology (2007), 61, pp. 132-140

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### **Zusammenfassung**

In dieser Studie wurde die Veränderung der intestinalen Laktobazillenpopulation beim Schwein um den Zeitpunkt des Absetzens untersucht. Hierfür wurden mittels Polymerasekettenreaktion amplifizierte V2-V3-Fragmente der 16SrRNA-Genfragemente in der denaturierenden Gradientengelelektrophorese (DGGE) aufgetrennt. Die DNS wurde aus dem Inhalt des Ileums von Ferkeln isoliert, die 1, 2, 5 bzw. 11 Tagen nach dem Absetzen (am 28. Lebenstag) getötet worden waren. Die dominierenden Banden wurden aus den Gelen ausgeschnitten und sequenziert. *Lactobacillus salivarius* wurde 1 und 2 Tage nach dem Absetzen beobachtet; *Lactobacillus crispatus* nur am Tag 1 und 11. *Lactobacillus sobrius* dominierte in allen Profilen. Die Zahl der Banden sank von  $13 \pm 3$  am Tag des Absetzens auf  $9 \pm 1$  am 5. Tag nach dem Absetzen, wobei die Anzahl der Banden das Ausgangsniveau 11 Tage nach dem Absetzen erreichte. Die Profile waren am Tag 1 und 2 nach dem Absetzen sehr ähnlich (>91%); die niedrigsten Übereinstimmungen ergaben sich am 11. Tag (<59%). Die Diversität der DGGE-Fingerabdrücke war 5 Tage nach dem Absetzen reduziert ( $0.83 \pm 0.076$  vs.  $1.02 \pm 0.127$  beim Absetzen,  $P = 0.042$ ), wobei sie am 11. Tag nach dem Absetzen sich wieder bei den Werten, die beim Absetzen vorlagen, einfand. Die Ergebnisse zeigen, dass die Laktobazillenpopulation im Ileum vom Schwein dramatischen, teilweise reversiblen Veränderungen unterstellt ist, welche eine Konsequenz des Absetzens zu sein scheint.

### Chapter 3

#### ***The intestinal microflora of piglets around weaning – with emphasis on lactobacilli.***

Pieper R., Janczyk P., Schumann R., Souffrant W.B.

Archiva Zootechnica (2006), 9, pp. 28-40

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#### **Zusammenfassung**

Diese Studie befasst sich mit der mikrobiellen Kolonisierung des Schweinedarms mit Fokus auf die ilealen Laktobazillen um den Zeitpunkt des Absetzens. Acht Ferkel wurden am Tag des Absetzens (28. Lebenstag) getötet, weitere 4 jeweils am 1., 2., 5. und 11. Tag nach dem Absetzen. Es wurden Digestaprobe aus dem distalen Drittel des Dünndarms, dem Zäkum und den ersten 40-50 cm des proximalen Kolon gewonnen. Die Proben wurden mittels selektiver Wachstumsmedien bakteriologisch untersucht. Die beobachteten Veränderungen waren sehr stark und zeitabhängig, dabei stärker ausgeprägt in Ileum als im Zäkum oder Kolon. Die Zahl der Kolonien von *Enterobacteriaceae* war bis Tag 5 stabil und am Tag 11 nach dem Absetzen signifikant reduziert. Die Enterokokken zeigten sich anfangs stabil, waren jedoch am 5. und 11. Tag nach dem Absetzen stark reduziert. Die Hefenzahl war am 5. Tag reduziert, erhöhte sich aber wieder am 11. Tag nach dem Absetzen. Die Laktobazillenzahl sank deutlich am 1. Tag nach dem Absetzen ( $P < 0.01$ ), aber erreichte das Anfangsniveau bereits am 5. Tag nach dem Absetzen.

Insgesamt 72 Laktobazilluskolonien wurden auf deren Kohlenhydratverwertung überprüft. Es dominierten die Stämme von *L. acidophilus* (44.4 %), *L. fermentum* (35.7 %) und *L. salivarius* (15.3 %). Des Weiteren wurde ein Zeiteffekt festgestellt. *L. salivarius*, *L. fermentum* und *L. acidophilus* dominierten in der Zeit vor dem Absetzen, während *L. fermentum*, *L. salivarius* am 1., 2. und 5. Tag nach Absetzen überwiegen; *L. acidophilus* wurde am 11. Tag nach dem Absetzen beobachtet. Die Ergebnisse geben einen Einblick in die Veränderungen, die in den Hauptgruppen der intestinalen Mikrobiota der abgesetzten Ferkel stattfinden. Diese Hinweise können sich für zukünftige Arbeiten auf dem Gebiet der Alternativen zu den Futterantibiotika als hilfreich erweisen. Des Weiteren können Veränderungen auch innerhalb der Gruppen vorkommen, was hier für die Laktobazillen dargestellt wurde. Jedoch sind noch weitere Untersuchungen notwendig, um die Bedeutung der intestinalen

## **Intestinal bacteria with emphasis on lactobacilli**

mikrobiellen Population auf den Wirtsorganismus während Absetzens feststellen zu können.



## Chapter 4

### ***Ecophysiology of the developing total bacterial and Lactobacillus communities in the terminal small intestine of weaning piglets.***

Pieper R., Janczyk P., Zeyner A., Smidt H., Guiard V., Souffrant W.B.

Microbial Ecology (2008), 56, pp. 474-483.

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## **Zusammenfassung**

Das Absetzen der Ferkel gilt gemeinhin als starker Stress, das auf Grund der Veränderungen im intestinalen Ökosystem zu klinischen Komplikationen führen kann. Die funktionellen Eigenschaften der Mikrobiota, die den Schweinedünndarm besiedelt, insbesondere die als gesundheitsfördernd betrachteten Laktobazillen, sind bislang unzureichend beschrieben. Darum wurden in dieser Studie die Ökophysiologie und der Einfluss der Fütterung auf die bakteriellen Gruppen, inklusive Genus *Lactobacillus*, untersucht, die im Dünndarm der Absetzferkel vorkommen. Hierfür wurde die Digesta aus dem Dünndarm von 20 Ferkel gesammelt, wovon 4 Tiere zum Absatzzeitpunkt getötet wurden (nur Sauenmilch, kein Beifutter) und jeweils 4 weitere Tiere am Tag 1, 2, 5 und 11 nach dem Absetzen (Getreide-basiertes Futter). Die Bakterienzahl wurde mittels "fluorescent *in situ* hybridisation" (FISH) bestimmt. Auch die bakteriellen Metabolite wurden untersucht. Abgesehen vom 1. Tag nach dem Absetzen, an dem eine starke Absenkung der Bakterienzahl zu verzeichnen war, bestand die dominierende Bakterienpopulation aus Laktobazillen. Am Tag 11 nach dem Absetzen wurden keine Bifidobakterien und *E. coli* detektiert, auch *Enterobacteriaceae* und die Mitglieder des Clusters *Clostridium coccooides/ Eubacterium rectale* wurden nur sporadisch gefunden. *L. sobrius/L. amylovorus* dominierte, während die Menge der *L. salivarius* und *L. gasseri/johnsonii* sank. Die Konzentration der Milchsäure erhöhte sich nach dem Absetzen der Ferkel, gleichzeitig sanken sowohl der pH als auch die Konzentration an flüchtigen Fettsäuren und Ammoniak. Die Untersuchung der Verwertung von Kohlenhydraten durch 76 Isolate des *Lactobacillus* spp. zeigte, dass eine Verschiebung von Laktose und Galaktose zu Stärke, Cellulose und Xylose erfolgt. Dies deutet an, dass die Bakterien, die den Dünndarm besiedeln, sich an die frisch, in der direkten Zeit nach dem Absatz, eingeführten Substrate adaptieren. Die Identifizierung der Isolate basierte auf partiellen Sequenzen des 16S r RNA-Gens.

