Characterization of the bacterial gut microbiota in new neonatal porcine diarrhoea

Hermann-Bank, Marie Louise

Publication date:
2014

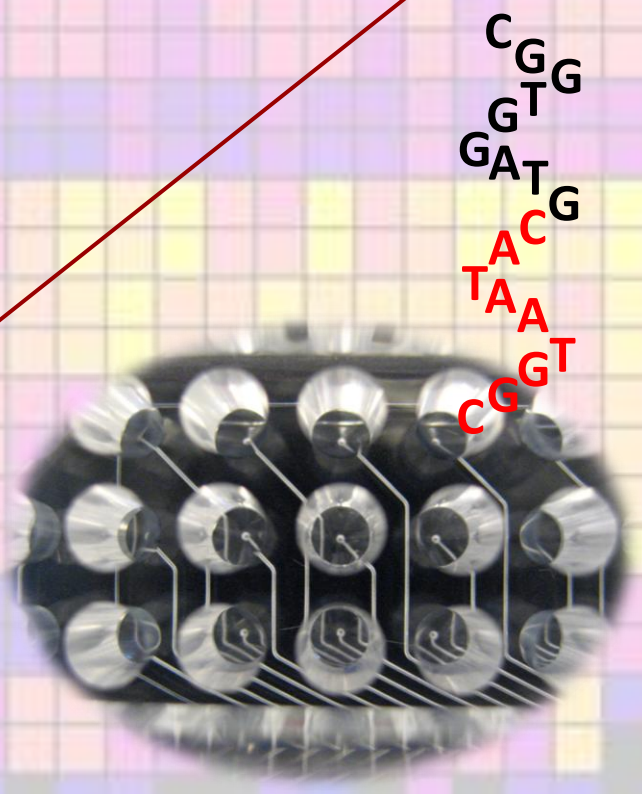
Document Version
Publisher's PDF, also known as Version of record

Citation (APA):
Hermann-Bank, M. L. (2014). *Characterization of the bacterial gut microbiota in new neonatal porcine diarrhoea*. Technical University of Denmark.

Characterization of the bacterial gut microbiota in new neonatal porcine diarrhoea

PhD Thesis by Marie Louise Hermann-Bank

November 2014



DTU Vet
National Veterinary Institute

Characterization of the bacterial gut microbiota in new neonatal porcine diarrhoea

PhD thesis by

Marie Louise Hermann-Bank

November 2014

Microbial Ecology Group
Section of Bacteriology, Pathology, and Parasitology
National Veterinary Institute
Technical University of Denmark

Supervisors

Senior scientist Kerstin Skovgaard
Innate Immunology Group
Section for Immunology and Vaccinology
Technical University of Denmark

Professor Mette Boye
Microbial Ecology Group
Section of Bacteriology, Pathology, and Parasitology
National Veterinary Institute
Technical University of Denmark

R&D Manager Lars Mølbak
Chr. Hansen A/S
Plant Health & Production
Denmark

Assessment committee

Senior scientist Kirstine Klitgaard Schou
Microbial Ecology Group
Section of Bacteriology, Pathology, and Parasitology
National Veterinary Institute
Technical University of Denmark

Professor Magdalena Jacobsen
Department of Clinical Sciences
Swedish University of Agricultural Sciences

Associate professor Dennis Sandris Nielsen
Department of Food Science
University of Copenhagen

Front page: piglet cuddling up with its siblings (from one of the herds included in the study); close-up of Access Array 48.48 sample inlets with partial 16S rRNA gene primer sequence and elongation (Enterococcus hirae). Photos: author. Background: heatmap generated from analyzing piglets' intestinal contents by use of the Gut Microbiotassay.

Preface

This thesis presents my research during my employment at Technical University of Denmark, DTU, The National Veterinary Institute, Section of Bacteriology, Pathology, and Parasitology, Microbial Ecology group. The entire work was conducted at The National Veterinary Institute through the period July 2010 to November 2014 under the academic supervision of senior scientist Lars Mølbak and Professor Mette Boye, from Section for Bacteriology, Pathology and Parasitology and from March 2012 senior scientist Kerstin Skovgaard from Section for Immunology and Vaccinology.

This thesis was part of an interdisciplinary research project investigating the epidemiology, aetiology, and pathology of 'new neonatal porcine diarrhoea' (NNPD) recently emerged in Denmark. The major project was established in collaboration between The National Veterinary Institute, Technical University of Denmark, DTU; Danish Pig Research Centre Danish Agriculture and Food Council; and Centre for Herd-oriented Education, Research and Development, HERD, University of Copenhagen, UCPH. The overall aim of this alliance was to detect and determine the aetiology behind NNPD in order to identify preventative interventions or treatment plans.

At no point has the search been trivial or boring. In a process like this, you come to admire the work of previous scientists, and acknowledge the hard toil which must underlie any diagnosis. It has truly been an educational journey all the way through!

The project was funded by the Act on Innovation no. 421 from 31/05/2000 granted by the Ministry of Food, Agriculture and Fisheries of Denmark, National Veterinary Institute, Technical University of Denmark, and the Danish Pig Research Centre.

The presented dissertation has been written and submitted as part of achieving the degree of Philosophiae Doctor, PhD, at the Technical University of Denmark, DTU.

28/11-2014 Marie Louise Hermann-Bank

Marie Louise Hermann-Bank, November 2014.

Acknowledgement

I would like to express my profound appreciation to each and everyone who have contributed and helped in any way to the conception of this thesis. First and foremost I would like to thank all my supervisors. Each of you has always had an optimistic attitude and a positive approach to my work in progress and to the project in general. This positivity has inspired me continuously. To my initial main supervisor Lars Mølbak thanks for great feedback and good discussions. I still benefit from the constructive and guided supervision you provided during your time at The National Veterinary Institute. A special thanks to Kerstin Skovgaard for offering to be my main supervisor when Lars Mølbak moved on to new challenges. I could not have done this without your help, your guidance and support. Great thanks to Mette Boye for her beneficial co-supervision, especially during the last six month of my project.

I owe thanks to all my co-authors for their essential contributions to the manuscripts included in this thesis. Special thanks to Anders Stockmarr for his time, patience, and effort helping me out with the statistics. Also great thanks to Niels Larsen for customizing BION-meta to my experimental setup.

I appreciate the help given by the technicians. Thanks to Sophia Rasmussen, for her thorough work and assistance in the laboratory and to Karin Tarp who also has been of great assistance in the laboratory.

Thanks to all people involved in the NNPD project for a fruitful collaboration. Also my colleagues that I have had the pleasure of coming to know during my time at the National Veterinary Institute deserve thanks for their support. I have appreciated our intellectual as well as non-intellectual discussions.

Last but not least I would like to thank my family and friend for being there to share my thoughts, successes and frustrations during my time as a PhD student. Thanks to my mother Anne and her husband Torben, for always believing in me, and encouraging me to follow my dreams. Always letting me know, that I as good as anybody can accomplish and reach my goals. Thanks to my daughter Gabriella for opening my eyes to the wonder of life, putting everything in perspectives, and reminding me that there is more to life than research. To my husband Henrik, thank you so much for supporting me through good and bad times, for cheering me on, and giving me valuable feedback and reflections whenever I needed it. I dedicate this thesis to you.

Summary

During the last decade farmers and veterinarians have reported the emergence of a new neonatal porcine diarrhoea (NNPD) affecting piglets up to 7 days old. Routine laboratory testing for common pathogens are inconclusive and vaccination and treatment with antibiotics or alternative zootechnical interventions have limited effect. NNPD is not associated with an increased mortality, but have been reported to cause significant morbidity within herds and litters. Piglets born to gilts are in particularly affected by NNPD. NNPD impairs the welfare of the piglets, and results in decreased weight gain which is of economic importance to the farmer. Despite the limited effect of antibiotics, farmers often treat affected piglets with antibiotics to prevent secondary infections to NNPD resulting in increased consumption of antibiotics. Thus, there are several encouraging reasons for identifying the aetiology behind NNPD. Consequently an interdisciplinary project called: "New neonatal porcine diarrhoea in Denmark. Elucidation of aetiology, diagnostics, and effect of treatments" (freely translated) was initiated. The project enrolled three PhD students with different approaches and hypotheses. The aim of this project was to investigate whether the aetiology to NNPD could be identified in the bacterial gut microbial changes.

In order to be able to characterize the bacterial gut microbiota of numerous samples simultaneously the Gut Microbiotassay was developed. This is an assembly of 24 different primer sets targeting 16S or 23S rRNA genes of the major bacterial groups constituting the gut microbiota. This approach was applied due to the limited number of intestinal bacteria that currently can be cultivated. Primers were found in published literature, tested *in silico* and modified or designed if necessary. The Gut Microbiotassay was optimized for the high-throughput quantitative real-time PCR-based 48.48 Access Array™ Integrated Fluidic Circuit (Fluidigm). The efficiency and sensitivity of the primer sets were tested against 15 different pure-cultured bacterial strains. Finally the Gut Microbiotassay was tested on DNA extracted from ileal or colonic contents from piglets with or without NNPD and verified via 454 next generation sequencing of the PCR amplicons. Bioinformatics was conducted using BION-meta customized for this specific setup.

With the Gut Microbiotassay in place gut microbial profiles of ileal and colonic contents of 50 control piglets and 52 case piglets from four Danish pig farms affected by NNPD were obtained and deeper taxonomic insight was acquired by sequencing the PCR amplicons. Statistic results from qPCR data revealed that the gut microbiota of NNPD-affected piglets differed from that of control piglets by a depletion of the phyla Firmicutes, Bacteroidetes, and Actinobacteria, while the numbers of genus *Enterococcus* and the class Beta- and Gammaproteobacteria (including family Enterobacteriaceae and species *Escherichia coli*), but also phylum Fusobacteria were elevated. Moreover, piglet born to gilts possessed more members from family Enterobacteriaceae including species *E. coli* and a reduced number of bacteria from phylum

Firmicutes. Piglets born to gilts were estimated to have 25 higher odds of being affected by NNPD. Sequence results revealed genus *Enterococcus* to be comprised of high read numbers of species *Enterococcus hirae* but also *Enterococcus durans.* Conversely, particularly *Lactobacillus acidophilus* was scarcely represented in piglets suffering from NNPD.

As part of one of the other enrolled PhD projects a NNPD-infection model was established by inoculating healthy neonate piglets with intestinal NNPD-material (case piglets) or healthy intestinal material (control piglets), while some piglets not were inoculated. Diarrhoea was successfully reproduced in case piglets while control piglets remained healthy. In order to assess whether the diarrhoea was characterized by similar gut microbial changes as detected for field cases of NNPD, ileal and colonic intestinal contents from 49 case piglets (13 un-inoculated) and 32 control piglets (18 un-inoculated) were analyzed using the Gut Microbiotassay. The corresponding regulation of selected intestinal genes involved in diarrhoea was examined for a subset of piglets by qPCR using the 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm). Similar to NNPD-field cases the gut microbiota of case piglets were characterized by reduced numbers of the phyla Firmicutes, Bacteroidetes, and Actinobacteria. Furthermore, they were inhabited by increased numbers of genus *Enterococcus* as well as class Beta- and Gammaproteobacteria including species *E. coli*. The expression of several genes involved in recognition of pathogen-associated molecular patterns, inflammation, and intestinal barrier function were significantly up- or down-regulated reflecting the complex immunological response to being inoculated and/or infected with NNPD-material. Finally, a high abundance of genus *Enterococcus* (characteristic of case piglets) was associated with high expressions of several transcripts involved in epithelial integrity.

Altogether, the results of the studies included in this thesis reveal that NNPD is associated with a disturbed gut microbial composition, and all points towards members from the genus *Enterococcus* are involved in the pathogenesis of NNPD.

Dansk sammendrag

Gennem det seneste årti har griseavlere og dyrlæger rapporteret om forekomsten af en spædgrisediarré (ny neonatal porcin diarré, NNPD), der afficerer spædgrise op til 7 dage gamle. Rutine laboratorietests er negative for kendte patogener, og vaccination samt behandling med antibiotika eller alternative zootekniske interventioner har begrænset effekt. NNPD er ikke associeret med en øget dødelighed, men kan forårsage betydelig morbiditet indenfor besætninger samt kuld. Det er i særdeleshed spædgrise født af gylte, der bliver afficeret af NNPD. NNPD forringer spædgrisenes velfærd og resulterer i nedsat tilvækst, som er af økonomisk betydning for griseavlerne. På trods af antibiotikas begrænsede effekt, behandler svineavlere ofte afficerede dyr for at forebygge sekundære infektioner til NNPD, hvilket resulterer i et øget antibiotikaforbrug. Således er der adskillige gode grunde til at identificere årsagen til NNPD. Følgelig blev et tværvidenskabeligt projekt startet op: ”Ny spædgrisediarre i Danmark. Afklaring af årsagsforhold og diagnostik samt effekt af behandlinger”. Projektet omfattede tre ph.d.-studier med hver deres tilgang og hypoteser. Formålet med dette projekt var at undersøge om årsagen til NNPD kunne findes i ændringer i tarmmikrobiotaens sammensætning.

For at kunne karakterisere tarmmikrobiotaen fra adskillige prøver samtidig, blev der udviklet et panel af 24 forskellige primersæt rettet mod 16S eller 23S rRNA generne fra de væsentligste bakterielle grupper, som udgør tarmmikrobiotaen. Denne tilgang blev anvendt på grund af det begrænsede antal tarmbakterier, som på nuværende tidspunkt kan detekteres ved dyrkning. Primerne blev fundet i publiceret litteratur, testet *in silico* og, om nødvendig, modificeret eller designet. Gut Microbiotassay blev optimeret til 48.48 Access Array™ Integrated Fluidic Circuit (Fluidigm), en høj-kapacitets kvantitativ PCR chip. Effektiviteten og sensitiviteten af primersættene blev testet imod 15 forskellige rendyrkede bakteriestammer. Endelig blev Gut Microbiotassay testet på DNA ekstraheret fra tarmindhold fra ileum og colon fra grise med og uden NNPD og verificeret ved 454 næste generations sekventering af PCR produkterne. BION-meta, tilpasset dette specifikke opsæt, blev anvendt til at analysere sekventeringsdata.

Efter at Gut Microbiotassay var blevet verificeret, blev tarmmikrobiotaen fra ileum- og colonindhold karakteriseret fra 50 kontrolgrise og 52 NNPD-grise fra fire forskellige besætninger afficeret af NNPD. Yderligere taksonomisk information blev genereret ved at sekventere PCR produkterne. Statistik resultater genereret fra qPCR-data afslørede, at tarmmikrobiotaen fra NNPD-afficerede grise adskilte sig fra kontrolgrisenes ved et formindsket antal af bakterier fra Firmicutes, Bacteroidetes og Aktinobakterier, mens antallet af Enterokokker, og phyla Gammaproteobakterier (inklusiv familie Enterobakteriaceae og species *Escherichia coli*), men også Fusobakterier var forhøjet. Ydermere havde grise født af gylte flere bakterier fra familie Enterobakteriaceae inklusiv species *E. coli* samt et reduceret antal fra phylum

Firmicutes. Grise født af gylte var estimeret til at have 25 gange højere odds for at være afficeret af NNPD. Sekvensresultater afslørede at genus *Enterococcus* bestod af et højt antal af sekvenser klassificeret som species *Enterococcus hirae*, men også *Enterococcus durans*. Omvendt var især *Lactobacillus acidophilus* sparsomt repræsenteret i NNPD-afficerede grise.

Som del af et af de andre ph.d.-projekter blev der etableret en NNPD-infektionsmodel ved at pode raske grise med NNPD-tarmmateriale (NNPD-inficerede grise) eller med raskt tarmmateriale (kontrolgrise), mens nogle grise ikke blev podet. Reproduktionen lykkedes, underforstået at NNPD-inficerede grise udviklede diarré, og kontrolgrise forblev raske. For at vurdere om diarréen var karakteriseret af lignende ændringer i tarmmikrobiotaen, som fundet i de naturligt NNPD-afficerede grise, blev tarmindhold fra ileum og colon fra 49 NNPD-inficerede grise (hvoraf 13 ikke var podet) og 32 kontrol grise (hvoraf 18 ikke var inokuleret) analyseret med Gut Microbiotassay. Derudover blev ekspressionen af udvalgte gener involveret i diarré undersøgt i et udpluk af grise ved hjælp af kvantitativ PCR ved brug af 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm). Ligesom hos NNPD-afficerede grise var tarmmikrobiotaen fra NNPD-inficerede grise karakteriseret af et øget antal af bakterier fra genus *Enterococcus* og klasse Beta- og Gammaproteobakterier inklusiv species *E. coli*. Ekspressionen af adskillige gener relateret til mønstergenkendende receptorer, inflammation og tarmepitelsintegritet var signifikant op- eller ned-reguleret, hvilket afspejler det komplekse immunologiske respons på at blive podet og/eller inficeret med NNPD-materiale. Endelig var et højt antal Enterokokker (karakteristisk for NNPD-inficerede grise) associeret med en høj ekspression af flere transskripter involveret i tarmepitelintegriteten i ileum og colon.

Samlet set viser resultaterne fra studierne inkluderet i denne afhandling, at NNPD er associeret med en ændring i sammensætningen af tarmmikrobiotaen, og det tyder på at bakterier fra genus *Enterococcus* er involveret i patogenesen bag NNPD.

Introduction and objectives

Would it not be nice if it was possible to link a specific microorganism to a disease of unknown aetiology and thereby be able to conclude: this is the traitor; this is the agent we have to get rid of and conquer in order to treat and prevent the disease? However, real life is not that simple. Many disorders are multifactorial which complicates the diagnosis. So what to do?

This was in fact the background of this thesis which is part of a bigger project that was initiated back in 2010, after several years of reported incidences of the emergence of a new type of neonatal porcine diarrhoea affecting piglets up to 7 days old. Common diagnostic tests were inconclusive, antibiotics and vaccination had limited effect, and additional zootechnical interventions did not prevent the occurrence of diarrhoea. The diarrhoea became known as 'new neonatal porcine diarrhoea' (NNPD) [1]. Various approaches were put in to use with the aim of elucidating the aetiology behind NNPD. So far the project has investigated the clinical, pathological, histopathological (including examination for parasites), viral, and microbiological features of NNPD [1,2,3; Larsen LE, Nielsen JP, unpublished results].

The objective of this thesis was to investigate the bacterial constituents of the gut microbiota of piglets with and without NNPD by use of DNA technology (Paper II). This approach was based on the hypothesis that NNPD may be caused by a new not yet detected or acknowledged microbial pathogen; or it may be a consequence of a general problem with the succession and establishment of the microbiota resulting in an imbalanced gut microbiota. In order to investigate this, an intermediate aim was to develop a quantitative method with the capacity to analyze numerous samples for multiple bacterial groups simultaneously, in order to compare bacterial profiles of luminal intestinal content from diarrhoeic and healthy piglets (Paper I). Finally, a NNPD infection model was established as part of another NNPD PhD project. In order to assess whether the NNPD infection model was characterized by similar gut microbial changes as detected in NNPD field cases, gut microbial profiles were obtained from case piglets (inoculated with NNPD-material or un-inoculated infected with NNPD) and control piglets (inoculated with healthy material or un-inoculated) using the Gut Microbiotassay. Furthermore, correlated immunological gene expression locally in the gut was investigated in order to study the effect of inoculation with NNPD-material on host transcriptional response (Paper III).

The first part of this thesis is a theoretical introduction which covers the following: basic anatomy and immunity of the gut; the normal bacterial colonization of the neonate gut; the pathophysiology, predisposing factors, and symptoms of diarrhoea; recognized bacterial diarrhoeic agents in piglets, and diarrhoea control measures. The second part describes the methodologies used in the experimental part of

the studies included in this thesis in addition to considerations en route. Finally, the research results are presented in three manuscripts, one of which is published. The last part includes discussion of the methods approach and the achieved results, conclusions, and perspectives/reflections drawn from the experience acquired during the conception of this thesis.

This thesis exclusively focuses on and refers to neonatal piglets as piglets up to 7 days old, since this is the age group affected by NNPD.

List of manuscripts

Paper I

Hermann-Bank ML, Skovgaard K, Stockmarr A, Larsen N, Mølbak L. (2013). The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity. *BMC Genomics*, 14:1-14.

Paper II

Hermann-Bank ML, Skovgaard K, Stockmarr A, Strube ML, Larsen N, Kongsted H, Ingerslev HC, Mølbak L, Boye M (2014). Characterization of the bacterial gut microbiota of piglets suffering from new neonatal porcine diarrhoea. Submitted to BMC Veterinary Research.

Paper III

Hermann-Bank ML, Stockmarr A, Jonach B, Jensen TK, Strube ML, Heegaard PMH, Boye M, Skovgaard K. The effect of fecal inoculation with NNPD-material on bacterial gut microbiota composition and immune-related gene expression in neonate piglets. Manuscript in preparation.

Not included in this thesis

Paper IV

Pedersen R, Andersen AD, **Hermann-Bank ML**, Stagsted J, Boye M. (2013). The effect of high-fat diet on the composition of the gut microbiota in cloned and non-cloned pigs of lean and obese phenotype. Gut Microbes, 4(5):371-381.

Abbreviations

454BL	454 Barcode Library
AA48.48	48.48 Access Array™ Integrated Fluidic Circuit
AA	aggregative adherence
AAD	antibiotic-associated diarrhoea
AAF	aggregative adherence fimbriae
A/E	attaching and effacing
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CHR	central husbandry register
CPE	*Clostridium perfringens* enterotoxin
CPB2	*Clostridium perfringens* beta2 toxin
Cq	quantification cycle
DA48.48	Dynamic Array 48.48
DAEC	diffusely adherent *E. coli*
DGGE	denaturing gradient gel electrophoresis
EAEC	enteroaggregative *E. coli*
EAF	EPEC adherent factor
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
FISH	Fluorescence in situ hybridization
GALT	gut-associated lymphoid tissue
GID	gastrointestinal disorders
ID	infectious dose
IFC	integrated fluidic circuit
IHC	immunohistochemistry
IP	incubation period
IPC	interplate calibrator
LPS	lipopolysaccharide
LT	heat-labile enterotoxin
NA	not available
NGS	next generation sequencing
NNPD	new neonatal porcine diarrhoea
NPD	neonatal porcine diarrhoea
NTC	non template control
PCR	polymerase chain reaction
PED	porcine epidemic diarrhoea
PRR	pattern recognition receptors
qPCR	quantitative real-time PCR
RDP	Ribosomal Database Project
RT-PCR	reverse-transcription PCR
SCFA	short chain fatty acids
ST	heat-stable enterotoxin
STEC	Shiga toxin producing *E. coli*
Stx	Shiga toxin
Tcd	*Cl. difficile* toxin
TGE	transmissible gastroenteritis virus
TGGE	temperature gradient gel electrophoresis
T_m	melting temperature

List of contents

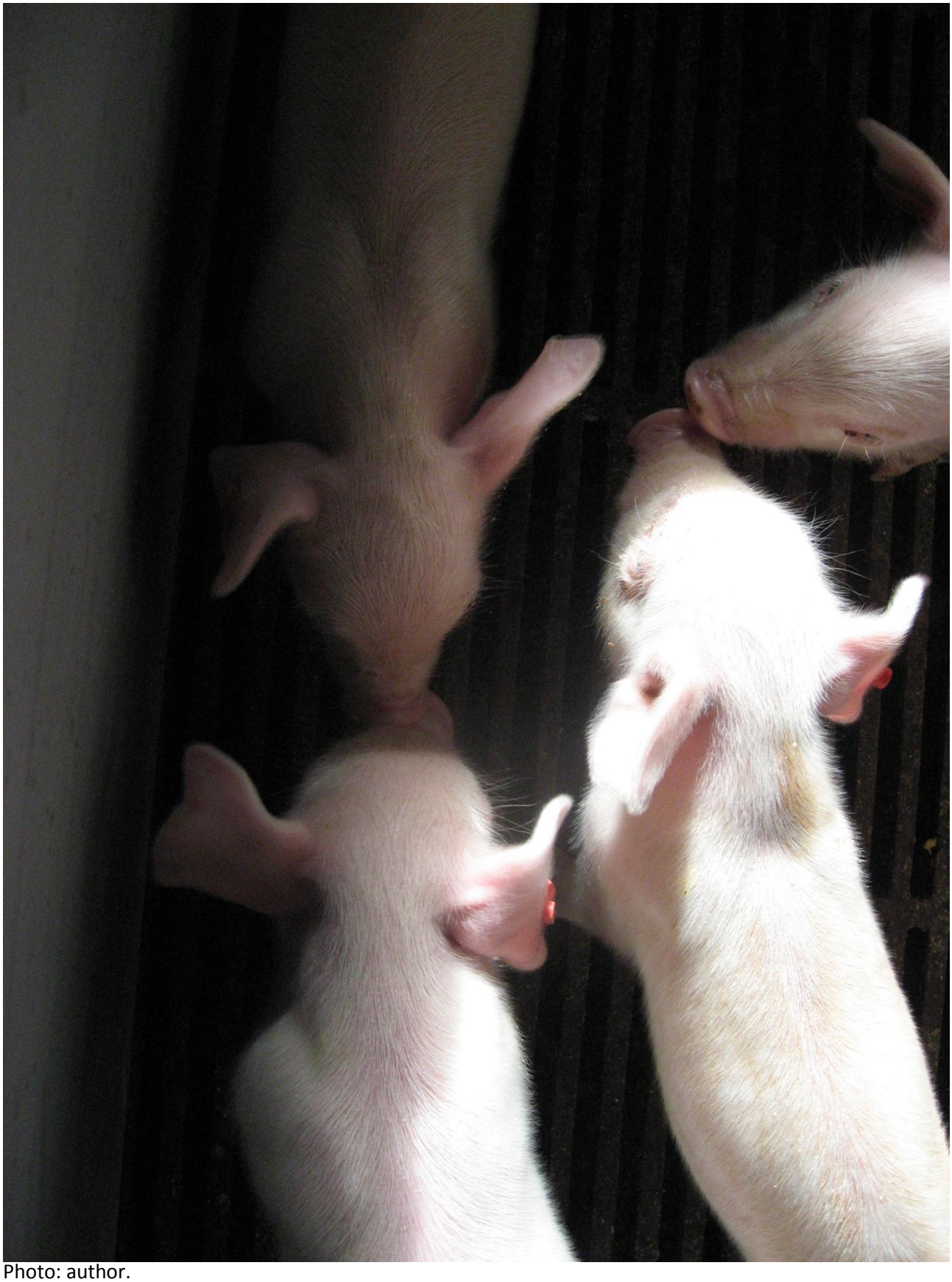


Photo: author.

Theoretical part

1. Intestinal structure and function

The intestine runs from the pylorus to the anus. It is divided into the small intestine proximately and the large intestine distally. The small intestine is further divided into three parts: the duodenum, jejunum, and the ileum (Figure 1). The caecum marks the transition from the small intestine to the large intestine [4].

The intestine exerts numerous functions through its complex cellular components. First and foremost it serves as an organ for digestion and absorption of nutrients. However, the intestine also participates in protecting the host from invading pathogens. Immune cells distributed in the lamina propria and the intraepithelial spaces as well as those constituting the gut-associated lymphoid tissue (GALT) are essential components of the defense mechanism [5]. Physical factors additionally contribute to the host's protection. These include continuous renewal of the enterocytes, the tight junctions linking the enterocytes together, the production of mucins as well as the secretion of anti-microbial peptides [6].

The intestinal wall is composed of several compartments (reviewed in [4,7-9]). The organization is as follows (Figure 1): the outer part of the intestine is covered by connective tissue, the serosa. Next is the muscularis externa. This is made up by a layer of muscle fibers arranged in a longitudinal layer followed by a circular layer. Between the two muscle-layers are the Meissner's plexus supplying sympathetic and parasympathetic innervations to the muscularis externa. The muscularis externa causes the peristaltic movements of the intestine. After the muscularis externa is the submucosa: a layer of loose connective tissue functioning as a supportive matrix for blood vessels, lymphatic vessels, glands, and parasympathetic ganglia, Auerbach's plexus, which also is part of the enteric nervous system. Separating the lamina propria and the submucosa is the muscularis mucosa; a thin layer of muscle fibers composing the outermost layer of the intestinal mucosa. The lamina propria holds capillaries, the lacteal (a central lymphatic capillary), glands, immune cells, and GALT in its loose connective tissue. The lamina propria supports the epithelium of the luminal surface. In the small intestine the epithelial layer is arranged in finger-like projections called villi that greatly increase the surface area of the epithelium, thereby improving the absorptive capacity of the intestine. In pigs, cell types constituting the epithelial layer are: enterocytes, undifferentiated crypt epithelial cells, Goblet cells, enteroendocrine cells, and M-cells.

The enterocytes are coated with a surface glycocalyx that is responsible for the digestive and absorptive properties of the enterocytes through the enzymatic activity. At the apical surface the enterocytes have cellular membranous protrusion called microvilli. These microvilli additionally contribute to an increased intestinal surface area, and constitute the brush boarder of the intestinal epithelium. The enterocytes are connected laterally by tight junctions consisting of transmembrane proteins like claudins and occludins [10]. Undifferentiated crypt epithelial cells are the progenitor cells of all of the other epithelial cells which

they continuously replace by migrating from the crypt to the top of the villi. Goblet cells produce mucin with a lubricating and protective function. Enteroendocrine cells are responsible for the synthesis of several gastrointestinal hormones. Finally, M-cells cover the underlying GALT areas and have an important function in capture and transcytosis of antigens across the intestinal epithelium from the lumen to the GALT, thereby enabling initiation of a mucosal immune response [4,7-9].

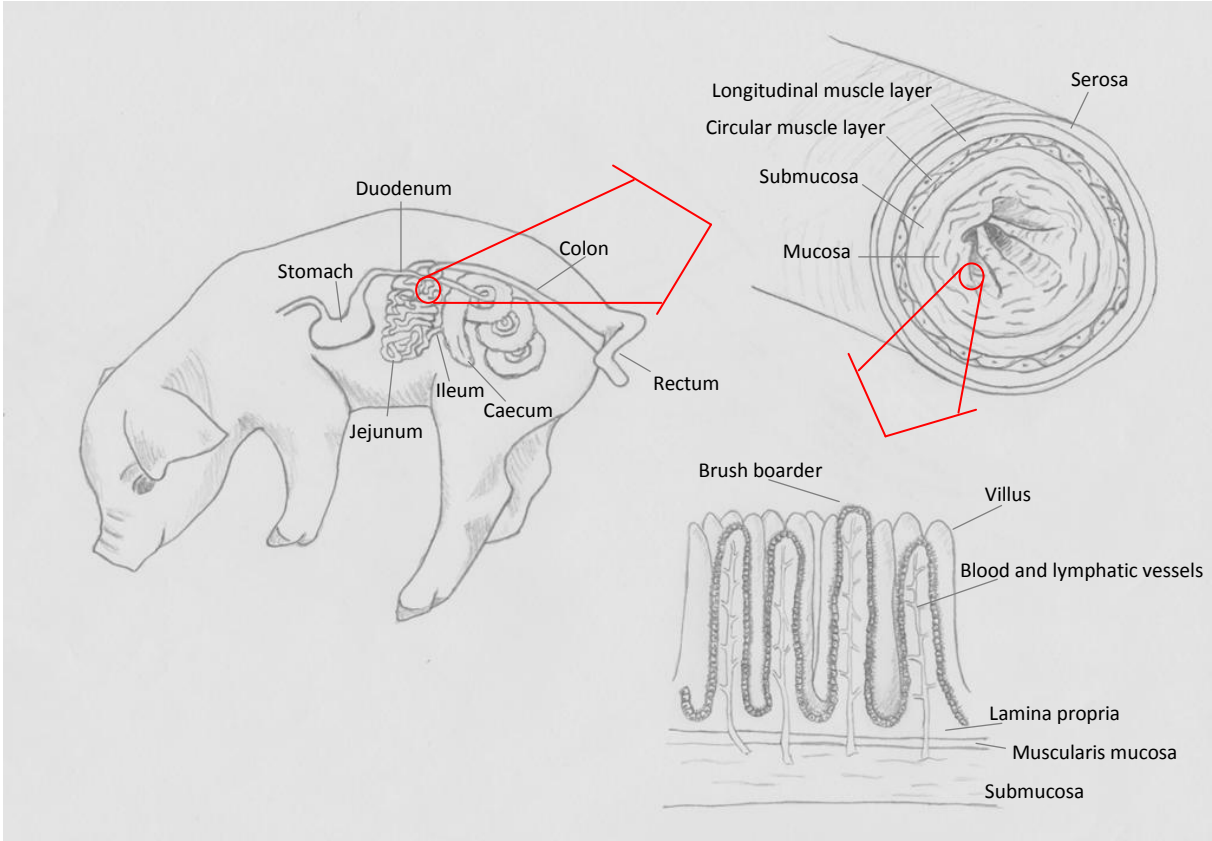


Figure 1: Schematic representation of the porcine gastrointestinal tract. Illustration: author, adapted from [7,11,12].

2. The neonate intestinal immune system

The gastrointestinal tract has an important immunological function and contains the largest accumulation of lymphoid tissue in the body [13]. Cells lining the gastrointestinal tract encounters enormous amount of foreign antigens and of these, the immune system has to be able to differentiate harmless from pathogenic. The intestinal epithelium itself comprises an essential physical barrier that together with the tight junctions prevent microorganisms from getting entry. The number of bacteria that reach the epithelial cells are further reduced by the mucus on top of the epithelium throughout the intestine [14].

The primary function of the immune system is to recognize and eliminate harmful microbial invaders. The immune system is composed of the innate immune system and the adaptive immune system [15]. The innate immune system is the first line of defense and is able to respond instantly to infections by immune cells equipped with a wide arsenal of pattern recognition receptors (PRR) capable of recognizing a variety of microbial pathogens. However, the innate immune system also activates and shapes the adaptive immune response [16]. The adaptive immune response takes time to develop, and unlike the innate immune response, exerts a specific enhanced response towards previously encountered pathogens through immunological memory [15].

Neonate piglets are immunologically immature as the adaptive immune system, in particular, is not fully developed. This manifests as a reduced number and activity of B- and T- cells in neonates compared to older piglets [17,18]. Therefore neonate piglets are fully dependent on the innate immune system and the passive immunity received through colostrum while the adaptive immune system matures [19,20]. Macrophages, neutrophils and natural killer cells are cellular participants of the innate immune response that are activated through the PPRs recognizing conserved molecular structures of pathogens such as bacterial cell wall components including lipopolysaccharide (LPS) and peptidoglycans [19].

3. The gut microbiota; a companion for life

The importance of a healthy, well-functioning gut environment has been acknowledged through thousands of years. As far back as in 400 BC Hippocrates, the father of modern medicine [21], should have uttered that "...death sits in the bowels..." and that "...bad digestion is the root of all evil..." [22]. Since then increasing attention has been given to the gastrointestinal tract and its inhabitants' co evolution with and impact on the host.

When mammals are born they come from a protective intra-uterine environment out to an extra-uterine environment rich in microorganisms. Now the colonization of the gastrointestinal tract begins. The first bacterial colonization depends on the type of delivery [23], but is generally said to be dominated by facultative anaerobic species, which within a few days are replaced by anaerobic species [24,25]. Hence, the gastrointestinal tract is a dynamic ecosystem evolving over time, and it is not only colonized by bacteria, but also additional microorganisms such as archaea, fungi, and protozoa. Collectively, these are known as the gut microbiota, referring to all the microorganisms present in the intestinal ecosystem [26]. However, throughout this thesis the gut microbiota will strictly refer to the bacterial gut microbiota.

The gut microbiota has coevolved with the host, to benefit from each other in a symbiotic way [27]. The beneficial effects of the gut microbiota on the host are numerous and include: 1) conversion of otherwise

indigestible food components into short chain fatty acids (SCFAs) that serves as a source of energy for the host; 2) synthesis of K- and B-vitamins that to some degree is used by the host [28]; 3) protection of the host from establishment of pathogenic bacteria through competitive exclusion and stimulation of enterocyte turnover [29-31]; and 4) assistance in development and maturation of the gastrointestinal tract and the mucosal immune system [17,31-33]. In return the gut microbiota is provided with a relatively stable environment rich in continuous nutritional supply [27].

3.1. Colonization of the intestinal tract

In utero the gastrointestinal tract of the fetus is regarded to be sterile [34]. The initial colonization of the intestinal tract begins as soon as this protective environment is disrupted; at birth. As described earlier, it is a common believe that the facultative anaerobic species are the pioneer bacteria that colonize the intestine [25]. By metabolizing oxygen in the intestinal atmosphere these bacteria reduce the oxygen tension in the intestinal atmosphere and thereby establish a favorable environment for the anaerobic species that subsequently gradually become the predominant bacteria of the intestine [35]. However, recently this thesis has been questioned by Vallès *et al.* (2014), rather, they propose that an anaerobic environment is established rapid after birth, and that the strict anaerobic species simply outnumber the facultative anaerobic species [36]. In any case, it appears that the 'normal' bacterial succession in the (porcine) gastrointestinal tract is characterized by the presence of certain bacteria, though it is a highly plastic event with great interindividual variability [37].

The bacteria first encountered by the neonate are the first to establish within the gastrointestinal tract, and these are mainly derived from the mother [36,38]. Due to a short period of relatively high gastric pH value (5.3 - 5.9) [39,40] in newborn piglets, bacteria from the surroundings have easy access to the small intestine as they can pass the stomach unharmed [39]. Studies on bacterial succession in the gastrointestinal tract are summarized in Table 1. Initially the gastrointestinal tract is flooded with the facultative anaerobic species belonging to *Escherichia coli, Lactobacillus* spp., and *Streptococcus* spp. / *Enterococcus* spp., but also the strict anaerobic *Clostridium perfringens.* While the three first mentioned bacterial species and genera exist throughout the entire channel, the former is mainly located in the distal small intestine and the large intestine. Within the first 48 hours after birth the pH of the stomach drops to as low as 2.0 with a following inhibiting effect on the proliferation of most bacteria except for Lactobacilli which becomes the predominant genera at this location. While Streptococci and Enterococci can persist to limited extend in the stomach, *E. coli* restricts its growth to the small and large intestine [39]. Figure 2 illustrates bacteria, bacterial numbers, and their typical location which is probably associated with the normal bacterial colonization of the piglet gastrointestinal tract, and which additionally is influenced by pH and digesta transit time.

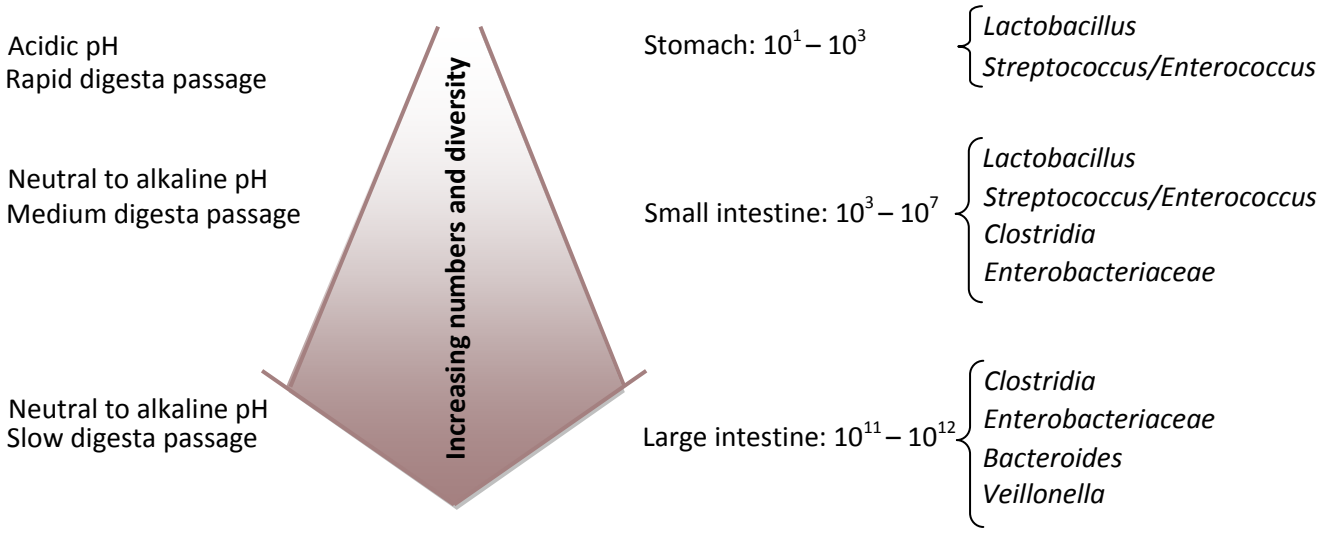


Figure 2: Gastrointestinal characteristics, bacterial cell numbers (cell forming units per ml), and dominating bacterial families and genera listed according to their typical location [39,41,42]. Altered from [43].

Table 1: Identified commensal bacteria in the gastrointestinal tract of piglet within the first week of life. Bacteria are listed according to highest abundance, and if equal; according to earliest detected during the first week of life. N is the number of piglets included in the study. Age is the age of the piglets when samples were collected for bacterial detection. PCR: polymerase chain reaction, TGGE: temperature gradient gel electrophoresis, BLAST: Basic Local Alignment Search Tool, RDP: Ribosomal Database Project.

Country, year [Reference]	N	Age	Gut Section	Diagnostic method	Commensal bacterial identified within the first week of life
United Kingdom, 1963 [39] †	26†	3 hrs – 150 days	Digesta from the stomach, seven succeeding portions of the small intestine, caecum, colon; and feces	Culturing	*E. coli* *Lactobacillus* spp. *Streptococcus* spp.* *Cl. perfringens* *Bacteroides* spp. *Veillonella* spp.
United Kingdom, 1965 [40]	27	3 hrs – 49 days	Digesta from the stomach, seven succeeding portions of the small intestine, caecum, colon; and feces	Culturing	*E. coli* *Cl. perfringens* *Streptococcus* spp.* *Lactobacillus* spp. *Bacteroides* spp. Micrococci *Veillonella* spp.
USA, 1993 [25]	25	1 min – 120 days	Digesta from proximal, mid, and distal colon	Culturing	*Clostridium* spp. *E. coli* *Lactobacillus* spp. *Bacteroides* spp.
Japan, 1995 [44]	18	1 – 45 days	Feces	Culturing	Enterobacteriaceae Streptococci *Lactobacillus reuteri* *Lactobacillus acidophilus* *Lactobacillus salivarius* *Lactobacillus casei*
Japan, 2005 [45]	6	1 – 49 days	Feces	DNA extraction → PCR using universal 16S rRNA gene primers → TGGE → PCR using universal 16S rRNA gene primers → cloned and sequenced → classified using BLAST (GenBank)	*E. coli* *Cl. perfringens*#
United Kingdom, 2006 [46]	(70) 2	2 (– 32) days	Digesta from ileum	DNA extraction → PCR using universal 16S rRNA gene primers → cloned and sequenced → classified using BLAST (GenBank)	*Shigella flexneri* *E. coli* *Lactobacillus sobrius* *L. reuteri* *L. acidophilus* *Pasteurella aerogenes* *Acinetobacter* sp. *Neisseria* sp.

Table 1 continued

Country, year [Reference]	N	Age	Gut Section	Diagnostic method	Commensal bacterial identified within the first week of life
Canada, 2010 [47]	48	0.25 – 20 days	Digesta from the stomach, proximal, mid, and distal small intestine, caecum and colon	DNA extraction → PCR using universal 16S rRNA gene primers → cloned and sequenced → classified with RDP	Streptococcaceae Clostridiaceae Lactobacillaceae Enterobacteriaceae Moraxellaceae Pasteurellaceae Peptostreptococcaceae Lachnospiraceae Veillonellaceae Micrococcaceae Bacteroidaceae Ruminococcaceae

* *Streptococcus* spp. were further determined to be *Streptococcus faecium* and *Streptococcus faecalis* which nowadays are known as *Enterococcus faecium* and *Enterococcus faecalis*, hence, it cannot be rejected that the *Streptococcus* spp. actually were *Enterococcus* spp..
[†]Reference is only to the part of the study investigating bacterial succession in the healthy porcine gastrointestinal tract.
[#]Though Inoue *et al.* obviously detected several other bacteria; unfortunately, these were the only bacteria that they mention.

4. Diarrhoea

4.1. Pathogenesis

Diarrhoea is a symptom of a malfunctioning gastrointestinal tract. Diarrhoea refers to: *"secretion of abnormally fluid feces accompanied by an increased volume of feces and an increased frequency of defecation"*[7], Figure 3. Diarrhoea results in loss of excess fluid, energy, and electrolytes which eventually leads to the animal becoming weak and dehydrated. The dehydration has several consequences which

Figure 3: Piglets with diarrhoea from one of the farms included in the study. Photo: author.

are connected in a vicious circle as summarized in Figure 4 [7,9]. The increased loss of fluids in feces reduces blood volume and results in inadequate circulation and impaired oxygenation of tissues. In circumstances of limited available oxygen cells turn to anaerobic glycolysis for energy production, however, this leads to diminished content of blood glucose. In shortage of glucose cells mobilize fatty acids which are transported in the blood stream to various sites including the liver. However, in lack of glucose the liver is unable to oxidize acetyl CoA from fatty acid oxidation and instead it releases ketone bodies. These can be used as energy, while in excess they induce the release of hydrogen ions with a subsequent drop in pH of blood and tissue. Moreover, the acidosis is deteriorated by loss of bicarbonate in feces and reduced renal secretion of hydrogen ions and absorption of bicarbonate. The resultant electrolyte imbalance impairs cellular function as hydrogen ions accumulate and potassium concentration decreases intracellularly. Eventually the neuromuscular control of myocardial contractions is disturbed and decreased heart function causes additional reduced tissue perfusion. In worst case scenario diarrhoea can lead to hypovolemic shock and be fatal.

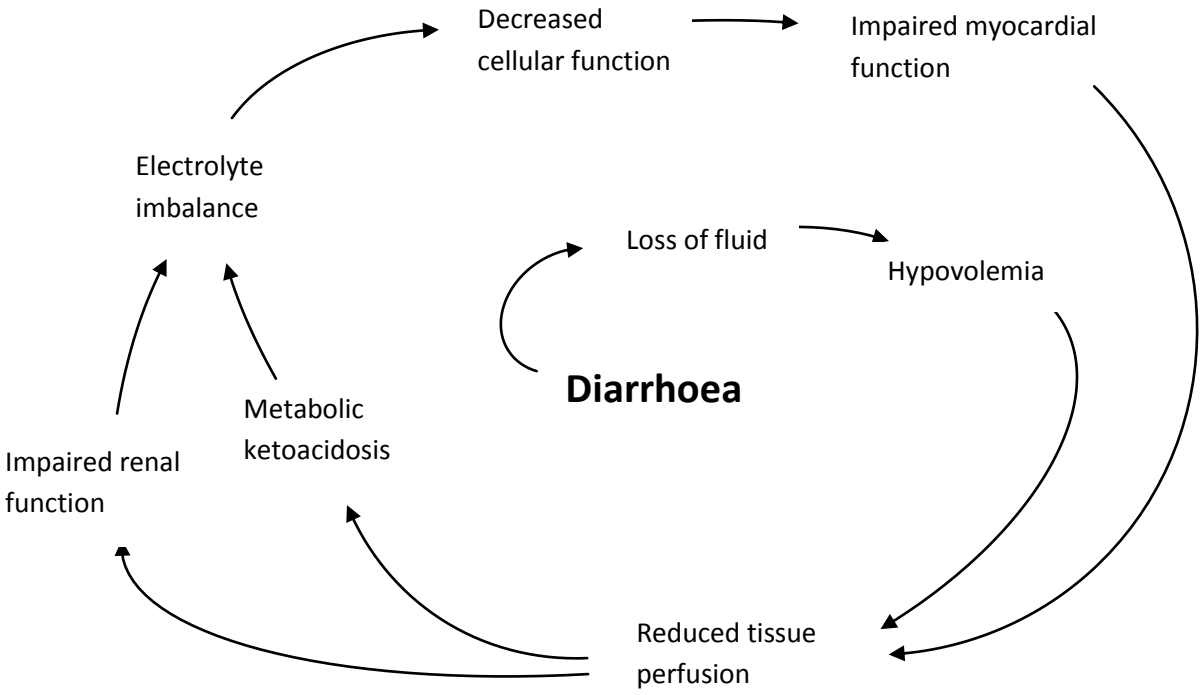


Figure 4: The pathophysiology of diarrhoea [7,9]. Illustration: author.

Four mechanisms can lead to diarrhoea these are the following: 1) osmotic; 2) secretion; 3) exudation; and 4) hypermotility. Osmotic diarrhoea is a sequlae to malabsorption as a consequence of epithelial injury. Secretory diarrhoea is characterized by intact intestinal mucosa and a result of an active secretion of fluid and electrolytes. Exudative diarrhoea is caused by inflammation which causes increased permeability of

capillaries and the intestinal mucosa. Finally, disturbed mobility, mainly hypermotility, generally has a diarrhoeic effect as a result of the decreased transit time for the luminal content [7,48].

4.2. Diarrhoea predisposing factors

Though diarrhoea is a symptom of numerous diseases and condition there are several factors that may predispose the neonate piglet to diarrhoea during the first week of life. Some of the important factors are listed and described in the following.

4.2.1. Colostrum

Colostrum is an essential source of dietary energy, however, it also supplies the piglets with passive immunity through its content of immunoglobulins and additional immune components [49]. The sow's placenta is of the epitheliochorial type which means that there are six layers separating the sow's blood from the blood of its fetuses [50]. Due to the anatomy of the epitheliochorial placenta there is no transmission of maternal immunoglobulins to the fetuses in the uterus. Consequently the neonatal piglet is born immunologically immature, and is entirely dependent on the protective immunoglobulins acquired through the ingestion of colostrum [51]. The enteric absorption of immunoglobulins is through pinocytosis, where enterocytes encapsulate immunoglobulins in vesicles to transfer these intact from the intestinal lumen to the lymphatic system and then the blood stream. This is a limited capability which ceases 24 to 36 hours post-partum, a phenomenon known as 'gut closure' [49]. Thus it is crucial that neonatal piglets ingest colostrum before gut closure in order to receive passive colostral immunity against pathogens encountered by the sow (Figure 5).

Figure 5: The piglet has a vital part to ensure its own health and survival through early and sufficient colostrum intake. Photo: author.

4.2.2. The immature intestine

There are many factors contributing to the increased risk of the newborn piglet being infected with diarrhoeic bacteria. In order to protect the colostral immunoglobulins from digestion (that is the breakdown of complex nutrient to simple molecules) the secretion of acids and enzymatic proteins by the stomach and the duodenum, respectively, are delayed for several days after birth. The less hostile environment due to a higher gastric pH value enables bacteria to more easily establish themselves in the gastrointestinal tract [9]. In addition, the lack of a stable microbiota means that there is less competition for nutrients and epithelial binding sites which permits the bacteria to proliferate and establish rather unrestrictedly. Finally, the immature intestine is insufficiently developed immunologically and must rely on the innate immune system until the adaptive system becomes fully functional. This inadequate immunological response against microorganisms presumably is an advantage for the intestinal bacteria [32,52].

4.2.3. Environment

In the wild the sow would demonstrate nesting behavior prior to farrowing, and build a nest for its young. The nest would function as a shelter to keep the piglets warm, dry, and safe. This is not possible in most modern conventional pig husbandry, where sows often is constraint by farrowing crates, with the main purpose of preventing piglets from getting crushed by the sow [53]. Consequently, the neonate piglets are depending on the farmer to ensure a suitable environment. This should be without draft wind, with adequate ambient temperature, and with the possibility to huddle up and get warmth under a heating lamp if chilled. It is crucial that piglets stay warm so they do not have to use their very limited energy-reserve to stay warm through shivering thermogenesis which subsequently could reduce piglet vigor and thereby intake of colostrum and milk and lead to fatal outcome for the piglets [54,55].

4.2.4. Breeding and cross-fostering

Today's intensified pig industry has for economic reasons favored sows producing the highest number of piglets born per litter. The placenta has a restricted nutrient supply, and an increasing litter size results in an uneven distribution between fetuses, and accordingly in piglets of varying birth weight [56]. Piglets of low birth weight by default have reduced survivability, as they are not as competitive as larger piglets, with a consequential reduced colostral intake [57].

Another tradeoff of the intensified production is the increasing litter size, which is about to reach its limits. Hence it is not uncommon that the number of piglets exceeds the number of functional teats. Pig breeders solve this by cross-fostering piglets by surrogate sows. Nonetheless, the time span from a piglet is born till it has been allocated to a surrogate sow is critical in regard to the absorption of immunoglobulins before gut closure. Furthermore, studies have proved varying immunoglobulin concentrations in colostrum

between sows, but also that the concentration tends to increase with increasing parity [49]. Additionally, there is an individual variation in the declining concentration within the first 24 hours, which is another risk factor influencing a sufficient immunoglobulin intake in time [58]. Finally, there is a risk of spreading diseases by cross-fostering, as piglets allocated to other litters can function as vehicles.

4.3.Clinical symptoms of diarrhoea

Irrespective of the aetiology there are some common clinical characteristics of diarrhoeic piglets besides diarrhoea. Dependent of the severity a diarrhoeic piglet shows decreased activity, and it may stand with hunched back and its hind legs positioned far under the body. It generally looks depressed with dropping tail, hanging ears, and erect hair coat. Piglets may still suck, but often appear shrunken, dehydrated, and may have protruding ribs and pelvic bones [59].

5. Recognized enteric pathogens causing diarrhoea in piglets

There are several recognized enteric pathogens known to cause diarrhoea in piglets. However, in this dissertation only enteric pathogens affecting piglets during the first week of life are described, since this is the age-group affected by NNPD. Diarrhoea causing agents include: *E. coli*, *Cl. perfringens* type A and C, *Clostridium difficile,* transmissible gastroenteritis virus, porcine epidemic diarrhoea, rotavirus, *Cryptosporidium spp.*, and *Isospora suis* [60,61]. However, it is important to emphasize that diarrhoea may be a result of infection with more than one pathogen [62,63].

5.1. Bacteria

5.1.1. *Escherichia coli*

E. coli is a facultative anaerobic Gram-negative bacteria with several surface antigens: O (somatic), H (flagellar), F (fimbrial), and K (capsular), while serotyping, used to differentiate *E. coli* isolates, primarily are defined by the O and H antigens [64,65]. Generally, *E. coli* is a harmless predominant commensal in the gut, and is commonly found in the distal part of the small intestine and in the proximal part of the large intestine [59]. Nonetheless, this species also comprise strains that are pathogenic to the host. The pathogenic strains are mainly characterized based on their pathogenic features and common for them all is that they have to establish contact with the intestinal mucosa in order to be pathogenic. The six major *E. coli* virulence groups are covered in the following section and summarized in Table 2 (revised in [65-67]):

5.1.1.1. Virulence groups of *E. coli*

Enterotoxigenic *E. coli* (ETEC): ETEC colonize the mucosa of the small intestine via fimbriae and release enterotoxins, either heat-labile (LT-I; LT-IIa; LT-IIb) and/or heat-stable (STa; STb, EAST1). Enterotoxins are taken up by the epithelial cells via endocytosis which initiates an intracellular cascade regulating ion channels of the cell. The result is secretory diarrhoea with a net efflux of fluid and electrolytes. Generally, the intestinal mucosa stays intact, while STb is exceptional as this enterotoxin causes damage to the intestinal epithelium and partial villus atrophy. ETEC infection results in watery diarrhoea.

Enteropathogenic *E. coli (*EPEC): EPEC is characterized by the histopathological lesions that they cause, known as attaching- and effacing (A/E). This mainly shows as *E. coli* adhering to cells of the mucosa with destroyed microvilli and disrupted cytoskeleton. Important virulence factors are: the EPEC adherent factor (EAF) plasmid that encodes a bundle-forming pilus enabling adherence of the bacterium to the intestinal mucosa; and the *eae* gene which encodes the membrane bound adhesin intimin which contributes to adherence as well as the characteristic A/E lesions. The mechanisms causing diarrhoea are the following 1) altered intracellular ion transport; 2) the effacement of microvilli results in malabsorption as a consequence of the reduced absorptive capacity; and 3) EPEC induces inflammation which causes increased mucosal permeability. The younger the individual the higher risk of being affected with EPEC. EPEC can spread with dust and aerosols, and the infectious dose in exposed animals is believed to be extremely low. The organism causes profuse watery diarrhoea of sudden onset, stool sporadically contains leucocytes.

Enterohemorrhagic *E. coli* (EHEC): These organisms belong under Shiga toxin producing *E. coli* (STEC). They produce Stx (Shiga toxins): Stx1 (highly conserved) and/or Stx2 (several variants: Stx2c, Stx2v etc., Stx2 is the most virulent of the two toxins). These toxins bind to a specific membrane receptor (Gb_3) of the enterocytes which facilitates endocytosis to the Golgi apparatus and then the endoplasmic reticulum. The toxin inhibits the protein synthesis leading to cell death. Diarrhoea is a result of the loss of intestinal epithelial cells. Like EPEC, EHEC also produces A/E lesions. Because the Stx toxins of EHEC can be lost it is important to use several virulence factor targets in the search for this organism. Watery diarrhoea with and without varying degrees of blood and mucus are common in EHEC infections.

Enteroaggregative *E. coli* (EAEC): Adherence of EAEC to enterocytes is mediated via the aggregative adherence fimbriae (AAF/I-III). EAEC facilitating intestinal colonization by stimulating the production of mucus which they bind to, and through aggregative adherence (AA) of the bacterial cells to each other EAEC produce a bacterium-mucus-biofilm. This organism exerts cytotoxic damages to the epithelium of the villi with hemorrhagic necrosis and shortening of the villi as well as inflammation of the mucosa. However, the cytoskeleton of the villi stays intact, thus, these lesions are not characterized A/E. Mucuid diarrhoea is characteristic for EAEC.

Enteroinvasive *E. coli* (EIEC): The pathogenesis of EIEC resembles that of *Shigella.* This group of bacteria is able to adhere to and penetrate enterocytes in which they multiply intracellularly. Moreover, EIEC can move through the cytosol and spread to adjacent enterocytes. This initiates an inflammatory response with increased permeability of the intestinal mucosa. The virulence factors of EIEC are encoded on the plasmid pINV. EIEC causes watery diarrhoea sometimes with blood, mucus, and leucocytes.

Diffusely adherent *E. coli* (DAEC): DAEC adhere to enterocytes in a disseminated pattern, and is potentially diarrhoeagenic in children. DAEC is able to induce extension of microvilli that seems to have a protective function for the bacteria. Due to its less virulent nature: only being potentially pathogenic, this organism has not gained as much focus as the other virulence groups of *E. coli.* The diarrhoea is watery without blood or leucocytes.

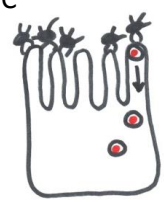
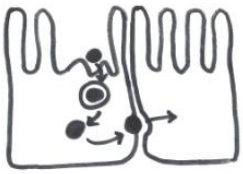
5.1.1.2. E. coli in piglets

Of the aforementioned *E. coli* virulence groups mainly ETEC is associated with diarrhoea in neonate piglets. Common isolates are: F4(K88), F5(K99), F6(987P), and F41, of which F4(K88):O149 and STa^{+}:F5(K99):non-hemolytic *E. coli* are some of the predominant isolates [59,67]. Piglets are infected orally from the sow's mammary glands and the surfaces of the farrowing crate, and ETEC is transmitted via feces from infected animals. The pathogenesis of ETEC is discussed in the previous section and in Table 2. Poor sanitary status of the environment and insufficient disinfection of farrowing crates increases the risk of infection with ETEC.

Clinically one or more piglets in a litter develop watery diarrhoea of varying degree within the first few days after birth [67]. These piglets have decreased daily weight gain and mortality is usually not high. The watery consistency of ETEC diarrhoea can make it hard to detect. However, indications are: dried crusts of diarrhoeic feces in the perineal area and on the thighs of the piglets but and also on the surfaces of the farrowing crate. Affected piglets may recover after three to six days or dye. ETEC is reported to be more common in gilt litters [59].

Diagnosis of ETEC is mainly through laboratory confirmation of some of the in Table 2 mentioned characteristics. This can be from culturing of pathogenic serotypes and enzyme-linked immunosorbent assay (ELISA) tests for surface antigens; both on intestinal content from the small intestine or feces. In addition PCR can be used to detect pathogenic determinants [59].

Table 2: Characteristics of different *E. coli* virulence groups. For each *E. coli* virulence group the pathogenesis is depicted for an enterocyte. See the text for detailed description of the histopathology. ID: Infectious dose (cell forming units); IP: Incubation period. NA: Not available. The table is related to human findings, there are several interspecies variations.

E. coli virulence group	Characteristics/pathogenesis	Primary location	Sequlae	Remarks
ETEC 	**Fimbriae** **Enterotoxins:** **LT–I, LT–II** **STa, Stb** **EAST1**	Small intestinal	Secretory diarrhoea	All age groups ID: 10^8 IP: hours
EPEC 	**A/E lesions** **EAF-plasmid** ***eae* gene** **EAST1**	Proximal small intestine (in piglets: small and large intestine)	Secretory and malabsorptive diarrhoea	Primarily in young individuals ID: 10^8-10^{10} IP: hours
EHEC (STEC) 	**A/E lesions** **Shiga toxin:** **Stx1, Stx2** (various subtypes) ***eae* gene** **EAST1** and **Enterohemolysin** (only some strains)	ascending and transverse colon	Malabsorptive diarrhoea	Primarily in young individuals ID: < 50-100 IP: days
EAEC 	**AA embedded in mucus** **Stimulates mucus production** and binds to mucus **Cytotoxic** (only some strains) **AAF/I -III** **EAST1 (**some strains)	Probably both small and large intestine	Malabsorptive diarrhoea	Primarily in young individuals ID: 10^{10} IP: hours
EIEC 	**pINV** (also characteristic for *Shigella*) **Invasive with intracellular multiplication and spreading to adjacent enterocytes**	Colon	Malabsorptive diarrhoea	All age groups ID: > 10^6 IP: hours
DAEC 	**DA pattern** histologically **Elongated villi**	NA	NA	Primarily in young individuals ID: NA IP: NA

E .coli; Endocytosis of enterotoxin; Intimate attaching *E. coli;*
E. coli with EAF; Cellular actin condensation; bacterium-mucus-biofilm

Drawings are reproduced by the author from J.P. Nataro and J.B. Kaper [65] with permission from American Society for Microbiology.

5.1.2. *Clostridium perfringens*

Clostridia are Gram-positive spore-forming anaerobic bacteria and a normal inhabitant in the intestine of healthy warm-blooded animals [68]. *Cl. perfringens* is divided into five different toxinotypes according to which of the four major toxin types that they produce: A: alpha; B: alpha, beta, epsilon; C: alpha, beta; D: alpha, epsilon; and E: alpha, iota. Additionally, *Cl. perfringens* can produce enterotoxin (CPE) and beta2-toxin (CPB2). Of all the enteric Clostridia inhabiting the intestine, *Cl. perfringens* is the species which causes most of the enteric diseases [68]. However, *Cl. perfringens* type A and type C are the toxinotypes that are most commonly associated with porcine neonatal diarrhoea [69]. Piglets are orally infected and the sow is most likely the source of infection [59]. Due to the production of spores that are highly resistant bodies formed to ensure bacterial survival under extreme environmental conditions, it is difficult to control clostridial infections [70].

5.1.2.1. *Clostridium perfringens* type A

This toxinotype is a commonly encountered cause of diarrhoea in newborn piglets and is reported from numerous countries (Table 3). It does not seem that CPB is a determining factor of diarrhoea because non-CPB *Cl. perfringens* mainly is isolated from diarrhoeic piglets [71]. There are conflicting evidences to whether the production of CPB2 contributes to the pathology of enteric diseases caused by *Cl. perfringens* Type A in pigs [71-73]. These organisms colonize and multiply in the jejunum, ileum, caecum, and colon and are mostly restricted to the intestinal lumen. Histopathologically the infection can cause mild villus atrophy, mucosal hyperemia (sometimes with hemorrhage), and occasionally slight mucosal necrosis. Lesions are especially located to the small intestine, however, lack of macro-and microscopic lesions have also been reported [59,68,73]. The pathogenesis is unknown but diarrhoea despite of intact intestinal mucosa suggests that *Cl. perfringens* type A causes secretory diarrhoea [68].

Clinically piglets develop yellowish, mucoid, creamy to watery diarrhoea which may contain fresh blood. Most piglets recover although death have been reported [59,68].

Diagnosis of *Cl. perfringens t*ype A is difficult, since it is part of the normal porcine gut microbiota, and no toxins have been proved to be strictly related to diarrhoea. However, isolation of large numbers of *Cl. perfringens* type A from the small intestine of affected piglets are indicative of this infection, and may be supported by detection of toxin genes, though these are not useful as diagnostic evidence alone [69,73]. The ambiguousness of the clinical signs and diagnosis makes it difficult to estimate the extend and significance of neonate porcine diarrhoea due to *Cl. perfringens* type A [74].

5.1.2.2. *Clostridium perfringens* type C

Contrary to toxinotype A, which generally cause somewhat ambiguous clinical symptoms, this toxinotype is very characteristic. *Cl. perfringens* type C typically produces acute or peracute fatal necrotic and hemorrhagic enteritis in piglets within the first week of life [59]. The source of infection is vegetative *Cl. perfringens* type C organisms from the feces of the dam, and this toxinotype is not regarded to be part of the normal gut microbiota [68,69]. The beta-toxin is the determining virulence factor of toxinotype C [75]. Following ingestion *Cl. perfringens* type C colonizes and adheres to the apex of the jejunal villi in particular. After adherence they release toxins, leading to desquamation of epithelial cells, necrosis of the subapical villous region which might progress to the muscularis mucosa, submucosa with resulting extensive hemorrhage. In affected regions there may be complete abolition of the villi and crypt architecture and the lumen contains large amounts of necrotic debris [76].

Clinically it is typically represented by depressed, pale, and thin piglets with abdominal pain and hindquarters smudged in bloody diarrhoea [59,68]. Piglets can be affected as early as 12 hours after birth, though the infection is more common in three days old piglets [68]. The susceptibility varies in a litter of which few piglets or close to the entire litter may be affected and high mortality may occur[77].

Diagnosis is straight forward: the macroscopic lesions seen at necropsy is pathognomonic and reveals massive necrosis and hemorrhage of the small intestine [75]. Macroscopic findings should be confirmed by isolation of a large number of *Cl. perfringens* cultured from intestinal content and subsequent genotyping and detection of the beta-toxin [68].

5.1.3. *Clostridium difficile*

Cl. difficile share features with *Cl. perfringens*: it is a Gram-positive, spore-forming, anaerobic bacterium, and it is also a normal inhabitant of the gastrointestinal tract in animals [68,78,79]. Clinical important toxins produced by *Cl. difficile* include: TcdB that is a cytotoxin and an enterotoxin and an enterotoxin called TcdA. TcdB is produced alone or together with TcdA, and it appears that TcdA needs the action of TcdB in order to be able to exert its virulent effect [69]. However, healthy piglets may also produce these toxins [80]. Following ingestion *Cl. difficile* adhere to enterocytes in the caecum and colon after which they release their toxins [80]. The toxins enter the enterocytes through endocytosis, and the virulent effects of the toxins include destruction of cytoskeleton and necrosis of epithelial cells and increased paracellular permeability by disruption of intracellular junctions [81]. Additionally, TcdA causes endothelial retraction that increase blood capillary permeability and allow escape of albumin and other plasma proteins into the surrounding tissue which results in extensive submucosal and mesocolonic edema [82]. *Cl. difficile* causes microscopic lesions seen as suppurative foci in the affected mucosa with exudation of neutrophils and

fibrin into the lumen known as 'volcano lesions' [83]. Piglets between 1-7 days olds are affected, and there is reason to believe that aerosols could be an important route of transmission between animals [70,78-80].

Clinically affected piglets may show signs of dyspnea, emaciation, mild abdominal distension, and scrotal edema with and without diarrhoea. The estimated mortality is 25 % though it may be as high as 90 % [82]. It is estimated that 2/3 of the litters and 1/3 of the piglets of an infected farrowing barn are affected by *Cl. difficile* [69].

Diagnosis is based upon detection of toxins; for example by use of ELISA or PCR, and from isolation of *Cl. difficile* by bacteriological culturing from colon and colonic luminal content [69].

5.2. Virus

5.2.1. Transmissible gastroenteritis virus

Transmissible gastroenteritis (TGE) virus is a group I coronavirus; an enveloped positive-sense, single-stranded RNA-virus. This virus is the aetiological agent of TGE in pigs. All age groups are affected, though clinical signs are most severe in younger piglets [84]. Pigs are affected by ingestion of infected droplets of fecal material, and the incubation period of 18-72 hours typically means that piglets between 1-4 days old are affected [59,85]. TGE virus attaches to surface receptors on the jejunal and ileal epithelial cells of the top of the villi via its surface protein S. This triggers membrane fusion between the virus' envelope and the membrane of the enterocyte resulting in viral entrance into the cell [85]. As a consequence of virus infection the epithelial cells are shed into the lumen, which results in villus atrophy and subsequently in watery diarrhoea. Crypt epithelial cells are unaffected, hence, migration of crypt epithelial cells to the affected areas restores villi integrity and function within 5-7 days after infection [86].

Clinically TGE virus cause initial vomiting followed by watery diarrhoea in young piglets, this results in dehydration and, consequently, piglets may die 2-4 days after infection [84,86].

Diagnosis of TGE virus can be from visualization of the virus in intestinal tissue by immunohistochemistry (IHC). Virus can be isolated in porcine thyroid or testicular cells and isolates can be identified by serology using ELISA [85]. Finally, virus can be detected in feces by reverse-transcription PCR (RT-PCR) [59].

5.2.2. Porcine epidemic diarrhoea

Symptoms caused by porcine epidemic diarrhoea (PED) virus are very similar to TGE. PED is also caused by a coronavirus with the same features as TGE virus but serologically unrelated to TGE virus [85]. Consequently, there are many parallels between infection, pathogenesis, and clinical characteristics between PED virus and TGE virus, thus, only characteristics which separate these two viruses will be mentioned.

The spread of PED virus is more slowly than for TGE virus. Clinically it typically causes primarily watery diarrhoea which occasionally may be preceded by vomiting; hence, vomiting is not as frequent as seen in infections with TGE virus. Mortality is not as common a sequela as it is for TGE virus, though it might reach 50 % [59].

5.2.3. *Rotavirus*

Rotaviruses are widespread in sense of causing host-specific diarrhoea in a variety of neonate animal species, though interspecies infectivity also is possible [87]. While rotaviral diarrhoea is more common in piglets more than one week old [88], it occasionally causes diarrhoea in piglets within the first week of life [89]. Members of the genus *Rotavirus* are non-enveloped, double-stranded RNA-viruses and the genus comprises seven antigenically diverse serogroups: A; B; C; D; E; F; and G. Of these only group A, B, C, and E include pathogens of pigs, and group A infections appear to be the major cause of early neonatal porcine diarrhoea [59].

Infection is through the oral-fecal route, though nasal transmission also have been demonstrated [90]. After ingestion rotavirus mainly binds to receptors of the jejuna and ileal epithelial cells at the top of the villi and this facilitates endocytosis of the virus. Once inside the cell virus replicate in the cytoplasm, eventually cells are shed and as a result villi become blunt and shortened [87]. The affected structural and functional integrity of the intestinal epithelium contribute to malabsorption and diarrhoea. However, additional diarrhoeic factors exists as diarrhoea can precede villus atrophy [91]. The incubation period is 24 hours or less [92].

Clinically piglets appear anorexic and depressed and after few hours they develop profuse diarrhoea [92]. Vomiting may occur. In uncomplicated cases piglets recover after 4-6 days while severe cases may be fatal, in which case mortality can reach 33-100 % [59]. The severity of the disease depends on the virulence of the virus, the inoculation dose, and the age of the animal [88].

Diagnosis of rotaviruses can be through electron microscopy, ELISA, RT-PCR all in feces material. Additionally IHC can be used to identify rotavirus antigens in infected cells [59].

5.3. Parasites

5.3.1. *Cryptosporidium spp.*

Cryptosporidia are coccidian parasites that cause cryptosporidiosis in a variety of animals. The genus *Cryptosporidium* contains several different species whereof those infectious for piglets include: *Cryptosporidium suis, Cryptosporidium* pig genotype II, and *Cryptosporidium parvum* [93]. Piglets are infected by ingesting infectious oocysts from the environment after which sporozoites (excysted from the

oocysts) target the brush boarder of the intestinal epithelium. Embedded in the membrane of the enterocytes, the sporozoites differentiate further and multiply [94,95]. The histological changes of affected areas (both the small and large intestine) include shortening of the affected villi and replacement of enterocytes by immature cuboidal cells [96,97].

Clinical signs appear 2-3 days after infection. Piglets may initially vomit and following develop diarrhoea, be anorexic and depressed, however, the clinical signs are not consistent and great variation is seen interindividually [97]. Though it is possible to infect neonatal piglets experimentally, it seems that natural acquired infections are more common in older piglets [98,99].

Diagnosis can be based on histological examination, fecal smears, and PCR [59,99].

5.3.2. *Isospora suis*

Like Cryptosporidia *I. suis* is also a coccidian parasite and the infectious route, pathogenesis, and the histological changes resembles those mentioned for *Cryptosporidium* spp.. The highest prevalence of isosporosis is mainly reported to occur among piglets of 2-3 weeks of age, and is detected as early as 5 days after birth [100,101]. Infection is through ingestion of infectious oocysts and once inside the host excystation results in release of sporozoites that particularly target the epithelium lining jejunum and ileum [102-104]. The intracellular infection with *I. suis* causes villus atrophy, fusion of villi and focal epithelial erosion of different degree in relation to infectious dose and age [105].

Clinically piglets develop watery diarrhoea, they become dehydrated, emaciated, and lethargic. Clinical signs are dose- and age-dependent; as with Cryptosporidia: the younger the piglet the more severely affected [104]. Isosporosis can infect up to 100 % of the piglets in a litter and mortality rates reach 20 % [59,102].

Diagnosis of isosporosis can be through demonstration of the characteristic *I. suis* oocysts in feces, detection of oocysts may be enhanced by flotation and use of fluorescence microscopy. Additionally, PCR can be used for diagnosis [59,100,101].

5.4. And then there is the rest…

Besides from the aforementioned well-known enteric pathogens, several other bacteria are associated with diarrhoea which also appears from Table 3. The fact that these bacteria have not been assigned to their own individual paragraph, does not mean that they are not as important as the others, only they are not as acknowledged in the literature. Bacteria that appear from Table 3 which have not been mentioned earlier will be described briefly in this section; however, *Enterococcus* will be dealt with in a separate paragraph due to its ability to cause neonatal diarrhoea.

5.4.1. *Enterococcus* spp.

Enterococcus spp. are Gram-positive facultative anaerobic bacteria that are ubiquitously distributed in nature and common inhabitants of the gastrointestinal tract [106]. The genus includes bacteria with probiotic qualities but also bacteria that are regarded opportunistic pathogens [107]. Some suggested virulence traits used by Enterococci are the following: adhesion to enterocytes through adhesins such as aggregation substance and enterococcal surface protein or via pili; and secretion of cellular toxins as for example cytolysin which augment virulence in animal models [108-111]. Moreover, Enterococci are resistant to a wide spectrum of antibiotics [112,113]. The establishment of the genus has a complicated history, and several species have been taxonomically reclassified through the years concurrently with advanced classification techniques [107]. This means that Enterococci-findings published in earlier studies possibly may refer to closely related *Enterococcus* spp. instead which should be kept in mind.

Enterococcus durans. Clinically piglets are not markedly affected by the infection and the mortality is insignificant. Nonetheless, affected piglets may exhibit decreased appetite, possible reduced weight gain and rough hair coat. Up to 80 % of the litters of a farrowing unit can be infected, and within the litters up to 90 % of the piglets. After entry into the body *E. durans* colonizes both the small and the large intestine, though primarily the jejunal and ileal mucosal surface. It adheres to the enterocytes with consequential villus atrophy and occasional necrosis. Watery to mucoid yellow diarrhoea develops within 24 hours after ingestion [114,115]. Piglets develop diarrhoea 3-5 days after birth. Apparently, litters by gilts and by second parity sows are mainly affected [58].

Enterococcus hirae. Literature on this species is scarce and especially in relation to piglets. Therefore the subsequent information on *E. hirae* is based on recent findings published by Larsson *et al.* (2014), which found it to be associated with neonatal porcine diarrhoea in Sweden. Piglets between 1-6 days old presented with watery to creamy diarrhoea and had milk-filled stomachs. This suggests that infected piglets continued suckling unaffectedly, or that diarrhoea was in its initial stage. *E. hirae* primarily colonized the villi of the small intestine, some adherent to the epithelium in association with epithelial lesions and villus atrophy [116].

Enterococcus faecium possesses probiotic properties that have been shown to positively affect intestinal health by reducing the duration and severity of diarrhoea as well as the presence of some potential pathogenic *E. coli* [117,118]. Additionally, administering *E. faecium* to piglets improves the average daily weight gain and the feed conversion ratio which may be an indirect effect of increasing the number of intestinal lactic acid bacteria [119]. The species has not been associated with piglet diarrhoea.

5.4.2. And the others….

Streptococci, *Lactobacillus* spp., *Actinobacillus suis,* and *Salmonella* spp. are briefly mentioned in the following, but will not be given much attention as these are not considered diarrhoeagenic agents in neonates.

Streptococci are facultative anaerobic Gram-positive bacteria. They are regarded part of the normal gut microbiota, and have previously not been linked to diarrhoea. Conversely, Larsson et *al.* (2014) do not attach any importance to *Streptococcus gallolyticus*, as this species only was detected in control piglets. The authors do not comment on either *Streptococcus equinus* or *Streptococcus lutetiensis* [116]. *S. gallolyticus* and *S. equinus* was also detected by Hermann-Bank *et al.* (Paper II, currently under submission). The number of sequence reads was slightly higher in healthy piglets compared to diarrhoeic piglets, and hence it seems reasonable to assume that these species are normal inhabitants of the porcine intestine. Likewise *S. lutetiensis* was among the sequence reads published by Hermann-Bank *et al.* (Paper I), it was detected with higher numbers of sequence reads in all of the control piglets compared with case piglets where it only was detected in 46 % [120].

***Lactobacillus* spp.** are Gram-positive facultative anaerobic bacteria that are part of the normal intestinal microbiota, and their numbers are often diminished in individuals suffering from gastrointestinal disorders and stress [46,121]. As mentioned earlier, *Lactobacillus* spp. are early intestinal colonizers, and it is therefore expected to find these bacteria in the intestinal tract of neonate piglets [34,47]. *Lactobacillus* spp. are mainly known for their beneficial effect on the gastrointestinal health and are commonly used probiotics [122].

Actinobacillus suis is a Gram-negative bacterium that has been isolated from the upper respiratory tract, the vagina and intestinal content from healthy pigs. This species has not been associated with diarrhoea, but sudden death, septicemia, fever, dyspnea, arthritis, and cyanosis are some of the sequela to *A. suis*, and younger animals are more severely affected [123-125].

***Salmonella* spp.** are Gram-negative enteropathogenic bacteria that cause inflammation and diarrhoea of the small and large intestine and most commonest in growing piglets from weaning till 4 months old. Nonetheless, *Salmonella* can also infect younger piglets with more severe outcome and cause atypical clinical symptoms compared with those of the older piglets which include: nervous signs, paralysis, polyarthritis, and death [59,126].

However, once all of these pathogens have been mentioned, it should be emphasized that it is not uncommon that the causative agent(s) is not identified, and the aetiology remains unknown [63,73]. Thus,

though the understanding and knowledge of intestinal infectious agents have grown considerable since Antony van Leeuwenhoek first described his observations on 'animalcules' (bacteria) back in 1676, we still have much to learn [127].

Table 3: Studies investigating the presence of bacterial agents in relation to neonate piglet diarrhoea within the first week of life. Bacteria are listed according to highest abundance, and if equal; according to earliest detection during the first week of life. N is the number of piglets included in the study. Age is the age of the piglets when samples were collected for bacterial detection. Grey studies: bacteria were not significantly associated with diarrhoea. FISH: Fluorescence in situ hybridization, qPCR: quantitative real-time PCR.

Country, year (study) [Reference]	N	Age	Gut Section	Diagnostic method	Bacterial agent(s) identified within the first week of life
South Korea, 1995 (case study) [114]	21	2-14 days	Jejunum, ileum, and colon	Culturing for *E. durans*	*E. durans*
USA, 2000 (case study) [83]	6	2-4 days	Small intestine and colon	Culturing; microscopy; ELISA for *Cl. difficile* toxins A and B; genotyping for *Cl. perfringens* toxinotype	*E. coli* *Cl. perfringens* type A *Cl. perfringens* type C *Cl. difficile*
Germany, 2001 (case study) [128]	41	1-7 days	Rectal swabs	Culturing for *E. coli* and colony blot hybridization for *E. coli* enterotoxin	ETEC
Slovakia, 2002 (case study) [129]	5	4-7 days	Small and large intestine	Culturing; microscopy; genotyping of *E. coli*; ELISA for *Cl. difficile* toxins A and B	ETEC *Cl. perfringens* type A *Cl. difficile*
USA, 2002 (case control study) [62]	100	1-7 days	Ileum, jejunum, and colon	Culturing for *E. coli, Salmonella* sp., *E. durans,* and *Cl. perfringens;* ELISA for *Cl. difficile* toxins A and B. Microscopy	*Cl. difficile* *E. durans* *E. coli* *Cl. perfringens* Type C
USA, 2007 (case control study) [130]	129	1-7 days	Jejunum, ileum, caecum and colon	Culturing and ELISA for *Cl. difficile* toxins (A and B)	*Cl. difficile* *Cl. perfringens* type A *E. coli* *E. durans* *Cl. perfringens* type C *A. suis*
Spain, 2009 (case control study) [78]	541	1-7 days	Rectal swabs	Culturing for *Cl. difficile* and DNA extraction → PCR for toxins A and B detection	*Cl. difficile*
Argentina, 2009 (case study) [131]	11	1-5 days	Small intestine, colon, and rectal swabs	Histopathology, PCR for *E. coli* virulence genes and optic immunoassay for *Cl. difficile* toxins (A and B)	*E. coli* *Cl. difficile*

Table 3 continued.

Country, year (study) [Reference]	N	Age	Gut Section	Diagnostic method	Bacterial agent(s) identified within the first week of life
Brazil, 2011 (case control study) [61]	276	1-7 days	Feces and rectal swabs	Culturing for *E. coli* and *Cl. perfringens:* PCR for type C or type A classification. ELISA for *Cl. difficile* toxin A and toxin B	*Cl. difficile* *Cl. perfringens* type A *E. coli*
USA, 2012 (case control study) [73]	48	1-13 days	Duodenum, jejunum, ileum, and colon	Culturing; histopathology; serotyping of *E. coli*; ELISA for *Cl. difficile* toxins A and B; qPCR for genotyping of *Cl. perfringens*	*Cl. perfringens* type A *Cl. difficile* *Salmonella* spp. ETEC
Canada, 2012 (case study) [63]	237	1-7 days	Small intestine and colon	Culturing; histopathology; gel-based PCR genotyping *Cl. perfringens* toxinotype A, B, C, D, E, and *cpb2* and agglutination serotyping for ETEC*;* ELISA for *Cl. difficile* toxins A and B	ETEC *Cl. perfringens* type A *Cl. difficile* *Salmonella* *E. durans*
Denmark, 2013 (case control study) [1]	101	3-7 days	Duodenum jejunum ileum, and colon	Culturing for *E. coli, Cl. difficile, and Cl. perfringens;* agglutination serotyping for *E. coli* and qPCR for virulence factor genes; Toxinotyping for *Cl. perfringens*	*E. coli* *Cl. perfringens* type A *Cl. difficile*
Denmark, 2014 (case control study) [2]	101	3-7 days	Duodenum jejunum ileum, and colon	FISH using universal, *E. coli, Enterococcus* spp., *Cl. perfringens,* and *Cl. difficile* probes	*E. coli* *Cl. perfringens* *Cl. difficile* *Enterococcus* spp.
Sweden, 2014 (case control study) [116]	29	1-6 days	Duodenum, jejunum, ileum, caecum, and colon	Culturing for Enterococci; microscopy; DNA extraction of mucosal-adherent bacteria → PCR using universal 16S rRNA gene primers → sequencing → classified using BLAST (GenBank)[†]	*E. hirae* *S. gallolyticus* *E. faecium* *S. equinus* *S. lutetiensis* *Lactobacillus* spp.

[†] In addition to several other supportive techniques.

6. Control of neonatal diarrhoea

Several preventative interventions and control measures have been employed against neonatal piglet diarrhoea. These include vaccination of sows during gestation to induce serum antibodies which later are passed on from the sow to the piglets through colostrum. Vaccination is commonly used against *E. coli* carrying fimbrial antigens [132,133] and *Cl. perfringens* beta-toxin [134]. In special cases customized autogenous vaccines can be prepared from farm-specific encountered pathogens [70]. Additionally, genetics has an influence, as resistance to *E. coli* F4 can be inherited in a Mendelian fashion through a homozygous recessive trait and this trait has been actively selected in breeding programs [135,136]. Furthermore, proper sanitation and disinfection should not be neglected or underestimated, as this step contributes to a significantly reduced contamination pressure [137,138]. All-in-all-out husbandry is preferable over continuous production as it allows thorough cleaning between groups of pigs and inhibits pathogen built-up [139]. Moreover, teat sanitation in lactating sows significantly reduces the bacterial count of the surface skin, and though laborious can be used as a prophylactic approach to decrease the piglets' encounter with bacteria [140]. As an alternative strategy which can be juxtaposed to vaccination, some farmers have implemented fecal backfeeding. By feeding fecal material or grinded intestines to gestating sows 7-8 weeks before farrowing, the goal is to initiate or enhance an immunological response in sows against 'herd-specific' pathogens. Subsequently the sow's piglets will receive protective 'herd-specific' antibodies via colostrum [141].

6.1. Antibiotics

Finally, antibiotics are a highly applied resort. Though the use of antibiotics in Danish piggeries accounts for more than 75 % of the total antibiotics used for all animals, the antibiotics used for treating sows and suckling piglets for gastrointestinal disorders (GID) accounts for less than 3 % and has actually decreased over the years since 2008 (Figure 6). Unfortunately it is not possible to assess the consumption of antibiotic exclusively for suckling piglets as this information is based on retrieval of data from the Central Husbandry Register (CHR), which collapse the antibiotic use for sows and suckling piglets [142]. Diarrhoea causes impaired animal welfare and as attempt to cure the underlying cause which may be infectious and/or to protect the susceptible piglets against secondary infectious agents, farmers and veterinarians resort to antibiotics for treatment of the piglets.

The most commonly used antibiotics for treatment of GID in Danish sows and suckling piglets are depicted in Figure 6. Active substrates are listed with the most prevalent at the bottom of the bars in the chart, thus the visible impression is in correct scale, though the logarithmic axis is deceptive. Among the depicted antibiotics the vast majority is broad-spectrum antibiotics: Sulfamethoxazole/trimethoprim, Tetracycline, Aminoglycosides, Penicillin, and Macrolides, while only two medical preparations are small-spectrum

antibiotics: Tiamulin and Lincomycin. Broad-spectrum antibiotics refer to antibiotics that are effective against a wide variety of microorganisms whereas small-spectrum antibiotics act specifically against a limited group of microorganisms [143]. Broad-spectrum antibiotics disrupt the normal bacterial gut microbiota and favor the overgrowth of antibiotic-insusceptible bacteria which may cause antibiotic-associated diarrhoea (AAD) [70]. Since neonate piglets have not yet established a stable gut microbiota neonate piglets may be particularly susceptible towards the microbial changes caused by broad-spectrum antibiotics. In humans *Cl. difficile* is a major cause of AAD, and while *Cl. difficile* has been associated with neonatal piglet diarrhoea, it is uncertain if this organism also is able to induces AAD in pigs [82,131,144].

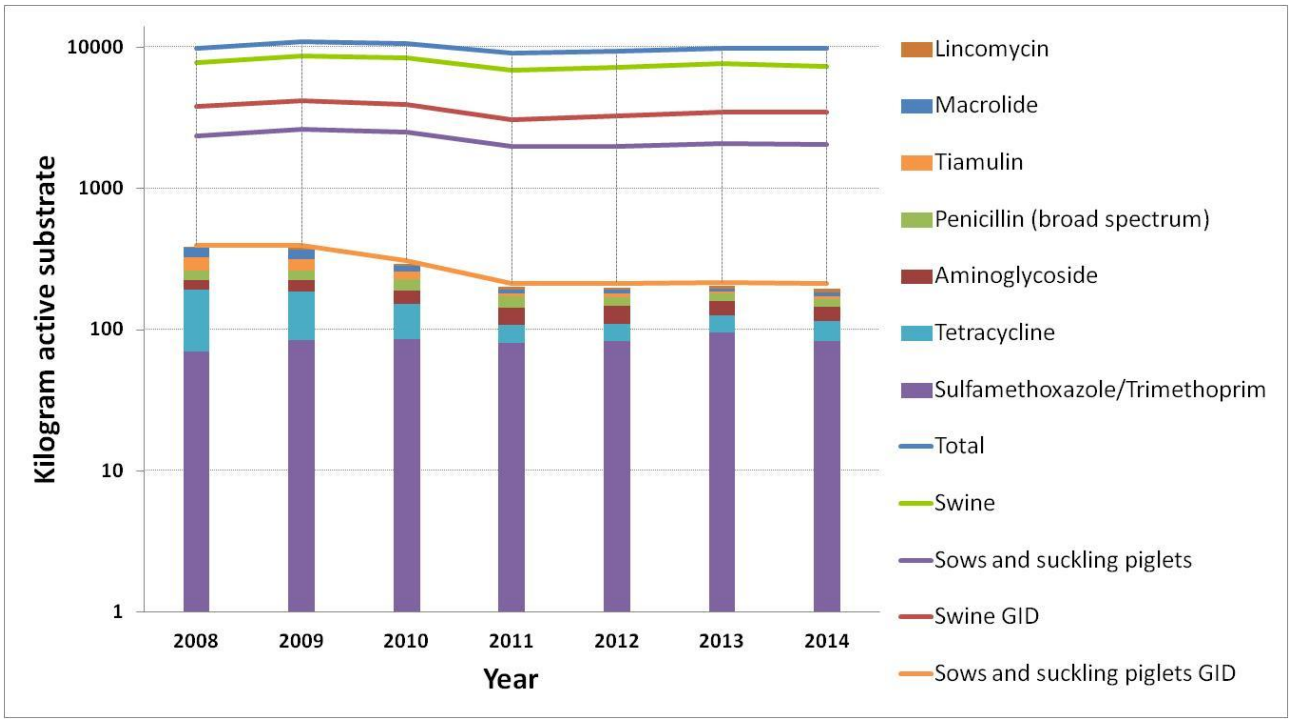


Figure 6: Average monthly veterinarian use of antibiotics in Denmark through the years 2008-2014 [142]. GID: gastrointestinal disorders.

Photo: author.

Methodology part

7. Methodologies, considerations and choices; the initial idea

The idea behind the methods applied in the experimental part of this thesis partly arose from studies published by Rajilic-Stojanovic *et al.* [145,146]. They investigated the microbiota of humans using their own designed phylogenetic microarray chip (the human intestinal tract chip, the HITChip) containing a high number of species-specific probes of intestinal relevance. This approach provided detailed information of the intestinal bacterial constituents present in the microbiota including those of scarce numbers.

In the search for a possible bacterial cause or involvement in the development of NNPD, we wanted to create an array after a similar concept. However, instead of only using species-specific probes, we wished to target groups of bacteria on various taxonomic levels ranging from domain down to species. As the bacterial load might have influence on the development of NNPD [147], we wanted the array to be quantitative. Furthermore, if the array was to be a potential future diagnostic tool a high throughput would be advantageous. Hence the goal was to develop an array which was able to analyze the bacterial constituents quantitatively in a large number of samples simultaneously.

The high throughput qPCR chip 48.48 Access Array (AA48.48) was first launched in December 2009 (personal communication, M. Lynch, Fluidigm Technical Support). At the time of planning the methodological approach of this thesis, the AA48.48 had exclusively been used for gene expression. To think innovatively we wanted to use the array to analyze DNA extracted from piglet-feces to get bacterial profiles from the individual piglets included in the studies. Paper I describes the development of the high throughput qPCR-based Gut Microbiotassay while trying to fulfill the MIQE guidelines [148]. The subsequent sections included in this chapter summarize theory and considerations concerning the individual methods which are part of this approach.

8. PCR and qPCR

In brief, PCR is a method that allows exponential amplification of a double-stranded target DNA by use of oligonucleotide primers flanking the target region, DNA polymerase, and free nucleotides. A thermal cycler executes the DNA amplification by increasing and lowering the temperature and a single PCR cycle includes the following steps: 94-98 °C: denaturation of the double-stranded DNA; 37-65 °C: hybridization of primers to complement DNA regions; 60-72 °C: DNA extension from the 3'- end of the primers via incorporation of free nucleotide. The end result after each PCR cycle is a doubling of the target DNA [149].

The main difference between PCR and qPCR is that during the qPCR the accumulation of specific products is detected in real-time by fluorescent dyes like Evagreen® or SYBR® Green. These dies binds selectively to double-stranded DNA after which they emit fluorescence, and at the end of each PCR cycle a camera

coupled to the thermal cycler registers the emitted fluorescence signal. Initially the number of target DNA is too low for the emitted signal to be distinguished from the background fluorescence. During the exponential amplification samples that contained high numbers of target DNA from start will rise above the background fluorescence after fewer PCR cycles than samples that initially contained lower number of target DNA. This, fluorescence will be equivalent to the amount of amplified product which will be equivalent to the target DNA in the sample.

The quantity of amplified DNA is given as the PCR cycle, Cq (quantification cycle, also known as the threshold cycle (Ct)), at which the emitted fluorescence signal can be registered from the background fluorescence, or the number of cycles needed to reach a specific threshold fluorescence. Furthermore, the Cq value has to be within the linear phase of the exponential amplification because this is where proportionality exists between the Cq value and the number of amplified target DNA copies [150,151].