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Deren Vergleich mit den Daten der Fermentierung deutete zusätzlich auf Adaptationsprozesse unter dem Spezieslevel. Die Ergebnisse helfen die Ökophysiologie der intestinalen Bakterien zu verstehen und neue Fütterungsregimes zu entwickeln, welche die mit dem Absetzen verbundenen Komplikationen vorbeugen oder eliminieren könnten.

### ***III.II Intestinal yeasts***

## Chapter 5

### ***Biological diversity of yeasts in the gastrointestinal tract of weaned piglets kept under different farm conditions.***

Urubschurov V., Janczyk P., Pieper R., Souffrant W.B.

FEMS Yeasts Research (2008), 8, pp. 1349-1356

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### **Zusammenfassung**

In dieser Studie wurden die Hefen untersucht, die im gastrointestinalen Trakt (GIT) der unter experimentellen (EF) bzw. kommerziellen (CF) Bedingungen gehaltenen Ferkel vorkommen. Hierfür wurden 95 Ferkel der Deutschen Landrasse, Voll- bzw. Halbgeschwister, im Alter von 39 Tagen getötet. Davon wurden 68 Ferkel am 28. Lebenstag abgesetzt, die restlichen Ferkel verblieben ohne Beifutter bei den Sauen. Die Digestaprobe wurden aus dem distalen Drittel des Dünndarms, Zäkum und Kolon gesammelt. Durch die Sequenzierung der mittels Polymerasekettenreaktion amplifizierten D1/D2 Fragmente des 26S rRNA-Gens und dem anschließenden Vergleich der Sequenzen mit den Einträgen in der GenBank wurden 173 Kolonien charakterisiert. Dabei wurden 17 Phylotypen identifiziert, darunter *Galactomyces geotrichum*, *Kazachstania slooffiae* und *Candida catenulata*, die den GIT der Ferkel dominiert haben. *Kazachstania slooffiae* und *Candida glabrata* dominierten in GIT der Ferkel aus der Gruppe EF. Die Sørensen und Morisita-Horn Ähnlichkeitsindexe zwischen den Farmen waren niedrig (entsprechend 0,44 und 0,54); der Simpson Diversitätsindex war höher in der Gruppe EF (7,58) als in CF (4,34). Diese Studie bringt neue Kenntnisse auf dem Gebiet der Hefebesiedlung im Darm der Schweine und zeigt, dass die Hefediversität sich unter verschiedenen Hygienebedingungen anders verhält.

## Chapter 6

### ***Establishment of intestinal microbiota with focus on yeasts of unweaned and weaned piglets kept under different farm conditions.***

Urubschurov V., Janczyk P., Souffrant W.B., Zeyner A.

FEMS Microbiology Ecology (2011), 77, pp. 493–502

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## **Zusammenfassung**

Die im Kapitel 6 beschriebene Studie diente der Charakterisierung der intestinalen Hefen, die beim Ferkel vorkommen. Des Weiteren wurden die möglichen Interaktionen mit den bakteriellen Hauptgruppen untersucht. Die Ferkel der Deutschen Landrasse wurden am 28. Lebenstag abgesetzt (WP, n=32) bzw. ohne Beifutter bei den Sauen gehalten (UP, n=32). Das Experiment wurde parallel auf einer experimentellen (EF) und kommerziellen Farm (CF) durchgeführt. Am Tag 28, 33 und 39 wurden Kotproben von den Ferkeln, den Sauen und dem Stallboden gesammelt, um die DNA zu isolieren und die Hefen, Enterobakterien, Enterokokken und Laktobazillen quantitativ durch Kultivierung zu bestimmen. Die Fragmente der D1-Domäne des 26s rRNA-Gens wurden amplifiziert und mittels der denaturierenden Gradientengelelektrophorese (DGGE) getrennt. Es wurden keine Hefen im Wasser und in den Futterproben gefunden. In den Proben aus der Gruppe UP wurde nur eine geringe Koloniezahl der Hefen nachgewiesen. In der Gruppe WP stieg die Hefenzahl mit dem Alter. Auf der Farm CF, aber nicht EF, war die Hefenzahl am Tag 39 höher als am Tag 33. Auf beiden Farmen sanken die Zahlen der Enterobakterien und Enterokokken in der Gruppe WP am Tag 33, während die Laktobazillen konstant blieben. In der Gruppe WP aus der Farm EF wurden bis Tag 39 keine Veränderungen festgestellt. Auf der Farm CF stiegen die Hefen ( $P < 0.004$ ) und Laktobazillen ( $P < 0.001$ ) an, während die Enterobakterien sanken ( $P < 0.001$ ).

Die Hefen in der Gruppe WP aus CF korrelierten negativ mit Enterobakterien ( $r = -0.582$ ,  $P < 0.0001$ ) und positiv mit Laktobazillen ( $r = 0.384$ ,  $P = 0.0071$ ). *Kazachstania slooffiae* war die einzige Spezies, welche mittels DGGE in der Gruppe WP detektiert werden konnte. Enterobakterien, Enterokokken und Hefen waren in höheren Zahlen (nicht signifikant) in Sauen aus EF im Vergleich zu den Tieren auf der Farm CF, wobei keine Unterschiede in der Laktobazillenzahl zwischen den Farmen festgestellt wurden. Die Entwicklung der intestinalen Hefepopulation hängt von den

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Haltungsbedingungen ab. *K. slooffiae* ist die am häufigsten gefundene Spezies, die den Darm nach der Einführung des festen Futters kolonisiert. Die im Darm vorkommenden Hefen können die bakterielle Besiedlung beeinflussen. Zur genauen Charakterisierung der Bedeutung und Ökologie der Hefen, insbesondere *K. slooffiae*, müssen weitere Untersuchungen, basierend auf molekularen und physiologischen Methoden, durchgeführt werden.

**IV. Porcine gastrointestinal microbial community around weaning –  
the impact of selected substances with potentially modifying  
properties (probiotics, essential oils, oligosaccharides)**

***IV.1 Changes in the intestinal microbiota caused by potential  
probiotic strains of *L. plantarum****

## Chapter 7

### ***Effect of single oral administration of Lactobacillus plantarum DSMZ 8862/8866 before and after weaning on intestinal microbial communities in piglets.***

Pieper R., Janczyk P., Urubschurov V., Korn U., Pieper B., Souffrant W.B.

International Journal of Food Microbiology (2009), 130 (3); pp. 227-232

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### **Zusammenfassung**

Diese Studie wurde durchgeführt, um zu prüfen, ob eine einmalige orale Gabe von zwei Stämmen des *Lactobacillus plantarum* (DSMZ 8862 and 8866) zu einem Zeitpunkt vor oder direkt beim Absetzen die intestinale Mikrobiota der Ferkel beeinflussen kann. Es wurden 176 Ferkel in 5 Gruppen aufgeteilt: die Kontrolle (LP0), niedrige ( $5 \times 10^9$  KBE) oder hohe Dosis ( $5 \times 10^{10}$  KBE) entweder am Tag 25 (LP1, LP2) oder beim Absetzen (Tag 28) (LP3, LP4). Am Tag 25 (LP1, LP2), 28 (LP0, LP3, LP4), 33 (alle Gruppen) und 39 (alle Gruppen) wurden 10-13 Ferkel pro Gruppe getötet und die Digesta aus dem Dünn- und Dickdarm für die DNA-Extraktion gesammelt. Die denaturierende Gradientengelelektrophorese zeigte einen signifikanten Einfluss des *L. plantarum* auf die intestinale Mikrobiota, welcher auf der Basis der berechneten Indexe der Simpson-Diversität und Dice-Ähnlichkeit bestimmt wurde. Dieser Effekt war in den Gruppen LP3 und LP4 deutlicher ausgeprägt. Es wurden die folgenden Phylotypen identifiziert, die abhängig von der Behandlung auftraten: *Clostridium glycolicum*, *Lactobacillus sobrius*, *Eubacterium rectale* und *Roseburia faecalis*. Die Ergebnisse zeigen, dass eine Verabreichung von *L. plantarum* am Tag des Absetzens die intestinale Mikrobiota der Ferkel beeinflussen kann, was wiederum eine positive Auswirkungen auf die Darmgesundheit haben könnte.



***IV.II Plant bioactive compounds and intestinal microbial community***

## Chapter 8

### ***Investigations on the effects of dietary essential oils and different husbandry conditions on the gut ecology in piglets after weaning.***

Janczyk P., Pieper R., Urubschurov V., Souffrant W.B.

International Journal of Microbiology (2009), Vol. 2009 Article ID 730809, doi:10.1155/2009/730809; <http://www.hindawi.com/journals/ijmb/aip.730809.html>

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### **Zusammenfassung**

Ätherische Öle (EO) werden als mögliche Alternative zu den wachstumsfördernden Futterantibiotika in der Schweinernährung betrachtet. In diesem Experiment wurde der Einfluss einer Mischung aus Limonen, Eugenol und Pinen (10.0, 2.0 and 4.8 mg /kg) auf die Physiologie und Ökologie des Darms der abgesetzten Ferkel untersucht. Um die potenziellen Wechselwirkungen zwischen den Halterungsbedingungen und den EO zu untersuchen, wurde der Versuch parallel auf einer experimentellen und einer kommerziellen Farm durchgeführt. Die Ferkel wurden am 28. Lebenstag abgesetzt und anschließend in eine Kontroll- (C) und Versuchsgruppe (C+EO) aufgeteilt. Am Tag 29, 30, 33 und 39 wurden je 4 Ferkel pro Gruppe getötet und die Digesta aus dem distalen Drittel des Dünndarmes und dem Kolon gesammelt, um den pH, die Trockenmasse und die Menge an Milchsäure, flüchtigen Fettsäuren und Ammoniak zu bestimmen. Enterobakterien, Enterokokken, Laktobazillen und Hefen wurden mittels Kultivierung quantifiziert. Aus der Digesta wurde die genomische DNA isoliert, und die Veränderungen in der Darmökologie wurden mittels Polymerasekettenreaktion-denaturierenden Gradientengelelektrophorese untersucht. Auf jeder Farm wurden individuelle mikrobielle Populationen festgestellt. Die intestinalen chemischen Parameter wurden durch das Alter beeinflusst. Abgesehen von der signifikanten Absenkung der kolonalen bakteriellen Diversität bei den Ferkeln aus der experimentellen Haltung am 39. Lebenstag konnten keine weiteren Effekte der eingesetzten Ölmischung festgestellt werden.

## Chapter 9

### ***Effect of thymol on microbial diversity in the porcine jejunum.***

Janczyk P., Trevisi P., Souffrant W.B., Bosi P.

International Journal of Food Microbiology (2008), 126 (1-2), pp. 258-261

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### **Zusammenfassung**

In der Studie wurde der Effekt von Thymol auf die intestinale Mikrobiota des Schweins in einem Infektionsmodell untersucht. Hierfür erhielten 32 abgesetzte Ferkel (am 24. Lebenstag) Futter ohne bzw. mit 1% (w/w) Thymol. Die Ferkel der beiden Fütterungsgruppen wurden in zwei weitere Gruppen geteilt, von denen eine am 29. Lebenstag mit *Salmonella enterica* serovar Typhimurium infiziert wurde. Die Ferkel wurden am 49. Lebenstag (20 Tage nach der Infektion) getötet, und die Digesta wurde aus dem mittleren Dünndarm gesammelt. Die mikrobielle Diversität wurde anhand der 16S rRNA-Genfragmente untersucht, mittels Polymerasekettenreaktion amplifiziert und in der denaturierenden Gradientengelelektrophorese (DGGE) getrennt. Die Shannon-Diversität wurde nicht beeinflusst, aber die Pearson Koeffizient Analyse zeigte eine klare Gruppierung der DGGE-Fingerabdrücke der Ferkel, die Thymol bekommen haben, unabhängig von der *Salmonella*-Infektion. Des Weiteren waren die Banden, die als *Actinobacillus minor* identifiziert wurden, in der Thymol-Gruppe nicht nachweisbar. Eine Bande, die als *Citrobacter freundii* identifiziert wurde, war in fast allen Profilen der Thymol-Ferkel und in nur 4 Proben der Ferkel, die kein Thymol bekamen, zu sehen. Die Fütterung der Ferkel mit Thymol hat deutliche Veränderungen in der Zusammensetzung der intestinalen Mikrobiota verursacht. Überraschenderweise hatte die Infektion der Ferkel mit *Salmonella* kaum Einfluss auf die Bakterienpopulation.

### ***IV.III Intestinal microbiota under exposition to oligossaccharides***

## Chapter 10

### ***Effect of alginate and inulin on intestinal microbial ecology of weanling pigs reared under different husbandry conditions.***

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FEMS Microbiology Ecology (2010), 72 (1), pp. 132-142

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### **Zusammenfassung**

In der in dem Kapitel 10 beschriebenen Studie wurde die Wirkung von Inulin und Alginat auf die intestinale mikrobielle Ökophysiologie untersucht. Die Ferkel bekamen das Futter (C) mit 0,1% Alginat (C+A) oder 1,5% Inulin (C+I) beginnend mit dem Absetzen am 28. Lebenstag. Das Experiment wurde auf einer experimentellen (EF) und einer kommerziellen Farm (CF) durchgeführt. Die Digesta wurde aus dem Ileum, Zäkum und Kolon von vier Ferkeln aus jeder Gruppe am Tag 29, 30, 33 und 39 entnommen. Die Metabolitkonzentrationen änderten sich mit dem Alter. Die Metaboliten aus dem Zäkum und Kolon wurden durch die präbiotische Behandlung beeinflusst. Die Veränderungen der mikrobiellen Zusammensetzung wurden durch die Kultivierung und denaturierende Gradienten-Gelelektrophorese (DGGE) der 16S rRNA-Gen-Fragmente beurteilt. Die Enterokokkenzahl war erhöht in der Gruppe C+A in EF und reduziert in der Gruppe C+I. Die Zahl der Laktobazillen sank in allen Segmenten in den experimentellen Gruppen am Tag 30 und 33. Am Tag 39 war die Kolonienzahl der Hefen in der Gruppe C+I 5-mal niedriger in CF als in EF. Der Umfang und die Diversität der DGGE-Profile waren in den experimentellen Gruppen erhöht. Die Gleichmäßigkeit der DGGE Fingerabdrücke aus kolonalen Digesta war höher in den Versuchsgruppen im Vergleich zu C; diese Situation war im distalen Dünndarm umgekehrt. Die multivariante Redundanz Analyse bestätigte die erzielten Effekte. Zusammenfassend haben beide Präbiotika die intestinale Mikrobiota beeinflusst, wobei die Veränderungen auf der kommerziellen Farm stärker ausgeprägt waren.