9. The 48.48 Access Array™ Integrated Fluidic Circuit (Fluidigm)

The 48.48 Fluidigm Access Array™ System is an Integrated Fluidic Circuit (IFC) consisting of 48 detector inlets and 48 sample inlets. These are joined in a mesh of interconnecting channels, which make up 2304 individual reaction chambers sealed by NanoFlex™ valves. Thereby every sample is combined with every primer set in individual reactions. The architecture of the chip makes it feasible to work with small volumes, as the final metric is in nanoliters. Before applying primers and samples into the respective inlets (Figure 7) the array is primed with Control Line Fluid and Harvest Reagent by loading the array into the IFC controller AX. This instrument employs pneumatic pressure to regulate the opening and closing of valves thereby managing the accurate fluidic inflow to the reaction chambers from the wells.

Figure 7: Loading of samples into AA48.48 sample inlets by use of multi-pipette. Photo: author.

The Biomark instrument from Fluidigm works as a thermal cycler executing 2304 individual qPCR reactions in just a few hours – but that is not all. If primers are tagged and a 454 barcode library (454BL) is added to the samples, amplicons will get unique barcodes incorporated corresponding to the respective sample (Appendix A). When the run is finished, the array is dismounted from the BioMark, old excess Harvest Reagent is replaced by fresh Reagent, and by use of an additional IFC Controller AX the amplicons are harvested. Likewise this is done by applying pneumatic pressure to the array, forcing the liquid from the reaction chambers back into the original sample well in which it came from.

As the samples are harvested separately from their respective inlets, it is possible to choose which samples to sequence based on the quantitatively informative heatmap generated by the Fluidigm Real-Time PCR Analysis software (Fluidigm Corporation). Following the amplicons can be sequenced after a purification step, excluding unwanted PCR-bi-products (Appendix B, described in details in Paper I [120]). This possibility offers deeper taxonomic information of the bacteria present in the individual samples [152].

Since AA48.48 is a qPCR-based array similar advantages and disadvantages goes for the AA48.48 as for every other qPCR methods. Some of the advantages of qPCR are that it is: quick; sensitive; reproducible; quantitative; it can detect non-culturable bacteria; and there is minimal risk of contamination as the quantification is done in closed reaction chambers. Limitations of qPCR includes: PCR is susceptible to inhibitors present in some biological samples; template to product ratio conversion of amplified target DNA is coupled with uncertainty; amplification efficiency varies among primer assays targeting different bacterial strains; it cannot discriminate between live or dead organisms; primers can only be designed to known target sequences. Thus, the outcome is only as good as the quality of the material investigated, the primers used, and the running conditions applied [120,151,153].

10. Primers

The original plan was to find all of the primer sets for the analysis of the gut microbiota in published literature in which case they all had been verified and tested in advance. Following, we would 'only' have to test their function in our own setup and either keep or discard them. However, after searching the literature and testing possible primer sets *in silico,* it soon became obvious that it was necessary to refine and design a considerable fraction of the primer sets (listed in Paper I [120]).

10.1. Design and selection

The AA48.48 has capacity for 48 primer sets, however, we wanted to run the array with technical duplicates, so we had to find 24 primer sets for the setup. The primer sets should target bacteria, and since we did not know which bacteria we were looking for, some of the included primer sets had to target bacteria in general (taxonomic level domain).

Most general bacterial primers are designed to target conserved regions of the 16S ribosomal RNA gene. This gene encodes for a ribosomal component that is essential for protein synthesis, consequently it is part of every bacterial genome. The 16S rRNA gene sequence is built up by conserved regions, semi-conserved, and hypervariable regions (V1-V9). The conserved regions are ideal for designing universal or general bacterial primers with a broad taxonomic target, whereas the hypervariable regions are used to distinguish between taxa [154]. There are some controversies to which position of the approximately 1550 base pairs (bp) long 16S rRNA gene the primer should complement to cover the greatest variety of bacteria [155]. To accommodate this issue, and acknowledge that no primers are truly universal, two general primer sets amplifying different hypervariable regions were included to increase the bacterial coverage [156]. These were "Domain Bacteria A V2-V3" (this primer set actually targets V1-V2, but the name is maintained to avoid confusion) and "Domain Bacteria B V4-V5" as recommended by Liu *et al.* [155] and Youssef *et al*. [157], respectively. Additionally, the two general bacterial primer sets were chosen because they complement each other: They both target a great variety of bacteria though with diverse coverage, still, they each contribute with different bacteria from some taxonomic groups. For instance: "Domain Bacteria A" targets approximately 10 % of all bacteria belonging to the family Lactobacillaceae whereas "Domain Bacteria B" targets 46 %. On the other hand "Domain Bacteria B" fails to amplify bacteria such as *Salmonella enterica* and *E. coli* while these are covered by "Domain Bacteria A".

Besides from the widely used 16S rRNA gene other genes or interspatial regions can be potential targets for bacterial detection, for instance: *cpn*60, *rpoB*, or the 16S-23S rRNA intergenic spacer region [158-160]. Studies exploiting these targets have shown that they provide more bacterial discriminating power compared with 16S rRNA gene targets [159]. However, the 16S rRNA gene databases encompass much more sequence information than the alternative targets which is a major advantage of using the 16S rRNA gene as target [161]. Nonetheless, an additional general bacterial primer sets targeting the *cpn*60 gene which had previously been verified to amplify DNA extracted from piglet feces was tested, confer Hill *et al.* (2006) [160]. Testing the *cpn*60 primer set on the AA48.48 clearly demonstrated a miserable performance under the given running conditions, consequently, this target region was discarded. No further effort was invested in order to find alternatives to the two 16S rRNA gene general bacterial primer sets, since both of these performed satisfactory.

All primer sets were checked *in silico* by use of Ribosomal Database Project release 10 (http://rdp.cme.msu.edu/), and ProbeCheck (http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home) using default settings with zero, one, and two mismatches to ensure that the primers not only were specific under perfect binding conditions. This was a subjective judgment characterized by tradeoffs between primers that were too restrictive thereby excluding some (possibly

important) bacteria, and primers that were too general and included (irrelevant) bacteria other than their intended targets (cross-reaction) with the risk of obscuring data from interesting bacteria. Primers that displayed an ideal target with zero mismatches became far too unspecific if one or two mismatches were introduced. On the other hand primers which had very limited targets and, consequently, excluded some bacteria from its intended target group when zero mismatches were allowed, turned out to have a perfect target group when one or two mismatches were introduced.

10.1.1. 'Go fish'

In the search for a possible bacterial cause to NNPD, it was important to ensure a high likelihood of targeting the unknown bacterial agent(s). This was accomplished by selecting primer sets that targeted different taxonomic levels. Theoretically, the setup is conceived as a hierarchical taxonomic approach that functions as fishing net. The primer sets with the highest taxonomic rank (domain) target all bacteria, the primer sets targeting lower taxonomic levels are restrictive and zoom in on the respective taxonomic lineages. Hence when amplicons are subsequently sequenced, the domain primer sets with a general and broad coverage will provide an open view of the diverse bacterial composition, however, the broad coverage compromises the degree of possible taxonomic resolution [162]. On the contrary specific primer sets will offer deeper taxonomic resolution, but of a restricted group of bacteria, varying according to the specificity of the primer set (Figure 8).

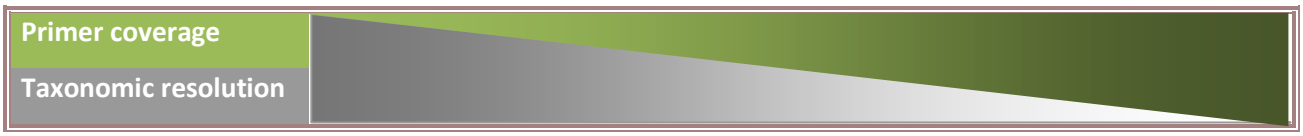


Figure 8: The balance between primer coverage and taxonomic resolution.

The bacterial hierarchical taxonomic targets of the Gut Microbiotassay are illustrated in Figure 9. The design increases the chances of detecting certain bacteria through its multi-target strategy. This means that highly represented bacteria will be detected by several primer sets targeting different taxonomic levels. Vice versa scarcely represented bacteria that might be overlooked at the higher taxonomic levels will be detected by the more restrictive primer sets. Either way, the detection level will be indicative of, where to search next, which can be an immense move in the right direction in case of searching for an unknown bacterial agent.

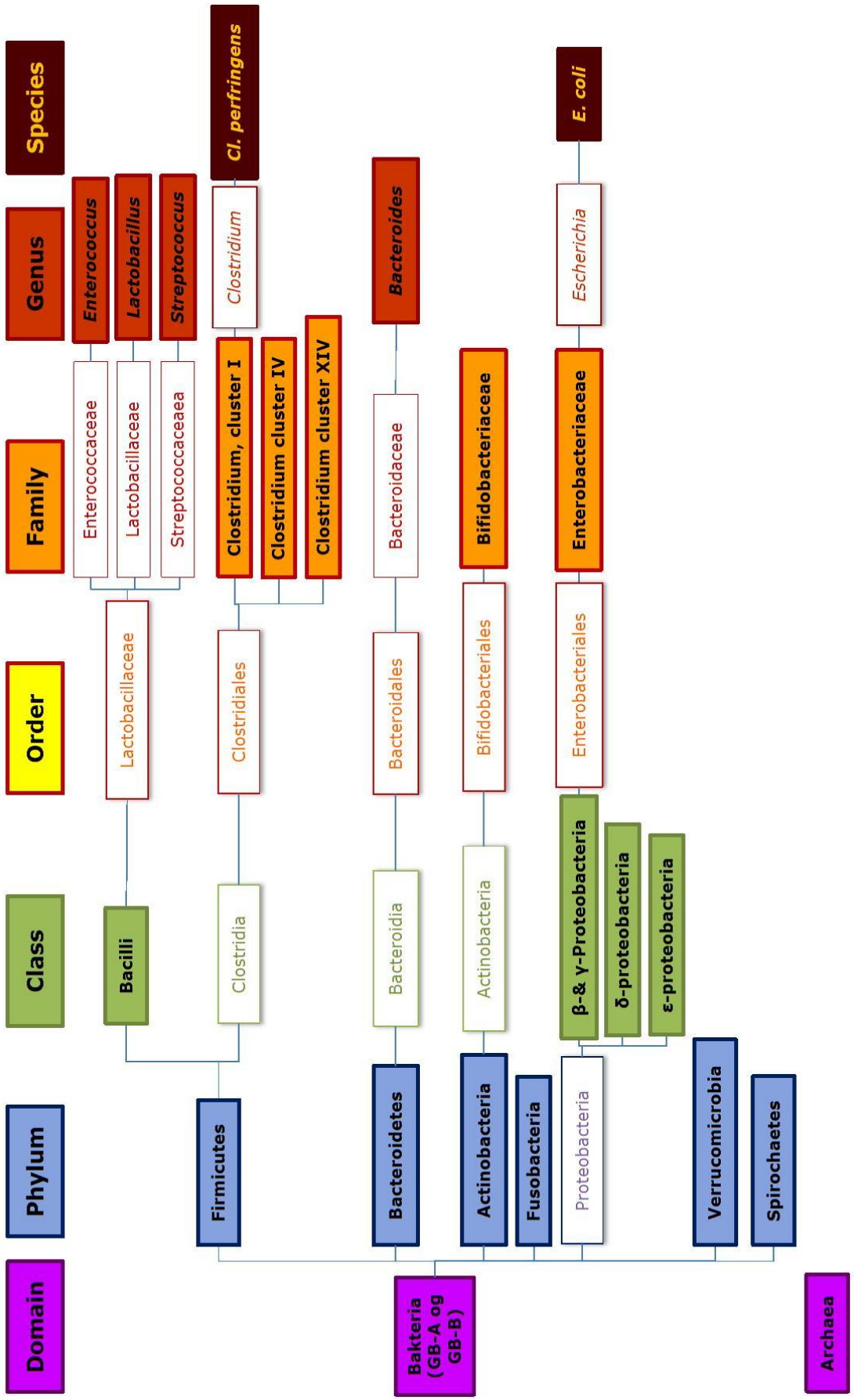


Figure 9: Primer targets of Gut Microbiotassay. Colored boxes represent the primer sets constituting the Gut Microbiotassay while the transparent boxes reflect the taxonomical lineage that the primer sets belong to.

10.2. Verification of the primers

A total of 100 primers were tested: 49 forward primers and 51 reverse primers, before settling with the final setup [120]. All primers were tested on the AA48.48 against standard curves of DNA extracted from pure cultures of representative reference bacteria ranging from 0.5 pg/µl to 50 ng/µl to test their amplification efficiency, dynamic range, and limit of detection, as well as to check if they exclusively amplified their target bacteria [120]. Appendix C lists the individual amplification efficiencies. Subsequently, all PCR amplicons were harvested and the specificity of the PCR reactions was assessed by visual inspection of the melting curves, but also by size verification of the amplicons using the Agilent DNA 1000 chip (Agilent Technologies, Waldbronn, Germany), Appendix D shows results for *E. coli* and *Cl. perfringens*.

The measurements revealed the expected number of PCR products as well as the expected size of the amplicons generated from each of the different primer sets when tested against 15 representative reference bacteria. Though, from the Agilent measurements on PCR amplicons with the 454BL included it was evident that there was some unwanted PCR by-products approximately 150 bp long.

11. Optimization of AA48.48 running conditions

In order to optimize the AA48.48 PCR reaction, the following parameters were tested, however, the recommendations from Fluidigm (*) were optimal in every case, and were thus applied [120,152]:

- Primer concentration: 1 , 2, and 4* µM
- $MgCl_2$ concentration: 4.5* and 5.5 mM
- 454BL concentration: 400* and 800 nM
- Sample concentration: 100; 50*; 25; 5; 2.5; 0.5; 0.25; 0.05; 0.025; 0.005; 0.0025; 0.0005; 0.00025 ng/µl

Furthermore, we would have liked to optimize different temperature and time settings, but unfortunately there was a bug in the Fluidigm/Biomark software that made it impossible to change the PCR thermal protocol (personal communication, M. Lynch, Fluidigm Technical Support).

12. A cheaper alternative to AA48.48

A counterpart to the AA48.48 is the Dynamic Array™ 48.48 IFCs (DA48.48). This is also a qPCR-based chip that separates from the AA48.48 by being reversed in architecture, and not offering the opportunity to harvest the PCR amplicons. Therefore it is cheaper than the AA48.48. Hence, we wanted to implement the successful running conditions from the AA48.48 to the DA48.48, and use the DA 48.48 as a cheaper alternative to the AA48.48, in case we did not want to harvest the PCR amplicons. This was even more appealing because Fluidigm has launched several dimensions of the Dynamic Array including 96 × 96 and

192 × 24 which offers an even higher throughput [163]. Nonetheless, it turned out that the two chips were not fully transposable after all. The DA48.48 appeared to be less sensitive compared with the AA48.48, and additionally, the reagents were more viscous, making it challenging not to introduce air bobbles when loading the reagents into the array inlets. It is critical to avoid air bobbles, as these clog the channels and as a result the entire lane following the air bobble fails. Consequentially DA48.48 was not used any further.

13. Choice of method for DNA extraction from fecal content

The choice of method for genomic DNA extraction has enormous impact on the final outcome, since the DNA extraction efficiency constitutes the basic material from which all further information is acquired. There are several concerns related to this issue including: DNA yield, DNA sharing, DNA contamination, and reproducibility. The ongoing challenge when analyzing microbial communities is how to ensure that all species are truly represented. The cell wall of Gram-positive bacteria is more rigid and strong compared with the cell wall of Gram-negative bacteria hence, Gram-positive bacteria are more resistant to cell lysis and can therefore be underrepresented [164].

The major challenge in extracting DNA from intestinal content from piglets included in this thesis was to obtain DNA yields high enough. Therefore several DNA extraction methods were tested in order to determine the method that provided the highest DNA concentrations and acceptable purity assessed by the 260/280 nm-ratio using the NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, Germany) spectrophotometer. In Paper I Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA) and QIAsymphony Virus/Bacteria Mini Kit (Qiagen, Hilden, Germany) were applied for DNA extraction of reference bacteria and intestinal content, respectively. These methods were recommended by the technicians that through experience had acknowledged their applicability. However, as extraction using Easy-DNA™ Kit was extremely laborious and time consuming, and there were difficulties getting DNA concentrations > 50 ng/µl using QIAsymphony Virus/Bacteria Mini Kit [120], other methods were tested in order to find a more optimal DNA extraction method for the hundreds of samples that was going to be analyzed in Paper II and Paper III. These were: QIAamp® DNA Stool Mini Kit (Qiagen, Hiden, Germany) with the QIAcube (Qiagen); Maxwell® 16 Blood DNA Purification Kit, AS1010; Maxwell® 16 Cell LEV DNA Purification Kit, AS1140; Maxwell® 16 LEV Blood DNA Kit, AS1290 (Promega, Madison, WI, USA) with the Maxwell® 16 Instrument (Promega), and MagMAX™-96 Viral RNA Isolation Kit, AM1836 (Applied Biosystems®, Foster City, CA, USA). Appendix E briefly describes preceding procedures for preparation of DNA for extraction with QIAcube, Maxwell, and MagMAX. The method of choice after comparing the DNA measurements from the NanoDrop® ND-1000 (NanoDrop Technologies Inc) spectrophotometer was Maxwell® 16 LEV Blood DNA Kit, AS1290 (Promega), as this method provided the highest DNA yields (Appendix F).

14. 454 GS FLX + Titanium sequencing

Fluidigm has customized the AA48.48 to the 454 GS FLX + Titanium sequencer. By adding a 454BL to the samples before processing the AA48.48 amplicon generation and library preparation are combined in the same reaction, a process called "the Access Array 4-primer amplicon tagging strategy" [152], schematically illustrated in Appendix A.

Sequencing has revolutionized our conception and understanding of microbial communities since the late 1990'ies [165]. The Golden standard was Sanger sequencing (first-generation sequencing) introduced in 1977 [166]. However, this method is time consuming and expensive compared to next generation sequencing techniques. Next generation sequencing techniques first launched in 2005 avoid the need for cloning, and enable sequencing of more samples at once and provides more sequence reads in a single run compared with Sanger sequencing [167,168]. Of the next generation sequencing techniques 454 GS FLX + Titanium sequencer (Roche) generates the longest sequence reads (up to 1000 bp) compared to the original classic platforms: Solexa (Illumina) (300 bp) and SOLiD (Applied Biosystems) (75 bp) (Table 4). That said, PacBio (Pacific Biosciences), a novel alternative platform, beats them all with its ability to generate read lengths up to 20 kb but at much higher expenses [169]. While the 454 GS FLX + Titanium sequencer is superior in read length to the Solexa and SOLiD these two platforms offer much higher throughput, hence, it is important to know in advance what is of most importance. In case of the studies in this thesis a longer read length increases the chances of identification and classification of unknown bacteria when sequence reads are subsequently aligned against known sequence databases like Greengenes Gene Database [161,167,170]. However, the read lengths provided by PacBio are overkill when investigating bacterial communities. Nonetheless, the 454 GS FLX + Titanium sequencer also possess some main drawbacks: its susceptibility towards homopolymers (that is repetitions of identical nucleotides), its relatively low throughput, and last but not least the announcement of its final in 2016 [169,171].

Table 4: **Comparison of different next generation sequencing platforms [169].**

Method	SOLiD Applied Biosystem	Solexa Illumina	454 GS FLX + Titanium Roche	PacBio Pacific Biosciences
Sequence length, max bp	75	300	1000	20000
Throughput per run, max Gb	320	1800	0.7	0.5
Run time, hours	336	240	23	3

Paper I

I

Highly accessed

The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity.

Marie Louise Hermann-Bank, Kerstin Skovgaard, Anders Stockmarr, Niels Larsen and Lars Mølbak

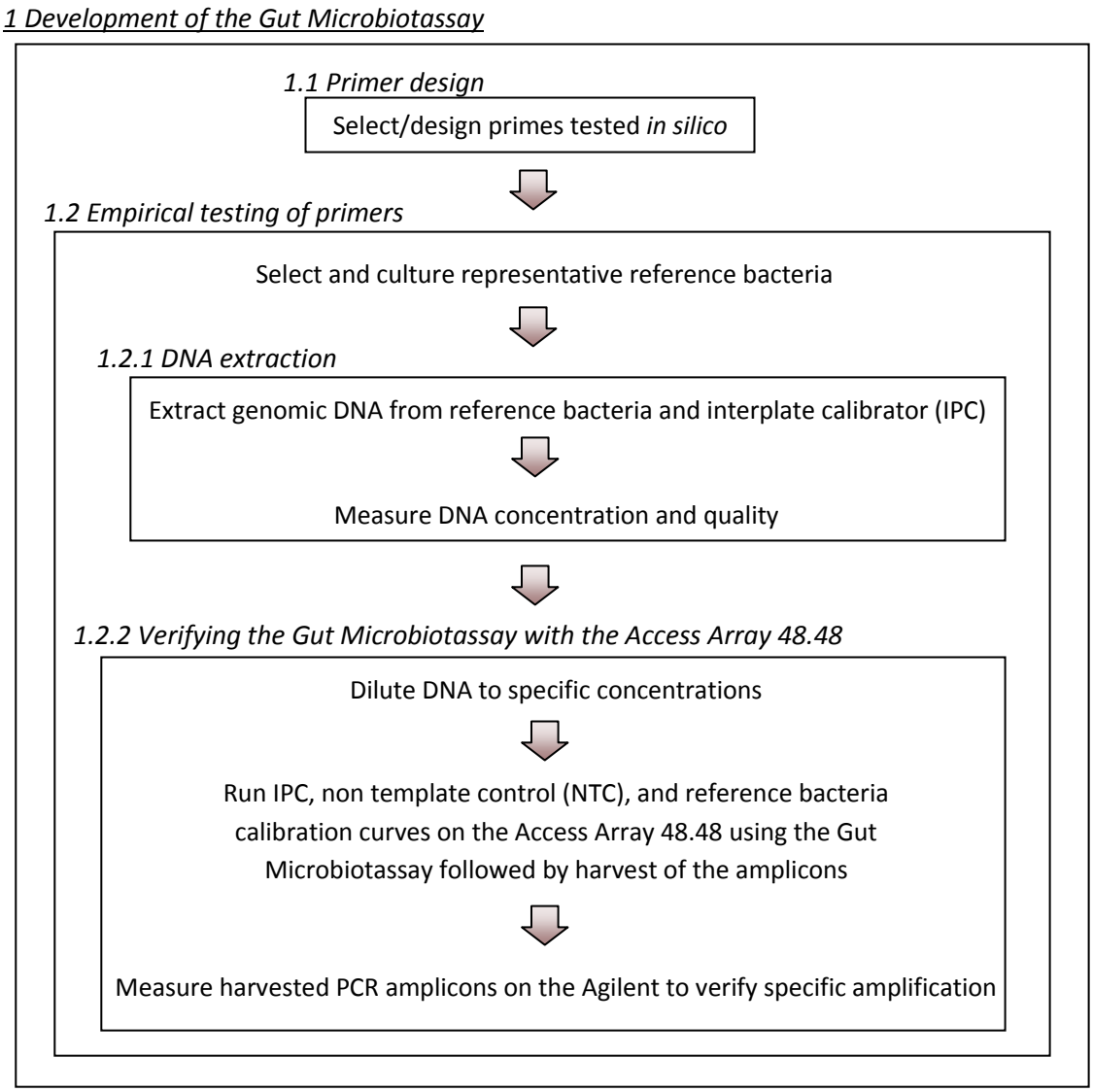
Published in

BMC Genomics

Introduction

The aim of this study was to design, test, optimize, and verify an assembly of 24 primer sets able to function with the Access Array 48.48 to detect and quantify the bacterial constituents of the gut microbiota (the Gut Microbiotassay).

Flow diagram



2 Testing the Gut Microbiotassay on complex samples

2.1 Samples and sampling

Samples: ileal and colonic luminal content from seven piglets with diarrhoea and five piglets without diarrhoea

2.2 DNA extraction

Extract genomic DNA

Measure DNA concentration and quality

2.3 Analyzing complex samples on the Access Array 48.48 with the Gut Microbiotassay

Dilute DNA to specific concentrations

Run sample-calibration curves on the Access Array 48.48 using the Gut Microbiotassay followed by harvest of the amplicons

Measure harvested PCR amplicons on the Agilent to verify specific amplification

Run individual samples on the Access Array 48.48 using the Gut Microbiotassay with and without a 454 barcode library added followed by harvest of the amplicons

2.4 Amplicon preparation for 454-sequencing

Measure harvested sample amplicons on the Agilent to verify specific amplification and to determine concentration

Pool all sample amplicons with a 454 barcode library incorporated in equal concentrations

Reduce volume of pooled amplicon with phenol chloroform

Purify pool of amplicons on agarose gel

Extract DNA from agarose gel and measure DNA concentration and quality

2.5 Sequence information

454 GS FLX + Titanium Sequencing, LGC Genomics

BION-meta for bioinformatics

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

BMC Genomics

METHODOLOGY ARTICLE Open Access

The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity

Marie Louise Hermann-Bank[1], Kerstin Skovgaard[2], Anders Stockmarr[3], Niels Larsen[4] and Lars Mølbak[1,5*]

Abstract

Background: The intestinal microbiota is a complex and diverse ecosystem that plays a significant role in maintaining the health and well-being of the mammalian host. During the last decade focus has increased on the importance of intestinal bacteria. Several molecular methods can be applied to describe the composition of the microbiota. This study used a new approach, the Gut Microbiotassay: an assembly of 24 primer sets targeting the main phyla and taxonomically related subgroups of the intestinal microbiota, to be used with the high-throughput qPCR chip 'Access Array 48.48', AA48.48, (Fluidigm®) followed by next generation sequencing. Primers were designed if necessary and all primer sets were screened against DNA extracted from pure cultures of 15 representative bacterial species. Subsequently the setup was tested on DNA extracted from small and large intestinal content from piglets with and without diarrhoea. The PCR amplicons from the 2304 reaction chambers were harvested from the AA48.48, purified, and sequenced using 454-technology.

Results: The Gut Microbiotassay was able to detect significant differences in the quantity and composition of the microbiota according to gut sections and diarrhoeic status. 454-sequencing confirmed the specificity of the primer sets. Diarrhoea was associated with a reduced number of members from the genus Streptococcus, and in particular S. alactolyticus.

Conclusion: The Gut Microbiotassay provides fast and affordable high-throughput quantification of the bacterial composition in many samples and enables further descriptive taxonomic information if combined with 454-sequencing.

Keywords: Access Array 48.48, Bacteria, Intestine, Microbiota, qPCR

Background

Immediately after birth the mammalian gastrointestinal tract is colonized by a complex and diverse microbial ecosystem. The bacterial invasion and the following gut microbial composition has an enormous impact on its host's health and well-being [1]. To gain a better understanding of this complex ecosystem, culture-independent methods are essential, as a considerable fraction of the intestinal microbiota has not yet been cultured [2]. One of the ongoing controversies is how to study the bacterial composition in complex ecosystems. To date, some of the widely used approaches to characterize the intestinal microbiota are: metagenomics, phylogenetic microarrays, DNA fingerprinting techniques, and qPCR [2-5]. These methods provide different degrees of taxonomic as well as quantitative information on the microbiota [5]. Nonetheless, variation in technical procedures and differences in data treatment and interpretation makes it challenging to compare results between studies. Also, the expenses and time consumption in relation to labour intensity and data analysis vary greatly. Especially metagenomic approaches are receiving increased attention in the study of microbial communities as a result of their shorter sequencing speed, extended read length, and lower costs [5,6]. However, the enormous amount of data generated becomes cumbersome to analyse, and requires lots of dedicated time as well as expertise to manage [6]. The Access Array 48.48, AA48.48, (Fluidigm Corporation, South San Francisco, CA, USA) creates an affordable link between high-throughput qPCR and next generation

* Correspondence: dklmb@chr-hansen.com
[1]Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark
[5]Present address: Chr. Hansen, Bøge Allé 10, 2970 Hørsholm, Denmark
Full list of author information is available at the end of the article

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

sequencing (NGS) and provides manageable data with valuable quantitative and taxonomic information.

Since the 1990's, qPCR has been applied widely due to its quantitative precision, high specificity and sensitivity, broad dynamic range, good reproducibility, and relatively low costs [7]. In general, qPCR is quick to perform with a low to medium throughput, since most qPCR platforms have a capability format of 96 or 384 [7]. The high-throughput qPCR chip AA48.48 combines 48 detector inlets and 48 sample inlets by interconnecting channels into 2304 individual reaction chambers (singleplex) http://www.fluidigm.com/access-array-system.html. In contrast to most qPCR platforms which require reaction volumes between 5 to 100 μl [7], the AA48.48 operates with a reaction volume of approximately 35 nl [8]. By tagging primers at their 5′-end (using the 'The Access Array 4-primer amplicon tagging strategy' [8]), and including a 454 Barcode Library (454BL) with the samples, amplicon generation and library preparation is achieved in the same reaction. Afterwards, amplicons can be harvested directly from the AA48.48 sample inlets where the respective samples were initially loaded. The unique barcodes and specific primers enable segregation of the pooled samples in NGS analysis later on. The AA48.48 process time from start to finish is approximately five hours. After each qPCR run Fluidigm Real-Time PCR Analysis software (Fluidigm Corporation) generates a heatmap constructed as a grid of 48 × 48 small squares presenting all the 2304 primer sample combinations. The heatmap depicts the Cq-value for each reaction according to a colour scale, providing a good overview of each sample and the possibility to compare the bacterial profiles across all the samples.

This study describes the design, optimization, and validation of the Gut Microbiotassay: a primer setup consisting of 24 primer sets targeting the main bacterial phyla of the intestinal microbiota at various taxonomic levels, to be used with the AA48.48 in combination with NGS. Furthermore, it demonstrates the applicability of the Gut Microbiotassay on luminal content collected from the small and large intestine of piglets of different diarrhoeic status. Finally, it validates the specificity of the Gut Microbiotassay by sequencing these amplicons. To our knowledge, the AA48.48 has not previously been used to investigate microbial communities.

Methods

Development of the Gut Microbiotassay

Primer design

Inspired by Rajilic-Stojanovic et al. [9], the primer setup was designed to target the ribosomal RNA genes (16S or 23S) of the major bacterial groups present in the mammalian intestinal microbiota, including the phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria [10]. To gain insight into the bacterial composition, different taxonomic levels were represented: domain, phylum, class, family, genus, and species-specific primers (Table 1).

Primer specificity was checked in silico with the 'probe match' facility of the Ribosomal Database Project, release 10 (http://rdp.cme.msu.edu/) [11] and the 'check' application of ProbeCheck (http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home) [12] using the programs' default settings. Judged by the search results from these online databases, primers were renamed according to their main target. Some of the published primers were slightly modified to improve their specificity. New primers were designed using the 'Probe Design Tool' of the ARB Software package (http://www.arb-home.de/) [13], or Primrose [14] using default options, and further validated based on BLAST search (NCBI). Primers were designed to have approximately same length of nucleotides, GC-content, a minimum number of degenerate bases, and to produce amplicons between 200–500 bp long, compatible with the read length of the 454 GS FLX Titanium Sequencer. Similar properties were important, given that all the primers had to function under the same conditions when run on an AA48.48.

All primers, except for the species-specific ones, were tagged at their 5′-end to enable incorporation of a 454BL necessary for 454-sequencing: forward tag: 5′-ACACTGACGACATGGTTCTACA-3′, and reverse tag: 5′-TACGGTAGCAGAGACTTGGTCT-3′ in accordance to the 'Access Array™ System User Guide' [8]. Primers were purchased from Eurofins MWG Synthesis GmbH (Ebersberg, Germany) and stored at −20°C.

Empirical testing of primers

All primers were tested against 15 strains of pure-cultured reference bacteria, representing targets for one or more of the different primer sets (Table 1). Thus the reference bacterial DNA functioned as both positive and negative controls for the individual primer sets.

DNA extraction Chromosomal DNA from cultured reference bacteria (Additional file 1: Table S1) was isolated using the Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA), for further details on the DNA extraction protocol see Additional file 2. DNA was precipitated with ethanol, and resuspended in 60 μl TE buffer. The reference bacteria were cultured as recommended by DSMZ (www.dsmz.de/).

An interplate calibrator (IPC) was included in all AA48.48, consisting of bacterial DNA extracted from ~ 100 mg colonic content from a healthy conventional pig, 14-week-old, Danish landrace. Intestinal content was collected immediately after euthanization and frozen at − 80°C. A 10% PBS suspension was made from the intestinal content, and from here on, the protocol was identical to the one used for the reference bacteria.

DNA concentration and purity were assessed by the 260/280 nm-ratio using the Nanodrop® ND-1000

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

Table 1 Target-specific primer sequences constituting the Gut Microbiotassay in order to target main bacterial phyla and bacterial groups and species of high interest in the mammalian intestine

Main target		rRNA gene	Sequence (5′ → 3′)	E. coli position	Size, bp	Reference	Reference bacterium
Domain Bacteria A V2-V3	Forward	16S	AGAGTTTGATCCTGGCTCAG	7	336	Liu et al. [30]	1,2,3,4,5,6,7,8,9, 10,11,13,14,15
	Reverse	16S	CTGCTGCCTYCCGTA	342		Liu et al. [30]	
Domain Bacteria B V4-V5	Forward	16S	CAGCAGCCGCGGTAATAC	518	389	Schwieger & Tebbe [31]	1,2,3,4,5,6,7,8,9, 10,11,13,14,15
	Reverse	16S	CCGTCAATTCCTTTGAGTTT	906		Schwieger & Tebbe [31]	
Phylum Firmicutes	Forward	16S	CTGATGGAGCAACGCCGCGT	385	429	Haakensen et al. [32]	6,7,9,11,13,14
	Reverse	16S	ACACYTAGYACTCATCGTTT	813		Mühling et al. [33]	
Class Bacilli	Forward	16S	GCAGTAGGGAATCTTCCGC	353	461	Felske et al. [34]	7,11,14
	Reverse	16S	ACACTTAGCACTCATCGTTT	813		Modified from Mühling et al. [33]	
Genus Enterococcus	Forward	16S	GGGTAACCTRCCCATCAGA	125	325	Modified from Behr et al. [35]	7
	Reverse*	16S 16S	GTTACTCTCATCCTTGTTC ACCGTCAGGGGACGTTCAG	449 466	342	Modified from Behr et al. [35] Modified from Behr et al. [35]	
Genus Lactobacillus	Forward	23S	GCGGTGAAATTCCAAACG	774	216	This study, Linux ARB [13]	11
	Reverse	23S	GGGACCTTAACTGGTGAT	989		This study, Linux ARB [13]	
Genus Streptococcus	Forward	16S	CTWACCAGAAAGGGACGGCT	488	337	This study, ClustalW2 [36]	14
	Reverse	16S	AAGGRYCYAACACCTAGC	824		This study, ClustalW2 [36]	
Family Clostridium cluster I	Forward	16S	AAAGGAAGATTAATACCGCATA	159	538	Modified from Rinttila et al. [37]	6
	Reverse	16S	TTCTTCCTAATCTCTACGCA	696		Hung et al. [38]	
Species Clostridium perfringens	Forward	16S	TGAAAGATGGCATCATCATTCAAC	183	258	Skånseng et al. [39]	6
	Reverse	16S	GGTACCGTCATTATCTTCCCCAAA	440		Skånseng et al. [39]	
Family Clostridium cluster IV	Forward	16S	ACAATAAGTAATCCACCTGG	866	298	Modified from Ramirez-Farias et al. [40]	9
	Reverse	16S	CTTCCTCCGTTTTGTCAA	1163		Matsuki et al. [41]	
Family Clostridium cluster XIV	Forward	16S	CGGTACCTGACTAAGAAGC	482	413	Rinttila et al. [37]	13
	Reverse	16S	CTTTGAGTTTCATTCTTGCGAA	894		Matsuki et al. [42]	
Phylum Bacteroidetes	Forward	16S	CCGGAWTYATTGGGTTTAAAGGG	554	414	Mühling et al. [33]	1
	Reverse	16S	GGTAAGGTTCCTCGCGTA	967		Mühling et al. [33]	
Genus Bacteroides	Forward	16S	AAGGTCCCCCACATTGG	302	300	Manz et al. [43]	1
	Reverse	16S	GAGCCGCAAACTTTCACAA	601		Franks et al. [44]	
Phylum Actinobacteria	Forward	16S	GCGKCCTATCAGCTTGTT	235	333	Modified from Glockner et al. [45] first published. They refer to Erhart [46]	2
	Reverse	16S	CCGCCTACGAGCYCTTTACGC	567		This study, ClustalW2 [36]	
Family Bifidobacteriaceae	Forward	16S	CTCCTGGAAACGGGTGG	152	442	Matsuki et al. [42]	2
	Reverse	16S	CTTTCACACCRGACGCG	593		Delroisse et al. [47]	
Class β- and γ-proteobacteria	Forward	23S	GTATAATGGGTCAGCGAC	569	673	This study, Linux ARB [13]	8
	Reverse	23S	CAGCATTCGCACTTCTGA	1241		This study, Linux ARB [13]	
Family Enterobacteriacea	Forward	16S	CGTCGCAAGMMCAAAGAG	182	333	Modified from Friedrich et al. [48]	8
	Reverse	16S	TTACCGCGGCTGCTGGCAC	514		Modified from Palmer et al. [49]	
Species Escherichia coli	Forward	16S	GTTAATACCTTTGCTCATTGA	461	320	Malinen et al. [50]	8
	Reverse	16S	ACCAGGGTATCTAATCCTGTT	780		Malinen et al. [50]	

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

Table 1 Target-specific primer sequences constituting the Gut Microbiotassay in order to target main bacterial phyla and bacterial groups and species of high interest in the mammalian intestine (Continued)

Class ε-proteobacteria	Forward	16S	TGGTGTAGGGGTAAAATCCG	680	286	Bui et al. [51]	5
	Reverse	16S	AGGTAAGGTTCTTCGYGTATC	965		This study, Primrose [14]	
Class δ-proteobacteria	Forward	16S	GGTGTAGGAGTGAARTCCGT	681	534	This study, Primrose [14]	3
	Reverse	16S	TACGTGTGTAGCCCTRGRC	1214		This study, Primrose [14]	
Phylum Fusobacteria	Forward	16S	GATCCAGCAATTCTGTGTGC	387	292	Sanguin et al. [52]	10
	Reverse	16S	CGAATTTCACCTCTACACTTGT	678		Walter et al. [53]	
Phylum Verrucomicrobia	Forward	16S	GAATTCTCGGTGTAGCA	673	551	Modified from Ranjan [54]	15
	Reverse	16S	GGCATTGTAGTACGTGTGCA	1223		This study, Primrose [14]	
Phylum Spirochaetes	Forward	16S	GTYTTAAGCATGCAAGTC	45	294	Choi et al. [55]	4
	Reverse	16S	TGCTGCCTCCCGTAGGAG	338		This study, ClustalW2 [36]	
Domain Archaea	Forward	16S	CAGCMGCCGCGGTAATWC	518	440	Giovannoni et al. [56]	12
	Reverse	16S	YCCGGCGTTGAMTCCAATT	957		Delong [57]	

Reference bacteria listed are target for the respective primer sets. Numbers represent: 1: Bacteroides fragilis (DJF_B083(EU728706)), 2: Bifidobacterium pseudolongum globosum (DMS 20092), 3: Bilophila wadsworthia (ATCC 49260), 4: Brachyspira pilosicoli (isolated from the intestine of slaughter pig at DTU-VET, 28-02-2000), 5: Campylobacter fetus (ATTC 10852), 6: Clostridium perfringens (NCTC 10240), 7: Enterococcus faecalis (ATCC 29212), 8: Escherichia coli (9711108–2), 9: Faecalibacterium prausnitzii (DSM 17677), 10: Fusobacterium Necrophorum (ATCC 25286), 11: Lactobacillus sakei (DSM 20017), 12: Methanocorpusculum labreanum (DSM 4855), 13: Roseburia sp. (DJF_VR77 (EU728794)), 14: Streptococcus suis (NCTC 10446), and 15: Verrucomicrobium spinosum (DSM 4136). Nucleotide explanation: Y, C/T ; R, A/G ; W, A/T ; K, G/T ; M, A/C.
*To improve coverage of the Genus Enterococcus, two reverse primers were mixed in equal concentrations.

(NanoDrop Technologies Inc., Wilmington, Germany) spectrophotometer (Additional file 2). DNA was stored at −20°C until needed.

Verifying the Gut Microbiotassay on the Access Array 48.48 Tenfold serial dilutions ranging from 0.5 pg/μl to 50 ng/μl were made from DNA extracted from 15 reference bacteria. The AA48.48 was processed following the 'Access Array System™ User Guide' [8]. In short: primer sets were mixed in equal concentrations and diluted to 4 μM with 20 × Access Array Loading Reagent (Fluidigm, South San Francisco, CA, USA) and nuclease-free water (Ambion Inc., Austin, USA). Master Mix was prepared as described in the instructions, and sample solutions were produced from 4 μl Master Mix and 1 μl DNA. The AA48.48 was primed in a 'pre-PCR' IFC controller AX (Fluidigm), before it was processed in the Biomark (Fluidigm) using the Fluidigm 'AA 48 × 48 Standard v1'-protocol listed in Table 2. Subsequently the amplicons were harvested in a 'post-PCR' IFC controller AX (Fluidigm).

Harvested amplicons were measured on the Agilent DNA 1000 chip (Agilent Technologies, Waldbronn, Germany) with the Agilent 2100 Bioanalyzer (Agilent Technologies) to verify the specificity of the Gut Microbiotassay. This was assessed from the size and number of amplicons generated by the primer sets listed in Table 1, with the reference bacteria as targets.

Three primer concentrations (1, 2 and 4 μM), and three primer:454BL-ratios (1:0.2, 1:0.4 and 1:0.8 μM) were tested, before settling on the final protocol.

Table 2 The Fluidigm 'AA 48 × 48 Standard v1' PCR thermal protocol [8]

PCR stages	Temperature	Number of Cycles
Thermal mixing and hot start phase	50°C 2 minutes	
	70°C 20 minutes	1
	95°C 10 minutes	
	95°C 15 seconds	
PCR cycle	60°C 30 seconds	10
	72°C 1 minute	
C0t cycle	95°C 15 seconds	2
	80°C 30 seconds	
	60°C 30 seconds	
PCR cycle	72°C 1 minute	8
	95°C 15 seconds	
	60°C 30 seconds	
	72°C 1 minute	
C0t cycle	95°C 15 seconds	2
	80°C 30 seconds	
	60°C 30 seconds	
	72°C 1 minute	
PCR cycle	95°C 15 seconds	8
	60°C 30 seconds	
	72°C 1 minute	
C0t cycle	95°C 15 seconds	5
	80°C 30 seconds	
	60°C 30 seconds	
Extension	72°C 1 minute	1

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

Testing the Gut Microbiotassay on complex samples

Samples and sampling

To explore the efficiency range of the Gut Microbiotassay, and test its sensitivities under varying circumstances, the study included 12 three-day-old piglets from conventional pig-farms (Danish landrace); seven with and five without clinical diarrhoea (Additional file 3: Table S2). None of the animals had received any treatment. Piglets were sacrificed and luminal content from the small and large intestine were obtained immediately after and frozen at −80°C. All handling of the animals was in accordance with regulations from The Danish Centre for Animal Welfare.

DNA extraction

100 mg intestinal content was suspended in 900 μl PBS and bead-beated in 2 ml microcentrifuge tubes containing a 5 mm steel bead (Qiagen, Hilden, Germany) at 15.0 hertz for 2.3 min (Tissuelyser II, Qiagen). Tubes were centrifuged at 10,000 × g, 90 s, 20°C, and 350 μl of supernatant were transferred to new tubes. These were placed in the QIAsymphony SP (Qiagen) for DNA extraction using the QIAsymphony Virus/Bacteria Mini Kit (Qiagen) with the protocol 'Pathogen complex 200' (Qiagen), elution volume: 60 μl. DNA was measured as previously mentioned and stored at −20°C until further processing (Additional file 3: Table S2).

Analysing complex samples on the AA48.48 with the Gut Microbiotassay

To test the amplification efficiency on DNA extracted from intestinal content 'sample-calibration-curves' were constructed from tenfold serial dilutions (0.25 pg/μl - 25 ng/μl). These were made from pooled sample material of 1 μl extracted DNA from each of the samples listed in Additional file 3: Table S2. 'Control-calibration-curves' were constructed from the IPC, and 'reference-calibration-curves' were generated by pooling all reference bacteria in equal concentrations.

DNA extracted from the pig samples included in the study was diluted to 50 ng/μl with nuclease-free water (Ambion). To test how the addition of a 454BL affected the Cq values, an AA48.48 with a 454BL and one without a 454BL were run under the exact same conditions. With a 454BL, each sample mixture consisted of 3 μl Master Mix, 1 μl sample (DNA, 50 ng/μl), and 1 μl Access Array Barcode Library for the 454 GS FLX Titanium Sequencer (Fluidigm), 2 μM. Afterwards the amplicons were harvested and stored at −20°C until needed.

Amplicon preparation for NGS

To normalize the harvested sample amplicons for NGS, they were measured as described earlier and pooled in equal concentrations. This resulted in a total volume of 144.27 μl of 16 ng/μl which was purified to remove any PCR by-products. First the volume was reduced to 15 μl by extracting the DNA with phenol chloroform using a standard procedure [15]. Next, the extracted DNA was run in a 0.7% Seakem® LE Agorose gel (Lonza Rockland, Rockland, ME, USA) for 86 min, 90 V, and incubated for 30 min in 0.0004% ethidium bromide. Bands were visualized with UV-radiation using the Bio-Rad Universal hood II (Segrate, Milan, Italy) and bands in the size range 200–900 bp were excised, equalling expected amplicon sizes. DNA was extracted from the excised gel using the Qiaquick Gel Extraction Kit (Qiagen) in accordance with the kit manual.

The final pool of purified DNA from the 12 piglets to be sequenced was 723.25 ng (260/280-nm ratio: 1.96). This was run on a quarter PicoTiterPlate™ on a 454 GS FLX Titanium Sequencer (Roche) by LGC Genomics (GmbH, Berlin, Germany).

Data analysis

From raw Cq values to relative quantitative data Cq values generated from the AA48.48 without a 454BL added were exported from the 'Fluidigm Real-Time PCR Analysis software', version 3.0.2 (Fluidigm), to Excel. Cq values were corrected to the IPC included in all runs, and those exceeding primer specific cut-off values, determined from the verification step of the Gut Microbiotassay (Additional file 4: Table S3), were excluded. Relative quantification was calculated from the mean of the technical replicates using the Livak-method [16]. This method was chosen based on the theory that the total amount of bacteria, targeted by the primer set domain Bacteria B, constituted 100% of the microbiota in the individual gut section at all times. Hence, the Cq values of all primer sets for each sample were normalised against the Cq value of their respective domain Bacteria B primer set: $R_{\text{primer set X}} = 2^{Cq_{\text{domain Bacteria B}} - Cq_{\text{primer set X}}}$. To compare total bacteria detected with the domain Bacteria B primer set, these Cq values were related to the final number of thermal cycles run, 35: $R_{\text{domain Bacteria B}} = 2^{35 - Cq_{\text{domain Bacteria B}}}$. Normalization was further done to total mean of all primer sets for each sample with similar results (data not shown).