## **V. General discussion**

### **V.1 Porcine gastrointestinal microbial community around weaning**

Weaning on a commercial pig farm is a very stressful moment in a piglet's life, involving an abrupt break of contact with the mother, a change of food, mixing with other litters, and the need to establish a new hierarchy – all two to three weeks before the natural weaning time. The animal's systems are still developing at this time, including the gut-associated immune system, which is not fully matured before 5–6 weeks of age (Bailey et al., 2005). In terms of gut morphology and physiology, weaning has been shown to cause shortening of villi with accompanying increased width of villi and deepening of crypts (Hampson, 1986; Spreeuwenberg et al., 2001; Boudry et al., 2004), together with alterations in the activity of digestive enzymes such as lactase and maltase and increased permeability of the epithelial barrier (Spreeuwenberg et al., 2001; Boudry et al., 2004). The young animal is therefore very sensitive to external influences at the time of weaning. The intestinal microbiota has to adapt to these changed conditions, and lactobacilli are one of the most abundant bacterial groups that may suffer at this time (Konstantinov & Smidt, 2006). A proper, balanced gut environment with well-established populations of commensal microbiota, including lactic acid bacteria (LAB) such as lactobacilli and bifidobacteria, is of importance in protecting the animal from gut infections (Berg, 1996). Broad spectrum antibiotics are often administered shortly after birth as prophylaxis of intestinal and systemic infections caused e.g. by enterobacteria (*E. coli*, *Salmonella* sp.). However, as it was shown in the **Chapter 1**, even parenteral antibiotics may affect the intestinal microbiota in an unexpected way. Control group received no antibiotics, and a treatment group received intramuscular 15mg/kg of amoxicillin. A bacterium belonging to *Clostridium* cluster XIVa that contains butyrate producing bacteria (Duncan et al., 2002, 2004), namely *Roseburia faecalis*-related population was strongly reduced in relative abundance in the treated group, while *Escherichia coli*-related bacterium was enriched. *Lactobacillus sobrius*, an abundant member of the porcine intestinal microbiota (Konstantinov et al., 2006a, b) was present only in the control group. Thus, the protective effect of prophylactic antibiotic administration may be outweighed by the long-lasting disturbance of the gut ecosystem. Therefore no antibiotics were administered in the following studies.

## General discussion

It was already mentioned, the lactobacilli are important beneficiary commensals. For this reason they were in focus of several investigations presented in this thesis. Weaning results in a transient decrease of lactobacilli counts (colony forming units, CFU) one day after weaning and their restoration to the initial level (9 log CFU/g digesta) 11 days post weaning (pw) (**Chapter 3, 4**). The diversity of lactobacilli was investigated using denaturing gradient gel electrophoresis (DGGE), a molecular 16S-rRNA gene based approach suitable for studies on complex bacterial ecosystems (Muyzer & Smalla., 1998). The general diversity of the lactobacilli community remains high one and two days after weaning, but a decrease of the diversity could be observed 5 days pw, what can be interpreted as negative for the host. Namely, increased diversity (i.e. degree of simplification) of microbial ecosystem links to their ability to respond to perturbations (McCann, 2000).

It was clearly shown that the weaning and change of the diet to solid food stimulated a shift in the lactobacilli profile (**Chapter 2**). Stress and lower feed intake resulted in increase of similarity of the DGGE fingerprints one and two days pw compared with the situation at weaning. Moreover, the reduced intra-day similarity of the DGGE profiles 5 and 11 days pw suggests significant inter-individual differences in the development of the lactobacilli microbiota in the gut, and large variations in the adaptation processes to the feed components. However, the decrease in the similarity of the profiles between time-points shows the age-dependent adaptation of the lactobacilli community. On the first day after weaning, when piglets take practically no or only very little solid food, several lactobacilli decreased their abundance, including *L. sobrius*, allowing the other species, such as *L. crispatus* or *L. johnsonii* to proliferate. These semi-quantitative data were confirmed after enumeration of *L. sobrius* cells by fluorescent in situ hybridization (FISH) from the same samples. A significant drop was observed from  $7.87 \pm 0.44$  log cells/g digesta at weaning to  $5.37 \pm 0.34$  log cells/g digesta one day later (**Chapter 4**). Eleven days post weaning, *L. sobrius* recovers and predominates the lactobacilli community (**Chapter 2, 4**).

Based on the cultivation-dependent and the molecular 16S rRNA gene approach several other lactobacilli were detected: *L. salivarius*, *L. amylovorus*, *L. johnsonii*, *L. acidophilus*, *L. fermentum*, *L. reuterii* and to a lesser extent *L. brevis*, *L. crispatus*, *L. cellobiosus* as well as *Lactococcus raffinolactis* (**Chapter 2, 3, 4**). Their abundance was dependent on the age of animals and the feed they were offered. The



## General discussion

adaptation of the lactobacilli to fermentation of solid feed components was another factor affecting their abundance (**Chapter 4**). Strains of starch utilizing species such as *L. sobrius*, *L. amylovorus*, and *L. reuteri* were the abundant ones 5 and 11 days pw, whereas the numbers of *L. johnsonii*, which is known for its proteolytic activity, were strongly reduced at this time.

Weaning results in changes not only in the lactobacilli community. The increasing lactic acid (LA) production observed in the distal small intestine (SI) (**Chapter 4**) helps to reduce the numbers of enterobacteria and enterococci (**Chapter 3, 4**). At the same time, the studies show the increase of yeast numbers in this part of GIT, what can be explained by the feasibility of these microorganisms to survive at low pH.

Yeasts have been taken into consideration in several studies on intestinal microbiota. However, there is still little information with respect to the diversity of pig intestinal yeasts expect from the old reports of Van Uden et al (1958) and van Uden & Sousa (1962). Therefore the development of yeasts in the porcine intestine was in focus of **Chapter 5** and **6**. Applying both classical cultivation and the modern molecular methods at least 17 different yeast species from *Ascomycetes* or *Basidiomycetes* could be determined in the GIT of the piglets, and the effect of the hygienic conditions on their diversity could be reported. In general, higher sanitary conditions led to higher yeast richness and diversity. In suckling piglets, isolates of *Galactomyces geotrichum* dominated the intestinal yeasts (**Chapter 5**) and in the following study *Geotrichum*-like colonies were isolated only from suckling piglets (**Chapter 6**). Its ability to grow on and to utilise milk products (Valdes-Stauber et al., 1997; Goerges et al., 2008), and its antimicrobial activity against other yeasts (Romano et al., 2006) are probably responsible for the advantage for this species in colonising the GIT of suckling piglets.