454-sequencing data Sequence data, available at NCBI Sequence Read Archive under Accession SRA061551, was analysed using BION, a yet unpublished open source program. For more information on the BION software, its functions, and the main statistics for the raw results see Additional file 5. In short: the sequence dataset was converted to FASTQ, split according to sample barcodes and primer sequences, and trimmed in the same process. Next, sequences were cleaned using a cut-off for minimum quality of 96%, and a minimum sequence length of 200 bp. The remaining sequences were clustered using UCLUST, based

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

on a minimum seed similarity of 99.5%. Query sequences were compared to Greengenes Gene Database [17] using the k-mer matching program Simrank2, an improved version of Simrank [18]. Simrank2 returns the n % best similarities, no matter how low they are. Also, it only produces 8-mers from regions above a given quality, and skips sequences with too few 8-mers. This last feature helps improve the data quality. Simrank2 was set to return the best 1% similarities with a similarity cut-off of 50%. Taxonomy was generated by transforming percentages for the Greengenes OTUs to scores, read densities, the sum of which was 1. This was done in a weighted manner, so that OTUs of the highest similarity scored a high number and vice versa, the sum of which was the original reads. Only phylotypes with a primer-specific read density of ≥ 1% were included in the statistical analysis.

Statistics Initially, all data were transformed with the natural logarithm. Primer sets with more than half of the data missing were removed from the dataset. If primer sets in taxonomic lineage showed pair-wise correlations above 0.99, only the primer set of the highest taxonomic level in the lineage was retained for analysis, and any conclusion drawn from this also accounted for the excluded sub-level primer sets. To include the information from remaining data-deficient primer sets, for which the fraction of missing data was low and never above 0.35, the EM-algorithm [19] was used to substitute missing values with imputed ones, by applying a multivariate Gaussian model. Each primer set was allowed to depend freely on the others and also for dependency on gut section, diarrhoeic status, and interaction between these. Model fit of the multivariate model for the primer sets after imputation was assessed by transforming the model residuals with the inverse of the square root of the estimated covariance matrix between the primer sets, and applying standard model control to these standardized residuals. These analyses were consistent with standard model behaviour.

Effect of gut section and diarrhoeic status was tested with the Likelihood Ratio Method using the Wilks test [20]. Sequence data were compared for effect of diarrhoeic status using the non-parametric Wilcoxon test [21].

P-values < 0.05 were considered statistically significant.

Results

Designing the Gut Microbiotassay

The Gut Microbiotassay was constructed from 49 primers constituting 24 primers sets (Table 1) in order to target the main bacterial Phyla (Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria and Verrucomicrobia) and some of the highly important bacterial groups and species reported in the mammalian intestinal tract [9]. 12 primers were designed de novo, and 37 primers were from published literature, of which ten were modified. Five of the 24 primer sets were unmodified pairs from published literature, whereas the rest were combinations composed in this study. As it was not possible to find or design specific primer sets targeting the 16S rRNA gene for class β- and γ-proteobacteria, and genus Lactobacillus, these were designed to target the 23S rRNA gene instead.

Sensitivity and specificity of the Gut Microbiotassay

Tenfold serial dilutions of DNA extracted from 15 reference bacteria were used to evaluate the specificity and sensitivity of the Gut Microbiotassay (Figure 1).

The specificity for each primer set was assessed from the Cq values obtained for their respective target bacteria in addition to any cross reaction. For 13 of the 24 primer sets, specific positive reactions were registered on various taxonomic levels in agreement with the reference bacteria listed for each primer set in Table 1. The remaining primer sets showed different degrees of cross reaction (Figure 1). On average, the lowest unspecific and the highest specific Cq of same concentration differed by 9 Cq values, equalling less than 1% cross reaction. Apparently, the primer set phylum Firmicutes did not have a complete coverage, as it only amplified few bacteria from the Clostridia clusters. The highest specific Cq value was determined for each primer set from the representative target bacterial species and used as cut-off value in the data analysis (Additional file 4: Table S3).

The dynamic range for the specific primer sets spanned from 50 ng to 50 pg DNA/μl for two primer sets, to 5 pg DNA/μl for 15 primer sets, and for seven primer sets down to 0.5 pg DNA/μl (lowest concentration tested), Table 3. Linear regression of log-concentration versus Cq values for the different primer sets demonstrated r^2 from 0.943 to 0.999 (SD 0.0074). Gel pictures and electropherograms confirmed specific amplification by demonstrating the expected number of amplicons corresponding to the number of taxonomic levels represented by the specific primer sets. Additionally, amplicon sizes (bp) were comparable to those listed in Table 1.

Fluidigm's recommendation on DNA sample concentration is 25–50 ng/μl [8]. When testing the amplification efficiency on 23 calibration curves (ranges: 0.25 pg/μl – 50 ng/μl), there was no PCR inhibition for either of the primer sets with DNA of 25 or 50 ng/μl.

Addition of a 454 Barcode Library

Including a 454BL with the reactions did not affect the Cq values drastically. The biggest deviation was less than 1 Cq compared to the respective Cq values without a 454BL added, and 90 percent of the corresponding values from the two data sets were correlated by $r^2 > 0.90$. Uncorrelated data were mainly seen if no or very few target bacteria were present in the reaction. In such cases the 454BL occasionally caused some interaction with itself or/and the present

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

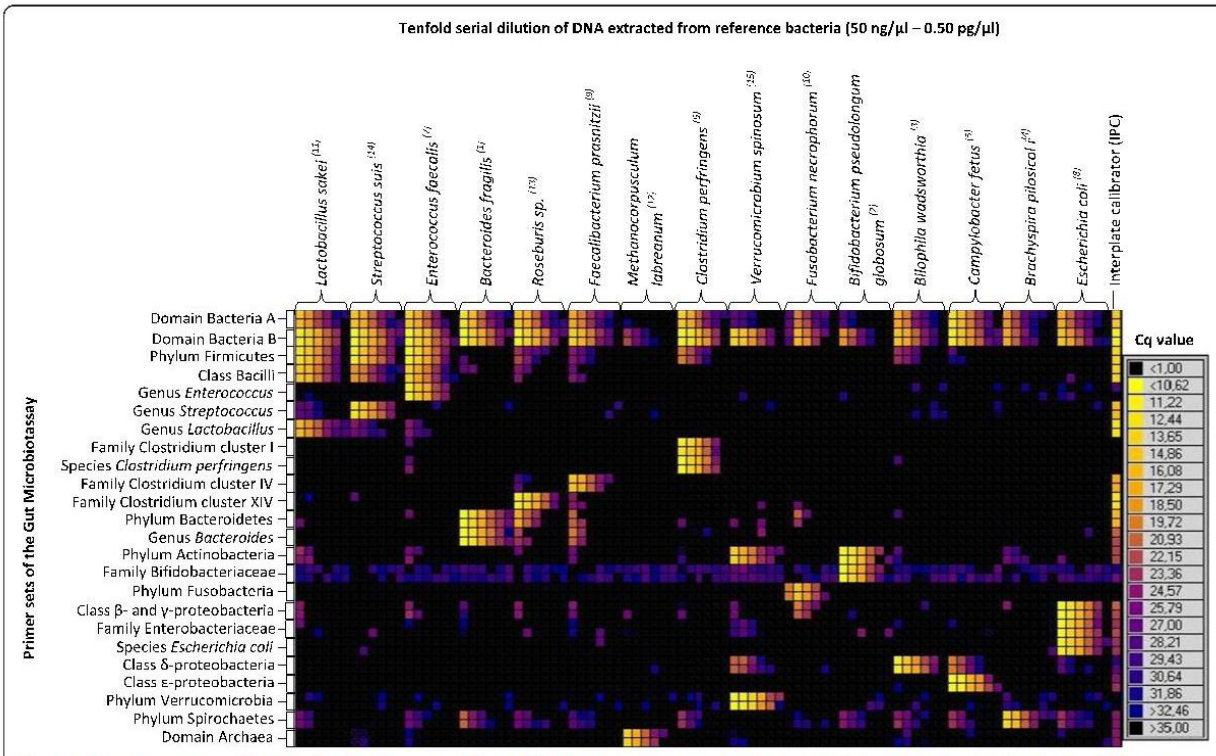


Figure 1 Heatmap generated by the Fluidigm Real-Time PCR Analysis program from raw Cq data. At the top horizontal: Tenfold serial dilutions of DNA (ranging from 50 ng/µl down to 0.5 pg/µl) extracted from 15 different reference bacteria. Raised numbers in parentheses represent the respective numbers given to each reference bacterium in Table 1. These were used to test the specificity and sensitivity of the primer sets included in the Gut Microbiotassay, listed vertically on the left. Primers were run in duplicates. On the right: a colour scale depicting the respective Cq values (the software uses commas instead of points as decimal separator for Cq values).

primer set, creating a signal that obscured the low or missing signal from the target bacteria. Because of this, samples were run twice: with and without a 454BL added. Cq values without a 454BL were used for data analysis, whereas amplicons with the 454BL incorporated were available for 454-sequencing.

The Gut Microbiotassay on complex samples

Piglets of different diarrhoeic status were sacrificed and luminal content was collected from the small and the large intestine. This resulted in a total of 23 samples divided into the following groups: small intestine without diarrhoea (S−) n = 5, small intestine with diarrhoea (S+) n = 7, large intestine without diarrhoea (L−) n = 4, and large intestine with diarrhoea (L+) n = 7.

AA48.48 general findings

The following primer sets were missing more than half of the data, and were consequently removed from the statistical data analysis: family Clostridium cluster IV, class ε-proteobacteria and δ-proteobacteria, phyla Verrucomicrobia, and domain Archeae. Primer sets showing pair-wise correlation above 0.99, were: class β- and γ- proteobacteria with family Enterobacteriacea, and species E. coli; and phylum Bacteroidetes with genus Bacteroides. Of the general bacteria primers only domain Bacteria B was tested as this was the primer set used for normalization. This left 15 primer sets for data analysis. The multivariate test for effect of gut section and diarrhoeic status, with effects allowed to differ with the values of gut section, revealed statistically significant effects (S/L $p = 0.01$, S+/S − $p = 0.002$, and L+/L − $p = 0.006$, respectively). However, effects were not limited to the primer sets that showed significant differences in the Gut Microbiotassay. If these were excluded, and the data analysis repeated, this still resulted in a significant effect from diarrhoeic status in the large intestine (L+/ L − $p = 0.01$), while it was borderline insignificant in the small intestine (S+/ S − $p = 0.056$).

Table 4 lists the estimated mean percentages and 95% confidence interval for each primer set included in the data analysis relative to total bacteria. Diarrhoeic gut sections contained fewer bacteria from phylum Firmicutes (genus Streptococcus and unclassified ($p < 0.05$)), but a higher fraction of genus Enterococcus (small intestine $p = 0.04$). There was a highly significant depletion of members from genus Streptococcus in the diseased compared to

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

Table 3 The primer efficiency, r^2-value, dynamic range and limit of detection of the Gut Microbiotassay

Primer set	Efficiency, %	r^2	Dynamic range	Limit of detection, ng/μl
Domain Bacteria B V4-V5	75-106	>0.968	4-6-fold	0.05-0.0005
Phylum Firmicutes*	86-97	>0.994	3-5-fold	0.05-0.005
Phylum Bacilli	99-101	>0.943	4-5-fold	0.05-0.005
Genus Enterococcus	87	>0.998	5-fold	0.005
Genus Lactobacillus	112	>0.992	5-fold	0.005
Genus Streptococcus	102	>0.999	5-fold	0.005
Family Clostridium cluster I	96	>0.995	4-fold	0.05
Species Clostridium perfringens	87	>0.999	5-fold	0.005
Family Clostridium cluster IV	103	>0.997	5-fold	0.005
Family Clostridium cluster XIV	96	>0.999	4-fold	0.05
Phylum Bacteroidetes	91	>0.991	6-fold	0.0005
Genus Bacteroides	85	>0.997	5-fold	0.005
Phylum Actinobacteria	92	>0.988	6-fold	0.0005
Family Bifidobacteriaceae	77	>0.995	5-fold	0.005
Class β- and γ-proteobacteria	87	>0.999	5-fold	0.005
Family Enterobacteriacea	84	>0.996	5-fold	0.005
Species Escherichia coli	91	>0.999	6-fold	0.0005
Class ε-proteobacteria	86	>0.995	6-fold	0.0005
Class δ-proteobacteria	91	>0.995	5-fold	0.005
Phylum Fusobacteria	85	>0.997	4-fold	0.005
Phylum Verrucomicrobia	107	>0.990	6-fold	0.0005
Phylum Spirochaetes	80	>0.996	5-fold	0.005
Domain Archaea	95	>0.996	5-fold	0.005

*Data from the reference bacteria Roseburia sp. and F. Prausnitzii has been excluded in primer set Phylum Firmicutes.
The Gut Microbiotassay was tested against tenfold dilution series of DNA extracted from individual reference bacteria without a 454 Barcode Library added.

the healthy intestines. The same tendency was seen for Clostridium cluster I (small intestine $p = 0.02$). The diarrhoeic small intestine harboured fewer members from class β- and γ-proteobacteria than the healthy one, while this was reversed for the large intestine ($p < 0.05$). Generally healthy piglets had a gut microbiota dominated by Gram-positive bacteria, which was partly displaced by Gram-negative bacteria in diarrhoeic piglets.

454-Sequencing results

Sequencing barcoded sample amplicons resulted in 275,133 unprocessed consensuses, which dropped to 164,055 after quality trimming. Amplicons generated by the primer sets domain Bacteria A and B encompassed 16S rRNA gene sequences from both Gram-positive and Gram-negative bacteria genera plus from unclassified bacteria. Good congruency was found between the primer sets of the Gut Microbiotassay and the 454-sequencing results generated from their respective amplicons (Figure 2).

Comparing significant findings from AA48.48 with 454-sequencing data

Based on the Gut Microbiotassay data for the individual bacterial groups, four groups showed significant differences: domain Bacteria B, genus Streptococcus, phylum Actinobacteria, and class β- and γ-proteobacteria, Table 4. In the small intestine, diarrhoea was associated with a significantly reduced bacterial load ($p = 0.003$). Sequence data generated by the domain Bacteria B primer set indicated that diarrhoea was associated with a decreased number of members from genus Streptococcus, including S. alactolyticus in both the small intestine ($p = 0.0061$, and $p = 0.0099$, respectively) and the large intestine ($p = 0.0061$ and $p = 0.013$). When comparing the results for genus Streptococcus, there were significantly fewer Streptococci in the diseased intestines (S+/ S − $p = 0.0003$, L+/ L − $p = 0.00002$). The sequences generated by genus Streptococcus confirmed the previous results (S+/ S − $p = 0.0061$, L+/ L − $p = 0.012$). These were classified to: genus Streptococcus, species S. hyointestinalis, S. suis, and S. alactolyticus. However, at species level, only S. alactolyticus, showed coherently significant results with genus Streptococcus (S+/ S − $p = 0.047$, L+/ L − $p = 0.012$). Phylum Actinobacteria was more abundant in the diarrhoeic compared to the healthy small intestine ($p = 0.034$), however, this was not reflected in the sequencing data for the small intestine. The diarrhoeic large intestine possessed significantly more members from the class β- and γ-proteobacteria, including family Enterobacteriaceae and species E. coli, than the healthy one ($p = 0.029$). Nonetheless, for this gut section no significant differences showed up in the sequencing data from the family Enterobacteriaceae primer set.

Specificity of the Gut Microbiotassay

After sequencing the amplicons generated by the Gut Microbiotassay the specificity of the primer sets was revaluated (Figure 2), except for the primer sets targeting the 23S rRNA gene (genus Lactobacillus and class β- and γ-proteobacteria) as the freeware used to analyze the sequence data is currently based on 16S rRNA gene databases.

16 of the 24 primer sets only generated sequences from their intended target group, four showed cross reaction with a single group, and two primer sets cross reacted with more than two other taxonomic groups. The primer set phylum Firmicutes did not reveal any clostridia, as predicted earlier. In conclusion, the sequencing data confirmed the specificity of the primer sets found in the verification process of the Gut Microbiotassay when tested against DNA extracted from pure-cultured reference bacteria.

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

Table 4 Estimated mean primer set values from the AA48.48 and the corresponding significant 454 GS FLX Titanium sequencing results

Primer set	Estimated mean percentages relative to total bacteria				P-values	
	S−	S+	L−	L+	S+/ S−	L+/ L−
Domain Bacteria B[1]	24.96	20.32	25.13	25.46	0.003	0.857
	[22.39;27.73]	[16.83;24.32]	[22.26;28.27]	[23.23;27.84]		
Genus Streptococcus					0.0061	0.0061
Species S. alactolyticus					0.0099	0.0134
Phylum Firmicutes	36.08	27.59	30.59	17.79	0.649	0.278
	[17.64;65.91]	[7.50;72.58]	[13.64;59.56]	[9.79;29.83]		
Genus Streptococcus					0.0242	0.0102
Unclassified					0.0462	0.0176
Class Bacilli	14.01	15.92	8.30	12.04	0.473	0.52
	[4.85;32.09]	[2.22;57.40]	[2.50;20.64]	[4.99;24.63]		
Genus Streptococcus					0.0287	0.0061
Unclassified					0.1516	0.029
Genus Enterococcus	0.036	0.39	0.04	0.11	0.208	0.34
	[0.0038;0.14]	[0.0049;2.51]	[0.0032;0.18]	[0.018;0.39]		
Genus Enterococcus					0.0424	0.9273
Genus Streptococcus	4.04	1.00	5.10	0.56	0.0003	0.00002
	[1.84;7.73]	[0.24;2.83]	[2.10;10.46]	[0.29;0.98]		
Genus Streptococcus					0.0061	0.0121
Species S. alactolyticus					0.0467	0.0121
Genus Lactobacillus	33.97	53.70	33.27	27.55	0.643	0.846
	[12.03;76.74]	[7.82;190.09]	[10.27;81.52]	[11.62;55.67]		
Family Clostridium cluster I	0.65	0.17	0.32	0.079	0.231	0.139
	[0.16;1.81]	[0.012;0.76]	[0.064;0.97]	[0.025;0.19]		
Genus Clostridium					0.0171	0.2558
Species Clostridium perfringens	3.03	2.82	2.33	0.63	0.81	0.219
	[0.62;9.24]	[0.14;14.31]	[0.38;7.86]	[0.17;1.66]		
Family Clostridium cluster XIV	0.89	16.84	10.82	5.02	0.818	0.672
	[0.098;3.53]	[0.22;106.79]	[0.86;47.63]	[0.82;16.95]		
Phylum Bacteroidetes and Genus Bacteroides	2.25	76.17	22.05	5.10	0.924	0.567
	[0.082;12.19]	[0.091;505.28]	[0.48;130.66]	[0.34;23.65]		
Species Bacteroides uniformis (generated from the primer set Genus Bacteroides)					0.0447	0.0985
Phylum Actinobacteria	0.32	3.92	1.02	0.32	0.034	0.068
	[0.13;0.68]	[0.70;12.70]	[0.35;2.32]	[0.15;0.61]		
Genus Actinomyces					1	0.0363
Species Actinomyces hyovaginalis					0.1042	0.0179
Species Bifidobacterium pseudolongum					1	0.0368
Unclassified					0.072	0.0104
Family Bifidobacteriaceae	0.0064	0.011	0.0063	0.012	0.399	0.24
	[0.0026;0.013]	[0.0022;0.036]	[0.0023;0.014]	[0.0056;0.022]		
Species Bifidobacterium pseudolongum					1	0.0424
Phylum Fusobacteria	1.59	32.60	6.28	9.71	0.413	0.604
	[0.18;6.17]	[0.48;203.87]	[0.54;27.06]	[1.66;32.13]		

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

Table 4 Estimated mean primer set values from the AA48.48 and the corresponding significant 454 GS FLX Titanium sequencing results (Continued)

Class β- and γ-proteobacteria; Family Enterobacteriaceae and Species Escherichia coli	2.96	0.54	0.61	3.17	0.6	0.029
	[0.83;7.69]	[0.049;2.28]	[0.14;1.72]	[1.10;7.25]		
Family Enterobacteriaceae (generated from the primer set Family Enterobacteriaceae)					0.0424	1
Species Escherichia fergusonii (generated from the primer set Family Enterobacteriaceae)					0.0368	0.7758
Genus Shigella (generated from the primer set Family Enterobacteriaceae)					0.0462	0.9244
Species Shigella flexneri (generated from the primer set Family Enterobacteriaceae)					0.0413	0.5626
Phylum Spirochaetes	0.0059	0.010	0.0091	0.0056	0.73	0.081
	[0.004;0.0085]	[0.005;0.019]	[0.0058;0.014]	[0.004;0.0076]		
Genus Streptococcus					0.0169	0.0127
Unclassified					0.0191	0.0694

[1]Domain Bacteria B is expressed in percentage relative to the final number of thermal cycles run, 35.
Primer set values from the AA48.48 are expressed as percentages relative to total bacteria, and beneath (indented) are listed the significant 454-sequencing results for the respective primer sets. Numbers in bold indicate significant p-values. L: Large intestine, S: small intestine, +: with diarrhoea, –: without diarrhoea. [95% lower level; 95% upper level].

Discussion

Most qPCR studies describing the gut microbiota typically do this by using a general bacteria primer and a few group-specific ones [4,22]. In contrast, this study developed the Gut Microbiotassay, an assay composed of 24 primer systems, capable of screening the microbiota for the most common bacteria in the mammalian intestine [9,10] at various taxonomic levels. The Gut Microbiotassay was tested against representative reference bacteria, and next on complex intestinal samples from piglets of different diarrhoeic status. The sample amplicons were harvested and sequenced, and functioned as a proof of concept, evaluated the specificity of the Gut Microbiotassay by further elucidating the components of the gut microbiota.

This approach offers an alternative to current molecular methods employed to characterize the gut microbiota such as phylogenetic microarrays [23], and NGS [24]. In contrast to phylogenetic microarrays, the AA48.48 is highly flexible because primer sets can readily be replaced to meet the needs of a current research study. In addition, the AA48.48 outmatches the phylogenetic microarray on sample capacity, as well as sensitivity [25]. Also, no pre-amplification is needed when running the Gut Microbiotassay with the AA48.48, which reduces the workload, and also the risk of introducing technical variation. The effect of such technical variation can be reduced by normalization. In the present study each sample were normalized against the Cq value of their respective domain Bacteria B primer set. Impact of normalization against their respective domain Bacteria B was tested by performing a second normalization procedure; at this point data was also normalized to total mean of all primer sets for each sample individually, with similar end results (data not shown). As the choice of normalization in the present study (Domain Bacteria B) or total mean did not have a great impact on final results we assume that the technical variation was low or that we managed to normalize efficiently using both methods. Further, data from domain Bacteria A and domain Bacteria B were highly correlated also pointing to domain Bacteria B as a reasonable reference primer efficiency of qPCR is of great importance and should ideally be within the range of 85% to 110%. The majority of the primers used in the Gut Microbiotassay (21 out of the 23 primers included in Table 3) had and acceptable efficiency between 80-110%. However, the primer Doman bacteria A was found to differ to much with regards to efficiency and dynamic range, thus data from this primer pair was only used to support data generated from the primer Doman bacteria B. Likewise primer set Phylum Firmicutes produced to high efficiency when tested on two reference bacteria Roseburia sp. and F. Prausnitzii. However Roseburia sp. and F. Prausnitzii are covered by several other well performing primers including Clostridium cluster XIV and Clostridium cluster IV respectively. Therefore the Gut Microbiotassay cannot be used as an absolute quantitative assay across the different primer sets. In order to make it a truly quantitative assay it will be necessary either to have a defined start sample regarding species composition or only to use specific primers on the array where high efficiency has been proven.

The heatmap generated by the software 'Fluidigm Real-Time PCR Analysis' (Fluidigm Corporation) depicts the raw Cq values for each reaction. This makes it feasible to quickly evaluate and visually compare the bacterial profiles across a large number of samples. As sample amplicons are harvested individually following qPCR, it is possible to pinpoint which samples to sequence for further taxonomic information. Selective sequencing reduces costs compared to non-selective sequencing. Also, the dataset generated from sequencing the Gut Microbiotassay PCR amplicons produces a much more manageable dataset compared to metagenomic approaches. The Gut Microbiotassay provides a quantitative picture

Hermann-Bank et al. BMC Genomics 2013, 14:788
http://www.biomedcentral.com/1471-2164/14/788

454-GS FLX Sequencer	Access Array 48.48																				
	Domain Bacteria A	Domain Bacteria B	Phylum Firmicutes	Class Bacilli	Genus Enterococcus	Genus Lactobacillus (235)	Genus Streptococcus	Family Clostridium cluster I	Family Clostridium cluster IV	Family Clostridium cluster XIV	Phylum Bacteroidetes	Genus Bacteroides	Phylum Actinobacteria	Family Bifidobacteriaceae	Class β &γ proteobacteria (235)	Family Enterobacteriaceae	Class ε-proteobacteria	Class δ-proteobacteria	Phylum Fusobacteria	Phylum Verrucomicrobia	Phylum Spirochaetes	Domain Archaea
Species Mitsuokella multacida	1																					
Genus Staphylococcus			1	2																		
Genus Enterococcus	2				22																5	
Species Enterococcus cecorum	1		1		14																1	
Species Enterococcus durans					5																1	
Species Enterococcus faecalis					30																	
Species Enterococcus hirae	2				22																5	
Species Enterococcus lactis					6																	
Genus Lactobacillus	29	10	55	62																	22	7
Species Lactobacillus acidophilus			1	1																		
Species Lactobacillus crispatus	2	2	9	12																	2	
Species Lactobacillus delbrueckii	7	3	1																		6	2
Species Lactobacillus jensenii			2	3																		
Species Lactobacillus mucosae	4		3	3																	3	
Species Lactobacillus pontis			1																			
Species Lactobacillus reuteri			1																			
Species Lactobacillus vaginalis	6		4	3																	5	
Genus Streptococcus	6	18	7	4			73														4	21
Species Streptococcus alactolyticus		1					4															1
Species Streptococcus hyointestinalis		3					12															3
Species Streptococcus suis		2					9															2
Genus Clostridium								16	14	9												
Species Clostridium perfringens	2	2						78													2	
Species Clostridium paraputrificum								3														
Family Ruminococcaceae									57													
Genus Ruminococcus									17													
Species Ruminococcus bromii									3													
Species Clostridium methylpentosum									4													
Species Faecalibacterium prausnitzii									1													
Family Lachnospiraceae	2									40											2	
Species Clostridium bolteae										2												
Genus Ruminococcus										26												
Species Ruminococcus gnavus										18												
Genus Blautia										2												
Species Blautia producta										1												
Order Bacteroidales											3											
Family Porphyromonadaceae											4						1					
Genus Parabacteroides											1											
Species Parabacteroides distasonis	1										7						2				1	
Genus Prevotella											7						2					
Genus Bacteroides	4	2									43	55					10				6	1
Species Bacteroides plebeius											1											
Species Bacteroides fragilis	3	1									23	33					5				4	
Species Bacteroides massiliensis											1	5										
Species Bacteroides uniformis											3	3										
Genus Actinomyces													1									
Species Actinomyces hyovaginalis													21									
Genus Arthrobacter													2									
Genus Cellulomonas													1									
Genus Corynebacterium													3									
Genus Rothia													9									
Species Rothia mucilaginosa													4									
Genus Streptomyces													11									
Genus Bifidobacterium													16	22								
Species Bifidobacterium pseudolongum													9	76								
Species Bifidobacterium thermacidophilum													8	2								
Species Bifidobacterium animalis													2									
Family Enterobacteriaceae																55						
Genus Citrobacter																2						
Genus Escherichia																25						
Species Escherichia fergusonii																7						
Genus Shigella																4						
Species Shigella flexneri																4						
Genus Helicobacter																	4					
Species Helicobacter canadensis																	4					
Genus Campylobacter																	48	8				
Species Campylobacter lanienae																	4					
Species Campylobacter mucosalis																	11					
Genus Desulfovibrio																		7				
Genus LE30																		9				
Species Bilophila wadsworthia																		76				
Genus Fusobacterium	9	3																	74		7	2
Genus J2-29 (Fusobacteriaceae)	3	1																	19		2	
Species Fusobacterium necrophorum																			3			
Genus Akkermansia																				53		
Species Akkermansia muciniphila																				47		
Unclassified	4	44	4	1					3		4	1	6			2	2		2		8	53

Figure 2 Specificity of the Gut Microbiotassay. The figure shows the sequencing results and corresponding read density scores (≥ 1%) for the individual primer sets of the Gut Microbiotassay targeting the 16S rRNA gene. Green colours indicate sequences corresponding to the primer sets' target, whereas red colours represents unspecific amplification. The darker the colour, the higher the read density score.

of the distribution of the known gut microbiota represented by the primers. Moreover, if combined with 454-sequencing, it enables detection of bacteria with unidentified sequences [25]. A limitation of the assay when running on complex bacterial samples is that the primers will have different efficiencies and dynamic ranges due to imperfect matches with some of the target sequences. The Gut Microbiotassay has therefore the most value for analysing high-throughput quantification of the bacterial composition in many samples or samples with defined biomarkers.

The validation of the Gut Microbiotassay by sequencing the amplicons from intestinal content of two different gut sections from piglets of different diarrhoeic status demonstrated the potential to further elucidate the components of the gut microbiota. This study used the results from the Gut Microbiotassay to quantify the taxonomical groups, and NGS to access the bacterial constituents.

Common intestinal bacteria in the neonatal piglet include members of: Clostridia, Streptococcaceae, Lactobacillaceae, Enterobacteriaceae, Fusobacteria and sometimes Bacteroidetes [26,27]. These bacterial groups were also found in the gut microbiota of three-day-old piglets using the Gut Microbiotassay. The Gut Microbiotassay indicated that a healthy gut microbiota was dominated by Gram-positive bacteria, which were partly replaced by Gram-negative bacteria in the large intestine of diarrhoeic piglets. Robinson et al. [28] came to a similar conclusion in a study investigating the intestinal microbiota of pig colons experimentally induced with swine dysentery. Consistent significant findings from the Gut Microbiotassay and the 454-sequencing results implied that diarrhoea was associated with a depletion of members from the genus Streptococcus, and previous research has shown that Streptococci is an important member of a healthy gut microbiota [26,28,29]. A detailed review of the aetiology behind the piglet diarrhoea is beyond the scope of this paper and prevented by the limited number of piglets analysed, as the primary focus of this paper has been on the verification and application of the Gut Microbiotassay.

Conclusions

The Gut Microbiotassay offers affordable quantitative screening of the microbiota with the AA48.48. It has been thoroughly tested and strict criteria for data analysis have been outlined. It provides a high sample capacity, a wide dynamic range, and it facilitates selective 454-sequencing afterwards. Hence, it is timesaving and economical due to the easy library preparation, the low consumption of master mix, and the optional selective sequencing. These features make the Gut Microbiotassay a worthy high-throughput competitor to the current alternative methods used for investigating diverse ecosystems.

Additional files

Additional file 1: Table S1. Concentration and purity of DNA extracted from the reference bacteria and the interplate calibrator.

Additional file 2: Protocol used for DNA extraction with the Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA).

Additional file 3: Table S2. Piglets included in the study.

Additional file 4: Table S3. Cross reactions detected between primer systems and reference bacteria tested. Highest specific Cq value determined from the respective target reference bacteria has been used as cut-off value for the different primer systems in the data analysis.

Additional file 5: BION analysis of 454-sequencing data. Detailed information on the BION software, its functions, and the main statistics for the raw results is included in the file Results_referee.zip. When the file is unpacked it creates the directory Results_referee. This contains 24 subfolders containing the main statistics for each primer pair. However, the species-folders are empty, since the species-specific primers were not tagged. The remaining subfolder 'Software' and files are explained in the README-file. The entire BION-meta package (200 Mb) is not included, and there is currently not a stable link to it. But if interested, it can be downloaded from the following link: https://www.dropbox.com/sh/fumscuqpanqaqvu/_4H–XBxHQ.

Abbreviations

AA48.48: Access array 48.48; 454BL: 454 Barcode library; IPC: Interplate calibrator; L: Large intestine; L+: Large intestine with diarrhoea; L−: Large intestine without diarrhoea; NGS: Next generation sequencing; S: Small intestine; S+: Small intestine with diarrhoea; S−: Small intestine without diarrhoea.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MLHB designed the Gut Microbiotassay, conducted all the experiments, and wrote the manuscript. KS supervised the study and contributed to the origin of this manuscript. AS performed the statistical calculations and provided all information on statistical issues. NL analysed the 454-sequence data using his BION software. LM has been the primus of this project and has guided and supervised the progress of both the project and the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors wish to thank Øystein Angen (Danish Veterinary Institute) for culturing the reference bacteria and Birgitta Svensmark (Pig Research Centre) for collecting the intestinal content samples. This work was supported by the Act on Innovation no. 421 of 31/05/2000 granted by the Ministry of Food, Agriculture and Fisheries of Denmark, the Danish Veterinary Institute (Technical University of Denmark), and the Pig Research Centre (Ministry of Food, Agriculture and Fisheries of Denmark).

Author details

[1]Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark. [2]Section for immunology and vaccinology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark. [3]Department of Informatics and Mathematical Modelling, Technical University of Denmark, Richard Petersens Plads, Building 305, room 126, 2800 Lyngby, Denmark. [4]Danish Genome Institute, Skt. Lucas Kirkeplads 8, 8000 Århus, Denmark. [5]Present address: Chr. Hansen, Bøge Allé 10, 2970 Hørsholm, Denmark.

Received: 7 January 2013 Accepted: 14 October 2013
Published: 14 November 2013

References

1. Sekirov I, Russell SL, Antunes LCM, Finlay BB: Gut microbiota in health and disease. Physiol Rev 2010, 90:859–904.
2. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Dore J: Direct analysis of genes encoding 16S rRNA from complex communities

reveals many novel molecular species within the human gut. Appl Environ Microbiol 1999, 65:4799–4807.
3. Dowd SF, Sun Y, Wolcott RD, Domingo A, Carroll JA: Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned Salmonella-infected pigs. Foodborne Pathog Dis 2008, 5:459–472.
4. van den Bogert B, de Vos WM, Zoetendal EG, Kleerebezem M: Microarray analysis and barcoded pyrosequencing provide consistent microbial profiles depending on the source of human intestinal samples. Appl Environ Microbiol 2011, 77:2071–2080.
5. Inglis G, Thomas MC, Thomas DK, Kalmokoff ML, Brooks SP, Selinger L: Molecular methods to measure intestinal bacteria: a review. J AOAC Int 2012, 95:5–23.
6. Lamendella R, VerBerkmoes N, Jansson JK: 'Omics' of the mammalian gut - new insights into function. Curr Opin Biotechnol 2012, 23:491–500.
7. Logan JMJ, Edwards KJ: An overview of real-time PCR platforms. In Real-Time PCR Current Technology and Application. Edited by Logan JMJ, Edwards KJ, Saunders NA. Norfolk, UK: Caister Academic Press; 2009:7–22.
8. Fluidigm: Access array system™ user guide v3. Part#: 68000158, Rev B 2010. http://www.fluidigm.com/user-document-request.html.
9. Rajilic-Stojanovic M, Smidt H, de Vos WM: Diversity of the human gastrointestinal tract microbiota revisited. Environ Microbiol 2007, 9:2125–2136.
10. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI: Evolution of mammals and their gut microbes. Science 2008, 320:1647–1651.
11. Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM, Tiedje JM: The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 2005, 33:D294–D296.
12. Loy A, Arnold R, Tischler P, Rattei T, Wagner M, Horn M: ProbeCheck - a central resource for evaluating oligonucleotide probe coverage and specificity. Environ Microbiol 2008, 10:2894–2896.
13. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, et al: ARB: a software environment for sequence data. Nucleic Acids Res 2004, 32:1363–1371.
14. Ashelford KE, Weightman AJ, Fry JC: Nucleic Acids Res 2002, 30(15):3481–9. Aug 1, PMID: 12140334 [PubMed - indexed for MEDLINE] Free PMC Article.
15. Sambrook J, Russell DW: Purification of nucleic acids by extraction with phenol:chloroform. Cold Spring Harb Protoc 2006.
16. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(−Delta Delta C) method. Methods 2001, 25:402–408.
17. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL: Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 2006, 72:5069–5072.
18. DeSantis T, Keller K, Karaoz U, Alekseyenko A, Singh N, Brodie E, Pei Z, Andersen G, Larsen N: Simrank: rapid and sensitive general-purpose k-mer search tool. BMC Ecol 2011, 11:11.
19. Little RJA, Rubin DB: Statistical analysis with missing data. New York: Wiley; 1987.
20. Anderson TW: An introduction to multivariate statistical analysis. 2nd edition. New York: John Wiley; 1984.
21. Lehmann EL: Nonparametrics. Statistical Methods Based on Ranks, Revised edition. New York: Springer; 2006.
22. Castillo M, Martin-Orue SM, Manzanilla EG, Badiola I, Martin M, Gasa J: Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet Microbiol 2006, 114:165–170.
23. Paliy O, Agans R: Application of phylogenetic microarrays to interrogation of human microbiota. Fems Microbiol Ecol 2012, 79:2–11.
24. Murray DC, Bunce M, Cannell BL, Oliver R, Houston J, White NE, Barrero RA, Bellgard MI, Haile J: DNA-based faecal dietary analysis: a comparison of qPCR and high throughput sequencing approaches. Plos One 2011, 6(10):e25776. doi: 10.1371/journal.pone.0025776.
25. Everett KR, Rees-George J, Pushparajah IPS, Janssen BJ, Luo Z: Advantages and disadvantages of microarrays to study microbial population dynamics - a minireview. New Zealand Plant Protection 2010, 63:1–6.
26. Petri D, Hill JE, Van Kessel AG: Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig. Livest Sci 2010, 133:107–109.
27. Ducluzeau R: Implantation and development of the gut flora in the newborn animal. Ann Rech Vet 1983, 14:354–359.
28. Robinson IM, Whipp SC, Bucklin JA, Allison MJ: Characterization of predominant bacteria from the colons of normal and dysenteric pigs. Appl Environ Microbiol 1984, 48:964–969.
29. Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Moller K: Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol 2002, 68:673–690.
30. Liu ZZ, DeSantis TZ, Andersen GL, Knight R: Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. Nucleic Acids Res 2008, 36:e120.
31. Schwieger F, Tebbe CC: A new approach to utilize PCR-single-strand-conformation polymorphism for 16s rRNA gene-based microbial community analysis. Appl Environ Microbiol 1998, 64:4870–4876.
32. Haakensen M, Dobson CM, Deneer H, Ziola B: Real-time PCR detection of bacteria belonging to the Firmicutes Phylum. Int J Food Microbiol 2008, 125:236–241.
33. Mühling M, Woolven-Allen J, Murrell JC, Joint I: Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. ISME J 2008, 2:379–392.
34. Felske A, Akkermans ADL, de Vos WM: In situ detection of an uncultured predominant bacillus in Dutch grassland soils. Appl Environ Microbiol 1998, 64:4588–4590.
35. Behr T, Koob C, Schedl M, Mehlen A, Meier H, Knopp D, Frahm E, Obst U, Schleifer KH, Niessner R, Ludwig W: A nested array of rRNA targeted probes for the detection and identification of enterococci by reverse hybridization. Syst Appl Microbiol 2000, 23:563–572.
36. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG: Clustal W and clustal X version 2.0. Bioinformatics 2007, 23:2947–2948.
37. Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A: Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 2004, 97:1166–1177.
38. Hung CH, Cheng CH, Cheng LH, Liang CM, Lin CY: Application of Clostridium-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. Int J Hydrog Energy 2008, 33:1586–1592.
39. Skånseng B, Kaldhusdal M, Rudi K: Comparison of chicken gut colonisation by the pathogens Campylobacter jejuni and Clostridium perfringens by real-time quantitative PCR. Mol Cell Probes 2006, 20:269–279.
40. Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P: Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. Br J Nutr 2009, 101:541–550.
41. Matsuki T, Watanabe K, Fujimoto J, Takada T, Tanaka R: Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl Environ Microbiol 2004, 70:7220–7228.
42. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R: Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 2002, 68:5445–5451.
43. Manz W, Amann R, Ludwig W, Vancanneyt M, Schleifer KH: Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology 1996, 142:1097–1106.
44. Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F, Welling GW: Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-Targeted oligonucleotide probes. Appl Environ Microbiol 1998, 64:3336–3345.
45. Glockner FO, Zaichikov E, Belkova N, Denissova L, Pernthaler J, Pernthaler A, Amann R: Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. Appl Environ Microbiol 2000, 66:5053–5065.
46. Erhart R: In situ Analyse mikrobieller Biozönosen in Abwasserreinigungsanlagen, PhD thesis. Technical University of Munich: Department of Microbiology; 1997.
47. Delroisse JM, Boulvin AL, Parmentier I, Dauphin RD, Vandenbol M, Portetelle D: Quantification of Bifidobacterium spp. and Lactobacillus spp. in rat fecal samples by real-time PCR. Microbiol Res 2008, 163:663–670.
48. Friedrich U, Van Langenhove H, Altendorf K, Lipski A: Microbial community and physicochemical analysis of an industrial waste gas biofilter and design of 16S rRNA-targeting oligonucleotide probes. Environ Microbiol 2003, 5:183–201.

49. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO: Development of the human infant intestinal microbiota. PLoS Biol 2007, 5:1556–1573.
50. Malinen E, Kassinen A, Rinttila T, Palva A: Comparison of real-time PCR with SYBR Green I or 5 '-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. Microbiology 2003, 149:269–277.
51. Bui XT, Wolff A, Madsen M, Bang DD: Reverse transcriptase real-time PCR for detection and quantification of viable Campylobacter jejuni directly from poultry faecal samples. Res Microbiol 2012, 163:64–72.
52. Sanguin H, Remenant B, Dechesne A, Thioulouse J, Vogel TM, Nesme X, Moenne-Loccoz Y, Grundmann GL: Potential of a 16S rRNA-based taxonomic microarray for analyzing the rhizosphere effects of maize on Agrobacterium spp. and bacterial communities. Appl Environ Microbiol 2006, 72:4302–4312.
53. Walter J, Margosch D, Hammes WP, Hertel C: Detection of Fusobacterium species in human feces using genus-specific PCR primers and denaturing gradient gel electrophoresis. Microb Ecol Health Dis 2002, 14:129–132.
54. Ranjan K: Verrucomicrobia: A model phylum to study the effects of deforestation on microbial diversity in the Amazon forest, Master's thesis. University of Texas at Arlington: Environmental & Earth Science; 2010.
55. Choi BK, Nattermann H, Grund S, Haider W, Gobel UB: Spirochetes from digital dermatitis lesions in cattle are closely related to treponemes associated with human periodontitis. Int J Syst Bacteriol 1997, 47:175–181.
56. Giovannoni SJ, Delong EF, Olsen GJ, Pace NR: Phylogenetic Group-Specific Oligodeoxynucleotide Probes for Identification of Single Microbial-Cells. J Bacteriol 1988, 170:720–726.
57. Delong EF: Archaea in Coastal Marine Environments. Proc Natl Acad Sci USA 1992, 89:5685–5689.

doi:10.1186/1471-2164-14-788
Cite this article as: Hermann-Bank et al.: The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity. BMC Genomics 2013 14:788.

Additional file 1: Concentration and purity of DNA extracted from the reference bacteria and the interplate calibrator.

Reference bacterium	Concentration, ng/µl	260/280 nm-ratio
Bacteroides fragilis (DJF_B083(EU728706))	1728.16	2.21
Bifidobacterium pseudolongum globosum (DMS 20092)	58.34	2.17
Bilophila wadsworthia (ATCC 49260)	29.39	2.07
Brachyspira pilosicoli (isolated from intestine of slaughter pig at DTU-VET, 28-02-2000)	487.44	2.18
Campylobacter fetus (ATTC 10852)	232.53	2.2
Clostridium perfringens (NCTC 10240)	417.35	2.21
Enterococcus faecalis (ATCC 29212)	233.57	2.22
Escherichia coli (9711108-2)	502.74	2.15
Faecalibacterium prausnitzii (DSM 17677)	190.59	2.17
Fusobacterium Necrophorum (ATCC 25286)	27.9	1.71
Lactobacillus sakei (DSM 20017)	219.39	2.28
Methanocorpusculum labreanum (DSM 4855)	21.26	1.69
Roseburia sp. (DJF_VR77(EU728794))	85.56	2.17
Streptococcus suis (NCTC 10446)	63.19	2.17
Verrucomicrobium spinosum (DSM 4136)	65.84	1.97
Interplate calibrator (IPC)	701.84	2.06

Additional file 2

Protocol used for DNA extraction with the Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA)

Pure-cultured colonies were suspended in 1000 µl PBS, and 175 µl of this was mixed with 250 µl Solution A, and incubated at 65 °C for 6 min. 450 µl chloroform was added to the lysate , vortexed until homogenised, and 100 µl of Solution B were transferred to each suspension and vortexed. After centrifuging the suspensions for 10 min, 10 000 *g*, 15 °C, the upper liquid phase containing the DNA was decanted to new tubes. The DNA was precipitated with 5 µl of 5 M NaCl and 1 ml of ice-cold 96% ethanol; next, tubes were centrifuged for 45 min, 20 000 *g,* 4 °C. Liquid was removed and 1 ml of ice-cold 70% ethanol was added, followed by centrifugation for 10 min, 20 000 *g,* 4 °C. The ethanol was discarded and the DNA-pellet was resuspended in 60 µl TE buffer.

Additional file 3: **Piglets included in the study.** All piglets were three days old.

Ear tag	Diarrhoea	Barcode		Gut section	DNA measurements	
		MID[1]	Sequence[2]		Concentration, ng/µl	260/280 nm-ratio
83183-1	yes	MID-16	TCACGTACTA	large	224.45	1.94
		MID-17	CGTCTAGTAC	small	26.15	2.27
83183-2	yes	MID-18	TCTACGTAGC	large	50.57	2.07
		MID-19	TGTACTACTC	small	120.08	1.98
83183-3	yes	MID-20	ACGACTACAG	large	60.71	2.06
		MID-21	CGTAGACTAG	small	31.85	2.11
83183-4	yes	MID-22	TACGAGTATG	large	51.6	2.05
		MID-23	TACTCTCGTG	small	81.24	1.98
83183-5	yes	MID-24	TAGAGACGAG	large	24.39	2.2
		MID-25	TCGTCGCTCG	small	186.96	1.96
83244-1	yes	MID-35	CAGTAGACGT	large	29.5	2.16
		MID-36	CGACGTGACT	small	104.26	2.01
83244-2	yes	MID-37	TACACACACT	large	44.7	2.08
		MID-38	TACACGTGAT	small	43.39	2.17
83184-1	no	MID-26	ACATACGCGT	large	57.43	2.07
		MID-27	ACGCGAGTAT	small	41.22	2.13
83184-2	no	MID-28	ACTACTATGT	large	43.58	2.11
		MID-29	ACTGTACAGT	small	82.5	1.95
83184-3	no	MID-30	AGACTATACT	large	89.85	1.98
		MID-31	AGCGTCGTCT	small	46.01	2.09
83184-4	no	MID-32	AGTACGCTAT	small	35.82	2.23
83184-5	no	MID-33	ATAGAGTACT	small	47.67	2.14
		MID-34	CACGCTACGT	large	36.19	2.09

[1] MID: Multiplexing Identifier, [2] Access Array Barcode Library for the 454 GS FLX Titanium Sequencer (Fluidigm, South San Francisco, CA, USA).

Additional file 4: Cq cut-off values: The highest specific Cq value was determined from the respective target reference bacteria and used as cut-off value for the different primer systems in the data analysis.

Primer system	Cq cut-off value
Domain Bacteria A V2-V3	31.45
Domain Bacteria B V4-V5	28.04
Phylum Firmicutes	30.03
Phylum Bacilli	31.33
Genus *Enterococcus*	24.59
Genus *Lactobacillus*	28.80
Genus *Streptococcus*	24.30
Family Clostridium cluster I	26.30
Species *Clostridium perfringens*	24.35
Family Clostridium cluster IV	24.10
Family Clostridium cluster XIV	25.85
Phylum Bacteroidetes	26.08
Genus *Bacteroides*	29.60
Phylum Actinobacteria	21.55
Family Bifidobacteriaceae	26.21
Class β- and γ-proteobacteria	24.45
Family Enterobacteriacea	25.48
Species *Escherichia coli*	25.32
Class ε-proteobacteria	23.95
Class δ-proteobacteria	26.03
Phylum Fusobacteria	22.82
Phylum Verrucomicrobia	21.91
Phylum Spirochaetes	25.28
Domain Archaea	23.53

Additional file 5

BION analysis of 454-sequencing data

Detailed information on the BION software, its functions, and the main statistics for the raw results is included in the file Results_referee.zip. When the file is unpacked it creates the directory Results_referee. This contains 24 subfolders containing the main statistics for each primer pair. However, the species-folders are empty, since the species-specific primers were not tagged. The remaining subfolder 'Software' and the additional files are explained in the README-file.

The entire BION-meta package (200 Mb) is not included, and there is currently not a stable link to it. But if interested, it can be downloaded from the following link:

https://www.dropbox.com/sh/fumscuqpanqaqvu/_4H--XBxHQ

Unfortunately supplementary information on the BION-meta generated output (Results-referee.zip) was not published, but if interested these results can be downloaded from the following link: https://www.dropbox.com/sh/j8obokhaelh003b/AAAK5WsCOTiYVSZzpkJg3v-va?dl=0.

As described in Additional file 5, the unpacking of the file will result in 24 subfolders, one for each primer set, the species-folders being empty, since these were not sequenced. Each folder contains a file named: "FDM.taxon.tab" which summarizes the mapped sequence results.

Paper II

II

Characterization of the bacterial gut microbiota of piglets suffering from new neonatal porcine diarrhoea

Marie Louise Hermann-Bank, Kerstin Skovgaard, Anders Stockmarr, Mikael Lenz Strube, Niels Larsen, Hanne Kongsted, Hans-Christian Ingerslev, Lars Mølbak, Mette Boye

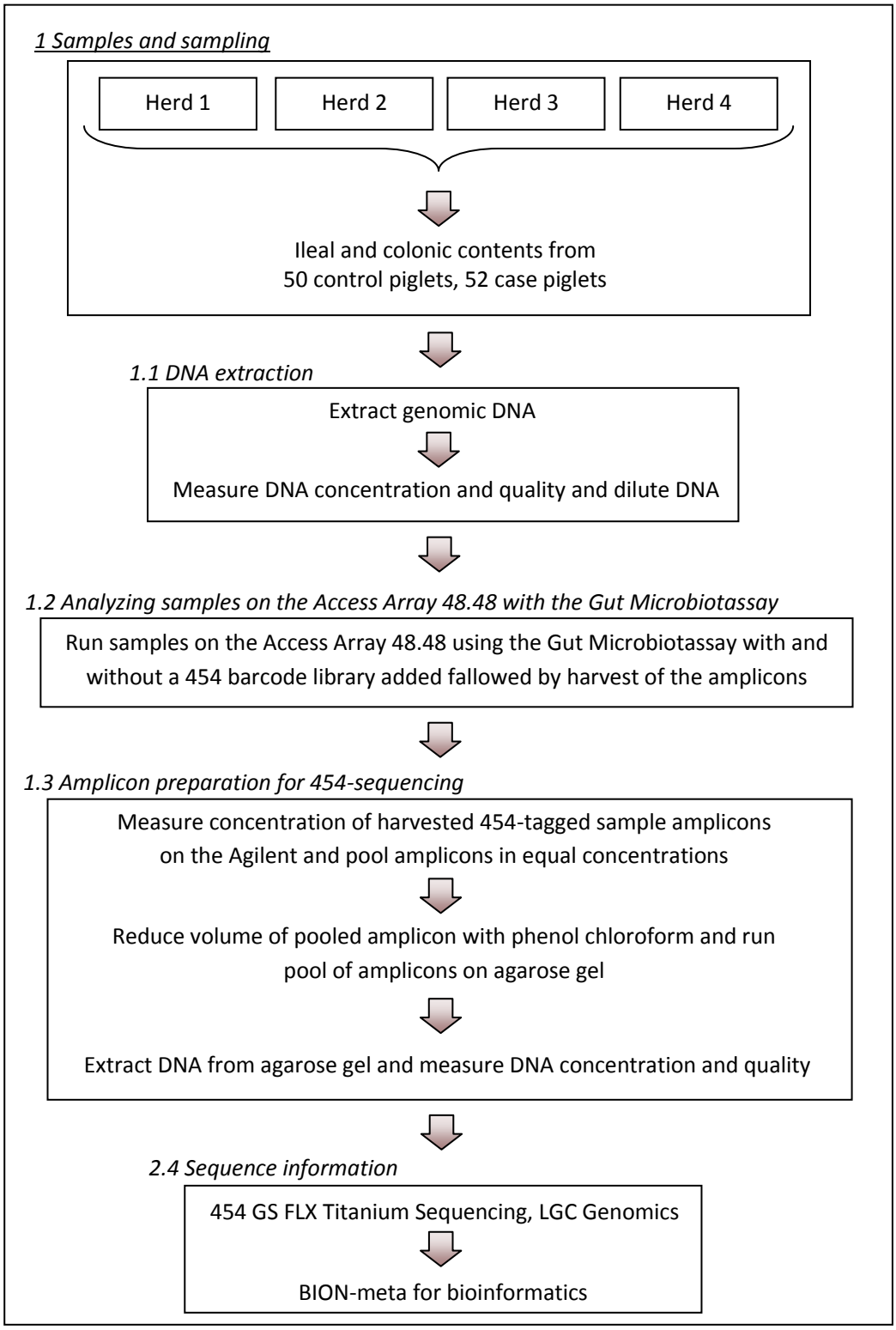
Submitted to

BMC Veterinary research

Introduction

The aim of this study was to compare the bacterial gut microbiota of piglets affected by NNPD (cases) with the gut microbiota of healthy piglets (controls) by use of the Gut Microbiotassay (developed in paper I), in order to search for a possible bacterial aetiology behind NNPD.

Flow diagram



Title page:

Characterization of the bacterial gut microbiota of piglets suffering from new neonatal porcine diarrhoea

Marie Louise Hermann-Bank[1], Kerstin Skovgaard[1], Anders Stockmarr[2], Mikael Lenz Strube[1], Niels Larsen[3], Hanne Kongsted[4], Hans-Christian Ingerslev[1], Lars Mølbak[1,5], Mette Boye[1§]

[1] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark.[2] Department of Applied Mathematics and Computer Science, Technical University of Denmark, Matematiktorvet, Building 324, DK-2800 Lyngby, Denmark. [3] Danish Genome Institute, Skt. Lucas Kirkeplads 8, DK-8000 Århus, Denmark. [4] Danish Pig Research Centre, Danish Agriculture and Food Council, Vinkelvej 13, DK-8620 Kjellerup, Denmark. [5] Present address: Chr. Hansen, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark.

[§] **Author of correspondence:** E-mail: mboy@vet.dtu.dk. Phone: +4535886263.

E-mail addresses:

MLHB: mlhh@vet.dtu.dk
KS: kesk@vet.dtu.dk
AS: anst@dtu.dk
MLS: milst@vet.dtu.dk
NL: niels@genomics.dk
HK: hko@lf.dk
HCI: hain@vet.dtu.dk
LM: dklmb@chr-hansen.com
[§]MB: mboy@vet.dtu.dk

Abstract

Background: In recent years, new neonatal porcine diarrhoea (NNPD) of unknown aetiology has emerged in Denmark and other countries. NNPD affects piglets during the first week of life and results in impaired welfare, decreased weight gain, and in the worst-case scenario death. Commonly used preventative interventions such as vaccination or treatment with antibiotics, have a limited effect on NNPD. Previous studies have investigated the clinical manifestations, histopathology, and to some extent, microbiological findings; however, these studies were either inconclusive or suggested that Enterococci, possibly in interaction with *Escherichia coli*, contribute to the aetiology of NNPD. This study examined ileal and colonic luminal contents of 50 control piglets and 52 NNPD piglets by means of the qPCR-based Gut Microbiotassay and 16 samples by 454-sequencing to study the composition of the bacterial gut microbiota in relation to NNPD.

Results: NNPD was associated with a diminished quantity of bacteria from the phyla Actinobacteria and Firmicutes while genus *Enterococcus* was more than 20 times more abundant in diarrhoeic piglets. The number of bacteria from the phylum Fusobacteria was also doubled in piglets suffering from diarrhoea. With increasing age, the gut microbiota of NNPD affected piglet and control piglets became more diverse. Independent of diarrhoeic status, piglets from first parity sows possessed significantly more bacteria from family Enterobacteriaceae and species *E. coli*, and fewer bacteria from phylum Firmicutes. Piglets born to gilts had 25 times higher odds of having NNPD compared with piglets born to multiparous sows. Finally, the co-occurrence of genus *Enterococcus* and species *E. coli* contributed to the risk of having NNPD.

Conclusion: The results of this study support previous findings that points towards genus *Enterococcus* and species *E. coli* to be involved in the pathogenesis. Moreover, the results showed that NNPD is associated with a disturbed bacterial composition and larger variation between the diarrhoeic piglets.

Keywords: NNPD, neonatal, piglet, diarrhoea, qPCR, microbiota, Gut Microbiotassay, 454-sequencing

Introduction

Neonatal piglet diarrhoea is of significant importance for the pig industry because it causes economic losses due to increased morbidity and mortality, decreased weight gain, and the need for extra medications [1,2]. Obviously, it impairs the welfare of the animals in the short term, but it may also affect their health in the longer term as a consequence of disrupting the normal bacterial succession in the gastrointestinal tract [3].

Bacterial colonization of the mammalian gastrointestinal tract begins at birth [4]. This colonization is a dynamic event, and the bacterial succession is influenced by a number of factors including: mode of delivery, surrounding environment, gestational age, and genetics [5,6]. The colonization of the gut has a major impact on the host's health and disease; for example, the microbiota helps in the maturation of the

gastrointestinal tract and immune system, protects against pathogen colonization through competitive exclusion, and converts otherwise indigestible substances into digestible components that benefit the host [3,5,7,8]. In accordance with these roles, studies of germ-free animals, deficient of normally developed gut microbiota, show animals with an impaired and immature intestinal immune system, in addition to changes in intestinal morphology [8-10].

During the last ten years, increasing attention has been focused on a new neonatal porcine diarrhoea [11-13]. In Denmark, this diarrhoea has been named 'New Neonatal Porcine Diarrhoea' (NNPD). What distinguishes NNPD from other types of neonatal piglet diarrhoea are the following: 1) The aetiology is unknown, however, routine diagnostic protocols show that it is not caused by known enteric pathogens such as hemolytic *Escherichia coli*, *Clostridium difficile, Clostridium perfringens* type A or C, coronavirus, rotavirus species A or C, *Cryptosporidium* spp., *Cystoisospora suis*, *Giardia* spp., or *Strongyloides ransomi.* 2) Typical strategies, such as vaccination or antibiotics, do not seem to have a noteworthy effect on the diarrhoea. 3) No obvious connection between NNPD and pig farm health status or management has been demonstrated [14,15, Larsen LE, Nielsen JP, unpublished results].

It is difficult to estimate how widespread NNPD is, mainly because of the unknown aetiology combined with a fluctuating clinical presentation [2], as well as differences in routine laboratory testing [13,15], but partly also because of the limited number of studies focusing on this issue. Nonetheless, a diarrhoea of much resemblance to NNPD has been described in Sweden and France [13,16,17].

This study is part of an interdisciplinary project investigating the aetiology of NNPD. Kongsted *et al.* (2013) suggested the following case-definition of NNPD: *"Non-hemorrhagic diarrhoea during the first week of life, without detection of known infectious pathogens, characterized by milk-filled stomachs and flaccid intestines at necropsy"* [15]. The same author found dissimilarities in the course and severity of NNPD among four pig farms and estimated that affected piglets had a negative average daily weight gain with an increased risk of dying, though this risk was not significant [2]. Jonach *et al.* (2014) examined the role of four enteric bacterial pathogens by fluorescence in situ hybridization (FISH) and found that simultaneous colonization of the intestinal mucosa with non-enterotoxigenic *E. coli* (non-ETEC) and *Enterococcus* spp. could be involved in the pathogenesis of NNPD [18]. Finally, several different viral assays tested negative on samples from some of the same NNPD animals that were examined in the aforementioned studies, indicating that common known viruses do not contribute to NNPD (Larsen LE, Nielsen JP, unpublished results).

This study investigates whether NNPD can be explained by the composition of the gut microbiota obtained from piglets with and without diarrhoea. This was examined by elucidating the overall bacterial composition and relative quantitative distribution of ileal and colonic intestinal content using the Gut

Microbiotassay: an assembly of 24 primer sets targeting ribosomal RNA (rRNA) genes, verified to function with the high-throughput quantitative real-time PCR (qPCR) chip Access Array™ Integrated Fluidic Circuit (AA48.48) from Fluidigm [19]. As the name implies, the assay is designed to target major bacteria phyla and selected taxonomic sub-groups of the gut microbiota. The Gut Microbiotassay provides a quick overview of the distribution, as well as the relative quantity of the gut microbiota in a large number of samples simultaneously. Subsequently, PCR amplicons from four case piglets and four control piglets were sequenced using 454-technology to acquire deeper taxonomic information. This approach revealed diverse gut microbial profiles dependent on piglet diarrhoeic status.

Materials and Methods

Animals and sample collection

Danish pig farms affected by NNPD were identified from conversations with veterinarians and farm managers. Four pig farms that fulfilled the inclusion criteria listed in Box 1 were included in the study. On each pig farm, approximately 15 randomly chosen sows from one farrowing batch (66 in total) were followed from the period of farrowing and for seven consecutive days afterwards. All newborn piglets were weighed at the beginning of the trial (average weight 1394 g, SD ± 335 g), and piglets weighing less than 800 g were excluded. All animals were subject to a daily clinical examination that paid special attention to fecal appearance on rectal swabs. Diarrhoea was defined as thin or watery feces. Based on these observations, piglets were characterized as either cases or controls: A case piglet had suffered from diarrhoea for at least two consecutive days, whereas a control piglet had never experienced diarrhoea. The inclusion criterion applied to case piglets resulted in a greater number of case piglets from gilts (approximately 66 %), as piglets born to multiparous sows did not suffer from diarrhoea to the same extent as piglets born to gilts. Control piglets were as far as possible collected from litters without diarrhoea. In total 50 control piglets and 52 case piglets were picked out (Table 1) and brought to the Danish Pig Research Centre, Kjellerup, for euthanization and necropsy. For ethical reasons, farmers were allowed to treat piglets for any disorder if necessary, on the condition that any such treatments were recorded for individual animals (for more information on the selection of pigs and herds included in this study, see Kongsted *et al.* (2013) [15]). During necropsy, intestinal contents were collected from the distal small intestine and the large intestine of each animal and stored at -80 °C until further analysis.

DNA extraction

Total DNA was extracted from intestinal content using the Maxwell® 16 LEV Blood DNA Kit (Promega, Madison, WI, USA) according to manufacturer's recommendations. A total of 200 mg of intestinal contents were suspended in 600 µl PBS and vortexed until visually homogeneous. The samples were centrifuged for

2 min at 200 × *g*, and the supernatant was transferred to new tubes. A volume of 350 µl of lysis buffer was then added, and the bacterial cells were lysed by bead beading (Tissuelyser II, Qiagen, Hilden, Germany) for 2.5 min at 20.0 hertz with a 5 mm steel bead (Qiagen). Subsequently, samples were centrifuged for 1 min at 1000 × *g* at 4 °C, after which the supernatant was transferred to novel 2 ml tubes and mixed manually with 30 µl Proteinase K (Promega). Next, samples were incubated for 30 min at 56 °C, and centrifuged at 13 000 × *g* for 1 min. The entire suspension was transferred to the sample inlets on the cartridge, and 50 µl of elution buffer was added to the bottom of the elution tubes. The cartridges were prepared according to the manufacturer's instructions [20] and the settings "Research Mode", "LEV Mode", "DNA", and "Blood/Cell" were selected for DNA extraction using the Maxwell® 16 Instrument (Promega). Finally, tubes were centrifuged at 20 000 x *g* for 3 min to settle any magnetic bead leftovers, and the DNA was moved to new tubes. DNA concentration and purity was measured using the Nanodrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, Germany) spectrophotometer, and DNA was stored at −20 °C until further analysis.

Analysis of bacterial profiles with use of the Gut Microbiotassay

To obtain a bacterial profile of the intestinal content, this study used the Gut Microbiotassay designed for the AA48.48 [19] with a few modifications. In order to improve the performance of the primer sets "Domain Bacteria B V4-V5" and "Phylum Firmicutes" degenerate nucleotides were introduced: Nucleotide 11 was changed from C to M (C/A), and nucleotide 5 from T to Y (T/C), (5'-3' direction), in the forward or reverse primer, respectively (Additional file 1). All remaining primer sets were identical to those previously published as 'The Gut Microbiotassay' [19]. Primers were purchased from Eurofins MWG Synthesis GmbH (Ebersberg, Germany) and stored at −20 °C.

In brief, the AA48.48 is a qPCR platform that is capable of running 48 × 48 = 2304 individual reactions simultaneously, and it enables quick and easy library preparation for 454-sequencing [21]. The AA48.48 was processed and prepared following the 'Access Array System™ User Guide' [21], with and without adding the Access Array Barcode Library for the 454 GS FLX Titanium Sequencer (454BL), as previously described [19]. Primers targeting bacteria at the taxonomic level of species were not tagged or included for sequencing, as these amplicons were regarded as contributing little information due to their specificity. However, analysis on species level was possible using the information obtained at higher taxonomic levels. All samples were diluted to 50 ng/µl with nuclease-free water (Ambion Inc., Austin, USA). Primers were diluted to 4 µM with 20 × Access Array Loading buffer and nuclease-free water (Ambion Inc.). Master mix was a mixture of: 10 × FastStart High Fidelity Reaction Buffer with 18 mM $MgCl_2$ (Roche Diagnostics, GmbH, Mannheim, Germany), 25 mM $MgCl_2$ (Roche), DMSO (Roche), 20 × Access Array Loading Reagent (Fluidigm Corporation, South San Francisco, CA, USA), 50 × ROX (Invitrogen Corporation, Carlsbad, CA, USA), 20 × EvaGreen®

(Biotium, Inc., Hayward, CA, USA), 10 mM PCR Grade Nucleotide Mix (Roche), and 5 U/µl FastStart High Fidelity Enzyme Blend (Roche), in final concentrations of 1 ×, 2.7 mM, 5 %, 1 ×, 0.5 ×, 1 ×, 200 µM, and 0.05 U/µl, respectively. Sample mix was prepared from 3 µl master mix, 1 µl 454BL (2 µM) (Fluidigm), and 1 µl DNA (50 ng/µl) as the very last step before running the array (without the 454BL: 4 µl master mix and 1 µl DNA (50 µg/µl)). When the PCR reaction had finished, the barcoded PCR amplicons were harvested and stored at −20 °C.

Next generation sequencing

Samples from two representative animals were selected from each farm: one piglet with NNPD and one piglet without NNPD, giving a total of 16 samples to be sequenced (when samples from both the ileum and colon of each animal were included). Piglets were chosen to be approximately the same age (5 or 6 days old). Concentrations of the respective PCR amplicons for each animal were determined using the Agilent 1000 chip (Agilent Technologies, Waldbronn, Germany). Amplicons were subsequently pooled in equal concentrations and size-separated by running the amplicons for 86 min, 90 V, in a 0.7 % Seakem® LE Agarose gel (Lonza Rockland, Rockland, ME, USA) followed by incubation for 30 min in 0.0004 % ethidium bromide for staining. By means of UV radiation from the Bio-Rad Universal hood II (Segrate, Milan, Italy) gel bands were visualized and bands spanning the size range of the primer products (200-900 base pairs) were excised. Finally, the Qiaquick Gel Extraction Kit (Qiagen) was used to purify DNA from the gel. This pool of 1615.7 ng DNA (260/280 nm-ratio: 1.97) derived from ileal and colonic luminal contents of 8 different animals was sequenced on a half PicoTiterPlate™ by a 454 GS FLX Titanium Sequencer (Roche) via LGC Genomics (GmbH, Berlin, Germany).

Data analysis

Relative quantification of Cq values

Data analysis was conducted as described in a previously published methodology article [19]: Raw Cq values were exported from 'Fluidigm Real-Time PCR Analysis' software version 3.0.2 to Microsoft Excel. To even out possible variation between the AA48.48 runs, all Cq values were normalized to an Interplate Calibrator. Next, Cq values from each sample were normalized to their respective "Domain Bacteria B V4-V5" primer set, thereby calculating the relative quantification.

Principal Component Analysis on normalized Cq data

Principal Component Analysis (PCA) was conducted in the software package R, version 3.1.0 [22]. All normalized data were initially transformed with the natural logarithm (ln). Next, primer sets with less than 50 % recorded Cq values were excluded from the analysis, and in the same manner, samples that resulted in less than 50 % registered Cq values were removed. For the remaining primer sets, missing values were

substituted with the lowest registered primer-specific value. Data were scaled by the individual primer mean and standard deviation for PCA. Samples used for 454-sequencing were pinpointed from the PCA results. In order for samples to be considered for sequencing the samples should be represented with luminal content from both ileum and colon, and none of these were allowed to have outlying coordinates in the PCA, but should be somewhat in the centre of its respective category (Control_colon; Case_colon; Control_ileum; and Case_ileum).

Statistics on the Gut Microbiotassay

Bacterial profiles obtained from the Gut Microbiotassay were used to study the gut microbiota of piglets with and without NNPD. For the statistical calculations all values were initially ln-transformed. A linear mixed-effect model was then applied with randomized effect of herd origin. The following variables were included as deterministic effects in the model: Gut section (ileum versus colon), Status (diarrhoeic versus healthy), Gilt (born to a gilt versus born to a multiparous sow), Percentage of diarrhoea (percentage points of life duration that a piglet has suffered from diarrhoea), Treatment (treated versus non-treated animal – this category only involves case piglets), Age (days). The interaction between Gut section and Status was also included to examine if possible diarrhoea-causing agents could be traced to a specific gut section. Additionally, the interaction between Status and Age was included to study whether the effect of age differed between case and control piglets. The effect of gender and birth weight in relation to status was also evaluated.

Previous studies have suggested that simultaneous colonization with *E. coli* and Enterococci species may be a cause of neonatal porcine diarrhoea [13,18,23,24]. This co-occurrence was also implied using the aforementioned model, thus, this was further investigated. A logistic regression model with randomized effect of herd origin, fit by penalized quasi-likelihood, was used to study if and how NNPD could be explained by the following variables: Gut section, Gilt, Age, genus *Enterococcus*, class Beta- and Gammaproteobacteria, family Enterobacteriaceae, species *E. coli*, as well as possible interaction between these bacteria.

A *p*-value of less than 0.05 was considered significant. Statistical analyses were performed in R.

Bioinformatics analysis of 454-sequencing data

Sequencing data available at NCBI Sequence Read Archive [NCBI:SRP044282] were analyzed using the as yet unpublished open source package BION-meta (in preparation, N. Larsen, Danish Genome Institute, Denmark) (Additional file 2). This program facilitates quick and easy bioinformatics analysis, and BION-meta has previously been applied to a similar dataset [19]. However, in addition to matching all sequences against the ribosomal small subunit (SSU) Silva dataset, suited for 16S rRNA gene targeting primer sets,

BION-meta was advanced to encompass searches in the ribosomal large subunit (LSU) Silva dataset, thereby accommodating the primer sets targeting the 23S rRNA gene used in the Gut Microbiotassay [25]. The BION-meta workflow included the following elements: 1) separation by sample barcodes and primer tags; 2) removal of primer remnants and low quality sequences at the ends, as well as filtering by length (200 nucleotides) and overall quality (96 %); 3) removal of chimeric sequences; 4) separation of each sample by matching it with the phylogenetic primer(s); 5) matching all sequences against the SSU and LSU Silva datasets and producing a table with the highest 1 % similarities for each query; and 6) mapping the similarities to the Silva SSU and LSU taxonomies, identifying consensus operational taxonomic units (OTUs).

To examine the gut microbiota in further details, BION-meta data were analyzed by Principal Coordinates Analysis (PCoA). This was conducted by applying the vegan package in R using Bray-Curtis distances on the untransformed sequence reads followed by k-means clustering [26].

Due to the hierarchical taxonomic design of the Gut Microbiota, where several primers in taxonomic lineage potentially target the same bacterial organism, a number of primer sets were chosen for this expanded diversity analysis (referred to as “grand data”): phylum: Actinobacteria, Bacteroidetes, Fusobacteria, and Spirochaetes; class: Deltaproteobacteria, and Epsilonproteobacteria; family: Clostridium cluster I, cluster IV, and cluster XIV, and Enterobacteriaceae; genus: *Enterococcus*, *Lactobacillus*, *Streptococcus*. These primer sets were selected because they provide the most comprehensive taxonomic information with the least taxonomic trespassing that would result in distortion and consequentially misinterpretation of the results.

Results

From four well-managed Danish conventional pig farms a total of 201 ileal and colonic intestinal content samples were collected from 50 control piglets without NNPD and 52 case piglets suffering from NNPD (3 samples were lost between sampling and the laboratory). Of the case piglets, 25 were treated with broad-spectrum antibiotics intramuscularly, depending on their diagnosis and according to herd treatment practices. Piglets were three to seven days old and were all reared with their biological dam and siblings [15].

A bacterial taxonomic profile was obtained from both gut sections of each animal using the Gut Microbiotassay targeting rRNA genes of major bacterial groups in the mammalian intestine. Normalized Cq data were used for PCA. After excluding the primer sets with less than 50 % recorded Cq values (“Class Epsilonproteobacteria”, “Phylum Verrucomicrobia”, and “Domain Archaea”), as well as the “Domain Bacteria A” and “Domain Bacteria B” primer sets (which did not contribute any information to the PCA analysis), 19 primer sets remained for the data analysis. In addition, 12 samples were removed from the

dataset as a consequence of having too few Cq registrations. From the PCA scores plot (see Figure 1), four case animals (three of which were treated with antibiotics) and four control animals were randomly chosen to be representatives for their respective herds. Detailed taxonomic information was acquired for these 16 samples using 454 next generation sequencing.

The Gut Microbiotassay

From the relative quantitative results generated by the Gut Microbiotassay, it was evident that luminal content from the large intestine possessed a significantly higher bacterial load than luminal content from the small intestine (expressed by domain Bacteria B, $p < 0.0001$), regardless of diarrhoeic status.

NNPD was associated with a diminished quantity of bacteria from the phyla Actinobacteria ($p < 0.0001$) and Firmicutes ($p = 0.02$). Firmicutes comprised the following: class Bacilli ($p < 0.0001$), genus *Lactobacillus* ($p < 0.0001$), genus *Streptococcus* ($p = 0.0002$), and family Cl. cluster IV ($p < 0.0001$). However, genus *Enterococcus* was estimated to be more than 20 times more abundant in diarrhoeic piglets ($p < 0.0001$). The number of bacteria from the phylum Fusobacteria ($p = 0.02$) was also doubled in piglets suffering from diarrhoea.

It was investigated whether the effect of diarrhoeic status differed between gut sections through a statistical interaction term. In both case and control piglets, there were generally more members from phylum Bacteroidetes and genus *Bacteroides* in the colon compared with the ileum. Nonetheless, case piglets had a reduced number of Bacteroidetes and *Bacteroides* compared to control piglets, and the depletion of these bacteria was located to the colon ($p = 0.0009$ and 0.04, respectively), while no significant depletion was detected in the ileum. Overall, there were greater numbers of family Cl. cluster I and species *Cl. perfringens* in control piglets compared with case piglets, except for *Cl. perfringens*, which was more numerous in the colon of case piglets, though not significantly. The numbers of Cl. cluster I and *Cl. perfringens* were significantly reduced in the ileum of case piglets compared with control piglets ($p = 0.01$, 0.04). Control piglets generally exhibited a disparity in the number of bacteria present in the ileum versus the colon, while the difference in bacterial abundance between gut sections was more negligible in case piglets. It should be noted that the preceding results all relate to circumstances of 'all other things being equal'.

Independent of diarrhoeic status, piglets from first parity sows possessed significantly more bacteria from family Enterobacteriaceae ($p = 0.03$) and species *E. coli* ($p = 0.004$), and fewer bacteria from phylum Firmicutes ($p = 0.01$), hereof genus *Streptococcus* ($p = 0.03$).

Bacteria from family Cl. cluster XIV (p = 0.001) and class Deltaproteobacteria (p = 0.04) were depleted, but bacteria belonging to genus *Streptococcus* (p = 0.008), and family Cl. cluster I (p = 0.04) were elevated in case piglets that suffered from an increasing percentage of diarrhoea in their lifetime. The estimated fold changes may be calculated as $\beta^{percentage\ points}$, where β is the effect parameter for diarrhoea that is estimated in Table 2 and where percentage points range from 0 to 100.

Diarrhoeic piglets treated with broad-spectrum antibiotics had a reduced presence of Fusobacteria (p = 0.002), and Deltaproteobacteria (p = 0.02).

Regardless of diarrhoeic status, the quantity of bacteria in the intestine generally increased with increasing age (p = 0.04). The older the piglet the more bacteria from the following groups: phylum Firmicutes (p = 0.001), class Bacilli (p = 0.0001), genus *Lactobacillus* (p < 0.0001), and class Cl. cluster IV (p = 0.04). Bacteria that diminished from the gut microbiota with increasing age were as follows: genus *Streptococcus* (p = 0.003), species *Cl. perfringens* (p = 0.009), phylum Actinobacteria (p = 0.04), and phylum Fusobacteria (p = 0.004).

In addition, the interaction between Status and Age was examined. There was a significant difference between control piglets and case piglets over time. As piglets aged, it was estimated that apparently increasing numbers of class Beta- and Gammaproteobacteria (slope: 0.21 log units/day), family Enterobacteriaceae (0.12) and species *Escherichia coli* (0.25) colonized the intestine of case piglets, while the numbers of these bacteria were estimated to decrease for control piglets (p = 0.004, p < 0.0001, p = 0.001, respectively). Figure 2 illustrates the abundance of class Beta- and Gammaproteobacteria in ileal and colonic luminal content in relation to piglet age, segregated according to diarrhoeic status. However, while the differences in age effects between control and case piglets were highly significant (p = 0.0007, 0.0001, 0.0004), the three estimated positive slope values for diarrhoeic piglets were not significantly greater than zero (p = 0.11, 0.39, 0.12). Table 2 summarizes the estimated fold changes, 95 % confidence intervals, and p-values of these results, and Table 3 lists the same parameters for all significant interactions.

The effects of birth weight and gender were investigated, and no significant results were discovered.

Table 4 lists the significant results calculated from the model investigating whether NNPD could be explained by genus *Enterococcus*, class Beta- and Gammaproteobacteria, family Enterobacteriaceae, species *E. coli*, possible co-occurrence of bacteria, Gut section, Gilt, and Age. The logistic regression model fitted the data according to standard model control measures. NNPD was found to be significantly associated with the presence of genus *Enterococcus* (p = 0.009), and there was also a significant effect of the co-occurrence of this genus and species *E. coli* (p = 0.02). Though the interaction apparently had a slightly diminishing effect on the probability of being recognized as an NNPD piglet, it actually contributed

to the risk in the majority of cases with increasing values of *E. coli.* This result is explained by the logged values of Enterococci, which were negative in 89 % of the data. Finally, there was a significant effect of the interaction between class Beta- and Gammaproteobacteria and the colon, suggesting that NNPD piglets are differentiated from control piglets by colonization of the colon with class Beta- and Gammaproteobacteria ($p = 0.001$). All things being equal, piglets born to gilts had 25 times higher odds of having NNPD compared with piglets born to multiparous sows ($p < 0.0001$).

PCA

Principal Component Analysis of data from the 19 primer sets showed no clustering in relation to pig farms (results not shown). Generally, the control large intestine was characterized by a gut microbiota that clustered together, whereas the bacterial composition of the small intestine from control piglets was more diverse (Figure 1). Diarrhoeic gut sections were more scattered compared to non-diarrhoeic ones in the PCA plot, demonstrating that NNPD is associated with a disturbed bacterial composition and larger variation between the diarrhoeic piglets. When looking at the microbial composition in piglets at different ages (three to seven days old), clustering became more defined with age, in relation to diarrhoeic status (Figure 3). Moreover, there was a trend towards the gut microbiota from control piglets becoming more clustered with age.

Next generation sequencing

BION-meta processing including de-multiplexing, cleaning, and chimera checking resulted in 279 103 reads for mapping (Additional file 2).

A Shannon's diversity index was calculated from the grand data: control ileum = 3.48; case ileum = 3.23; control colon = 3.43; and case colon = 3.18. Additionally, a PCoA was generated from the grand data that showed separation of the sequenced samples according to NNPD status (first canonical axis) and gut section (second canonical axis), as shown in Figure 4. The two major loading scores in the PCoA were explained by the following: Control: genus *Lactobacillus* and species *Fusobacterium varium*; Case: species *E. coli* and genus *Enterococcus*; Ileum: genus *Lactobacillus* and species *Fusobacterium ulcerans*; and Colon: species *F. varium* and species *Fusobacterium mortiferum.*

Discussion

All piglets included in the study had previously been tested for hemolytic *E. coli*, *Cl. difficile, Cl. perfringens* type A and C, coronavirus, rotavirus species A and C, and microscopically inspected for parasites, with the conclusion that none of these agents were related to NNPD [15]. In this study it was generally expected that bacteria associated with diarrhoea would exhibit more or less consistent tendencies in case piglets

compared with control piglets. Consequently, the Cq results and the sequencing results displaying similar trends will be discussed in relation to diarrhoea in the subsequent section, with the main focus being on potential NNPD-causing bacteria with high statistical estimates (Tables 2-4). Sequencing results are based on a small subset of case and control piglets. The mapped sequences are mainly referred to as most similar species or isolate identified, while acknowledging the limits of classifying bacteria at these taxonomic levels. Nonetheless, even though mapped sequencing results at species and particularly isolate level are questionable, these are still construed as indicative and possibly important bacteria, which is why they are highlighted in the consecutive text.

Genus *Enterococcus*

Enterococci were 21 times more abundant in diarrhoeic piglets than in healthy piglets ($p < 0.0001$), and moreover genus *Enterococcus* was one of the bacterial groups positively correlated with status, meaning that the presence of Enterococci increases the risk of suffering from NNPD ($p = 0.009$). Genus *Enterococcus* was classified to 27 different OTUs, of which *Enterococcus hirae*, *Enterococcus faecium*, *Enterococcus durans*, and *Enterococcus mundtii* were the predominant.

Enterococci are ubiquitous distributed in nature and a consistent finding in the gastrointestinal tract of several animal species, including the pig [13,18,27,28]. Enterococci with regard to neonatal porcine diarrhoea has been reported in previous studies [13,18,29,30]. Another noteworthy feature is that this genus is reported to be resistance to several antibiotics [27,28], which could explain why the efficiency of antibiotic treatment of NNPD is variable.

Larsson *et al.* (2014) found neonatal porcine diarrhoea (NPD) in Swedish pig farms to be associated with enteroadherent *E. hirae* colonizing the small intestine accompanied by mucosal lesions [29]. This conforms to observations published by Jonach *et al.* (2014) using FISH to investigate the same piglets as the present study. The authors demonstrated small intestinal colonization by adherent *Enterococcus* spp. and often found them occurring together with adherent *E. coli* [18]. According to sequencing results, *E. hirae* was the most abundant species, consistent with results from a former study on Enterococcal communities in pig feces [27]. Nonetheless, this species displayed the smallest difference in numbers of OTU between diarrhoeic and healthy piglets' gut microbiota. However, the numerical occurrence of reads may not be related to the development of diarrhoea, especially not if diarrhoea is a result of co-occurrence of different bacteria such as *E. hirae* and *E. coli*.

E. durans could be a contributor to NNPD, as it has previously been reported to co-occur with *E. coli* in cases of neonatal piglet diarrhoea [13,23]. In a study from 1984 in which foals and gnotobiotic piglets were experimentally inoculated with *E. durans*, this species was found to promote the proliferation of *E. coli*, and

to adhere to the mucosa of the small intestine in either case [24]. In fact, *E. durans* has been hypothesized to act as a primary enteric pathogen with the ability to clear the way for other pathogens [23]. Although the ratio of *E. durans* between case and control gut sections is largest for colonic content, there were 40 % more reads of this species in the ileum compared with the colon, in line with previous findings [13,23].

The preceding information implies an interesting co-occurrence of Enterococci and *E. coli* [18]. Similarly, this study also found a significant effect of the interaction between species *E. coli* and genus *Enterococcus,* which contributed to the risk of piglets suffering from NNPD ($p = 0.02$). Furthermore, there were generally more Enterococci in the ileum compared with the colon, which supports previous findings ($p = 0.02$) [13,18,23,29]. Both *E. hirae* and *E. durans* have been reported to cause villous atrophy, which was frequently found in the small intestine of piglets included in this study [18,23,29].

E. mundtii was almost exclusively found in the gut microbiota of case piglets, primarily in antibiotic-treated piglets, indicating that antibiotic treatment created a favorable environment for this species. Interestingly, this finding did not account for one of the antibiotic-treated animals, in which no *E. mundtii* was detected. However, it seems more likely that this divergence was due to a herd effect rather than the treatment.

Phylum Fusobacteria

Piglets with diarrhoea harbored more bacteria classified to phylum Fusobacterium ($p = 0.02$). A similar trend has been associated with acute hemorrhagic diarrhoea in dogs and ulcerative colitis in humans [31,32]. In this study, 16 OTUs were identified. Species *F. mortiferum* was a consistent finding in the digesta of all piglets, but it was less abundant in control piglets compared to case piglets. Nonetheless, Portrait *et al.* (2000) demonstrated that this species was able to produce bacteriocin-like substance(s) with an inhibitory effect on a number of both Gram-positive and Gram-negative species [33]. Therefore, it is difficult to say whether the increased abundance of *F. mortiferum* in case piglets is due to its potential pathogenicity or if the increase is a type of defense mechanism mediated by the gut microbiota. Phylum Fusobacterium was one of the two bacterial groups that were significantly reduced by antibiotic treatment ($p = 0.002$).

Class Beta- and Gammaproteobacteria, family Enterobacteriaceae and species *Escherichia coli.*

E. coli are commonly found in the gastrointestinal tract. This species is normally a harmless commensal in the host, nonetheless some *E. coli* possess pathogenic features [34,35].

This study found a highly significant effect of the interaction between NNPD status and this bacterial taxonomic lineage in relation to age in days (Figure 2 and Table 3). Thus, with increasing age, these bacteria proliferated in piglets with diarrhoea (though not significantly), whereas control piglets were significantly better at limiting this group. With age, the estimated difference increased, indicating that the diarrhoeic

piglets have impaired ability to control and clear this group of bacteria. However, it also suggests that NNPD causes an imbalance in the gut microbiota, creating a favorable environment for these bacteria.

Kongsted *et al.* (2013) thoroughly examined the same animals for *E. coli* by aerobic culturing, serogrouping, and testing for virulence factor genes. The most prevalent finding was the presence of non-hemolytic *E. coli,* independent of diarrhoeic status. Fimbrial genes were evenly distributed among the genes investigated in approximately 25 % of the tested *E. coli* from both case and control piglets. Of all piglets, only one case of classic Enterotoxigenic *E*. c*oli* and six cases of non-typable hemolytic *E. coli* were detected, all in diarrhoeic piglets [15].

NNPD piglets investigated by 454-sequencing from different pig farms resulted in the identification of 109 OTUs and revealed a remarkably large number of reads classified to DEC8A and *E. coli* HQ219945.1.1457, both with the highest prevalence in case animals in all but one pig. The single pig that stood out was from pig farm four, which generally differed from the others. DEC8A is the name of a diarrhoeagenic *E. coli* (DEC) isolate classified as EHEC 2 (enterohemorrhagic *E. coli* 2 clonal complex), serotype O111a:NM [36]. EHEC 2 comprises a group of pathogenic *E. coli* reported to cause various disorders in mammals, including diarrhoea. The O111a was not part of the serogroups investigated by Kongsted *et al.* (2013) [15].

Except for one nucleotide positioned in the 5'-end of the *E. coli* oligonucleotide probe used for FISH analysis by Jonach *et al.* (2014), this probe matches the DEC8A 23S rRNA gene [18,37]. Hence, the possibility cannot be dismissed that the *E. coli* adherent to the small intestinal epithelium observed by Jonach *et al.* (2014) could be DEC8A. This could also explain the frequent finding of villous atrophy previously found to characterize diarrhoeic piglets [15,18].

E. coli HQ219945.1.1457 isolate was detected in ileal and colonic content of all animals sequenced, though in much higher numbers in diarrhoeic piglets, indicating that it might contribute to the pathogenesis of NNPD.

Piglets born to gilts had a significantly higher abundance of family Enterobacteriaceae and species *E. coli* than piglets born to multiparous sows (p = 0.03, 0.004, respectively). These were mainly classified to be the aforementioned isolates: DEC8A and *E. coli* HQ219945.1.1457. The increased risk of having NNPD if born to a gilt is consistent with farmers' and veterinarians' anamnesis reports, as they describe NNPD to be most prevalent among litters from first parity sows [2,13,14,17]. While the frequency of piglets born to gilts in the data material was 38 % as a product of the randomized selection of sows, the frequency of case piglets born to gilts was 66 %, reflecting the strongly significant effect of being born to a gilt (Table 4). This model also found that piglets of different NNPD status could be separated based on colonic colonization with species *E. coli* from class Gammaproteobacteria according to 454-sequencing results.

Phylum Firmicutes, class Bacilli, genus *Lactobacillus,* and genus *Streptococcus*

These bacterial groups are all part of the normal gut microbiota of pre-weaned piglets [4,38]. The gut microbiota of case piglets was inhabited by fewer *Lactobacillus acidophilus, Streptococcus gallolyticus subsp. gallolyticus, Streptococcus alactolyticus,* and *Streptococcus uberis* compared with the gut microbiota of control piglets.

Lactobacilli have been shown to colonize the intestines of piglets soon after birth and to be a stable member of the gut microbiota throughout the intestinal tract [4]. A low abundance of species *L. acidophilus,* which is regarded as a beneficial bacterium, could be an indicator of a troubled gastrointestinal milieu in pigs, as it was also diminished in diarrhoeic piglets included in this study. *S. gallolyticus subsp. gallolyticus* has previously been isolated from the gastrointestinal tract of numerous animal species, including pigs, and is therefore most likely included in the normal gut microbiota [39,40]. Additionally, *S. alactolyticus* is a member of the normal gut microbiota in pigs. There are few reports of *S. uberis* being isolated from pigs [41,42], and it does not seem to play a significant role in the development of NNPD, as the abundance of *S. uberis* is significantly lower in case piglets versus control piglets.

Phylum Firmicutes, family Clostridium cluster I, species *Clostridium perfringens*, family Clostridium cluster IV, and family Clostridium cluster XIV

In general, there were fewer of these bacteria in intestinal content from both the ileum and colon of case piglets compared with control piglets, except that colonic content possessed a higher number of *Cl. perfringens* (not statistically significant). It can be speculated whether the increased abundance in the colon reflects the reduction of Cl. cluster I and *Cl. perfringens* in the ileum (p = 0.01, 0.04, respectively). Nonetheless, 454-sequencing of amplicons generated by primer sets targeting Cl. cluster I and higher taxonomical levels failed to demonstrate this trend. *Cl. perfringens* is a normal finding in the gastrointestinal tract but it is also a potential pathogen [43]. Sequencing amplicons generated by the primer set targeting family Cl. cluster I revealed reads from 17 OTUs, of which only *Cl. perfringens* ATCC 13124 was worthy of notice. *Cl. perfringens* ATCC 13124 is a type A strain that is a potential diarrhoea-causing agent [43]. However, because piglets included in this study have all been tested for *Cl. perfringens* type A (among others) previously [15], where it was a frequent finding with higher prevalence in control piglets versus case piglets, this species is not considered to be essential to the development of NNPD.

The fact that all remaining bacterial groups were diminished in diarrhoeic piglets is most likely due to an imbalance in the gut microbiota because all of these groups have been demonstrated in digesta from healthy piglets [38]. Various bacteria from family Cl. cluster IV and Cl. cluster XIV are regarded to be beneficial due to their ability to produce short chain fatty acids (SCFA) such as acetate, propionate, and butyrate [44].

Of the 64 OTUs classified to family Cl. cluster IV, deficient bacteria mainly comprised species *Ruminococcus bromii*, which have previously been found in the porcine gastrointestinal tract [40]. A reduced number of *R. bromii* have also been reported in patients suffering from ulcerative colitis [32].

Family Cl. cluster XIV was not significantly related to the NNPD status of the pigs, but there was a significant effect of suffering from diarrhoea for a prolonged period of life (percentage of diarrhoea) that resulted in a reduction in the abundance of this family ($p = 0.001$). Thus, this bacterial group was indirectly affected by NNPD, as results indicate that family Cl. cluster XIV was vulnerable to the continuously flushing effect of diarrhoea. A scarce population of bacteria from family Cl. cluster XIV has also been described in previous studies on gut microbial communities in intestinal disorders [31,45]. *Roseburia inulinivorans* was the central bacterium missing from the 81 OTUs classified to family Cl. cluster XIV. This species is able to degrade oligofructose to free fructose, which can function as a fuel for other members of the gut microbiota, a phenomenon called cross-feeding [46].

Phylum Bacteroidetes and genus *Bacteroides*

Members of phylum Bacteroidetes are a common finding in the gastrointestinal tract of mammals, and they are also an early intestinal colonizer of the healthy piglet [4,5,38,40]. Culture studies found bacteria belonging to this phylum in piglets older than 48 hours, most frequently in the large intestine [4]. This result is consistent with the findings of the present study, which found phylum Bacteroidetes to be more abundant in colonic contents of both case piglets and control piglets. However, diarrhoea resulted in a significant depletion of these bacteria in the colon (phylum Bacteroidetes $p = 0.0009$, and genus *Bacteroides* $p = 0.04$). Several studies have also found a reduced presence of these bacteria in different enteric disorders, such as acute non-hemorrhagic diarrhoea in dogs, experimentally induced swine dysentery in pigs, and inflammatory bowel disease in humans [31,45,47]. A total of 126 OTUs were identified. Unfortunately, sequencing results did not reveal any consistent tendencies for this taxonomic lineage. Disregarding the criterion that piglets from all herds should exhibit the same trends, case piglets harbored particularly lower numbers of species *Bacteroides pyogenes, Bacteroides rodentium, Bacteroides xylanisolvens*, and the unclassified *Porphyromonadaceae bacterium* C941 in their intestinal luminal content versus control piglets.