*Kazachstania slooffiae* was identified to be the dominating yeast in porcine intestine. The source of this species was identified to be the faeces of the sows, and the solid feed, providing complex nutrients seems to be necessary for this species to further grow and colonise the GIT (**Chapter 6**). Noteworthy, its abundance was higher at lower sanitary conditions, what raises a question on the interplay between the microorganisms. This yeast has been isolated only from healthy animals (Gedek, 1968, **Chapter 5**) and it can be assumed that *K. slooffiae* commensally settles the pig intestine and probably interacts with intestinal bacteria to establish a balanced ecosystem. This species raises interest for further research, as there is only known

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its morphology and the fact that under laboratory conditions *K. slooffiae* metabolizes only glucose (Kurtzman et al., 2005).

*Candida catenulata* and *Candida glabrata* were the other most frequent isolates among the yeast species detected in the porcine GIT, present mostly in the suckling piglets (**Chapter 5**). These are commensal inhabitants of the porcine GIT, but they can cause an infection in immune-suppressed humans (Fidel et al., 1999; Radosavljevic et al., 1999; Ahmad, 2005).

Further studies on effects of feed components, prebiotics and probiotics on the composition of the intestinal yeasts population would be needed to improve our knowledge on the interactions between the microbiota and the host, and if some of the yeasts could have the potential to stabilize and balance the intestinal microbiota. However, in face of the results presented in **Chapter 6**, other molecular approaches than DGGE shall be used or be developed, because this method appeared to be not sensitive enough to investigate the yeasts present in the complex intestinal microbial ecosystem. It seems that an old, but nevertheless a novel, area of research is now being opened for the new generation of researchers.

### ***V.II Changes in the intestinal microbiota caused by potential probiotic strains of L. plantarum***

After the use of antibiotics as growth promoters had been banned in the European Union, much interest has been raised on potential use of probiotics, microorganisms with specific function in the GIT of farm animals, instead. LAB are in focus of the research as they are traditionally used in a range of industrial food fermentations and have a long history of safe use (Saxelin et al., 2004). The administration of any potential probiotic must be easy to perform and practically relevant. The intake of appropriate numbers of bacteria with a minimum of stress for the young animal must be assured. Therefore, in the study presented in **Chapter 7** a single oral dose of *L. plantarum* strains DSMZ 8862 and 8866 was chosen and administered either before or at weaning.

The changes in the microbial community were studied by DGGE, the method applied also in **Chapter 1, 2, 4 and 6**. An effect of *L. plantarum* DSMZ 8862/8866 on the distal small intestinal and colon microbial communities could be observed. The

## General discussion

treatment with *L. plantarum* DSMZ 8862/8866 increased the Simpson's diversity index, what can be interpreted as beneficial for the weaned animal.

The administration of *L. plantarum* DSMZ 8862/8866 at weaning resulted in increased intensity of the DGGE-band representing *Lactobacillus sobrius*-like phylotypes, showing indirectly the increase of the abundance of *Lactobacillus sobrius*. This porcine-specific lactobacillus (Konstantinov et al., 2006b; **Chapter 2, 4**) was shown to protect the host against an infection with *Escherichia coli* (Konstantinov et al., 2008; Roselli et al., 2007). Thus, an indirect beneficial effect of *L. plantarum* cannot be excluded. Furthermore, the recorded shift in the DGGE-profiles towards clostridia from the cluster XIV (Chapter 7), which produce butyrate, the primary energy source used by the colonocytes (Wong and Jenkins, 2007), can be additionally interpreted as a beneficial effect of the *L. plantarum*.

### **V.III Plant bioactive compounds and intestinal microbial community**

Plants have been used for hundreds of years in the natural medicine and they seem to provide alternatives to antibiotics also in animal nutrition. Among the plant bioactive compounds the essential oils play major role and therefore stay in focus of many research groups. These volatile, aromatic mixtures consist principally of terpenes and phenylpropane derivatives, and were used for the studies presented in **Chapter 8 and 9**. They are present in many plant tissues protecting the plant against bacteria and parasites. The composition of essential oils may vary depending on the plant species (Aligiannis et al., 2001), its geographical origin or vegetative stage (Hudaib et al., 2002). Many essential oils have strong antibacterial effects *in vitro* (Burt, 2004). The ability of these compounds to kill bacteria depends on their chemical structure (Si et al., 2006b). Thymol, a phenolic molecule present as the main component in thyme oil, is highly active against *Salmonella Typhimurium in vitro*, presumably damaging the cytoplasmic membrane integrity of the pathogen (Si et al., 2006a). However the results of *in vivo* experiments are contradictory, probably due to a combination of poorly controlled experimental designs, disparate sources of essential oils (containing different proportions of active ingredients) and a wide range of doses (Lalles et al., 2009).

Positive effects of essential oils on the nutrient digestibility (Stoni et al., 2006; Cho et al., 2006) and growth performance (Cho et al., 2006) in piglets have been reported.

## General discussion

In the study presented in **Chapter 8** a mixture of limonene, eugenol and pinene was fed to weaned piglets but resulted in no effect on piglet production parameters. Also thymol did not affect the growth parameters in the study from **Chapter 9** (Trevisi et al., 2007).

Previously, an increase in lactobacilli and the ratio of lactobacilli and enterobacteria in the jejunum and caecum of early weaned pigs, respectively, has been reported for dietary application of carvacrol, cinnamaldehyde and capsicum oleoresin (Manzanilla et al., 2004; Castillo et al., 2006). However, no such an effect could be found in the study presented in **Chapter 8**.

Simulation of the gastric environment (artificial substrate containing several different sugars, casein, soybean oil, vitamins, minerals etc. in a buffer solution of pH 3) to test anaerobic bacteria inhibition by essential oils revealed relatively high minimal effect concentrations (MEC) for carvacrol, thymol, eugenol and trans-cinnamaldehyde (Michiels *et al.*, 2007). Trans-cinnamaldehyde displayed a strong inhibitory activity in the small intestinal environment (containing the same substrate as for the gastric environment with bile salts in a buffer of pH 6.5 inoculated with fresh small intestine supernatant of a weaned piglet). Synergistic action of carvacrol and thymol against total anaerobic bacteria was observed at ratios >1. Obviously, the reason for this was the different, dose-dependent mode of action of the essential oils components against *E. coli* and other coliform bacteria.

Whereas the EO used in the **Chapter 8** resulted in no effects on the intestinal microbiota of weaning pigs, thymol caused shift in the DGGE fingerprint profiles (**Chapter 9**). The dosage applied in the **Chapter 8** was chosen based on concentrations of EO commonly used in feed industry and scientific studies (Manzanilla et al., 2004), which would be economically feasible and therefore the essential oil content was much lower than in **Chapter 9**, where thymol was added to the diet at 1%. In a test of thymol inhibition of *S. Typhimurium*, it was shown that the *in-vitro* dose was 700-fold higher than that required with the antibiotic gentamycin that was also able to inhibit *Salmonella* translocation to pig mesenteric lymph nodes (Modesto *et al.*, 2007). On the basis of the practical concentration of gentamycin in weaning pigs, that of thymol to be added to the diet could be estimated at 4%, but such a high level of thymol would reduce the palatability of the diet, and thus the daily feed intake.

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The DGGE-band corresponding to *Citrobacter freundii* was present in most pigs from groups fed thymol (10 of 15) and only in four pigs (of 14) from the thymol-free groups (**Chapter 9**). Furthermore, the absence of a band corresponding to *Actinobacillus minor* was observed when 1% thymol was in the diet. Interestingly, no effect of thymol on *Salmonella* infection could be noticed, which was in opposite to *in vitro* data (Si et al., 2006a). Thus, limonene, eugenol and pinene fed in amount of 16.8 mg essential oils per kg diet had no effect on the microbiota, and thymol (10 g in kg diet) affected the intestinal microbiota in an unexpected way. The reasons for this are unclear, the absorption time, transit time or interactions with other digesta components all could play a role. Si et al. (2006b) noticed strong antibacterial activity of carvacrol, thymol, cinnamon oil and eugenol *in-vitro*. When added to pig caecal digesta, all the tested oils reduced the growth of the indigenous *E. coli*, exogenous *E. coli* O157:H7 and coliforms present in the digesta, with no effect on lactobacilli (Si et al., 2006b). Sads and Bilkei (2003) and Molnar and Bilkei (2005) observed positive effects of feeding high levels of essential oils in pig diet (60 - 180 mg carvacrol and 55 – 165 mg thymol in kg diet) whereas others observed no effects feeding low levels of phytogetic products (Manzanilla et al., 2004; Kommera et al., 2006; Schöne et al., 2006). Therefore, certain effects occurring first at higher dosage of the essential oil mixture tested in **Chapter 8** can not be excluded.

### ***V.IV Intestinal microbiota under exposition to oligosaccharides***

In the study presented in **Chapter 10** either fructooligosaccharides (1.5% of long chain inulin with 10-60 degree of polymerization) or oligosaccharides from marine algae (0.1% of alginate) were fed to weaning pigs. Similarly to other studies, also here two farms with different sanitary conditions were chosen to follow up the effects of different initial bacterial load, i.e. the commercial (CF) and experimental farm (EF). There were changes in the pH, volatile fatty acids (VFA), LA and ammonia concentrations observed with age, which remained comparable to previously reported findings (Canibe et al., 2001; Franklin et al., 2002; Kluess, 2004). However, the inclusion of inulin or alginate in the diet did not cause significant changes in pH, VFA, LA or ammonia concentrations in the distal SI. Similar to the findings recorded in this study, also other groups reported no changes in the intestinal chemical

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composition after addition of non-fermentable carbohydrates to pigs' diet (Hoebler et al., 2000; Pierce et al., 2005; 2006; Bhandari et al., 2009; Mair et al., 2010).

According to existing literature, inulin is postulated to support LAB such as bifidobacteria and lactobacilli (Tako et al., 2007; Mølbak et al., 2007). In the study presented in the **Chapter 10**, differences in the intestinal lactobacilli from pigs reared either at a commercial or at experimental farm could be observed. When inulin and alginate were added to the diet at CF, the lactobacilli counts remained low up to 5 days after weaning, whereas at EF they increased already on d2 post weaning. These observations confirm in a way the tested hypothesis that different sanitary conditions could affect the outcome of the incorporated prebiotics. Additional confirmation of this thesis provided the finding that the lactobacilli counts measured on d 39 were about 1 log higher at EF than at CF.

The intestinal bifidobacteria seem not be as important for pig as for humans (Mikkelsen et al., 2003; Loh et al., 2006; Eberhard et al., 2007), therefore only the effect of inulin and alginate on overall lactobacilli and selected main intestinal microbial groups such as enterobacteria, enterococci, and yeasts were investigated. The tested prebiotics resulted in no changes in the total counts of these groups, as it has also been reported by other researchers (Howard et al., 1995; Mountzouris et al., 2006; Eberhard et al., 2007; Mair et al., 2010). In opposite to the findings in studies performed on pigs, when 2.5% alginate oligosaccharides was fed to rats, an increase of the number of lactobacilli and bifidobacteria was recorded (Wang et al., 2006), providing further evidence that the porcine intestinal microbiota might be much more diverse than in other species (Böhmer et al., 2005). Obviously, the tested amounts of the oligosaccharides were far too low to result in noticeably shift of the intestinal microbial population.

Interestingly, a strong reduction in the yeast counts was observed in pigs from CF fed inulin. In face of no differences in intestinal lactic acid and pH between groups, there is no plausible explanation for this finding, as increased acidity and lactic acid concentration could explain the inhibition of the growth of yeasts. Furthermore, as it has been already discussed in **Chapters 5** and **6**, the knowledge on the presence and role of yeasts in the pig intestine remains scarce, therefore no conclusions on the impact of these changes for the host can be drawn yet.

## **V.V Husbandry conditions**

## General discussion

Reading the reports on the changes within the intestinal microbial community caused by prebiotics, probiotics or plant active compounds many discrepancies in the provided results can be found (e.g. a review by Lallés et al., 2009). Furthermore, the literature provides evidences that the alternatives to the in-feed antibiotics may result in improvement of the overall health status and performance of a herd with chronic infectious problems (Sads and Bilkei, 2003; Molnar and Bilkei, 2005) but no beneficiary effects can be observed when studies are performed under experimental conditions (Lin, 2011). In the studies presented in **Chapters 5, 6, 8 and 10** this issue was a particular part of the studies. It could be demonstrated that different results can be obtained, when the piglets are reared under experimental or commercial conditions, while keeping the same experimental design. For instance, the microbiota in the intestine of the piglets from a CF seemed to be more stable and less affected by the EO than at the EF, where higher husbandry conditions could be recorded. The dendrogram of the DGGE fingerprints representing the intestinal microbial profiles from the individual piglets also showed clusters distinct for each farm (**Chapter 8**). In face of these findings a question arises if an increase of the sanitary conditions by implementation of improved feeding and cleaning regimes would provide more beneficiary effects for the health and performance of weaning animal than wide use of pre-, probiotics or plant active components in the feed.

### ***V.VI Future perspectives***

Mammals are born with sterile internal body surfaces. Bacterial colonisation takes place immediately at birth with most of the organisms being derived from the mother's vagina and faeces, but also from mother's milk (Bauer et al., 2006) and undergoes dynamic changes caused by stress and diet. It has been shown that gut bacteria directly modulate the utilisation of food (Hooper et al., 2002), implying that the true "calorific value" of food is variable depending on the microbiota composition and its ability to mobilise potential energy sources for the host. The commensal microbiota is pivotal for degradation of otherwise indigestible complex carbohydrates, education and programming of the neonatal immune system, competitive exclusion of pathogens, production of antimicrobials, production of vitamins etc. Therefore the

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intestinal microbiota and its dynamics caused by dietary components shall be further in the focus of the future research.

After a decade of cultivation-independent surveys using 16S rRNA-targeted molecular approaches, we have now started to appreciate the immense complexity of the GIT microbiota in its full extent, acknowledging that the microbiota is in constant interplay with its host, and can thus be considered as an extra, ancillary organ (Bäckhed et al., 2005; Zoetendal et al., 2006). High-throughput approaches have been continuously developed, which allow even better and more detailed investigating of the impact of dietary compounds on the intestinal microbiota (He et al., 2010). In example, a recently developed microarray allowing characterization of pig GIT microbiota, detecting over 600 phylotypes, and being continuously updated, would further help to get much better insight into the changes of the microbial population caused by dietary components (Perez Gutierrez, 2010).