Phylum Actinobacteria

Several studies have found phylum Actinobacteria to be part of the normal gut microbiota and to be scarce in the gut microbiota of different gastrointestinal disorders, such as irritable bowel syndrome in humans and acute hemorrhagic diarrhoea in dogs [31,48]. The phylum includes the genus *Bifidobacterium*, which is considered to be beneficial to its host, and a number of species from this genus are recognized probiotics [49,50]. There was a significant depletion of members from this phylum in the intestinal content of NNPD-

affected piglets compared with those of control piglets ($p < 0.0001$). This was supported by OTU counts from all diarrhoeic piglets, except for the piglet that originated from the atypical herd mentioned earlier. Of the 74 OTUs the species scarcely represented were *Bifidobacterium boum*, and *Corynebacterium kutscheri* and they were mainly diminished from the colon. *Bifidobacterium* as probiotic has been demonstrated to protect piglets against weaning diarrhoea associated with *E. coli*, and *B. boum* to inhibit Shiga toxigenic *E. coli* virulence gene expression experimentally [51,52]. This fits the fact that case piglets harbored an inverse proportion of members from the taxonomical lineage class Beta- and Gammaproteobacteria, family Enterobacteriaceae and species *E. coli,* and from phylum Actinobacteria, having a lot more of the former bacteria and fewer of the latter.

C. kutscheri has primarily been reported to be a common bacterium on the mucous membranes of rodents including the gastrointestinal tract [53]. Interestingly, *C. kutscheri* was more plentiful in the intestinal content of healthy piglets than in that of diarrhoeic piglets, irrespective of gut section. The bacterium does not count as part of the normal porcine gut microbiota in the existing literature. Thus, even though this cannot be ruled out for certain, it seems more likely that piglets may have acquired this species from foraging rodents directly or indirectly.

Class Deltaproteobacteria

This bacterial group was not associated with the diarrhoeic status of the piglets, but there was a significant reduction in numbers of members of this class in case piglets suffering from prolonged diarrhoea (increasing percentage of lifetime). Moreover, Deltaproteobacteria were also diminished by administration of antibiotics to the piglets. These facts are intertwined, as case piglets were mainly given antibiotics in an attempt to cure prolonged diarrhoea. Finally, both results were not strongly significant ($p = 0.04$, $p = 0.02$, respectively). The only OTU found by sequencing amplicons generated by the primer targeting this class was species *Desulfovibrio piger,* and this was only identified in healthy piglets from two herds. This species has primarily been described as an opportunistic pathogen in humans but has also been detected in the feces of wild ducks [54,55]. While the species was primarily found in colonic content of the control piglets, it is postulated that this species constitutes part of the normal gut microbiota of piglets.

PCoA of the gut microbiota

From the BION-meta processed sequencing data a PCoA was generated (Figure 4). This investigation supported the preliminary PCA that was produced from the Gut Microbiotassay data, and additionally the major loading scores confirm the findings in the section above concerning the different bacterial groups. It diverges from the preceding discussion by taking all bacteria into account, and the clustering strongly suggests that it is possible to associate certain bacteria with a healthy gut microbiota.

The intestinal microbiota of the aging piglet

When the data were subset according to age in days, this study found that, with age, the gut microbiota clustered in relation to status, but also that clustering became more defined with age (Figure 3). The former probably reflects the significance of *E. coli* in NNPD. Control piglets had significantly fewer of this species compared with case piglets, and over time the abundance of *E. coli* diminished in control piglets whilst it increased in case piglets (Figure 2). A more defined microbiota with increasing age is in line with conclusions from a previous study [56], which found that seven- to nine-day-old piglets (youngest piglets included in the study) had the lowest individual average similarity, independent of environmental or maternal relationship, compared with piglets from older age groups. The more distinctive clustering of NNPD status with increasing age could hint age to be a relevant factor in the diagnosis of NNPD.

Antibiotics

The NNPD piglets investigated in this study included antibiotic-treated piglets. Antibiotic-treated piglets were included because they typically suffered the worst cases of NNPD and were medicated for ethical reasons. It is evident that antibiotics have an impact on the gut microbial composition [6]. However, one of the characteristics of NNPD is that it responds poorly to antibiotics. Additionally, Figure 1, 3-4 illustrate how it is possible to cluster piglets according to NNPD status despite the fact that some of the NNPD cases are treated with different antibiotics, indicating that the effect of NNPD on the gut microbial distribution and composition is more significant than the effect exerted by antibiotics in piglets of this age group.

NNPD might be a multi-factorial condition

Most likely NNPD is a multi-factorial disorder, requiring different factors to be present in order to manifest. Examples of contributing factors could be as follows: 1) Immunologically depressed animals, which would be the case if they were deprived of colostrum [7]. 2) presence of the necessary circumstances or sequence of events, such as a preceding primary aetiology, like viruses or certain bacteria, allowing secondary bacterial pathogens to establish themselves [23]; and 3) management practices in which the farmer himself might introduce the disorder through zootechnical interventions. This could be as a result of antibiotic treatment. On the other hand, it could also be by acting as a vector, transmitting the agent with the equipment used for the animals [13]. However, irrespective of aetiology, the results of this study show that diarrhoeic piglets suffer from an imbalanced gut microbiota with diminished bacterial diversity. At present, it is impossible to conclude whether diarrhoea is a consequence of an absence of beneficial bacteria or if diarrhoea is an outcome of invading pathogenic bacteria or an overgrowth of opportunistic pathogenic bacteria.

Considerations on study approach

Case and control piglets included in this study all originate from herds affected with NNPD, hence the study lack the inclusion of true control piglets from none-affected herds. Also, despite the efforts to ensure correct inclusion of NNPD herds through predefined criteria there is still a risk that piglets actually did not suffer from NNPD.

It is acknowledged that this study only examines the bacterial composition of the luminal content, and not bacteria from the gastrointestinal wall. However, bacteria associated with the mucosa are not restricted to this site, as they are shed into the lumen together with intestinal epithelial cells and mucus. Thus, it is assumed that this study also detects these or at least a sub-fraction of these.

Additionally, results from this study are affected by the various steps involved in preparing the samples, for example DNA extraction, PCR, and sequencing, which all introduce variation into the final outcome [57]. Furthermore, the results do not reflect bacterial numbers but relative values of 16S and 23S rRNA gene abundance or OTUs (consensus sequences).

Finally, conclusions drawn from sequencing data are based on OTUs displaying similar trends to data obtained from the Gut Microbiotassay. Moreover, sequences had to be represented in a noteworthy number, and with somewhat consistent findings in piglets, irrespective of the variables in relation to diarrhoea. This means that there might be a risk that important bacteria could be overlooked if they do not meet the aforementioned criteria.

Conclusion

The results of this study on NNPD indicate that bacteria could be the aetiology of this diarrhoea that affects piglets during the first week of life. *E. coli* appears to be a contributing factor to NNPD, and this might even be narrowed down to one or two isolates, namely O111a:NM and *E. coli* HQ219945.1.1457. Additionally, NNPD piglets differ from healthy piglets by colonic colonization of bacteria from class Beta- and Gammaproteobacteria, primarily being species *E. coli*, and it appears that there is an important effect of age, both of which might be relevant in the characterization and diagnosis of NNPD. There is reason to believe that genus *Enterococcus* is a participating factor, most likely species *E. hirae* or species *E. durans.* Furthermore, diarrhoeic piglets suffer from an imbalanced gut microbiota, in which bacteria regarded as beneficial are diminished, which particularly accounts for *L. acidophilus*.

Ethical

According to Danish laws no ethical approval is required for studies not including treatment groups or needle injections/blood testing. Therefore this study was not subject to ethical approval, but fulfilled the regulations from the Danish Ministry of Justice. Hence, all handling of animals was performed by trained personnel and veterinarians, and euthanization was executed exclusively by veterinarians. Procedures

concerning the animals were all part of routine examinations and diagnosis of animals normally used in practice.

List of abbreviations used

AA48.48: Access Array 48.48, **454BL:** Access Array Barcode Library for the 454 GS FLX Titanium Sequencer, **Cq**: quantification cycle, **DEC**: diarrhoeagenic *E. coli,* **EHEC 2**: enterohemorrhagic *E. coli* 2 clonal complex, **ETEC**: enterotoxigenic *E. coli,* **FISH**: fluorescence in situ hybridization, **ln**: natural logarithm, **LSU**: large subunit, **NNPD**: new neonatal porcine diarrhoea, **NPD**: neonatal porcine diarrhoea, **OTU**: operational taxonomic units, **PCA**: principal component analysis, **PCoA**: principal coordinates analysis, **PRRS:** Porcine reproductive and respiratory syndrome, **qPCR**: quantitative real-time polymerase chain reaction, **rRNA**: ribosomal RNA, **SCFA**: short chain fatty acids, **SSU**: small subunit.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MLHB, LM, MB and KS participated in conceiving and designing this study. The inclusion of pig farms and clinical examination of animals was performed by HK. MLHB completed all the laboratory experiments. NL advanced BION-meta to also include search results from ribosomal LSU databases, and HCI assisted in the improvement of BION-meta for this study, and critically revised the manuscript. AS conducted all statistical calculations related to the qPCR results and MLS contributed with the multivariate analysis of the qPCR data and the sequencing data. MLHB drafted the manuscript, and LM, MB and KS helped along by revising and proofreading the manuscript for appropriate content. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by the Act on Innovation no. 421 of 31/05/2000 granted by the Ministry of Food, Agriculture and Fisheries of Denmark, National Veterinary Institute (Technical University of Denmark), and the Pig Research Centre (Ministry of Food, Agriculture and Fisheries of Denmark). The authors wish to thank Birgitta Svensmark (Pig Research Centre) for collecting the intestinal content samples and Sophia Rasmussen for assistance in the laboratory.

References

1. Wittum TE, Dewey CE, Hurd S, Dargatz DA, Hill GW: **Herd- and litter-level factors associated with the incidence of diarrhea morbidity and mortality in piglets 1 to 3 days of age.** *J Swine Health Prod* 1995, **3:**99-104.

2. Kongsted H, Stege H, Toft N, Nielsen JP: **The effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain and mortality in 4 Danish pig herds.** *BMC Veterinary Research* 2014, **10:**90.

3. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE: **Succession of microbial consortia in the developing infant gut microbiome.** *Proc Natl Acad Sci U S A* 2011, **108:**4578-4585.

4. Kenworthy R, Crabb WE: **The intestinal flora of young pigs, with reference to early weaning, *Escherichia coli* and scours.** *Journal of Comparative Pathiology and Therapeutics* 1963, **73:**215-228.

5. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI: **Evolution of mammals and their gut microbes.** *Science* 2008, **320:**1647-1651.

6. Bezirtzoglou E: **The intestinal microflora during the first weeks of life.** *Anaerobe* 1997, **3:**173-177.

7. Bauer E, Williams BA, Smidt H, Verstegen MWA, Mosenthin R: **Influence of the gastrointestinal microbiota on development of the immune system in young animals.** *Current issues in intestinal microbiology* 2006, **7:**35-51.

8. Guarner F, Malagelada JR: **Gut flora in health and disease.** *Lancet* 2003, **361:**512-519.

9. Willing BP, Van Kessel AG: **Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig.** *J Anim Sci* 2007, **85:**3256-3266.

10. Pabst R, Geist M, Rothkötter HJ, Fritz FJ: **Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnobiotic pigs.** *Immunology* 1988, **64:**539-544.

11. Svensmark S: **New neonatal diarrhoea syndrome in Denmark.** In *Proceedings of the 1st ESPHM*. Edited by Stege H, Kristensen CS. Copenhagen, Denmark: Faculty of Life Sciences, University of Copenhagen; 2009:27.

12. Melin L, Wallgren P, Mattsson S, Stampe M, Löfstedt M: **Neonatal diarrhoea in piglets from *E.coli* vaccinated sows in Sweden.** In *Proceedings of the 21st IPVS Congres*. Edited by D'Allaire S, Friendship R. Vancouver, British Columbia, Canada; 2010:290.

13. Gin T: **Diagnostic des nouveaux cas de diarrhées néonatales anzootiques du porcelet: évaluation de la prise colostrale.** *PhD Thesis.* Ecole Nationale Vétérinaire de Toulouse - ENVT; 2008.

14. Astrup P, Larsen KV, Jorsal SE, Larsen LE: **Neonatal Diarrhea in Danish pig farms - a questionnaire among veterinarians.** In *Proceedings of the 21st IPVS Congres*. Edited by D'Allaire S, Friendship R. Vancouver, British Columbia, Canada; 2010:751.

15. Kongsted H, Jonach B, Haugegaard S, Angen O, Jorsal SE, Kokotovic B, Larsen LE, Jensen TK, Nielsen JP: **Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome.** *BMC Veterinary Research* 2013, **9:**206.

16. Wallgren P, Mattson S, Merza M: **New neonatal porcine diarrhoea. II. aspects on etiology.** In *Proceedings of the 22nd IPVS Congres*. Jeju, Korea; 2012:76.

17. Sialelli JN, Lautrou Y, Oswald I, Quiniou N: **Peut-on établir une relation entre les caractéristiques de la truie et de sa portée et l'apparition de diarrhées néonatales? Proposition de réponse à partir de mesures réalisées en élevage de production.** *Journées Recherche Porcine* 2009, **41:**167-172.

18. Jonach B, Boye M, Stockmarr A, Jensen TK: **Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea.** *BMC Veterinary Research* 2014, **10:**68.

19. Hermann-Bank ML, Skovgaard K, Stockmarr A, Larsen N, Mølbak L: **The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity.** *BMC Genomics* 2013, **14:**1-14.

20. Promega: ***Maxwell® 16 Cell LEV DNA Purification Kit. Instructions for Use of Product AS1140.*** USA; 2010.

21. Fluidigm: ***Access Array System™ User Guide v3.*** 2010.

22. R Core Team. **R: A language and environment for statistical computing.** Version 3.1.0. [http://www.R-project.org], 2014.

23. Johnson DD: **Enteric *Streptococcus durans*--An adhering *Streptococcus* as a cause of diarrhea in suckling piglets?** In *Twenty Fifth Annual George A. Young Conference*. Lincoln, Nebraska; 1984:1-8.

24. Tzipori S, Hayes J, Sims L, Withers M: ***Streptococcus durans:* An Unexpected Enteropathogen of Foals.** *J Infect Dis* 1984, **150:**589-593.

25. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO: **The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools.** *Nucleic Acids Res* 2013, **41:**D590-D596.

26. Oksanen, Jari, Blanchet, Guillaume, Kindt, Roeland, Legendre, Pierre, Minchin, Peter, O'Hara, R. B., Simpson, Gavin, Solymos, Peter, Stevens, and Wagner, Helene. **vegan: Community Ecology Package.** Version 2.0-2. [http://CRAN.R-project.org/package=vegan], 2011.

27. Ahmad A, Ghosh A, Zurek L, Schal C: **Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community.** *BMC Microbiology* 2011, **11**.

28. Ramos S, Igrejas G, Capelo-Martinez JL, Poeta P: **Antibiotic resistance and mechanisms implicated in fecal enterococci recovered from pigs, cattle and sheep in a Portuguese slaughterhouse.** *Ann Microbiol* 2012, **62:**1485-1494.

29. Larsson J, Lindberg R, Aspán A, Grandon R, Westergren E, Jacobson M: **Neonatal Piglet Diarrhoea Associated with Enteroadherent *Enterococcus hirae*.** *J Comp Pathol* 2014, **151:**137-147.

30. Cheon DS, Chae CH: **Outbreak of diarrhea associated with *Enterococcus durans* in piglets.** *J Vet Diagn Invest* 1996, **8:**123-124.

31. Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L: **The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease.** *Plos One* 2012, **7**.

32. Rajilic-Stojanovic M, Shanahan F, Guarner F, de Vos WM: **Phylogenetic Analysis of Dysbiosis in Ulcerative Colitis During Remission.** *Inflamm Bowel Dis* 2013, **19:**481-488.

33. Portrait V, Cottenceau G, Pons AM: **A *Fusobacterium mortiferum* strain produces a bacteriocin-like substance(s) inhibiting *Salmonella enteritidis*.** *Lett Appl Microbiol* 2000, **31:**115-117.

34. Rendon MA, Saldana Z, Erdem AL, Monteiro-Neto V, Vazquez A, Kaper JB, Puente JL, Giron JA: **Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization.** *Proc Natl Acad Sci U S A* 2007, **104:**10637-10642.

35. Taras D, Vahjen W, Macha M, Simon O: **Performance, diarrhea incidence, and occurrence of *Escherichia coli* virulence genes during long-term administration of a probiotic *Enterococcus faecium* strain to sows and piglets.** *J Anim Sci* 2006, **84:**608-617.

36. **STEC Center at Michigan State University** [http://www.shigatox.net/]

37. Hazen TH, Sahl JW, Redman JC, Morris CR, Daugherty SC, Chibucos MC, Sengamalay NA, Fraser-Liggett CM, Land HS, Whittam TS, Whittam B, Manning SD, Rasko DA: **Draft Genome Sequences of the Diarrheagenic *Escherichia coli* Collection.** *J Bacteriol* 2012, **194:**3026-3027.

38. Petri D, Hill JE, Van Kessel AG: **Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig.** *Livestock Science* 2010, **133:**107-109.

39. Osawa R, Fujisawa T, SLY LI: ***Streptococcus gallolyticus* sp. nov.*;* Gallate Degrading Organisms Formerly Assigned to *Streptococcus bovis*.** *Syst Appl Microbiol* 1995, **18:**74-78.

40. Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Møller K: **Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited.** *Appl Environ Microbiol* 2002, **68:**673-690.

41. Vaissaire J, Marcon C, Kobisch M, Menec M, Carnero R, Laroche M, Mirial G: **Importance of streptococcal disease (*Streptococcus suis*, group R) in France.** *Annales De Zootechnie* 1985, **34:**372-373.

42. Bramley AJ: **Beecham Mastitis Series- *Streptococcus uberis* udder infection - a major barrier to reducing mastitis incidence.** *Br Vet J* 1984, **140:**328-335.

43. Lippke RT, Borowski SM, Marques SMT, Paesi SO, Almeida LL, Moreno AM, Corbellini LG, de Barcellos DESN: **Matched case-control study evaluating the frequency of the main agents associated with neonatal diarrhea in piglets.** *Pesq Vet Bras* 2011, **31:**505-510.

44. Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ: **The microbiology of butyrate formation in the human colon.** *FEMS Microbiol Lett* 2002, **217:**133-139.

45. Frank DN, Amand ALS, Feldman RA, Boedeker EC, Harpaz N, Pace NR: **Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases.** *Proc Natl Acad Sci U S A* 2007, **104:**13780-13785.

46. Sarbini SR, Rastall RA: **Prebiotics: Metabolism, Structure, and Function.** *Functional Food Reviews* 2011, **3:**93-106.

47. Robinson IM, Whipp SC, Bucklin JA, Allison MJ: **Characterization of Predominant Bacteria from the Colons of Normal and Dysenteric Pigs.** *Appl Environ Microbiol* 1984, **48:**964-969.

48. Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K, Palva A: **Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers.** *BMC Gastroenterology* 2009, **9**.

49. Cho JH, Zhao PY, Kim IH: **Probiotics as a Dietary Additive for Pigs: A Review.** *J Anim Vet Adv* 2011, **10:**2127-2134.

50. Fuller R: **Probiotics in man and animals.** *J Appl Bacteriol* 1989, **66:**365-378.

51. Carey CM, Kostrzynska M, Ojha S, Thompson S: **The effect of probiotics and organic acids on Shiga-toxin 2 gene expression in enterohemorrhagic *Escherichia coli* O157 : H7.** *J Microbiol Methods* 2008, **73:**125-132.

52. Gill HS, Shu Q, Qu F: **Probiotic treatment using *Bifidobacterium lactis* HN019 reduces weanling diarrhea associated with rotavirus and *Escherichia coli* infection in a piglet model.** *J Pediatr Gastroenterol Nutr* 2001, **33:**171-177.

53. Amao H, Akimoto T, Komukai Y, Sawada T, Saito M, Takahashi KW: **Detection of *Corynebacterium kutscheri* from the oral cavity of rats.** *Exp Anim* 2002, **51:**99-102.

54. Murphy J, Devane ML, Robson B, Gilpin BJ: **Genotypic characterization of bacteria cultured from duck faeces.** *J Appl Microbiol* 2005, **99:**301-309.

55. Loubinoux J, Bronowicki JP, Pereira IAC, Mougenel JL, Le Faou AE: **Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases.** *Fems Microbiol Ecol* 2002, **40:**107-112.

56. Thompson CL, Wang B, Holmes AJ: **The immediate environment during postnatal development has long-term impact on gut community structure in pigs.** *ISME J* 2008, **2:**739-748.

57. Inglis G, Thomas MC, Thomas DK, Kalmokoff ML, Brooks SP, Selinger L: **Molecular Methods to Measure Intestinal Bacteria: A Review.** *J AOAC Int* 2012, **95:**5-23.

Illustrations and figures

Box 1: Pig farm inclusion criteria for studying NNPD [15].

- A stock of more than 400 sows
- Routinely vaccinate against ETEC[1] and *Cl. perfringens* type C
- Farrowing units proven to be PRRS[2] virus negative
- NNPD has been a problem for a period of minimum 3-6 months
- Management-related diarrhoea has been excluded
- Traditional interventions such as vaccines and antibiotics have limited effect on the diarrhoea
- At least 30 % of the litter is affected
- The piglets suffer from diarrhoea during the first week of life
- Random check of five case piglets should test negative for ETEC, *Cl. perfringens* type C and Rotavirus by routine diagnostic examination

[1] ETEC: Enterotoxigenic *Escherichia coli*, [2] PRRS: Porcine reproductive and respiratory syndrome

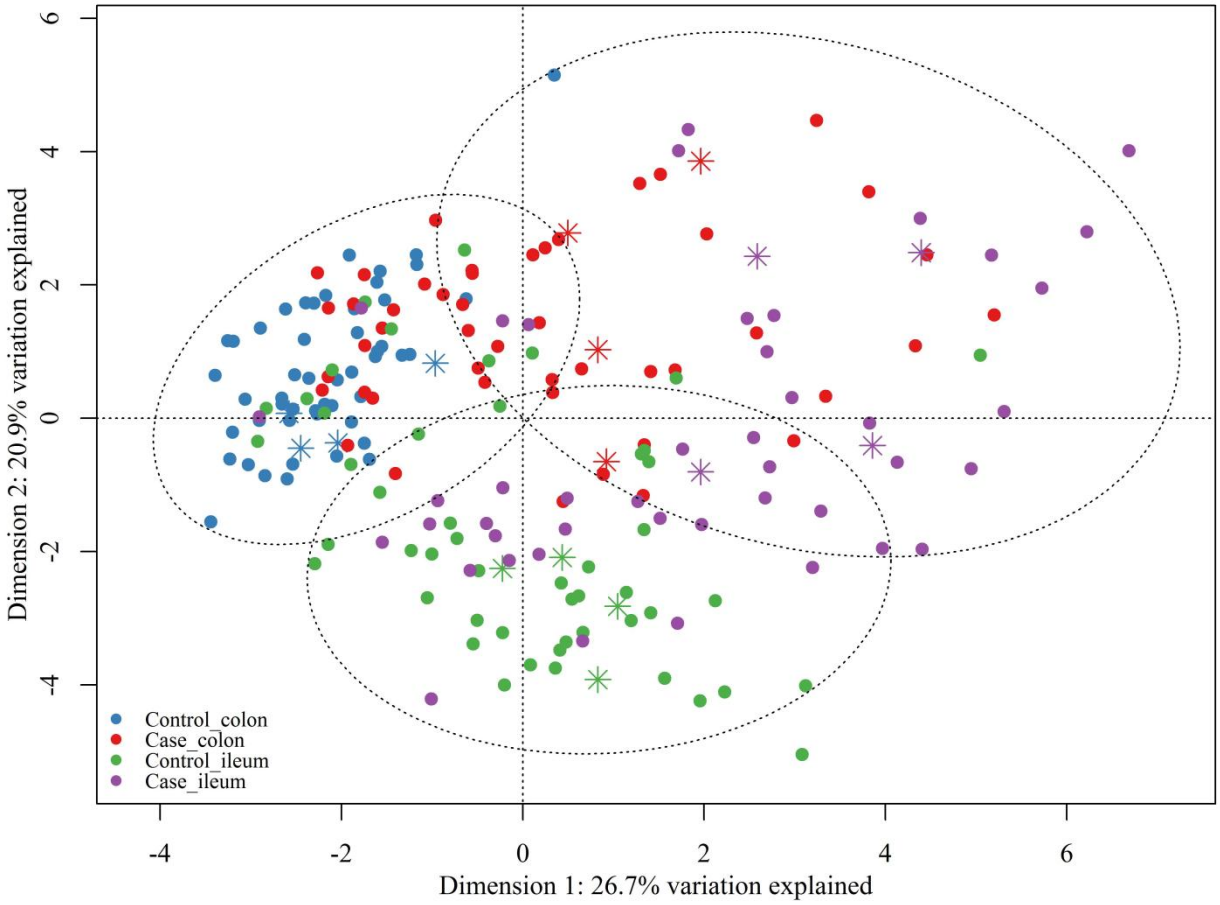


Figure 1: PCA scores of ileal and colonic content of piglets with (case) and without (control) NNPD. PCA scores are generated from normalized relative values of ileal and colonic content of case and control piglets obtained from the Gut Microbiotassay. Asterisks symbolize samples selected for 454-sequencing. Circles represent K-means clustering.

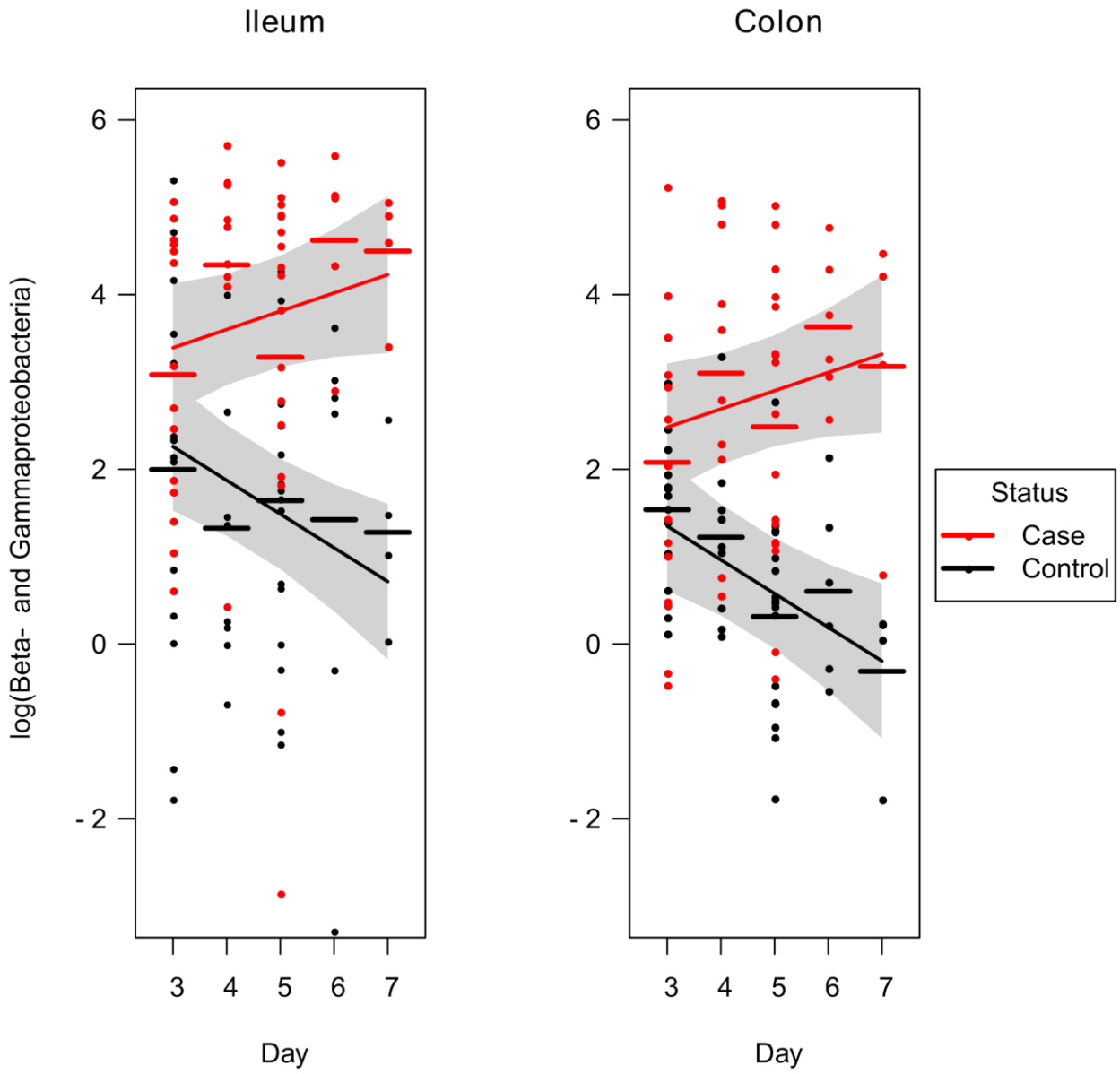


Figure 2: Age-related changes of Beta- and Gammaproteobacteria in luminal content of piglets with and without NNPD. Relative amount of class Beta- and Gammaproteobacteria (transformed with the natural logarithm) in ileal and colonic content of all piglets included in the study, plotted as function of the age of the piglets. Case and controls are piglets with and without diarrhoea, respectively. Age-related model regression lines are depicted with corresponding 95 % confidence intervals as shaded areas. Horizontal bars show means for a given age and group.

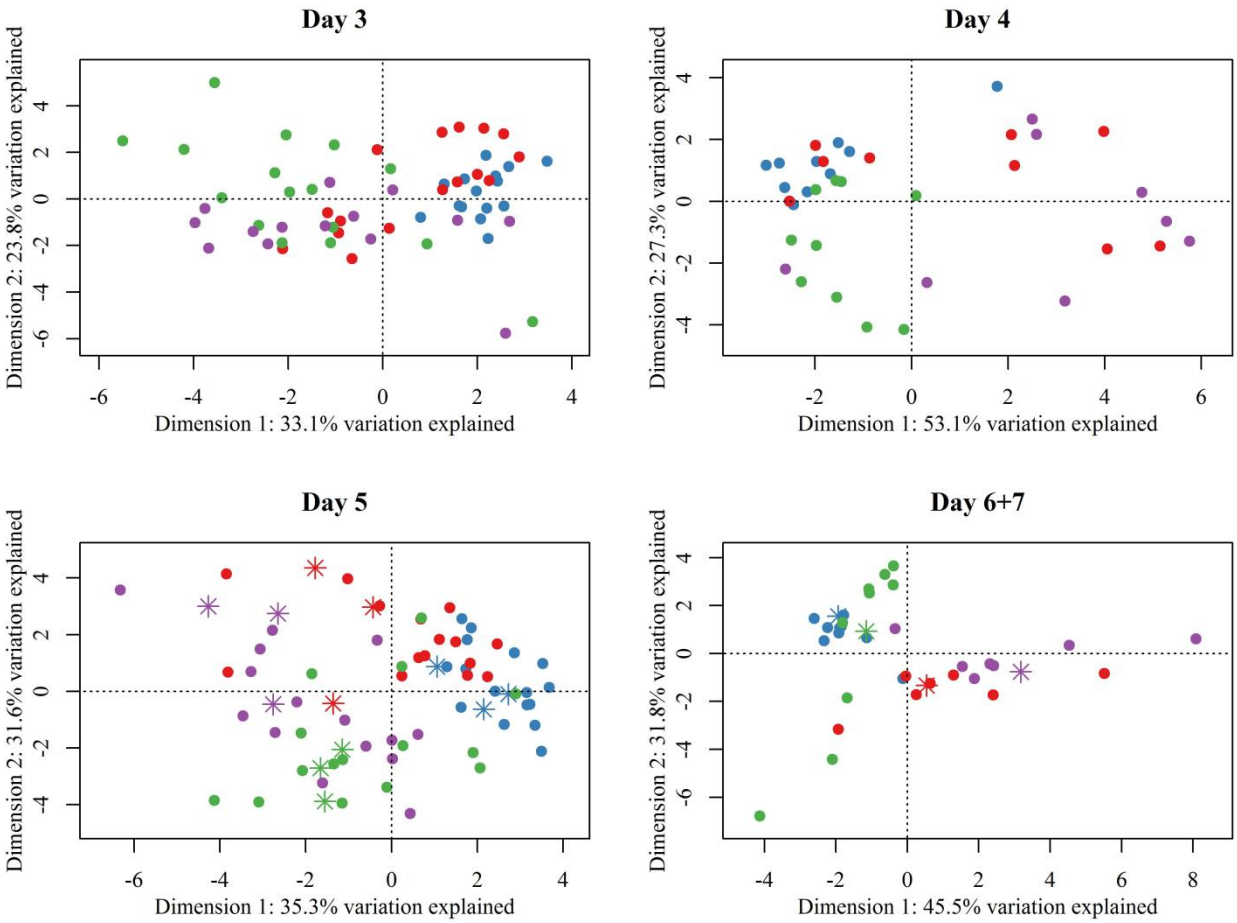


Figure 3: PCA scores of the gut microbiota segregated on gut section according to age in days. PCA scores from normalized relative values of ileal and colonic content of case and control piglets in relation to age in days obtained from the Gut Microbiotassay. Blue: Control colon, Purple: Case colon, Green: Control ileum, Red: Case ileum. Asterisks symbolize samples selected for 454-sequencing. Samples cluster primarily according to diarrhoeic status with increasing age

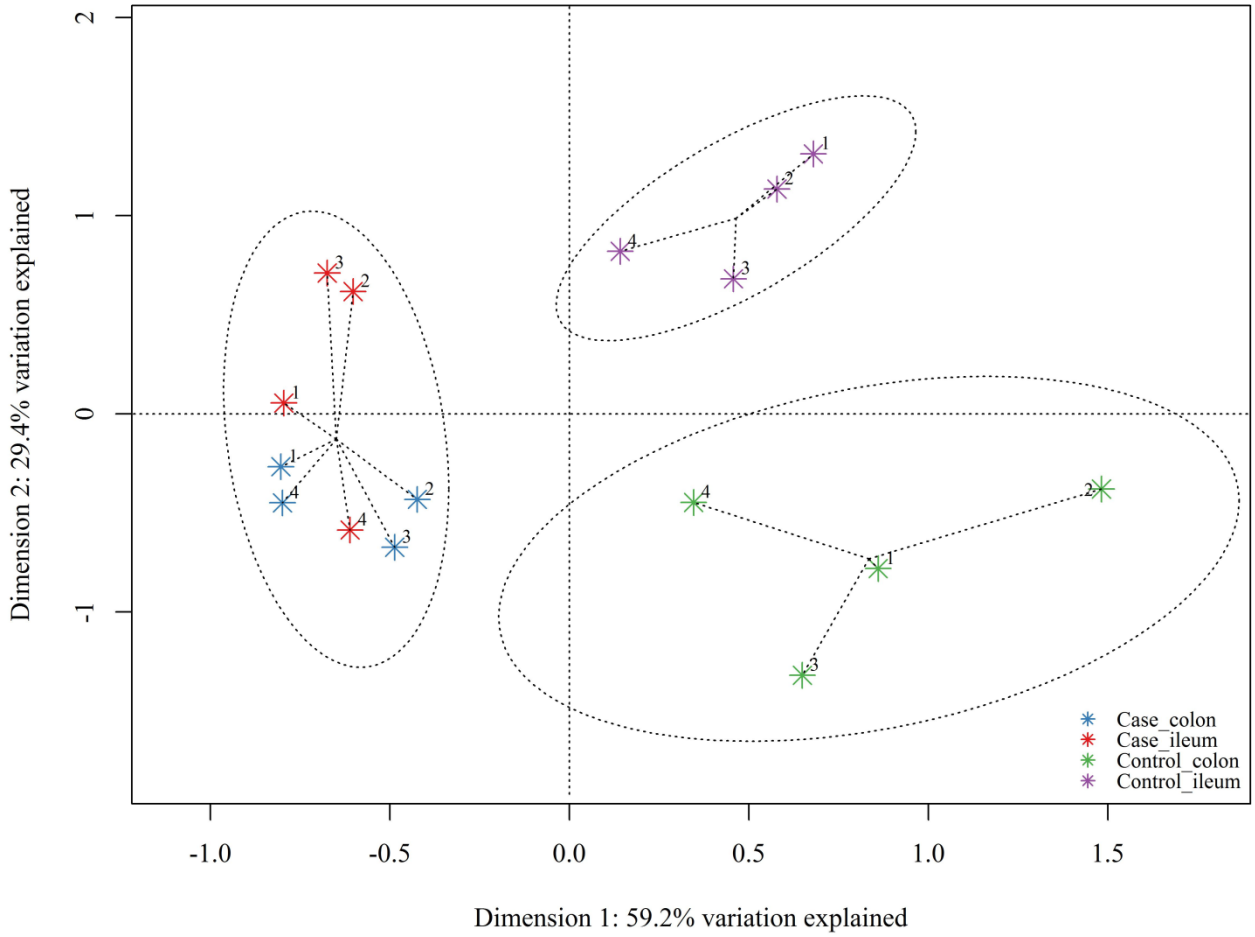


Figure 4: PCoA plot showing similarity of composition of the gut microbiota from sequenced samples. Grand data of ileal and colonic luminal content of a case piglet and a control piglet from each herd included in the study. Numbers refer to herd origin. Samples were randomly chosen for 454-sequencing based on PCA scores generated from normalized Cq data obtained through the Gut Microbiotassay for 454-sequencing (see Figure 1). Circles represent K-means clustering.

Tables

Table 1: Piglets included in the study. The number of piglets is listed for each pig farm in accordance with age in days: (−) Control piglets without diarrhoea, (+) Case piglets with diarrhoea for ≥2 days.

Age in days	3		4		5		6		7		Total	
Diarrhoea	**−**	**+**	**−**	**+**	**−**	**+**	**−**	**+**	**−**	**+**	**−**	**+**
Pig farm 1	6	7			7	6					**13**	**13**
Pig farm 2			2	2	6	6			4	4	**12**	**12**
Pig farm 3			7	8			6	6			**13**	**14**
Pig farm 4	8	8			4	5					**12**	**13**
Total	**14**	**15**	**9**	**10**	**17**	**17**	**6**	**6**	**4**	**4**	**50**	**52**

Table 2: Significant findings from the Gut Microbiotassay and 454-sequencing results displaying similar trends. Estimated fold changes, corresponding 95 % confidence intervals [], and *p*-values. Superscripted numbers equal the total number of OTUs belonging to the respective taxonomic group identified by sequencing amplicons generated by the Gut Microbiotassay**.** OTU-ratio between diarrhoeic and healthy piglets for ileum and colon, respectively, listed in parenthesis. Dashes indicate a significant effect of interaction (Table 3). Shaded species are not consistent findings. #Estimated fold changes for this category are calculated as: $\beta^{percentage\ points}$, where *β* is the estimated effect parameter for diarrhoea, and percentage points ranges from 0 to 100.

Primer set and respective 454-sequencing findings	Gut section estimate (ileum vs. colon)	Gut section *p*-value	Status estimate (diarrhoeic vs. healthy)	Status *p*-value	Gilt estimate (gilt vs. multiparous sow)	Gilt *p*-value	Percentage of diarrhoea estimate#	Percentage of diarrhoea *p*-value	Age estimate	Age *p*-value	Antibiotics estimate (treated vs. untreated)	Antibiotics *p*-value
Domain Bacteria B [268]	0.11 [0.08,0.17]	< 0.0001		NS		NS		NS	1.21 [1.01,1.44]	0.04		NS
Phylum Firmicutes [183]	2.33 [1.63,3.33]	< 0.0001	0.57 [0.36,0.89]	0.02	0.53 [0.33,0.87]	0.01		NS	1.34 [1.13,1.59]	0.001		NS
Class Bacilli [125]	3.21 [1.98,5.20]	< 0.0001	0.28 [0.18,0.46]	< 0.0001		NS		NS	1.61 [1.27,2.03]	0.0001		NS
Genus *Enterococcus* [27] species *E. hirae* (3.36/11.11) species *E. faecium* (9.87/129.60) species *E. durans* (15.57/255.00) species *E. mundtii* (113.00/179.00)	3.14 [1.25,7.91]	0.02	21.57 [8.50,54.73]	< 0.0001		NS		NS		NS		NS
Genus *Streptococcus* [47] species *S. gallolyticus subsp. gallolyticus* (0.29/0.22) species *S. alactolyticus* (0.27/0.18) species *S. uberis* (0.68/0.33)	2.42 [1.53,3.82]	0.0002	0.07 [0.02,0.27]	0.0002	0.52 [0.29,0.93]	0.03	1.03 [1.01,1.05]	0.008	0.75 [0.62,0.90]	0.003		NS
Genus *Lactobacillus* [79] species *L. acidophilus* (0.35/0.06)	4.81 [2.91,7.94]	< 0.0001	0.28 [0.17,0.46]	< 0.0001		NS		NS	1.73 [1.36,2.20]	< 0.0001		NS
Family Clostridium cluster I [17] species *Cl. perfringens;* ATCC 13124 (1.18/0.47)	3.05 [1.37,6.79]	-	0.24 [0.04,1.39]	-		NS	1.03 [1.00,1.05]	0.04		NS		NS

Table 2 continued.

Species *Cl. perfringens*	8.38 [3.40,20.66]	-	1.27 [0.51,3.16]	-		NS		NS	0.66 [0.49,0.90]	0.009		NS
Family Clostridium cluster IV [64]	0.11 [0.05,0.25]	< 0.0001	0.12 [0.06,0.25]	< 0.0001		NS		NS	1.39 [1.02,1.91]	0.04		NS
species *Ruminococcus bromii* (0.00/0.26)												
Family Clostridium cluster XIV [81]	0.08 [0.05,0.14]	< 0.0001		NS		NS	0.99 [0.98,0.99]	0.001		NS		NS
species *Roseburia inulinivorans* (1.59/0.02)												
Phylum Bacteroidetes [126]	0.02 [0.01,0.05]	-	0.11 [0.05,0.26]	-		NS		NS		NS		NS
Genus *Bacteroides* [65]	0.03 [0.01,0.07]	-	0.18 [0.08,0.43]	-		NS		NS		NS		NS
species *Bacteroides pyogenes* (0.02/0.33)												
species *Bacteroides rodentium* (0.46/0.00)												
species *Bacteroides xylanisolvens* (0.94/0.43)												
species *Porphyromonadaceae bacterium* C941 (0.00/0.01)												
Phylum Actinobacteria [74]	2.74 [1.64,4.60]	< 0.0001	0.28 [0.17,0.46]	< 0.0001		NS		NS	0.80 [0.65,0.99]	0.04		NS
species *Bifidobacterium boum* (0.41/0.06)												
species *Corynebacterium kutscheri* (0.39/0.34)												
Family Bifidobacteriaceae [12]	2.43 [1.28,4.61]	0.007		NS		NS		NS		NS		NS
Phylum Fusobacteria [16]	0.19 [0.10,0.34]	< 0.0001	2.12 [1.11,4.05]	0.02		NS		NS	0.68 [0.53,0.88]	0.004	0.17 [0.05,0.52]	0.002
species *F. mortiferum* (2.25/1.21)												
Class Beta- and Gammaproteobacteria [109]	2.49 [1.63,3.79]	< 0.0001	0.52 [0.12,2.55]	-		NS		NS	0.68 [0.52,0.88]	-		NS
Family Enterobacteriaceae [89]	3.93 [2.50,6.16]	< 0.0001	0.19 [0.04,1.02]	-	2.02 [1.09,3.75]	0.03		NS	0.53 [0.40,0.71]	-		NS
species *Escherichia coli* (8.61/6.22)												
species *E.coli;* HQ219945.1.1457 (11.46/5.00)												
species *E. coli* DEC8A (6.22/5.16)												
Species *E. coli*	3.44 [2.05,5.76]	< 0.0001	0.17 [0.02,1.26]	-	2.78 [1.39,5.56]	0.004		NS	0.57 [0.41,0.80]	-		NS
Class Deltaproteobacteria [1]		NS		NS		NS	0.98 [0.97,1.00]	0.04		NS	0.02 [0.00,0.48]	0.02
species *Desulfovibrio piger* (0.00/0.00)												

Table 3: Estimated fold changes, 95 % confidence intervals [], and *p*-values of significant interactions from the Gut Microbiotassay. This table is an extension of Table 2.

Primer set	Gut section: Status estimate (ileum, diarrhoeic)	Gut section: Status estimate (colon, diarrhoeic)	Gut section: Status *p*-value	Status: Age Estimate, day 3	Status: Age estimate, day 4	Status: Age estimate, day 5	Status: Age Estimate, day 6	Status: Age Estimate, day 7	Status: Age Estimate, mean age	Status: Age *p*-value
Family Clostridium cluster I	0.05 [0.01,0.31]	0.24 [0.04,1.39]	0.01							NS
Species *Cl. perfringens*	0.32 [0.13,0.80]	1.27 [0.51,3.16]	0.04							NS
Phylum Bacteroidetes	0.97 [0.39,2.42]	0.11 [0.05,0.26]	0.0009							NS
Genus *Bacteroides*	0.71 [0.13,0.80]	0.18 [0.08,0.43]	0.04							NS
Class Beta- and Gammaproteobacteria			NS	3.10	5.62	10.20	18.49	33.52	4.52 [5.05,11.64]	0.0007
Family Enterobacteriaceae			NS	1.79	3.79	8.03	17.02	36.08	4.52 [3.19,9.69]	< 0.0001
Species *E. coli*			NS	1.95	4.35	9.74	21.79	48.74	4.47 [3.39,11.85]	0.0004

Table 4: NNPD status explained by selected bacteria and relevant variables. Of the following variables tested, genus *Enterococcus*, class Beta- and Gammaproteobacteria, family Enterobacteriaceae, species *Escherichia coli*, Gut section, Gilt, and Age, as well as possible interactions between bacteria, NNPD was found to be significantly related to the variables mentioned below. Numbers are transformed with the natural logarithm (ln).

ln(variable)	Estimated fold change	95 % confidence interval	*p*-value
Intercept	-0.98	[-3.10,1.13]	-
Species *E. coli*	-0.11	[-0.42,0.65]	-
Genus *Enterococcus*	0.34	[0.09,0.59]	0.009
Enterococcus: *E. coli*	-0.12	[-0.23,-0.02]	0.02
Beta- and Gammaproteobacteria: colon	0.82	[0.33,1.30]	0.001
Gilt	3.23	[1.99,4.46]	< 0.0001

Additional files

Additional file 1: Primer modifications introduced to improve their performance and accompanying Ribosomal Database Project (RDP) search results. Nucleotide explanation: M = C/A, Y = T/C.

Primer	Sequence (5'→ 3')	RDP search[1], % coverage	
		Domain Bacteria	Phylum Firmicutes
Domain Bacteria B V4-V5 *(r)*	CCGTCAATTCCTTTGAGTTT [58]	51.3	-
	CCGTCAATTCMTTTGAGTTT	58.9	-
Phylum Firmicutes *(f)*	CTGATGGAGCAACGCCGCGT [59]	-	6
	CTGAYGGAGCAACGCCGCGT	-	37.4

[1]: RDP searches were performed using default settings with no mismatches allowed.

As the legends states, this table summarizes the modifications introduced in two primer sets from the Gut Microbiotassay: "Domain Bacteria B V4-V5" and "Phylum Firmicutes", respectively. Ribosomal Database Project search results are given as percentage coverage of intended target group to demonstrate the improved performance of the modified primers tested *in silico.*

References:

68. Schwieger F, Tebbe CC: **A new approach to utilize PCR-single-strand-conformation polymorphism for 16s rRNA gene-based microbial community analysis.** *Appl Environ Microbiol* 1998, **64:**4870-4876.