In the presented thesis new data on the composition of the porcine intestinal microbiota has been collected. Especially the changes within the lactobacilli population were in the focus. Intestinal yeasts were characterized and shown to correlate with the lactobacilli, and the relationships between these microbial groups should be further elucidated. Based on the findings presented in the thesis novel target organisms as markers of health status of pigs, e.g. *Lactobacillus sobrius* or *Kazachstania sloofiae*, can be selected.

Husbandry conditions affecting the intestinal microbiota in the pig herd must be taken into consideration when animal studies on alternatives to in-feed antibiotics are to be performed. It would be the best to perform more parallel trials under different husbandry conditions with well defined sanitary regimes to elucidate the appropriateness of use of the alternatives to in-feed antibiotics, and their needed dietary levels.

Alternatives to in-feed antibiotics do not necessarily have to act on the intestinal microbiota in order to increase the performance of growing animals. Direct action on e.g. animals' biochemical pathways, activation of different receptors with effects on the neuroendocrine pathways can not be excluded. Therefore, combined studies



## **General discussion**

covering additional research fields such as biochemistry, physiology, immunology, microbiology, or histology are needed, to elucidate the effects of alternatives to in-feed antibiotics and the potential interrelationships between the microbiota and host, modulated by these substances.

Finally, with regard to the practice, which expects healthy pigs with high performance, the economy of the use of such alternatives must be calculated and compared with the economy of other approaches that provide the same result, such as improved husbandry regimes.

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## Summary

### Summary

The gastrointestinal tract of pigs is inhabited by a diverse, very complex and dynamic microbiota, which plays a major role for the host health. It builds the first barrier against pathogens, supplies the host with nutrients and simulates the development of its intestinal and systemic immunity. After the colonization shortly after birth, a co-evolution takes place, resulting in individual communities. Disturbances in this process can challenge the integrity of the microbial ecosystem, and weaning is one of the time-points, when enormous imbalance of the microbiota can occur. The in-feed antibiotics helped to stabilize the intestinal microbiota, but in face of the increased resistance they had to be banned from the pig feeding and alternatives are to be found.

This thesis provides data for reconsideration of the use of antibiotics as prophylactic agents at birth, as the intestinal microbiota remains affected even 6 weeks after the parenteral administration of a broad-spectrum antibiotic. The composition of pig intestinal lactobacilli population is provided in more detail based on molecular studies. The application of group-specific denaturing gradient gel electrophoresis (DGGE) showed that the *Lactobacillus* community within the porcine ileum undergoes dramatic, partly reversible changes as a consequence of weaning. The adaptation of the lactobacilli to carbohydrates is presented at species level. The thesis describes the yeasts resident in the porcine intestine, their diversity and development. Dominating species of yeasts are determined.

With regard to the search after promising alternatives to in-feed antibiotics, prebiotics (inulin and sodium alginate), potential probiotic *Lactobacillus plantarum* and active plant components such as essential oils (thymol, eugenol, limonene, and pinene) and their effects on the microbial diversity are presented. Cultivation and application of molecular techniques allowed following the microbial changes caused by these dietary supplements. Supplementation of pig diet with thymol caused clear changes in small intestine microbial community without effects on *Salmonella* infection. Moreover, it was postulated that husbandry conditions could affect the outcome of the studies, which was confirmed by the thesis, as the tested prebiotics caused more pronounced changes on a commercial farm, with lower sanitary conditions than at an experimental farm.



## Summary

High-throughput approaches have been continuously developed, which allow even better and more detailed investigating of the impact of dietary compounds on the intestinal microbiota. However, the consequences of the dietary supplements on the biochemistry, physiology of microbiota, the intestinal morphology and immunity still remain unexplored. Combined studies covering multiple research areas including next to the intestinal ecology the intestinal physiology, immunology, or histology are needed to elucidate the dietary effects and interrelationships between the microbiota and the host. Finally, the economy of the use of the active components in pig industry must not be forgotten, and compared with improved sanitary regimes in order to improve the health of weanling piglets.

## Zusammenfassung

### Zusammenfassung

Der Magen-Darm-Trakt von Schweinen ist durch ein vielfältiges, sehr komplexes und dynamisches Mikrobiota bewohnt, das eine wichtige Rolle für die Gesundheit des Wirtes spielt. Die Mikroorganismen bilden die erste Barriere gegen die Krankheitserreger, liefern dem Gastgeber Nährstoffen und stimulieren die Entwicklung des mukosalen und systemischen Immunsystems. Nach der Kolonisation kurz nach der Geburt findet eine Co-Evolution statt, die zu für Einzeltiere spezifischen mikrobiellen Gemeinschaften führt. Jegliche Ablenkungen in diesem Prozess bedeuten für das Mikrobiota eine Herausforderung, die Integrität des mikrobiellen Ökosystems zu erhalten. Das Absetzen gehört zu einem der Zeitpunkte, wenn ein enormes Ungleichgewicht des Darmmikrobiota auftreten kann. Die den Futtermitteln beigefügte Antibiotika haben über lange Zeit dazu beigetragen, die Darmflora zu stabilisieren, aber angesichts der Entwicklung der Resistenzen wurden sie in der Schweinefütterung verboten. Deshalb begann die Suche nach Alternativen.

Diese Thesis liefert Daten zur Überprüfung der prophylaktischen Verwendung von Antibiotika bei der Geburt, da das Darmmikrobiota kann sogar bis 6 Wochen nach der parenteralen Gabe eines Breitband-Antibiotikum betroffen bleiben. Die Zusammensetzung der Population der Laktobazillen in porcinem Darm wurde in Untersuchungen auf Molekularbasis untersucht. Die Anwendung der gruppenspezifischen denaturierenden Gradienten-Gelelektrophorese (DGGE) zeigte, dass die Lactobacillen, die im Ileum der Schweine vorkommen, dramatischen, teils reversiblen Veränderungen als Folge des Absetzens Erleben. Die Anpassung der Laktobazillen zu Kohlenhydraten ist am Spezies-Level präsentiert. Die Thesis beschreibt weiterhin die Zusammensetzung, die Vielfalt und Entwicklung der Hefen, die den Darm der Schweine besiedeln. Die dominierenden Spezies der Hefen wurden bestimmt.

Im Hinblick auf die Suche nach vielversprechenden Alternativen zu Antibiotika in Futtermitteln, Präbiotika (Inulin und Natriumalginat), potenziell probiotischwirkendes *Lactobacillus plantarum* und aktive Pflanzenprodukte wie ätherische Öle (Thymol, Eugenol, Limonen und Pinen) und ihre Auswirkungen auf die mikrobielle Vielfalt werden dargestellt. Die Anwendung von molekularen Verfahren erlaubt das Verfolgen der mikrobiellen Veränderungen, die durch diese

## Zusammenfassung

Nahrungsergänzungsmittel verursacht wurden. Die Supplementierung der Nahrung der Ferkel mit Thymol verursachte deutliche Veränderungen der mikrobiellen Gemeinschaft im Dünndarm, ohne Auswirkungen auf die Infektion mit Salmonellen. Eine der Thesen war es, dass Haltungsbedingungen das Ergebnis der Studien beeinflussen könnten. Diese These bestätigte diese Vermutung, da die getesteten Präbiotika verursachten stärker ausgeprägten Veränderungen wenn die Ferkel auf kommerzieller Farm, mit geringeren hygienischen Bedingungen, gehalten wurden, mit Vergleich zu einer experimentellen Farm.

High-Throughput-Ansätze werden kontinuierlich weiterentwickelt, somit auch die Auswirkungen der Ernährung auf die Zusammensetzung der Darmmikrobiota noch besser und detaillierter untersucht werden können. Jedoch die Folgen der Nahrungsergänzungsmittel auf die Biochemie, Physiologie der Mikroorganismen, die Morphologie des Darmes und die Immunität bleiben immer noch unerforscht. Kombinierte Studien, die über mehrere Forschungsbereiche hinausgehen, die neben der Ökologie des Darmes und der intestinalen Physiologie z.B. auch die Immunologie, Histologie erfassen, sind nötig, um die diätetischen Effekte und Interaktionen zwischen der Mikrobiota und dem Wirt aufzuklären. Schließlich darf die Wirtschaftlichkeit des Einsatzes der aktiven Komponenten in der Schweineindustrie nicht vergessen werden, und mit verbesserten sanitären Regimes verglichen werden, um die Gesundheit der abgesetzten Ferkel stets zu verbessern.

**Alternatives to in-feed antibiotics  
and their effects on development and stability of intestinal  
microbial community in weaning piglets**

Thesen zur  
Habilitationsschrift

Aus der Agrar- und Umweltwissenschaftlichen Fakultät  
der Universität Rostock

zur Erlangung des akademischen Grades  
Doctor medicinae veterinariae habilitatus (Dr. med. vet. habil.)

vorgelegt von

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Rostock, 08.04.2011

## Theses

It is a common practice on a pig farm to wean the piglets from their dams abruptly at early age of 21 or 28 days of life. The piglets are susceptible to many sub-clinical and clinical infections at this time, as their immune system is still not mature, and additionally the change from liquid feed (the milk) to a solid diet is associated with anatomical and physiological changes in the gastrointestinal tract (GIT). As result, diarrhoea with accompanying reduction of performance occurs. Thus far, the disorders of the GIT during the weaning period were controlled by introducing sub-therapeutic levels of antibiotics in the feed. Many pathogens have developed cross-resistance to the antibiotics, what led to the ban of the in-feed antibiotics in the European Union since 1 January 2006. The antibiotics have been further widely used as prophylaxis and they are commonly applied to piglets at or shortly after birth. This practice can lead to changes in the intestinal microbial population and thus bias the outcome of dietary regimes.

Despite years of use of in-feed antibiotics, relatively little knowledge on the composition of the microbiota harbouring the porcine GIT has been collected. Quantitative changes within main microbial groups have been investigated, but qualitative changes remained beyond the scope of many studies. Furthermore, the underlying mechanisms explaining why the in-feed antibiotics promoted the growth of animals still remains unclear. This was an obvious disadvantage when the search after alternatives to in-feed antibiotics started among very different groups of components, such as probiotics, prebiotics, plant bioactive components. Furthermore, controversial results have been recorded between different *in-vivo* studies, as well as in comparison to *in-vitro* results, without clear clues for the reasons.

The aim of the studies presented in this thesis was to obtain more detailed insight into the qualitative and quantitative changes within the microbial population harbouring the porcine intestine, with emphasis on lactobacilli, which are agreed to beneficially affect the host. Furthermore, it was to investigate if potential alternatives to in-feed antibiotics would show any effects on the intestinal ecology. The following questions were to be answered:

## Theses

- Do parenteral antibiotics affect the gastrointestinal microbiota in piglets, when the prophylactic administration is performed at birth?
- What are the physiological changes in the lactobacilli community of the porcine intestine around weaning? Which species are affected?
- Are the particular species of the gastrointestinal lactobacilli able to adapt to ferment different dietary carbohydrates introduced after weaning?
- What is the kinetics of the changes of other dominating bacteria caused by weaning?
- What is the diversity of porcine intestinal yeasts and are there correlations to bacteria?
- Does *Lactobacillus plantarum* affect the intestinal microbiota? If yes, then would the changes allow considering it as a possible probiotic? What would be the best time-point to administer it to the weaning piglets?
- Can the *in-vitro* observation on antimicrobial activity of essential oils be confirmed *in-vivo*? Do the essential oils affect the microbial activity with concomitant changes in the intestinal chemical composition?
- Do different oligosaccharides have effect on the intestinal microbial activity and composition?
- Do sanitary conditions affect the outcome of the dietary studies?

To collect data allowing answering these questions studies on pigs were performed. The contents from different segments of the small and large intestine were collected, at different time-points around weaning. Chemical analyses, cultivation of main microbial groups as well as cultivation-independent molecular techniques were applied. Regarding the results from the performed studies following theses can be drawn:

1. Prophylactic administration of antibiotics in newborn pigs shall be reconsidered for its sense as it may result in destruction of the intestinal microbial balance and thus result in the opposite to expected effect.
2. The intestinal *Lactobacillus* community undergoes dramatic quantitative and qualitative, partly reversible changes caused by weaning. These changes can help the pathogens to colonize the gastrointestinal tract.

## Theses

3. The porcine intestinal populations of enterobacteria, enterococci and yeasts undergo quantitative changes caused by weaning and adaptation to solid feed.
4. Intestinal lactobacilli adapt their abilities to ferment complex carbohydrates after introduction of solid feed. This could be a target for dietary strategies aiming at stabilization and selective support of the members of *Lactobacillus* community.
5. Porcine intestine harbours very diverse population of yeasts. *Kazachstania sloofiae* dominates this community and possibly positively interacts with intestinal lactobacilli.
6. The development of the yeast community in the porcine intestine depends on the introduction of solid feed.
7. There are possible further interactions between intestinal yeast and bacterial populations which need to be explored in more detail.
8. *Lactobacillus plantarum* administered to piglets at weaning can affect the intestinal microbial community with potential positive effect on the gastrointestinal health.
9. Effect of essential oils on intestinal microbial community depends on the origin of the oils and the administered amount.
10. Dietary oligosaccharides can result in increase of richness and diversity of colonic microbiota which can be regarded as beneficiary for the host. However, they do not necessarily improve the performance of the weaning piglets.
11. Development of the intestinal microbial community and its changes caused by dietary components depend on husbandry and sanitary conditions.

## Theses

In the presented thesis new data on the composition of the porcine intestinal microbiota has been collected. Based on these findings novel target organisms for markers of health status can be selected. Combination of the classic cultivation methods and cultivation-independent high-throughput molecular techniques provide a good tool for the investigations on effects on dietary supplements and their use should be further improved and developed.

Further studies are needed to establish a proper dosage of different alternatives to in-feed antibiotics. Combined studies on the biochemistry and physiology of the microbiota in face of the nutrients or supplements will provide better understanding of underlying mechanisms and mode of action. Furthermore, more data is needed on interaction between the microorganisms and intestinal physiology, immunology, or histological changes. Finally, the economy of the use of the active components in pig industry must not be forgotten, and compared with improved sanitary regimes in order to improve the health of weanling piglets.



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## Selbstständigkeitserklärung

Hiermit versichere ich, die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst zu haben, keine anderen als die von mir angegebene Quellen und Hilfsmittel benutzt und die herangezogenen Werke wörtlich oder sinngemäß entnommenen Stellen als solche gekennzeichnet zu haben.

Berlin, 08.03.2011

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