69. Haakensen M, Dobson CM, Deneer H, Ziola B: **Real-time PCR detection of bacteria belonging to the Firmicutes Phylum.** *Int J Food Microbiol* 2008, **125:**236-241.

Additional file 2: BION-meta generated output. The workflow recipe, parameters, inputs and outputs can be easily navigated via the static web site: http://vet.dtu.dk/NNPD.

Silva profile tables summarize the mapped sequences. The MID numbers corresponds to the following:

MID	Status	Age	Gut section	Herd
18	case	5	ileum	1
19	case	5	colon	1
23	control	5	ileum	1
25	control	5	colon	1
26	case	6	ileum	3
27	case	6	colon	3
28	control	6	ileum	3
30	control	6	colon	3
31	case	5	ileum	2
32	case	5	colon	2
33	control	5	ileum	2
34	control	5	colon	2
35	case	5	ileum	4
36	case	5	colon	4
37	control	5	ileum	4
38	control	5	colon	4

Briefly, the workflow steps were as follows: 1) separation by sample barcodes and primers; 2) removal of primer remnants and low quality sequence at the ends, as well as filtering by length (200 nucleotides) and overall quality (96 %); 3) removal of chimeric sequences. The following steps were performed for the forward and reverse phylogenetic primer separately, as well as in combination: 4) separation of each sample by matching with the phylogenetic primer(s); 5) matching all sequences against the SSU and LSU Silva datasets and producing a table with the highest 1 % similarities for each query; 6) mapping the similarities to the Silva SSU and LSU taxonomies by summing up the original read numbers to create scores for each taxon; and 7) formatting these scores and producing tables that show them. The end result is a table for each combination of sample primers and phylogenetic primers.

The as yet unpublished open source package BION-meta can be downloaded from http://box.com/bion (Larsen N *et al.*, in prep.).

Paper III

The effect of fecal inoculation with NNPD-material on bacterial gut microbiota composition and immune-related gene expression in neonate piglets

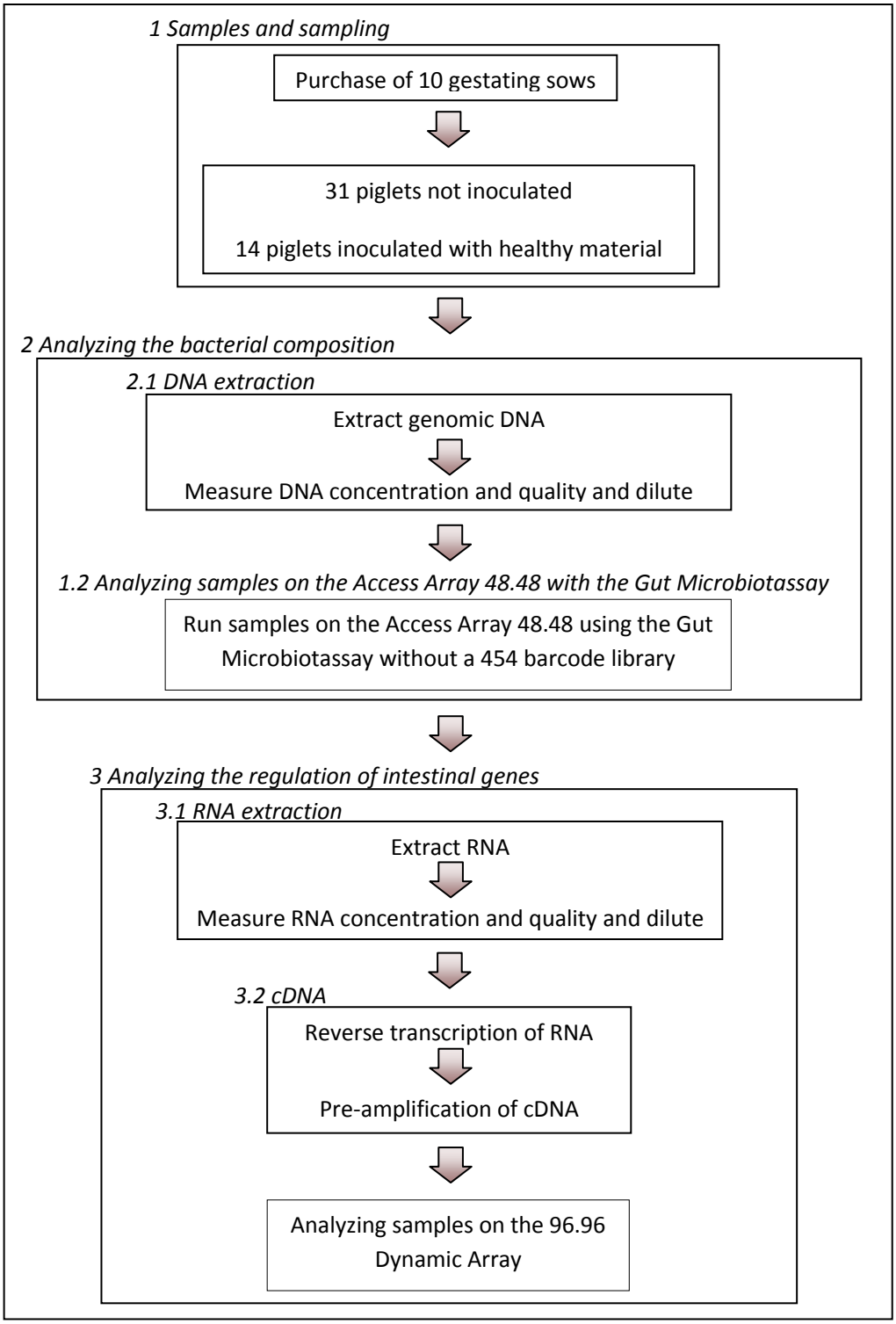
Marie Louise Hermann-Bank, Anders Stockmarr, Beata Jonach, Tim K. Jensen, Mikael L. Strube, Peter M. H. Heegaard, Mette Boye, Kerstin Skovgaard

Manuscript in preparation

Introduction

The aim of this study was to investigate whether the bacterial gut microbiota profile characteristic of piglets affected by NNPD (found in Paper II) could be recovered in experimentally NNPD-infected piglets. Additionally, regulations of local intestinal genes were assessed for a subset of samples and possible associations between bacterial groups and gene expression was examined.

Flow diagram



Title page:

The effect of fecal inoculation with NNPD-material on bacterial gut microbiota composition and immune-related gene expression in neonate piglets

Marie Louise Hermann-Bank[1], Anders Stockmarr[2], Beata Jonach[1], Tim K. Jensen[1], Mikael L. Strube[1], Peter M. H. Heegaard[1], Mette Boye[1], Kerstin Skovgaard[$1]

[1] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark. [2] **Department of Applied Mathematics and Computer Science, Technical University of Denmark, Matematiktorvet, Building 324, DK-2800 Lyngby, Denmark.**

[$] **Author of correspondence: E-mail:** kesk@vet.dtu.dk. Phone: +45 35 88 63 62

E-mail addresses:	**MLHB:**	mlhh@vet.dtu.dk
	AS:	anst@dtu.dk
	BJ:	jobea@vet.dtu.dk
	TKJ:	tije@vet.dtu.dk
	MLS:	milst@vet.dtu.dk
	PMHH:	pmhh@vet.dtu.dk
	MB:	mboy@vet.dtu.dk
	[§]KS:	kesk@vet.dtu.dk

Abstract

During the last decade a new type of neonatal porcine diarrhoea (NNPD) has emerged in Denmark and other countries. The exact aetiology is not determined, although results from different studies suggest that bacteria from the genus *Enterococcus* are involved in NNPD. This study examined whether a NNPD-disease model of piglets inoculated with intestinal NNPD-material from NNPD-affected piglets was characterized by similar changes in microbial composition of ileal and colonic contents as previously established for NNPD-field cases. Moreover, the subsequent expression of intestinal genes involved in inflammation, intestinal barrier function, and pathogen recognition was explored in a subset of piglets. This was tested using high-throughput qPCR on DNA to detect bacteria and on cDNA to detect regulation of mRNA transcription, respectively. The gut microbiota of piglets inoculated with NNPD material possessed a significant diminished number of bacteria from the phyla Firmicutes, Bacteroidetes, and Actinobacteria. Contrary, the number of genus *Enterococcus* and class Beta-and Gammaproteobacteria, family Enterobacteriaceae, species *E. coli,* family Bifidobacteriaceae, and class Epsilonproteobacteria were significantly higher compared with the gut microbiota of control piglets. Besides from the Bifidobacteria and Epsilonproteobacteria these findings are consistent with the bacterial changes previously found to characterize NNPD in field cases. The transcriptional analysis demonstrated a complex regulation of genes involved in the innate immune response in response to inoculation with fecal NNPD-material. Both significant up- and down-regulations were detected for genes involved in inflammation, intestinal barrier function and pathogen recognition. Linear regression modeling of major changes in the microbiota of NNPD-infected piglets and correlated expression of intestinal genes revealed that high numbers of Enterococci were associated with high expression of genes involved in intestinal barrier function reflecting impaired intestinal epithelium integrity of case piglets compared with control piglets. Altogether these results support the successful establishment of a NNPD-disease model, and confirmed the involvement of genus *Enterococcus* in NNPD.

Keywords: NNPD, piglet, neonatal, immune system, qPCR, microbiota, Gut Microbiotassay

Introduction

Neonatal porcine diarrhoea is a common problem in the pig industry, and numerous defined pathogens have been associated with it. However, it is not unusual that piglet diarrhoea remains undiagnosed because no aetiological agent can be identified. This is in fact the problem for numerous pig breeders in Denmark and other countries [1-4]. In recent years there have been several reported incidences of a new type of neonatal piglet diarrhoea. Veterinary practitioners and pig farmers describe it as being indifferent to zootechnical interventions or antibiotics, nor is it preventable with vaccination, and routine laboratory tests rule out common enteric pathogens [3-6]. This study is part of an interdisciplinary project investigating the

still undiagnosed piglet diarrhoea affecting Danish piggeries, known as New Neonatal Porcine Diarrhoea (NNPD) [7]. So far the project has investigated clinical, pathological, histopathological, viral, and microbiological features of NNPD [7-10]. NNPD affects piglets during the first week of life. Affected piglets become weak and develop non-hemorrhagic diarrhoea which results in reduced weight gain and in worst case scenario death, however, NNPD is not as such associated with mortality [8]. Routine laboratory analyses are inconclusive, still they have excluded the following enteric pathogens: Enterotoxigenic *Escherichia coli, Clostridium perfringens* type A or C*, Clostridium difficile,* rotavirus group A, and coronavirus. Necropsy exposes NNPD piglets of poor body condition, with flaccid intestines, and milk-filled stomachs [7]. Histopathological examinations reveal villous atrophy with crypt hyperplasia in the small intestine. Villous atrophy was associated with the presence of *Enterococcus* spp., some adherent to the small intestinal epithelium lining of the villi coexisted with adherent *E. coli*. No parasites were detected in the intestinal mucosa [7,9]. Quantitative real-time PCR (qPCR) confirmed that piglets affected by NNPD had more than 20 times higher numbers of genus *Enterococcus* compared with control piglets, and an apparent co-occurrence with *E. coli* contributed to the risk of piglets suffering from NNPD. With age, NNPD-affected piglets possessed increasing numbers of bacteria from class Gammaproteobacteria, family Enterobacteriaceae, and species *E. coli,* while the numbers of these bacteria were reduced in control piglets over time [10]. NNPD-affected piglets were also characterized by a reduced number of bacteria regarded to be beneficial members of the microbiota including members of the phyla Actinobacteria, Firmicutes and Bacteroidetes [10]. Comprehensive viral testing has not contributed with further understanding of the aetiology of NNPD (unpublished results, L.E. Larsen *et al.*). Nonetheless, despite great effort indefinite conclusions have been drawn, mainly suggesting that NNPD contributing agents should be found within the genus *Enterococcus* spp. and possibly in interaction with species *E. coli* [1,9-11].

Microbial colonization of the neonate gut is highly important in activation and development of the mucosal as well as the systemic immune system of the pig. This central role of the gut microbiota is not surprising as the intestine comprise a prominent part of the immune system [12,13]. Neonate piglets are immunologically naive and rely completely on the innate immune system and the passive immunity which they receive through colostrum until the adaptive immune system matures [14,15]. The innate immune system is activated through pattern recognition receptors (PRRs) on intestinal epithelium and immune cells. PPRs recognize conserved molecular structures of pathogens such as the bacterial cell wall components lipopolysaccharides (LPS) and peptidoglycans [15], or nucleic acids derived from viruses. This first line of defense is based on receptors capable of sensing and responding instantly to infectious agents initiating signal pathways, which leads to secretion of pro-inflammatory cytokines and recruitment of immune cells. Moreover, these receptors are also involved in maintenance of epithelial barrier function [16,17]. This balance of host-microbial interaction is highly complex and depends on the gut microbiota, and the ability

of the mucosal immune cells to tolerate harmless commensal bacteria and to react against and eliminate pathogenic bacteria. Disruption of this dynamic interaction as seen during infectious diarrhoea might have fatal consequences for the neonate piglet.

One way to learn if a disease is of infectious nature is through experimental reproduction in animal models. This is an acknowledged approach which has been used to investigate various pig disorders in previous studies for example the pathogenesis and infectious nature of *Brachyspira hampsonii,* and porcine reproductive and respiratory syndrome virus (PRRSV) [18,19]. Likewise, the early immune response to infection can be characterized under these highly controlled experimental conditions. Such experimental infection studies provide comprehensive data on clinical symptoms and high quality sample material for the study of microbiology, pathology, and immune related gene expression.

With the aim of gaining further insight into the aetiology behind NNPD as well as the subsequent regulation of local immune host response, this study examined intestinal samples from piglets involved in the establishment of a NNPD-infection model (unpublished results, B. Jonach *et al.*). The focus of the present study was to examine the microbiological changes and the intestinal expression of immune-related genes in corresponding gut sections of case and control piglets within the first week of life. It was hypothesized that if NNPD was successfully reproduced the bacterial gut microbiota of case piglets would be characterized by the presence or absence of the same bacterial groups as previously demonstrated [10]. Furthermore, it was expected that subsequent local intestinal gene expression involved in recognition of pathogen-associated molecular patterns, inflammation, and intestinal barrier function, would be regulated in accordance with NNPD. Finally, the association between a selection of bacteria (based on previous results on NNPD [10]) and gene regulation was examined.

Materials and methods

Ethical statements

The piglets included in this study were part of an experimental trail subject to ethical approval by the Danish Animal Experiments Inspectorate (J.nr. 2012-15-2934-00319 and J.nr. 2012-15-2934-00318).

Background

As result of being part of a greater interdisciplinary project investigating NNPD in Denmark the sample material used in this study originate from the same piglets previous used in the establishment of the NNPD-disease model (Unpublished results, B. Jonach *et al.*). A brief description of the experimental design will be given in the following.

Experimental design

From four Danish pig farms affected by NNPD (Herd 1-4) and one Danish pig farm with no history of unknown piglet diarrhoea (Trial) 51 case piglets and 4 control piglets from 3 to 7 days old were brought to the laboratory and euthanized (Figure 1). For further details on NNPD herds and inclusion criteria, reference is made to Kongsted *et al.* (2013). Intestines were collected and frozen at – 80 °C instantly. This material was used for production of NNPD-inocula (Inoculum 1-4 and 1F) and Inoculum 0 made from homogenized intestinal tissue, explained in details latter.

Four weeks ante partum six healthy gestating sows were purchased from a Danish well-managed conventional pig farm without any history of undiagnosed piglet diarrhoea. The sows were acclimatized and housed in separate pens at the facility stables at the National Veterinary Institute, Technical University of Denmark. All of the sows farrowed naturally at term, and piglets were reared with their biological dams. Within the first 6 - 48 hours of life, piglets were orally inoculated with 5 ml of freshly prepared 30 % inoculum obtained from either NNPD-affected piglets (Inoculum 1-4 and 1-F) or control piglet (Inoculum C1-2) using sterile plastic syringes. This concentration was determined in a pilot study testing three different concentrations: 0 %; 10 %; and 30 %, respectively. In each litter a few number of piglets remained un-inoculated as a result of the limited amount of inoculum, but also to observe if piglets infected each other. Besides from the inoculum piglets received no treatments. The inoculum was made from batches from each farm of the aforementioned intestines stored at – 80 °C which were thawed just before each trial: A 30 % solution was composed of a mixture of 21 g pooled NNPD intestines or control intestines and 49 ml of sucrose-potassium-glutamate (SPG, pH 7.0), mixed in a kitchen blender for 2 min. A single litter was not inoculated at all, to serve as control for the piglets inoculated with healthy-material. The study design and the number of animals included in the study appear from Figure 1 and Table 1. Table 2 summarizes the subset of piglets of which both bacterial data and immunology data were obtained.

Following inoculation piglets were subject to daily clinical examination giving special attention to fecal appearance on rectal swabs. Control piglets never had diarrhoea, whereas piglets with watery feces for at least two consecutive days were characterized as case piglets (as previously defined [7]). If piglets suffering from diarrhoea showed significant impaired welfare they were euthanized immediately, otherwise piglets were euthanized after zero to seven days (Figure 1 and Table 1). Euthanization was executed using 20 % pentobarbital intravenously. During post-mortem examinations intestinal content from ileum and colon were collected and frozen at – 80 °C. Tissue samples from ileum and colon were stabilized in RNAlater (Ambion, UK) and stored at −20 °C until RNA extraction.

Piglets that were euthanized before planned were not included in the study.

The gut microbiota

DNA extraction

DNA was extracted from 200 mg intestinal content obtained from ileum and colon of each animal. Intestinal content was suspended in 600 µl PBS and vortexed until visually homogenous. After centrifugation of the sample at 200 × *g* for 2 min, the supernatant was transferred to new tubes and bead beated with a 5 mm steel bead (Qiagen, Hilden, Germany) for 2.5 min at 20 hertz with an added volume of 350 µl of lysis buffer (Tissuelyser II, Qiagen). Following, samples were centrifuged for 1 min at 1000 × *g* at 4 °C, and the supernatant was transferred to new tubes and mixed manually with 30 µl Proteinase K (Promega, Madison, WI, USA). After an incubation for 30 min at 56 °C, this was centrifuged for 1 min at 13000 × *g*, and the resulting supernatant was used for DNA extraction using Maxwell® 16 LEV Blood DNA Kit, AS1290 (Promega, Madison, WI, USA) with the DNA protocol for feces in accordance to manufacturer's instructions [20]. DNA concentration and purity was assessed via NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Germany), and values with a 260/280 nm-ratio (generally in the range 1.8 − 2.1). DNA was stored at −20 °C until further analysis.

qPCR

The Gut Microbiotassay was used with high-throughput qPCR chip 48.48 Access Array™ Integrated Fluidic Circuit (Fluidigm Corporation, CA, USA), to generate semi-quantitative bacterial profiles of intestinal content from ileum and colon from each piglet. The Gut Microbiotassay is an assembly of 24 primer sets targeting the major intestinal bacterial groups. All samples were analyzed in a concentration of 50 ng/µl, and further details on primers, procedure, PCR protocol, and data treatment has previously been published [21]. No 454 barcode library was included as amplicons were not to be sequenced, and slightly modified primer sets replaced "Domain Bacteria B V4-V5", and "Phylum Firmicutes", as these had been tested *in silico* to be improved compared with those published in the Gut Microbiotassay by Hermann-Bank *et al.* [10]. To validate specific amplification and ensure successful qPCR reactions a non template control (NTC) as well as a positive control (one specific sample of DNA 50 ng/µl extracted from intestinal content of a healthy adult pig) were included in all runs.

Immunology

Intestinal RNA extraction

Frozen intestinal tissue samples (approximately 50 mg) were transferred to gentleMACS M tubes (Miltenyi Biotec, Lund, Sweden) containing QIAzol (Qiagen, Ballerup, Denmark) and homogenized using the gentleMACS Dissociator (Miltenyi Biotec). RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen,

Hombrechtikon, Switzerland) according to manufacturer's instructions including on-column digestion of DNA using the RNase-Free DNAse set (Qiagen). RNA concentration and purity was measured using a NanoDrop 1000 spectrophotometer (Saveen and Werner AB, Limhamn, Sweden). RNA integrity was measured on an Agilent 2100 Bioanalyzer (Agilent Technologies, Glostrup, Denmark) using the RNA 6000 Nano Kit (Agilent) and kept at −80 °C until reverse transcription into cDNA. Average RNA integrity values (RIN) above 8 was accepted for further analysis.

cDNA synthesis and pre-amplification

First-strand cDNA synthesis was performed with 500 ng total RNA per sample using the QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's protocol. For assay validation, two cDNA synthesis reactions were prepared per samples. Non-reverse transcriptase controls were prepared for assessment of potential genomic DNA contamination. 15 cycles of pre-amplification was performed as described previously [22] using TaqMan PreAmp Master Mix (Applied Biosystems, Naerum, Denmark). Pre-amplified cDNA was incubated with 4 µL of 4U/µL exonuclease (30 min at 37 °C, followed by 15 min at 80 °C) and diluted 1:8 in low EDTA TE-buffer before quantitative real-time PCR (qPCR).

qPCR

qPCR was performed in 96.96 Dynamic Array Integrated Fluidic Circuits (Fluidigm). Pre sample mix were prepared using the following components for 96 reactions: 3 µl ABI TaqMan Gene Expression Master Mix (Applied Biosystems), 0.3 µl 20× DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.3 µl 20× EvaGreen (Biotium, VWR – Bie & Berntsen, Soeborg, Denmark), and 0.9 µl low EDTA TE Buffer (VWR – Bie & Berntsen). Pre sample mix (4.5 µl) was mixed with 1.5 µl pre amplified cDNA. Primer mix (96 reactions) were prepared using 3 µl of 20 µM forward and reverse primer and 3 µl of 2× Assay Loading Reagent (Fluidigm). Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) as described by Skovgaard *et al.*, 2010 [23], and purchased from Sigma-Aldrich, Broendby, Denmark. Several of the selected genes were targeted by two or more primer assays to strengthen the expression analysis. Primer sequences, efficiencies and amplicon length are shown in Supplementary File 1.

The 96.96 Dynamic Array was primed in the IFC controller (Fluidigm) prior to loading of samples and primers. Sample mix, including cDNA (5 µl) and primer mix (5 µl) were dispensed into appropriate inlets and loaded into the chip in the IFC Controller (Fluidigm). The Dynamic Array was placed in the BioMark real-time PCR instrument (Fluidigm) and the following cycle parameter was used after an initial step of thermal mixing (2 min at 50 °C, 30 min at 70 °C, 10 min at 25 °C) and hot start (2 min at 50 °C, 10 min at 95 °C) 35 cycles with denaturing for 15 s at 95 °C and annealing/ elongation for 1 min at 60 °C. Melting curves were generated after each run to confirm a single PCR product (from 60 °C to 95 °C, increasing 1 °C/ 3 s).

Reactions were performed in duplicates (cDNA replicates). NTCs were included to indicate potential problems with non-specific amplification, non-reverse transcriptase controls were included to assess potential genomic DNA contamination.

Data analysis

The Gut Microbiotassay

Data analysis of Gut Microbiotassay data was processed similarly as previously published [21]. Briefly: Cq values were exported from the software Real-Time PCA Analysis version 3.0.2 (Fluidigm) to Microsoft Excel. From three separate standard curves (generated from three individually prepared pools containing all samples) the primer efficiencies were calculated. The primer sets were corrected to their corresponding efficiency. Subsequently, all samples and primer sets with less than 50 % registered Cq-values were excluded from the data set, before calculating the mean of the technical replicates. Finally, each sample was normalized against its corresponding “Domain Bacteria B” primer set (whose efficiency was validated by the “Domain Bacteria A” primer set), thereby calculating the relative fraction of different bacterial constituents relative to the total intestinal bacterial load in ileum or colon.

Immunology assay

Expression analysis of genes involved in inflammation, intestinal barrier function, and pathogen recognition was analyzed separately for each gut section. Primer efficiencies were calculated based on linear regression plots from all primer sets individually, using a pool of all samples to prepare four individual standard curves. Data were exported from Fluidigm Real-Time PCR Analysis software version 4.1.3 to GenEx5 (Tataa Biocenter, Göteborg, Sweden) for data pre-processing. All primer sets were efficiency corrected individually. Subsequently Cq values were normalized against the four most suitable reference genes. For ileum the reference genes were: GAPDH, TBP, HPRT1, and PPIA which were mutually identified by the two algorithms Normfinder [24] and geNorm [25] to be top four in a panel of eight reference genes tested in total. In the same manner a panel of eight reference genes was tested for colon samples, and the six best genes mutually identified by the two algorithms suitable for normalization were: ACTB, GAPDH, HPRT1, PPIA, TBP, and YWHAE. Following cDNA replicates and primer sets exhibiting variation of > 15 % were excluded from the dataset, before calculating the mean of all technical replicates (cDNA synthesis). Missing values (less than 1 %) were replaced with the highest primer specific Cq value plus 1 prior to statistical analysis.

Statistics

For statistical analysis Gut Microbiotassay generated data were transformed with the natural logarithm and investigated using a linear mixed-effect model where litter entered as a random effect. Variables investigated as deterministic effects included: Gut section (ileum versus colon), Status (sick versus healthy), Age (days), Inoculum (NNPD-inoculum versus healthy inoculum), and Day of inoculation. Moreover, the interaction between Gut section and Status in addition to the interaction between Status and Age were included. These interactions were examined in order to determine whether the location of certain bacteria associated with diarrhoea could be related to either the ileum or the colon, but also to assess age-related gut microbial changes of diarrhoea-associated bacteria.

A logistic regression model was used to study if the aforementioned variables and a selection of bacterial groups: genus *Enterococcus*, class Beta- and Gammaproteobacteria, family Enterobacteriaceae, and species *E. coli*, in addition to interaction between these bacterial groups, could explain the induced diarrhoea.

For each gut section individually, interaction between any of the bacterial groups and the regulation of intestinal genes in ileum and colon, respectively, was explored on a subset of data from litter 1; 1.1; 2; C1; and C2 (Table 2). Due to the low amount of available data, a standard linear regression model for the log-transformed bacteria data was applied with the explanatory factors from the mixed effect model and each gene variable separately. *P*-values were adjusted for simultaneous testing using Bonferroni-correction according to the number of genes investigated. Furthermore, in order to investigate which bacteria that had an impact on up- or down-regulation of the genes, the regulation on each intestinal gene was modeled with a standard linear regression model, with the explanatory variables including Gut section, Inoculum, Day of inoculation, Age, for a selection of bacterial groups according to Table 3.

Finally, the expression of genes was examined between control piglets (C1 + C2) and case piglets from litter 1; 1.1; 2 (Table 2). For this analysis gene expression data were log transformed to approach a normal distribution prior to two-tailed Student's t-test. Only fold-changes close to 2 or above were regarded biologically relevant.

P-values < 0.05 were considered significant.

Results

Table 4-6 summarize the statistical findings related to the Gut Microbiotassay.

NNPD Status: The total bacterial number was higher in the ileum but lower in the colon of case piglets compared with control piglets ($p < 0.0001$), which is opposite to the normal bacterial load with higher numbers in colon compared with ileum. Piglets that developed diarrhoea were estimated to have more

than 30 times higher numbers of Enterococci compared with piglets that remained healthy ($p < 0.0001$). Additionally, diarrhoeic piglets possessed higher numbers of bacteria belonging to the taxonomic lineage class Beta- and Gammaproteobacteria (14 times more in ileum, $p = 0.007$ and 4 times more in colon, $p < 0.0001$, respectively) and its represented subgroups: family Enterobacteriaceae ($p < 0.0001$) and species *E. coli* (29 times more in ileum, $p < 0.0001$, and 4 times more in colon, $p = 0.03$). Both class Beta- and Gammaproteobacteria and *E. coli* demonstrated a significant effect of interaction with gastrointestinal location ($p = 0.01$ and $p = 0.003$, respectively). Class Epsilonproteobacteria was also estimated to be 11 times more abundant in the colon of case piglets compared with control piglets, while there was no difference when comparing ileum ($p = 0.01$). Diarrhoea was also associated with an increased quantity of family Bifidobacteriaceae, with a 5 fold change ($p < 0.0001$).

Bacterial groups which were scarcely represented in diarrhoeic piglets included: phylum Firmicutes, class Bacilli, and species *Lactobacillus.* Class Bacilli and species *Lactobacillus* were significantly reduced in both gut sections though mainly from the ileum of case piglets compared with control piglets ($p = 0.03$, $= 0.02$, respectively). Family Clostridium cluster I ($p < 0.0001$), family Clostridium cluster IV ($p < 0.0001$), family Clostridium cluster XIV ($p = 0.0003$, for piglets up to 6 days old), phylum Bacteroidetes ($p = 0.004$), genus *Bacteroides* ($p = 0.03$, for piglets up to 5 days old), and phylum Actinobacteria ($p = 0.01$, for piglets up to 6 days old) were also diminished in case of diarrhoea.

Age: With age the number of intestinal bacteria increased ($p = 0.03$), thus the estimated daily average percentile bacterial gain was approximately 18 % compared with the preceding day, irrespective of diarrhoeic status. Bacterial groups which significantly proliferated in the intestine over time included: phylum Firmicutes ($p = 0.001$), class Bacilli ($p = 0.007$), species *Lactobacillus* ($p < 0.0001$), family Clostridium cluster IV ($p = 0.0002$), family Clostridium cluster XIV ($p = 0.002$), phylum Bacteroidetes ($p = 0.001$), genus *Bacteroides* ($p = 0.02$), and phylum Actinobacteria ($p = 0.002$). Meanwhile, bacterial groups which were depleted included: species *Clostridium perfringens* ($p = 0.0002$), class Beta- and Gammaproteobacteria, family Enterobacteriaceae, and species *E. coli* ($p = 0.005$; $= 0.02$; $= 0.01$, respectively). However, family Clostridium cluster XIV, genus *Bacteroides*, and phylum Actinobacteria demonstrated significant effect of interaction between status and age, and their numbers were estimated to increase by roughly 70 %; 50 %; and 60 % per day, respectively, in case piglets compared with control piglets, thereby approximating the levels of control piglets over time ($p = 0.05$; $= 0.02$; $= 0.02$, respectively). Accordingly these bacterial groups also displayed the largest variation depended on NNPD-status, as these were 47; 16; and 37 fold lower, respectively, in case piglets compared with control piglets.

Inoculum: The effect of inoculating piglets with inoculum prepared from NNPD-material was associated with a significant estimated reduction of bacteria belonging to phylum Actinobacteria in general ($p = 0.01$).

On the contrary members of family Bifidobacteriaceae were estimated to increase in piglets inoculated with healthy material (p = 0.0008). Both estimates are relative to un-inoculated control piglets.

Day of inoculation: Inoculating the piglets at different time point (age in days) had a significant effects on the numbers of phylum Firmicutes, class Bacilli, and genus *Lactobacillus* (p = 0.009; 0.04; 0.007, respectively). Hence, waiting to inoculate the piglets for one day had a diminishing effect on the number of these bacterial groups estimated to be approximately 60 % compared to inoculating piglets one day earlier.

Diarrhoea explained by selected variables: The model exploring if diarrhoea could be explained by a selection of bacterial groups: genus *Enterococcus*, class Beta- and Gammaproteobacteria, family Enterobacteriaceae, and species *E. coli* revealed that the presence of *E. coli* (p = 0.003) and Enterococci (p = 0.01) in ileum, as well as Enterococci in colon (p < 0.0001), and the effect of age, all increased the risk of having diarrhoea. However, at the same time the risk of having diarrhoea was reduced with an increasing number of class Beta- and Gammaproteobacteria in ileum (p = 0.004).

Expression of genes involved in inflammation and intestinal integrity: A very complex expression pattern of genes involved in inflammation was found in response to different inocula. Mean expressions detected for piglets administrated inoculum 1 (n = 9), inoculum 1.1 (n = 5), and Inoculum 2 (n = 8) are illustrated in Figure 2 relative to control piglets (n = 6) administered inoculum C1 (n=3) or C2 (n = 3), respectively. In the ileum mRNA expression levels of the pro-inflammatory interleukin (IL)- 1A and IL1B as well as the acute phase proteins haptoglobin (HP) and serum amyloid A (SAA) were up-regulated whereas IL6, tumor necrosis factor alpha (TNFA), transforming growth factor beta (TGFB), chemokine (C-C Motif) ligand 3 (CCL3), and interferon gamma (IFNG) were all significantly down-regulated in the ileum of all or of several of the case piglets (Figure 2A). Cluster of differentiation (CD) 163, induced by anti-inflammatory mediators, and caspase 1 (CASP1) were significantly up-regulated in ileum from case piglets. In the colon only IL1A and CASP1 were up-regulated whereas several pro-inflammatory and important mediators, responsible for recruiting leukocytes to the inflamed intestine, were down-regulated (Figure 2B).

Several genes involved in intestinal integrity and barrier function were found to be regulated in agreement with diarrhoeic status in both gut sections (Figure 2C and 2D). mRNA expression levels of occludin (OCLN), claudin (CLDN) 3, mucin (MUC) 1, and trefoil factor (TFF)2 and TFF3 were up-regulated in case piglets while CLDN1 was down-regulated compared with control piglets. Data obtained from two or three primer assays targeting different regions of the same mRNA transcript were highly correlated (data not shown).

Intestinal expression of genes involved in pathogen recognition: Inoculation with NNPD-material induced transcriptional changes of mRNA coding for several PRR, illustrated in Figure 2E for ileum and Figure 2F for colon. In the ileum, Toll-like receptor (TLR) 2 which mainly recognizes Gram-positive bacteria, was up-

regulated in litter 1 case piglets, and though the TLR4 co-receptors CD14 and Lymphocyte antigen 96 (MD2) were both significantly up-regulated, TLR4 was not found to be regulated. Likewise in the ileum TLR3, 5, 6, 7, 8, 9, and nucleotide-binding oligomerization domain containing protein (NOD) 1 were also not regulated (data not shown). The C-type lectin receptor DC-SIGN, which can be stimulated by a broad range of ligands, was down-regulated in case piglets from litter 1 and 1.1. The two cytoplasmic viral RNA detecting PRR: Melanoma Differentiation-Associated Protein 5 (MDA5) and Retinoic Acid-Inducible Gene 1 (RIG-1) were significantly up-regulated in both gut sections from case piglets from litter 1 and 2. In contrast, TLR7 and TLR8 which also detect viral and to some extend bacterial nucleic acid were significantly down-regulated in the colon of all case piglets (Figure 2F).

Associations between bacteria and gene expressions: A greater number of associations were found in ileum which generally displayed a more diverse host response compared with colon. In ileum, a high regulation of TFF2 and MUC1 was associated with high numbers of genus *Enterococcus* which was found in case piglets ($p = 0.0009$ and $p = 0.03$). A high expression of TGFB in the control group was associated with an increased number of family Clostridium cluster IV, family Clostridium cluster XIV, phylum Bacteroidetes, and phylum Actinobacteria ($p = 0.01$; = 0.05; = 0.01; and = 0.03, respectively). High numbers of these bacterial groups were all related to a non-diarrhoeic gut microbiota. A high expression level of NOD1 was related to a reduced number of the Gram-negative phylum Fusobacteria ($p = 0.008$). Increased numbers of Lactobacilli, characteristic for control piglets, was associated with expression of IL1A ($p = 0.04$). IL1A is a pro-inflammatory cytokine which is continuously expressed in epithelial cells including those of the gastrointestinal tract. A high abundance of class Clostridium cluster I was associated with the highest expression level of IL6 ($p = 0.02$), which also was found in the control piglets. In colon a high number of genus *Enterococcus* was associated with a high expression of CLDN3 ($p = 0.005$) and MD2 ($p = 0.0001$) in some of the piglets, while NOD1 was negatively correlated with Enterococci ($p = 0.01$). Finally, a high number of species *E. coli* was associated with a low expression of NOD2 ($p = 0.009$).

Discussion

The success of experimentally reproducing NNPD can be evaluated from several aspects. The first obvious one is the aforementioned: do piglets develop diarrhoea, and are the findings associated with diarrhoea similar to the previous findings described for field cases. Jonach *et al.* (2014) found that none of the control piglets developed diarrhoea and of the case piglets (including un-inoculated siblings) 75 % developed diarrhoea. Histopathologically the diarrhoea was associated with villus atrophy in the small intestine accompanied by mild epithelial lesions in the colon characterized by sloughing of epithelial cells, erosions and necrosis, Figure 3 (unpublished results). Since NNPD is still poorly defined, further methods should be implied in order to assess whether it seems reasonable to conclude that the diarrhoea was related to

NNPD. The Gut Microbiotassay was used to obtain semi-quantitative bacterial profiles of case piglets, control piglets and of healthy piglets not inoculated to serve as controls for the control piglets. Additionally, the host transcriptional response to NNPD-material in the ileum and colon was studied in order to measure any regulation of intestinal genes involved in recognition of pathogen-associated molecular patterns, inflammation, and intestinal integrity. These genes were examined with the aim to gain further knowledge of the local immune response to NNPD, but also to search for possible associations between gene expression and bacterial groups detected by the Gut Microbiotassay.

When comparing the bacterial gut microbial profiles of case piglets with the bacterial gut microbial profiles of NNPD-field case piglets found previously [10] there was a high degree of convergence. Like the field cases the case piglets possessed a high number of members from genus *Enterococcus* and species *E. coli* which each individually contributed to the risk of having NNPD. Likewise, case piglets had a reduced number of bacteria from the phyla: Actinobacteria, Firmicutes, and Bacteroidetes in concordance with NNPD-field cases. These three phyla all contribute to the host's defense through colonization resistance against several intestinal pathogens [26].

The next question that arises is whether these bacterial changes are related to NNPD. Gorkiewicz *et al.* (2013) examining the gut microbial alterations in human stool in relation to non-infectious diarrhoea, and detected some of the same changes. They reported that the effect of diarrhoea caused a reduced abundance of the phyla Bacteroidetes and Firmicutes, but an increase in the number of Proteobacteria [27]. This suggests that a shift in the abundance of these bacterial groups could be related to diarrhoea in general, and that the depleted bacteria are susceptible to the flushing effect of diarrhoea, while Proteobacteria are able to take advantage of this gut microbial disturbance. An important member of phylum Proteobacteria is species *E. coli* that was found to be increased in case piglets. Many *E. coli* strains express surface proteins, pili, which facilitates adherence to intestinal epithelium, bacterial aggregation, and biofilm formation [28]. This feature enables *E. coli* to remain part of the gut microbiota during diarrhoea. Conversely, members belonging to phylum Bacteroidetes are susceptible to the pathophysiological effect of diarrhoea, because they bind to luminal particles (shed epithelial cells and mucus as well as food particles) for extraction of energy [29]. If disregarding the phyla that seem to be related to diarrhoea in general: Bacteroidetes, Firmicutes, and Proteobacteria, this leaves a reduced abundance of Actinobacteria and an increased quantity of genus *Enterococcus* as possible NNPD-related bacteria. The aforementioned study, [10], thoroughly discuss the gut microbial changes related to NNPD so this will not be the focus in the present study. Instead focus will be on the gut microbial similarities and differences compared with previously established NNPD-gut microbial changes, expression of intestinal

mucosal genes, and associations between bacterial groups and mucosal host response in case and control piglets.

Previous results implied that NNPD piglets differed from control piglets by an age-related increased colonization of bacteria from class Gammaproteobacteria, family Enterobacteriaceae, and species *E. coli*. Moreover the number of phylum Fusobacteria was doubled in piglets suffering from NNPD compared with control piglets [10]. In the present study, none of these findings counted for case piglets. On the contrary, increased numbers of class Epsilonproteobacteria and family Bifidobacteriaceae were detected in case piglets. One explanation for this discrepancy could be that a considerable number of the piglets included in the NNPD-field studies were treated with broad-spectrum antibiotics. Antibiotics affect the gut microbial composition and quantity and has been shown to significantly reduce the number of class Clostridium cluster IV and cluster XIV, while the abundance of family Enterobacteriaceae was increased [30]. These bacterial shifts were also found in NNPD-affected piglets [10]. However, similar findings in case piglets from the present study, where piglets or sows were not treated with antibiotics, confirm that in this situation these bacterial shifts are not caused by the effect of antibiotics. Contrary, the significant age-related increase of members belonging to the taxonomic lineage Gammaproteobacteria found previously in field case piglets [10], but not in case piglets from this study, could be an effect of antibiotics. The increased abundance of Epsilonproteobacteria and Bifidobacteriaceae detected in piglets inoculated with NNPD-material seen in the present study but not in NNPD-field cases [10] could be a results of not treating the piglets with antibiotics. *Bifidobacterium* spp., have been demonstrated to be reduced after antibiotic treatment [30].

Expression of genes involved in inflammation and intestinal integrity

Host transcriptional expression of mRNA coding for inflammatory cytokines as well as locally expressed acute phase proteins revealed a quite complex pattern of expression after inoculation with NNPD-material. In agreement with infection studies of epithelial neonatal porcine cells (IPEC-J2) with enterotoxigenic *E. coli* [31,32] there was a significant up-regulation of IL1A and IL1B in case piglets. Ileal expression of acute phase proteins HP and SAA further points to a moderate local inflammatory state in the case piglets from litter 1. Interestingly, many other pro-inflammatory cytokines and chemokines were down-regulated in response to inoculation with NNPD-material. The homeostasis of intestinal immunity is of great importance as inoculation with non-invasive bacteria should not trigger the cascade of gene expression leading to inflammation. This balance of host-microbial interaction is highly complex and dependent on the ability of the mucosal immune cells to tolerate harmless commensal bacteria. Firm regulation of pro-inflammatory cytokines as seen after inoculation with NNPD-material is important as these cytokines may contribute to epithelial barrier damage and loss of absorptive capacity which deteriorates the diarrhoea [33,34]. CD163

has a central role in anti-inflammatory response [35], the fact that this receptor was highly induced in all of the case piglets, suggests that the host aims to establish a more tolerant mucosal environment after inoculation.

Recently Larsson and co-workers reported CASP3 to be expressed in the small intestine of neonatal piglet with diarrhoea (NPD) associated with *Enterococcus hirae* [11], in the present study CASP1 was significantly up-regulated in piglets from litter 1 and 2 compared with control piglets in both ileum and colon. Caspases play an important role during apoptosis and it could be speculated that these proteins are involved in the villus atrophy related with NNPD [7,9,11]. CASP1 is involved in the activation of IL1B which also was up-regulated in case piglets of litter 1.

The extent of the gene regulation reveals that the effect of the inoculum is impaired after one passage through piglets when comparing case piglets from litter 1 with case piglets from litter 1.1. Host expression levels of both up- and down-regulated genes of case piglets from litter 1 were more pronounced compared with case piglets from litter 1.1. The very diverse pattern of inflammatory gene regulation might be a result of sampling. Whole ileal or colonic samples were collected; future studies using laser capture microdissection on specific population of immune cells or tissue might provide more distinct expression profiles related to mucosal immune response following NNPD-infection.

Tight junctions are involved in epithelial cell-cell adhesion. In the healthy intestine tight junctions and intact microvilli play a critical role in intestinal integrity and permeability, both known to be compromised during infectious diarrhoea [36]. To investigate if different expression of tight junction proteins were implicated in NNPD; OCLN, CLDN1, and CLDN3 were investigated. CLDN1 was down-regulated while OCLN, CLDN3, TFF2, and TFF3 were up-regulated in case piglets compared with control piglets. Several other studies have shown a significant regulation or redistribution of OCLN and CLDN after mucosal infection with enteropathogenic *E. coli* [37-39], or enterohemorrhagic *E. coli* and subsequent impairment of intestinal barrier function has been reported [36,40]. TFF2 and TFF3 are involved in mucosal repair and integrity [41,42], and seem also to play an important role during host response to NNPD.

Intestinal expression of genes involved in pathogen recognition

Activation of the innate immune system after inoculation with NNPD-material was studied by measuring mRNA levels of a wide range of PPRs including TLR, NOD-like receptors, RIG-1-like receptors, and a single C-type lectin receptor. Many of these receptors were either not regulated or in fact down-regulated as seen for DC-SIGN, or TLR5, 7, and 8 in the colon. This lack of PRR responsiveness in case piglets could be a consequence of establishing a tolerant environment during microbial colonization in the neonate gut [43,44]. Inoculation with NNPD-material induced a minor but significant up-regulation of mRNA encoding

for TLR2 in case piglets from litter 1. In addition to recognizing many Gram-positive bacteria, TLR2 also has an important role in maintaining the integrity of the intestinal epithelial barrier [44] and has previously been found to be up-regulated in the ileum of pigs infected with *Salmonella* or sow's feces [45,46]. The two cytoplasmic viral RNA detecting PRRs: MD5A and RIG-1 were significantly up-regulated in both gut sections of case piglets from litter 1 and 2. Though virus apparently is not involved in natural occurring NNPD, this implies that a viral component could be involved in the NNPD-infection model. Ongoing studies suggest that it might be a rotavirus (personal communication, LE Larsen).

Bacterial groups and associated gene expressions

The subsequent piece discusses the significant associations found between a selection of bacteria detected in intestinal luminal content and the mucosal expression of genes. The correlations may be indicative, but also speculative, and probably be better reflected and related if bacteria associated with the gut mucosa instead of free luminal bacteria were investigated. Nonetheless, epithelial cells and mucus (as well as associated bacteria) is continuously shed from the intestinal wall into the lumen, and are consequently assumed to be represented in the luminal content [47].

Enterococci and related gene expression

In agreement with this study, previous studies have reported Enterococci to be related with neonatal piglet diarrhoea [11,48]. The expression of pili by some strains of Enterococci contribute to the resistance to the flushing effect of diarrhoea [11,46]. The expression of genes involved in pilus production has been demonstrated to be enhanced in the presence of bicarbonate, and increased intestinal concentration of bicarbonate is a common sequela to diarrhoea [49,50]. Hence, it is likely that diarrhoea stimulates the expression of virulence genes that promotes the colonization of genus *Enterococcus.* In ileum a high expression of the host intestinal epithelium surface mucin, MUC1, and of TFF2 was both significantly correlated with high numbers of genus *Enterococcus*. MUC1 is one of several genes coding for mucin that provides the intestinal epithelium with a lubricating and protective layer which act as part of the host's defense against pathogens. High expression of this gene protects the intestinal epithelium from bacterial infections [51]. Likewise, Trefoil factors are a family of proteins that often is co-expressed in response to gastrointestinal inflammatory injury. TFF2 is rapidly up-regulated upon mucosal injury as it participates in epithelial restitutions by inducing cell migration to restore epithelium continuity [41]. The co-expression of MUC1 and TTF2 support the theory that these might exert an innate synergistic protective effect in response to an early exposure to Enterococci [41].

In colon, a high abundance of genus *Enterococcus* was associated with the highest expression level of CLDN3 and MD2, but with the lowest expression level of NOD1.The correlation between a high number of

genus *Enterococcus* and CLDN3 was also present in ileum, but it did not turn out significant because of a single control piglet. In colon this correlation was mainly driven by piglets from litter 1. Claudins are key components of the tight junctions that are vital in retaining intestinal epithelium integrity. CLDN3 have been demonstrated to be significantly increased in rats suffering from necrotizing enterocolitis with severe intestinal epithelial lesions [52]. The fact that a high number of Enterococci were associated with a high expression of CLDN3 in colon, where histopathology revealed epithelial erosions (Figure 3), supports the link between genus *Enterococcus* and NNPD. Enterococci are Gram-positive bacteria which mean that they mainly will be detected by TLR2. While no associations were found for TLR2 (though this receptor was up-regulated in ileum) an association was detected between high numbers of genus *Enterococcus* and the highest expression level of MD2. Besides being a helper-molecule for TLR4, MD2 enhances the responsiveness of TLR2 and broadens its receptor-mediated response to a variety of stimuli [53], hence, this correlation might reflect an indirect response to Enterococci in case piglets.

Remaining bacterial groups and related gene expression

The phyla Actinobacteria and Bacteroidetes in addition to the classes Clostridium cluster IV and cluster XIV are regarded to be part of the normal gut microbiota also of neonate piglets [54,55]. Numerous bacteria from these groups benefit to the health of the host [56,57]. Like NNPD-field cases, NNPD-infected piglets had significantly fewer of these bacteria, which have also been reported to be scarce in several other gastrointestinal disorders [58-60]. Probiotic bacteria can modulate the immune system [61] as seen in a study on cytokine response from mainly human dendritic cells after stimulation with *Bifidobacterium infantis* (phylum Actinobacteria). O'Mahony and co-workers found that this commensal bacterium increased the secretion of TGFB; contrariwise, the secretion of TGFB could not be stimulated with the pathogenic *Salmonella typhimurium* [62]. In the present study a high expression of TGFB seen in control piglets inoculated with healthy intestinal material was associated with high numbers of the aforementioned bacterial groups.

As well as being a gut commensal, *E. coli* is a well-known enteric pathogen that is able to cause diarrhoea in newborn piglets [63]. *E. coli* has been suggested to be involved in the pathogenesis of NNPD [9,10]. Being a Gram-negative bacterium, it was expected that the presence of pathogenic *E. coli* would activate the TLR4 pathway. Although the TLR4 co-receptors CD14 and MD2 were up-regulated, only one association was found and this was between Enterococci and MD2. Instead, a high occurrence of *E. coli* was associated with a low colonic expression of NOD2 detecting peptidoglycans from both Gram-positive and Gram-negative bacteria. In addition to protecting the intestine from infection by pathogenic bacteria, NOD2 also participates in the regulation of commensal bacteria [64]. Still, this PRR seems to be of less importance in sensing of *E. coli.*

In chickens, the gut microbial composition after inoculation has been demonstrated to depend on the bacterial content of the inocula, and also to differ dependent on geography [65]. This can be a possible explanation for the divergence in the relative gene expression between the litters illustrated in Figure 2, since inoculum 1 and inoculum 2 originate from two different pig farms. Inoculating piglets one day later had a major diminishing effect on the number of members from phylum Firmicutes, class Bacilli, and genus *Lactobacillus* colonizing the intestine. This reflects the importance of the piglet's age at inoculation, and the possibility to influence and alter the composition of the gut microbiota at such early time points [65]. It is likely that inoculating piglets with beneficial bacteria (probiotics) can be used as a step towards promoting the piglets' health and resistance to enteric pathogens [56].

Conclusion

Piglets inoculated with NNPD-material were characterized by much similar bacterial changes as NNPD-field case piglets, namely: a high abundance of genus *Enterococcus* and species *E. coli*, while the phyla Firmicutes, Bacteroidetes, and Actinobacteria were diminished. Even though whole intestinal samples, containing very different cell subsets, were used for transcriptional analysis, it was possible to demonstrate a significant regulation of transcripts involved in the innate immune response including pathogen recognition receptors, inflammation, and intestinal barrier function according to diarrhoeic status and origin of inoculum. The results of the present study confirm that a NNPD-disease model has been established, and supports previous findings suggesting that bacteria belonging to genus *Enterococcus* are involved in NNPD.

List of abbreviations used

CASP: caspase; **CCL3**: chemokine (C-C Motif) ligand 3; **CD**: Cluster of differentiation; **CLDN**: claudin; **Cq**: quantification cycle, **DC-SIGN**: C-type lectin receptor; **IL**: interleukin; **IPEC-J2**: epithelial neonatal porcine cells; **HP**: haptoglobin; **IFNG**: interferon gamma; **LPS**: lipopolysaccharide; **MD2**: Lymphocyte antigen 96; **MDA5**: Melanoma Differentiation-Associated Protein 5; **MUC**: mucin; **NNPD**: new neonatal porcine diarrhoea, **NOD**: nucleotide-binding oligomerization domain containing protein; NPD: neonatal piglet diarrhoea; **NTC**: non template control, **OCLN**: occluding; **PRRs**: pattern recognition receptors; **PRRSV**: porcine reproductive and respiratory syndrome virus, **qPCR**: quantitative real-time PCR; **RIG-1**: Retinoic Acid-Inducible Gene 1; **TGFB**: transforming growth factor beta; **TLR**: Toll-like receptor; **TNFA**: tumor necrosis factor alpha; **TFF**: trefoil factor; **SAA:** serum amyloid A.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

BJ and TKJ established the NNPD-infection model and provided the sample material. MLHB wrote the manuscript, and the immunology-related text was written in close collaboration with KS. KS, MB and MLHB designed this study. AS conducted statistical analyses on bacteria and correlations between bacteria and genes, and KS performed the analyses on genes. KS, MB, MLS, and PMH thoroughly read the manuscript and provided valuable feedback.

Acknowledgement

This study was supported by the Act on Innovation no. 421 of31/05/2000 granted by the Ministry of Food Agriculture and Fisheries of Denmark, the National Veterinary Institute (Technical University of Denmark), and the Pig Research Centre (Ministry of Food Agriculture and Fisheries of Denmark). The authors wish to thank Sophia Rasmussen and Karin Tarp for excellent support in the laboratory. Likewise, Kaja Alicja Plewnia and Lina Gruzinskyte are thanked for initial screening of gene expressions.

Reference lists

1. Gin T: **Diagnostic des nouveaux cas de diarrhées néonatales anzootiques du porcelet: évaluation de la prise colostrale.** *PhD Thesis.* Ecole Nationale Vétérinaire de Toulouse - ENVT; 2008.

2. Chan G, Farzan A, DeLay J, McEwen B, Prescott JF, Friendship RM: **A retrospective study on the etiological diagnoses of diarrhea in neonatal piglets in Ontario, Canada, between 2001 and 2010.** *Can J Vet Res* 2013, **77:**254-260.

3. Melin L, Wallgren P, Mattsson S, Stampe M, Löfstedt M: **Neonatal diarrhoea in piglets from *E.coli* vaccinated sows in Sweden.** In *Proceedings of the 21st IPVS Congres*. Edited by D'Allaire S, Friendship R. Vancouver, British Columbia, Canada; 2010:290.

4. Svensmark S: **New neonatal diarrhoea syndrome in Denmark.** In *Proceedings of the 1st ESPHM*. Edited by Stege H, Kristensen CS. Copenhagen, Denmark: Faculty of Life Sciences, University of Copenhagen; 2009:27.

5. Astrup P, Larsen KV, Jorsal SE, Larsen LE: **Neonatal Diarrhea in Danish pig farms - a questionnaire among veterinarians.** In *Proceedings of the 21st IPVS Congres*. Edited by D'Allaire S, Friendship R. Vancouver, British Columbia, Canada; 2010:751.

6. Sialelli JN, Vautrin F, Lautrou Y, Oswald I, Quiniou N, Martineau G: **Farrowing progress (chronopart) and enzootic neonatal diarrhoea: Observational study in nine commercial herds.** In *Proceedings of the 21st IPVS Congres*. Edited by D'Allaire S, Friendship R. Vancouver, British Columbia, Canada; 2010:752.

7. Kongsted H, Jonach B, Haugegaard S, Angen O, Jorsal SE, Kokotovic B, Larsen LE, Jensen TK, Nielsen JP: **Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome.** *BMC Vet Res* 2013, **9:**206.

8. Kongsted H, Stege H, Toft N, Nielsen JP: **The effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain and mortality in 4 Danish pig herds.** *BMC Vet Res* 2014, **10:**90.

9. Jonach B, Boye M, Stockmarr A, Jensen TK: **Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea.** *BMC Vet Res* 2014, **10:**68.

10. Hermann-Bank ML, Skovgaard K, Stockmarr A, Strube LM, Larsen N, Kongsted H, Ingerslev H, Mølbak L, Boye M: **Characterization of the bacterial gut microbiota of piglets suffering from new neonatal porcine diarrhoea.** *Submitted to Microbial Ecology* 2014.

11. Larsson J, Lindberg R, Aspán A, Grandon R, Westergren E, Jacobson M: **Neonatal Piglet Diarrhoea Associated with Enteroadherent *Enterococcus hirae*.** *J Comp Pathol* 2014, **151:**137-147.

12. Bauer E, Williams BA, Smidt H, Verstegen MWA, Mosenthin R: **Influence of the gastrointestinal microbiota on development of the immune system in young animals.** *Curr Issues Intest Microbiol* 2006, **7:**35-51.

13. Cebra JJ: **Influences of microbiota on intestinal immune system development.** *Am J Clin Nutr* 1999, **69**.

14. Butler JE, Sinkora M: **The enigma of the lower gut-associated lymphoid tissue (GALT).** *J Leukoc Biol* 2013, **94:**259-270.

15. Kelly D, Coutts AGP: **Development of digestive and immunological function in neonates: role of early nutrition.** *Livest Prod Sci* 2000, **66:**161-167.

16. Abreu MT: **Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function.** *Nat Rev Immunol* 2010, **10:**131-143.

17. Lavelle EC, Murphy C, O'Neill LAJ, Creagh EM: **The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis.** *Mucosal Immunol* 2010, **3:**17-28.

18. Rubin JE, Costa MO, Hill JE, Kittrell HE, Fernando C, Huang Y, O'Connor B, Harding JCS: **Reproduction of Mucohaemorrhagic Diarrhea and Colitis Indistinguishable from Swine Dysentery following Experimental Inoculation with "*Brachyspira hampsonii*" Strain 30446.** *Plos One* 2013, **8:**e57146.

19. Terpstra C, Wensvoort G, Pol JMA: **Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled.** *Vet Q* 1991, **13:**131-136.

20. Promega: ***Maxwell® 16 Cell LEV DNA Purification Kit. Instructions for Use of Product AS1140.*** USA; 2010.

21. Hermann-Bank ML, Skovgaard K, Stockmarr A, Larsen N, Mølbak L: **The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity.** *BMC Genomics* 2013, **14:**1-14.

22. Skovgaard K, Cirera S, Vasby D, Podolska A, Breum SØ, Dürrwald R, Schlegel M, Heegaard PMH: **Expression of innate immune genes, proteins and microRNAs in lung tissue of pigs infected experimentally with influenza virus (H1N2).** *Innate Immunity* 2013, **19:**531-544.

23. Skovgaard K, Mortensen S, Boye M, Hedegaard J, Heegaard PMH: **Hepatic gene expression changes in pigs experimentally infected with the lung pathogen Actinobacillus pleuropneumoniae as analysed with an innate immunity focused microarray.** *Innate Immunity* 2010, **16:**343-353.

24. Andersen CL, Jensen JL, Orntoft TF: **Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets.** *Cancer Res* 2004, **64:**5245-5250.

25. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** *Genome Biol* 2002, **3**.

26. Buffie CG, Pamer EG: **Microbiota-mediated colonization resistance against intestinal pathogens.** *Nat Rev Immunol* 2013, **13:**790-801.

27. Gorkiewicz G, Thallinger GG, Trajanoski S, Lackner S, Stocker G, Hinterleitner T, Guelly C, Hoegenauer C: **Alterations in the Colonic Microbiota in Response to Osmotic Diarrhea.** *Plos One* 2013, **8**.

28. Rendon MA, Saldana Z, Erdem AL, Monteiro-Neto V, Vazquez A, Kaper JB, Puente JL, Giron JA: **Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization.** *Proc Natl Acad Sci U S A* 2007, **104:**10637-10642.

29. Wexler HM: **Bacteroides: The good, the bad, and the nitty-gritty.** *Clin Microbiol Rev* 2007, **20:**593-621.

30. Pirker A, Stockenhuber A, Remely M, Harrant A, Hippe B, Kamhuber C, Adelmann K, Stockenhuber F, Haslberger AG: **Effects of antibiotic therapy on the gastrointestinal microbiota and the influence of *Lactobacillus casei*.** *Food Agric Immunol* 2013, **24:**315-330.

31. Hermes RG, Manzanilla EG, Martin-Orue SM, Perez JF, Klasing KC: **Influence of dietary ingredients on in vitro inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic *Escherichia coli* (K88).** *Comp Immunol Microb* 2011, **34:**479-488.

32. Zhou C, Liu Z, Jiang J, Yu Y, Zhang Q: **Differential gene expression profiling of porcine epithelial cells infected with three enterotoxigenic *Escherichia coli* strains.** *BMC Genomics* 2012, **13**.

33. Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM, Nusrat A: **Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms.** *J Immunol* 2003, **171:**6164-6172.

34. Li Q, Zhang Q, Wang M, Zhao S, Ma J, Luo N, Li N, Li Y, Xu G, Li J: **Interferon-gamma and tumor necrosis factor-alpha disrupt epithelial barrier function by altering lipid composition in membrane microdomains of tight junction.** *Clin Immunol* 2008, **126:**67-80.

35. Moestrup SK, Møller HJ: **CD163: A regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response.** *Ann Med* 2004,347-354.

36. Noth R, Lange-Grumfeld J, Stueber E, Kruse ML, Ellrichmann M, Haesler R, Hampe J, Bewig B, Rosenstiel P, Schreiber S, Arlt A: **Increased intestinal permeability and tight junction disruption by altered expression and localization of occludin in a murine graft versus host disease model.** *BMC Gastroenterology* 2011, **11**.

37. Hecht G: **Microbes and microbial toxins: Paradigms for microbial-mucosal interactions - VII. Enteropathogenic *Escherichia coli:* physiological alterations from an extracellular position.** *Am J Physiol-Gastro L* 2001, **281**.

38. Lapointe TK, O'Connor PM, Buret AG: **The role of epithelial malfunction in the pathogenesis of enteropathogenic *E. coli*-induced diarrhea.** *Lab Invest* 2009, **89:**964-970.

39. Li X, Wang Q, Xu H, Tao L, Lu J, Cai L, Wang C: **Somatostatin regulates tight junction proteins expression in colitis mice.** *Int J Clin Exp Pathol* 2014, **7:**2153-2162.

40. Roxas JL, Koutsouris A, Bellmeyer A, Tesfay S, Royan S, Falzari K, Harris A, Cheng H, Rhee KJ, Hecht G: **Enterohemorrhagic *E. coli* alters murine intestinal epithelial tight junction protein expression and barrier function in a Shiga toxin independent manner.** *Lab Invest* 2010, **90:**1152-1168.

41. Taupin D, Podolsky DK: **Trefoil factors: Initiators of mucosal healing.** *Nat Rev Immunol* 2003, **4**.

42. Kinoshita K, Taupin DR, Itoh H, Podolsky DK: **Distinct pathways of cell migration and antiapoptotic response to epithelial injury: Structure-function analysis of human intestinal trefoil factor.** *Mol Cell Biol* 2000, **20:**4680-4690.

43. Otte JM, Cario E, Podolsky DK: **Mechanisms of Cross Hyporesponsiveness to Toll-Like Receptor Bacterial Ligands in Intestinal Epithelial Cells.** *Gastroenterology* 2004, **126:**1054-1070.

44. Cario E, Podolsky DK: **Intestinal epithelial TOLLerance versus inTOLLerance of commensals.** *Mol Immunol* 2005, **42:**887-893.

45. Collado-Romero M, Arce C, Ramirez-Boo M, Carvajal A, Garrido JJ: **Quantitative analysis of the immune response upon *Salmonella typhimurium* infection along the porcine intestinal gut.** *Vet Res* 2010, **41**.

46. Willems RJL, van Luit-Asbroek M, Schapendonk CME, Bonten MJM, Kragten AHM, Hendrickx APA: **Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate.** *Microbiology* 2008, **154:**3212-3223.

47. Kim M, Ashida H, Ogawa M, Yoshikawa Y, Mimuro H, Sasakawa C: **Bacterial interactions with the host epithelium.** *Cell Host Microbe* 2010, **8:**20-35.

48. Cheon DS, Chae CH: **Outbreak of diarrhea associated with *Enterococcus durans* in piglets.** *J Vet Diagn Invest* 1996, **8:**123-124.

49. Bourgogne A, Thomson LC, Murray BE: **Bicarbonate enhances expression of the endocarditis and biofilm associated pilus locus, ebpR-ebpABC, in *Enterococcus faecalis*.** *BMC Microbiol* 2010, **10**.

50. Perex G, Oster J, Roger A: **Acid-base disturbances in gastrointestinal disease.** *Dig Dis Sci* 1987, **32:**1033-1043.

51. McAuley JL, Linden SK, Png CW, King RM, Pennington HL, Gendler SJ, Florin TH, Hill GR, Korolik V, McGuckin MA: **MUC1 cell surface mucin is a critical element of the mucosal barrier to infection.** *J Clin Invest* 2007, **117:**2313-2324.

52. Khailova L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, Yajima M, Dvorak B: ***Bifidobacterium bifidum* improves intestinal integrity in a rat model of necrotizing enterocolitis.** *Am J Physiol-Gastro L* 2009, **297**.

53. Dziarski R, Wang Q, Miyake K, Kirschning CJ, Gupta D: **MD-2 enables Toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to gram-positive and gram-negative bacteria and their cell wall components.** *J Immunol* 2001, **166:**1938-1944.

54. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI: **Evolution of mammals and their gut microbes.** *Science* 2008, **320:**1647-1651.

55. Petri D, Hill JE, Van Kessel AG: **Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig.** *Livest Sci* 2010, **133:**107-109.

56. Cho JH, Zhao PY, Kim IH: **Probiotics as a Dietary Additive for Pigs: A Review.** *J Anim Vet Adv* 2011, **10:**2127-2134.

57. Sarbini SR, Rastall RA: **Prebiotics: Metabolism, Structure, and Function.** *Functional Food Reviews* 2011, **3:**93-106.

58. Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L: **The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease.** *Plos One* 2012, **7**.

59. Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K, Palva A: **Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers.** *BMC Gastroenterology* 2009, **9**.

60. Robinson IM, Whipp SC, Bucklin JA, Allison MJ: **Characterization of Predominant Bacteria from the Colons of Normal and Dysenteric Pigs.** *Appl Environ Microbiol* 1984, **48:**964-969.

61. Isolauri E, Sutas Y, Kankaanpaa P, Arvilommi H, Salminen S: **Probiotics: effects on immunity.** *Am J Clin Nutr* 2001, **73**.

62. O'Mahony L, O'Callaghan L, McCarthy J, Shilling D, Scully P, Sibartie S, Kavanagh E, Kirwan WO, Redmond HP, Collins JK, Shanahan F: **Differential cytokine response from dendritic cells to commensal and pathogenic bacteria in different lymphoid compartments in humans.** *Am J Physiol-Gastr L* 2006, **290**.

63. Flores-Abuxapqui JJ, Suárez-Hoil G, Puc-Franco MA, Heredia-Navarrete MR, Vivas-Rosel M, Oberhelman RA: **Frequency of adhesive factors and entero-toxins in strains of *Escherichia coli* isolated from piglets with diarrhea.** *Rev Latinoam Microbiol* 1997, **39:**145-151.

64. Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, Kobayashi KS: **Nod2 is required for the regulation of commensal microbiota in the intestine.** *Proc Natl Acad Sci U S A* 2009, **106:**15813-15818.

65. Yin Y, Lei F, Zhu L, Li S, Wu Z, Zhang R, Gao GF, Zhu B, Wang X: **Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression.** *ISME J* 2010, **4:**367-376.

Table 1: Piglets analyzed in the Gut Microbiotassay.

Litter	Age when inoculated	Inoculated	No treatment	Age in days							
				0	1	2	3	4	5	6	7
0	1	2	2					4			
1	1	9	3					12			
1.1	0	4	0					3	1		
1F	1	6*	2				4			4*	
2	0	6	4*				4*			6	
3	2	5	2					3		4	
4	2	6	2					4		4	
Control	-	0	12*	2*	3	3	4				
C1	0	6	0				3		3		
C2	0	6	4					5			5
Total	-	50	31	2	3	3	15	31	4	18	5

* One gut section was lost between sampling and laboratory

Table 2: Piglets of which corresponding gut immune response was investigated.

Gut section	Ileum						Colon					
Litter	Inoculated	No treatment	Age in days				Inoculated	No treatment	Age in days			
			3	4	5	6			3	4	5	6
1	8	0		8			9	0		9		
1.1	5	0		3	2		5	0		3	2	
2	6	2	3			5	6	1	3			4
Control	0	4	4				0	4	4			
C1	3	0	3				3	0	3			
C2	3	0		3			3	0		3		
Total	25	6	10	14	2	5	26	5	10	15	2	4

Table 3: Bacterial groups and associated gene expression examined in ileum and colon. All bacterial groups were tested against all genes. Symbols reflect different linear regression analysis performed dependent on the listed gene functions: pattern recognition (PRR), inflammation (INFL), and intestinal barrier function (IBF), in order to examine correlations between bacterial groups and gene expression. All genes were included in the qPCR analysis, but not all genes were expressed in both gut sections.

Bacterial groups	Tested combination	Gene function	Gene abbreviation : gene name
Phylum Bacteroidetes †	‡	PRR	CD163 : Cluster of differentiation 163
Phylum Actinobacteria †	‡	PRR	CD14 : Cluster of differentiation 14
Phylum Fusobacteria	‡	PRR	MRC1 : Mannose receptor c1
Class Clostridium cluster I	‡	PRR	NOD1 : Nucleotide-binding oligomerization domain containing protein 1
Class Clostridium cluster IV †			
Class Clostridium cluster XIV †	‡	PRR	NOD2 : Nucleotide-binding oligomerization domain containing protein 2
Genus *Enterococcus* ‡, £, §			
Genus *Lactobacillus* †	‡	PRR	TLR1 : Toll-like receptor 1
Genus *Streptococcus* †	‡	PRR	TLR2 : Toll-like receptor 2
Species *Escherichia coli* ‡, £, §	‡	PRR	TLR4 : Toll-like receptor 4
	‡	PRR	TLR5 : Toll-like receptor 5
	‡	PRR	TLR6 : Toll-like receptor 6
	‡	PRR	TLR9 : Toll-like receptor 9
	‡	PRR	MD2 : Lymphocyte antigen 96
	†,£	INFL	IL-1RN : Interleukin 1 receptor antagonist
	†,£	INFL	IL1A : Interleukin 1 alpha
	†,£	INFL	IL1B : Interleukin 1 beta
	†,£	INFL	IL6 : Interleukin 6
	†,£	INFL	IL10 : Interleukin 10
	†,£	INFL	IL18 : Interleukin 18
	†,£	INFL	SAA : Serum amyloid A
	†,£	INFL	TGFB: Transforming growth factor beta
	†,£	INFL	TNFA : Tumor necrosis factor alpha
	§	IBF	CLDN1 : Claudin 1
	§	IBF	CLDN3 : Claudin 3
	§	IBF	MUC1 : Mucin 1
	§	IBF	MUC2 : Mucin 2
	§	IBF	OCLN : Occludin
	§	IBF	TFF2 : Trefoil factor 2
	§	IBF	TFF3 : Trefoil factor 3

Table 4: Significant findings from the Gut Microbiotassay generated data. Estimated fold changes and *p*-values. If there was a significant effect if interaction, only this *p*-value is given. Dashes indicate a significant effect of interaction. Age refers to age in days.

Primer set	Gut section estimate (ileum vs. colon)	Gut Section *p*-value	Status estimate (diarrhoeic vs. healthy)	Status *p*-value	Age estimate	Age *p*-value	Inoculum healthy estimate	Inoculum healthy *p*-value	Inoculum NNPD estimate	Inoculum NNPD *p*-value	Day of inoculation estimate	Day of inoculation *p*-value	Gut section : Status estimate (ileum, diarrhoeic)	Gut section : Status *p*-value	Status : Age estimate	Status : Age *p*-value
Domain Bacteria B	0.03	-	0.61	-	1.18	0.03							6.92	< 0.0001		
Phylum Firmicutes	3.20	< 0.0001	0.57	0.006	1.23	0.001					0.62	0.009				
Class Bacilli	5.64	-	0.83	-	1.24	0.007					0.59	0.04	0.41	0.03		
Genus *Enterococcus*	2.92	0.0002	30.42	< 0.0001												
Genus *Streptococcus*																
Genus *Lactobacillus*	5.92	-	0.61	-	1.39	< 0.0001					0.55	0.007	0.41	0.02		
Family Clostridium cluster I			0.09	< 0.0001												
Species *Clostridium perfringens*					0.54	0.0002										
Family Clostridium cluster IV	0.18	< 0.0001	0.07	< 0.0001	1.74	0.0002										
Family Clostridium cluster XIV	0.06	< 0.0001	0.02	-	1.44	-									1.72	0.05
Phylum Bacteroidetes	0.04	< 0.0001	0.23	0.004	1.52	0.001										
Genus *Bacteroides*	0.02	< 0.0001	0.06	-	1.02	-									1.52	0.02
Phylum Actinobacteria			0.03	-	1.00	-			0.49	0.01					1.62	0.02
Family Bifidobacteriaceae	1.94	0.001	5.27	< 0.0001			2.88	0.0008								
Phylum Fusobacteria	0.04	< 0.0001														
Class Beta-and Gammaproteobacteria	0.49	-	4.25	-	0.74	0.005							3.48	0.01		
Family Enterobacteriaceae	3.08	< 0.0001	5.04	< 0.0001	0.79	0.02										
Species *Escherichia coli*	0.42	-	4.34	-	0.71	0.01							6.87	0.003		
Class Epsilonproteobacteria	4.80	-	11.62	-									0.03	0.0006		

Table 4: Estimated fold changes for significant effect of interaction between Status (diarrhoeic vs. healthy) and Age found in Table 4. Age refers to age in days.

Primer set	0	1	2	3	4	5	6	7	*p-value*
Family Clostridium cluster XIV	0.02	0.04	0.06	0.11	0.18	0.31	0.53	0.92	0.002
Genus *Bacteroides*	0.06	0.09	0.14	0.21	0.32	0.48	0.73	1.11	0.02
Phylum Actinobacteria	0.03	0.05	0.08	0.13	0.20	0.33	0.53	0.86	0.002

Table 5: Estimated fold changes for significant effect of interaction between Gut section and Status (diarrhoeic vs. healthy) found in Table 4.

Primer set	Gut section : Status estimate	
	Ileum, diarrhoeic	Colon, diarrhoeic
Domain Bacteria B	4.2	0.6
Class Bacilli	0.3	0.8
Genus *Lactobacillus*	0.2	0.6
Class Beta-and Gammaproteobacteria	14.8	4.3
Species *Escherichia coli*	29.8	4.3
Class Epsilonproteobacteria	0.4	11.6

Table 6: NNPD status explained by selected bacteria and relevant variables. Of the following variables tested: genus *Enterococcus*, class Beta- and Gammaproteobacteria, family Enterobacteriaceae, species *Escherichia coli*, Gut section, and Age, as well as possible interactions between bacteria, NNPD was found to be significantly related to the variables mentioned below. Numbers are transformed with the natural logarithm (ln).

ln(variable)	Gut section	Estimated fold change	95 % confidence interval	*p*-value
Intercept	-	1.1	-	-
Age	-	0.74	[0.14 ; 1.34]	0.02
Species *Escherichia coli*	ileum	3.61	[1.21 ; 6.00]	0.003
Class Beta- and Gammaproteobacteria	ileum	-3.62	[-6.04 ; -1.19]	0.004
Genus *Enterococcus*	ileum	0.81	[0.19 ; 1.43]	0.01
Genus *Enterococcus*	colon	1.73	[1.00 ; 2.46]	< 0.0001

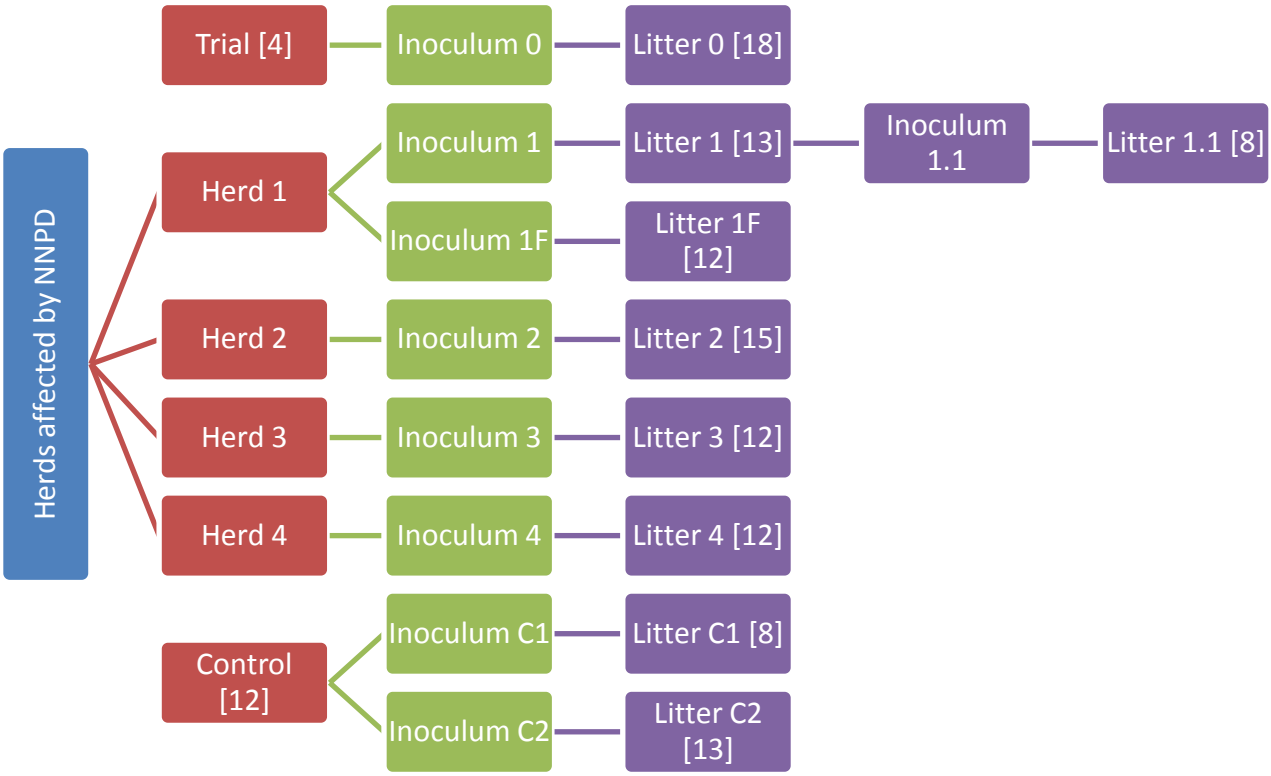
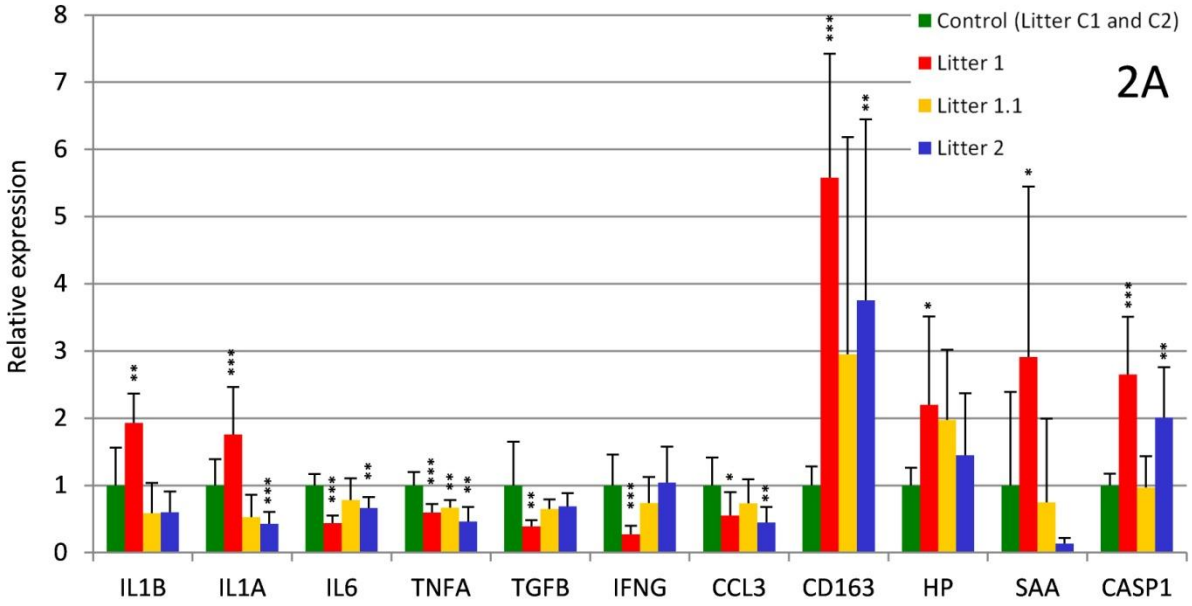
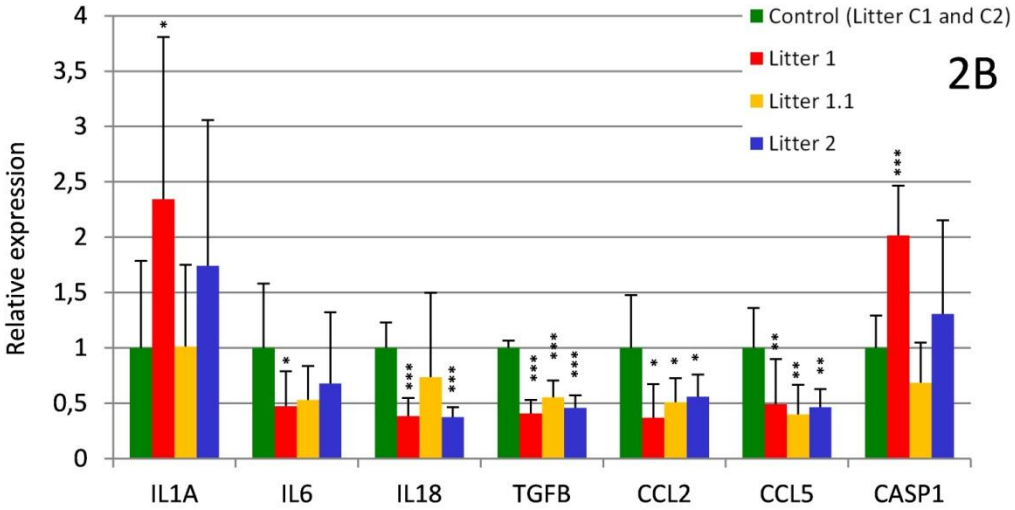


Figure 1: Study design. Through the initial Trial the inoculums concentration was determined to be 30 % for all subsequent inocula. Herds affected by NNPD were included from a previous study [7]. Red boxes depict origin of the inoculum. Green boxes represent inoculum. Purple boxes symbolize the litters inoculated with the respective inocula. Numbers in square brackets: number of piglets included in the study from each litter.





(Figure continues on the next page)

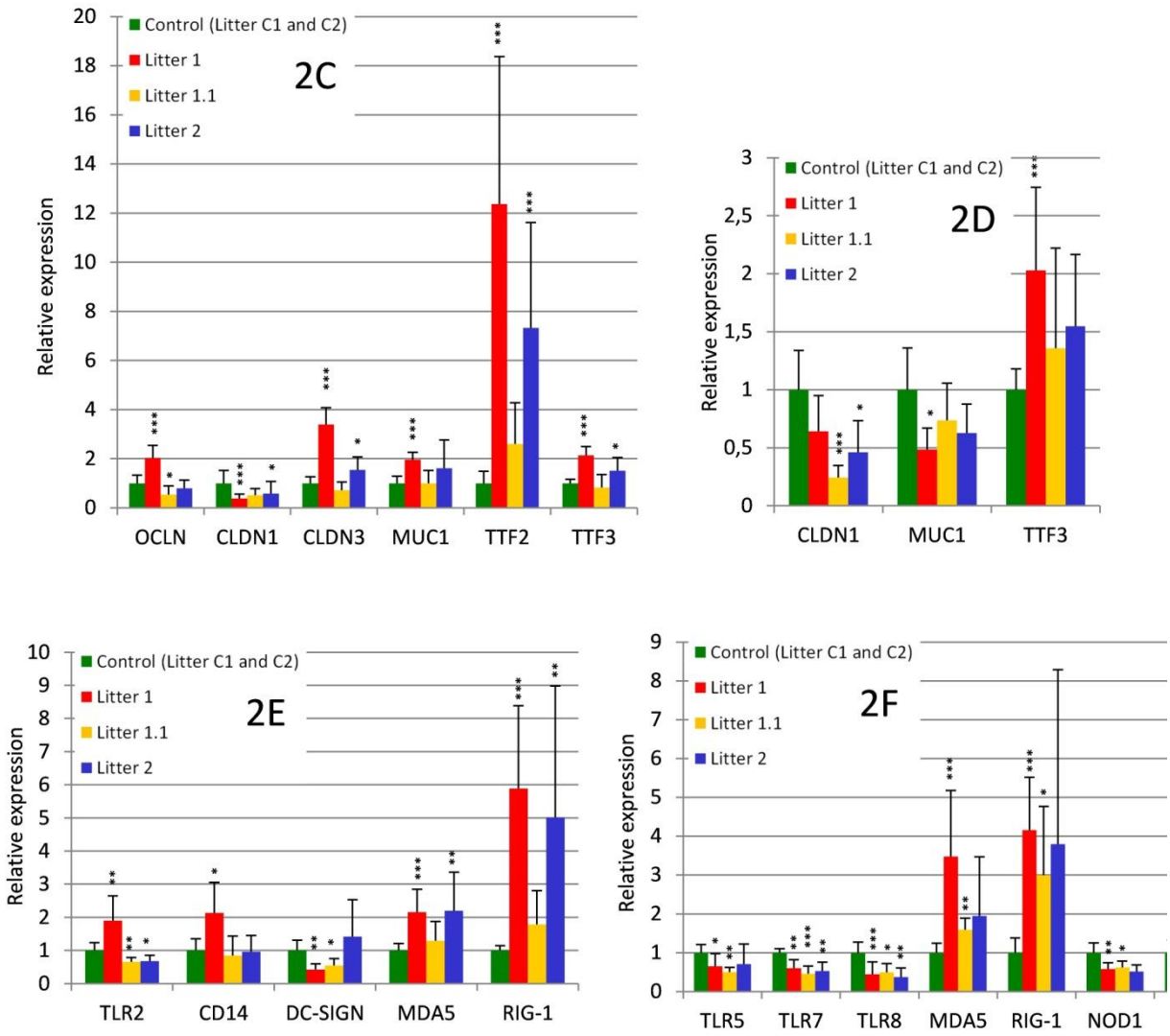


Figure 2 Relative expression visualized as mean fold change of mRNA levels relative to control samples. 2A: inflammatory genes of the ileum; **2B:** inflammatory genes of the colon; **2C:** genes related to intestinal integrity within the ileum; **2D:** genes related to intestinal integrity within the colon; and finally, genes coding for pattern recognition receptors expressed in the ileum (**2E**) and colon (**2F**). Mean expression seen in the case piglets from litter 1 (n = 9), litter 1.1 (n = 5), and litter 2 (n = 8) are illustrated relative to the control group inoculated with healthy material (n = 6). Error bars represent SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-tailed Student's t-test).

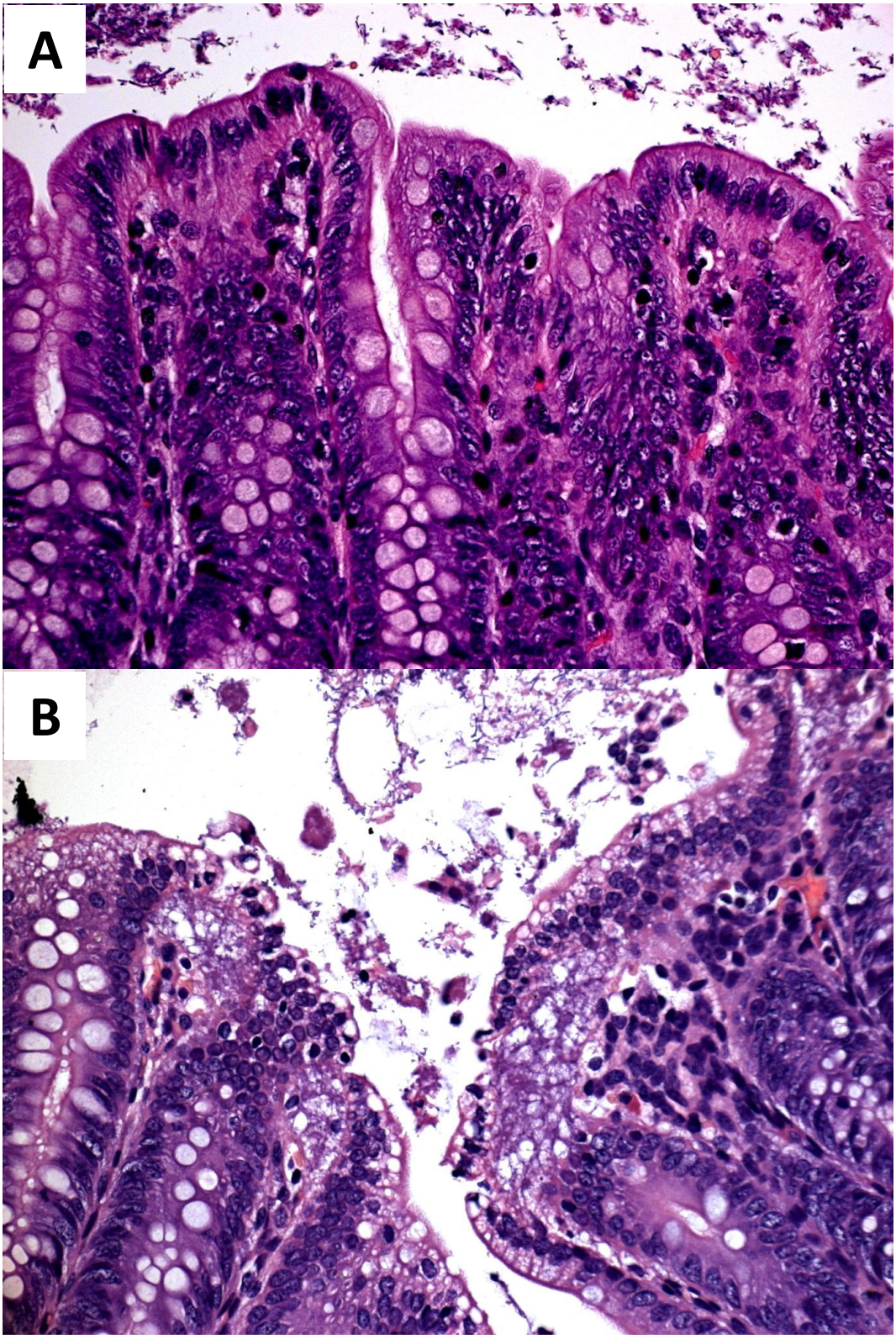


Figure 3: Section of colon. A: Normal colonic epithelium from non-diarrhoeic control piglet inoculated with intestinal material from healthy piglets. **B**: Sloughing of colonic epithelial cells from diarrhoeic case piglet inoculated with NNPD-material. Hematoxylin and eosin stain, magnification 400× (Pictures: B. Jonach).

Supplementary File 1: Primer assays used for porcine gene expression analysis.

Gene	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'	Product size (bp)	PCR efficiency Ileum (%)	PCR efficiency colon (%)	Name
ACTB	CTACGTCGCCCTGGACTTC	GCAGCTCGTAGCTCTTCTCC	76	104	112	Actin, beta
B2M	TGAAGCACGTGACTCTCGAT	CTCTGTGATGCCGGTTAGTG	70	100	107	Beta-2-microglobulin
CASP1	GAAGGACAAACCCAAGGTGA	TGGGCTTTCTTAATGGCATC	147	102	107	Caspase 1
CCL2	GCAAGTGTCCTAAAGAAGCAGTG	TCCAGGTGGCTTATGGAGTC	103	104	103	Chemokine (C-C motif) ligand 2
CCL3	CCAGGTCTTCTCTGCACCAC	GCTACGAATTTGCGAGGAAG	90	96	NA	Chemokine (C-C motif) ligand 3
CCL5	CTCCATGGCAGCAGTCGT	AAGGCTTCCTCCATCCTAGC	121	99	107	Chemokine (C-C motif) ligand 5
CD14	GGGTTCCTGCTCAGATTCTG	CCCACGACACATTACGGAGT	164	104	109	CD14 Molecule
CD163	CACATGTGCCAACAAAATAAGAC	CACCACCTGAGCATCTTCAA	130	103	100	CD163 Molecule
CLDN1	TGATGAGGTGCAGAAGATGC	CCATGCTGTGGCAACTAAGA	88	90*	90*	Claudin 1
CLDN3	TTATCACAGCGCAGATCACC	ACACTTTGCACTGCATCTGG	81	102	107	Claudin 3
DC-SIGN	GGCAATGGGTGGACAACAG	CACAGATCCAGGCGTTTTCT	144	101	98	C-Type Lectin Domain Family 4, Member L
GAPDH	ACCCAGAAGACTGTGGATGG	AAGCAGGGATGATGTTCTGG	79	105	107	Glyceraldehyde-3-phosphate dehydrogenase
HP	ACAGATGCCACAGATGACAGC	CGTGCGCAGTTTGTAGTAGG	105	93	97	Haptoglobin
HPRT1	ACACTGGCAAAACAATGCAA	TGCAACCTTGACCATCTTTG	71	97	102	Hypoxanthine phosphoribisyl.transferase I
IFNG	CCATTCAAAGGAGCATGGAT	TTCAGTTTCCCAGAGCTACCA	76	89	NA	Interferon gamma
IL10	TACAACAGGGGCTTGCTCTT	GCCAGGAAGATCAGGCAATA	110	96	NA	Interleukin 10
IL18	CTGCTGAACCGGAAGACAAT	TCCGATTCCAGGTCTTCATC	100	101	104	Interleukin 18
IL1A	GACGAACCCGTGTTGCTG	CCATATTGCCATGCTTTTCC	97	101	104	Interleukin 1 alpha
IL1B	TCTCTCACCCCTTCTCCTCA	GACCCTAGTGTGCCATGGTT	60	95	89	Interleukin 1b
IL1RN	TGCCTGTCCTGTGTCAAGTC	GTCCTGCTCGCTGTTCTTTC	90	89	91	Interleukin 1 receptor antagonist
IL33	GTAAACCTGAGCCCCACAAA	CTGTTCTGGCAGTGGGTTTT	102	93	104	Interleukin 33
IL6	CCTCTCCGGACAAAACTGAA	TCTGCCAGTACCTCCTTGCT	118	90	115	Interleukin 6
MD2	CAGTAAAGGTTGAGCCCTGTG	TTTGCGCATTGGTAAAGTCA	140	96	107	Lymphocyte antigen 96
MDA5	CAGTGTGCTAGCCTGCTCTG	GCAGTGCCTTGTTTCCTCTC	113	104	109	Interferon Induced With Helicase C Domain 1

Supplementary File 1 continued.

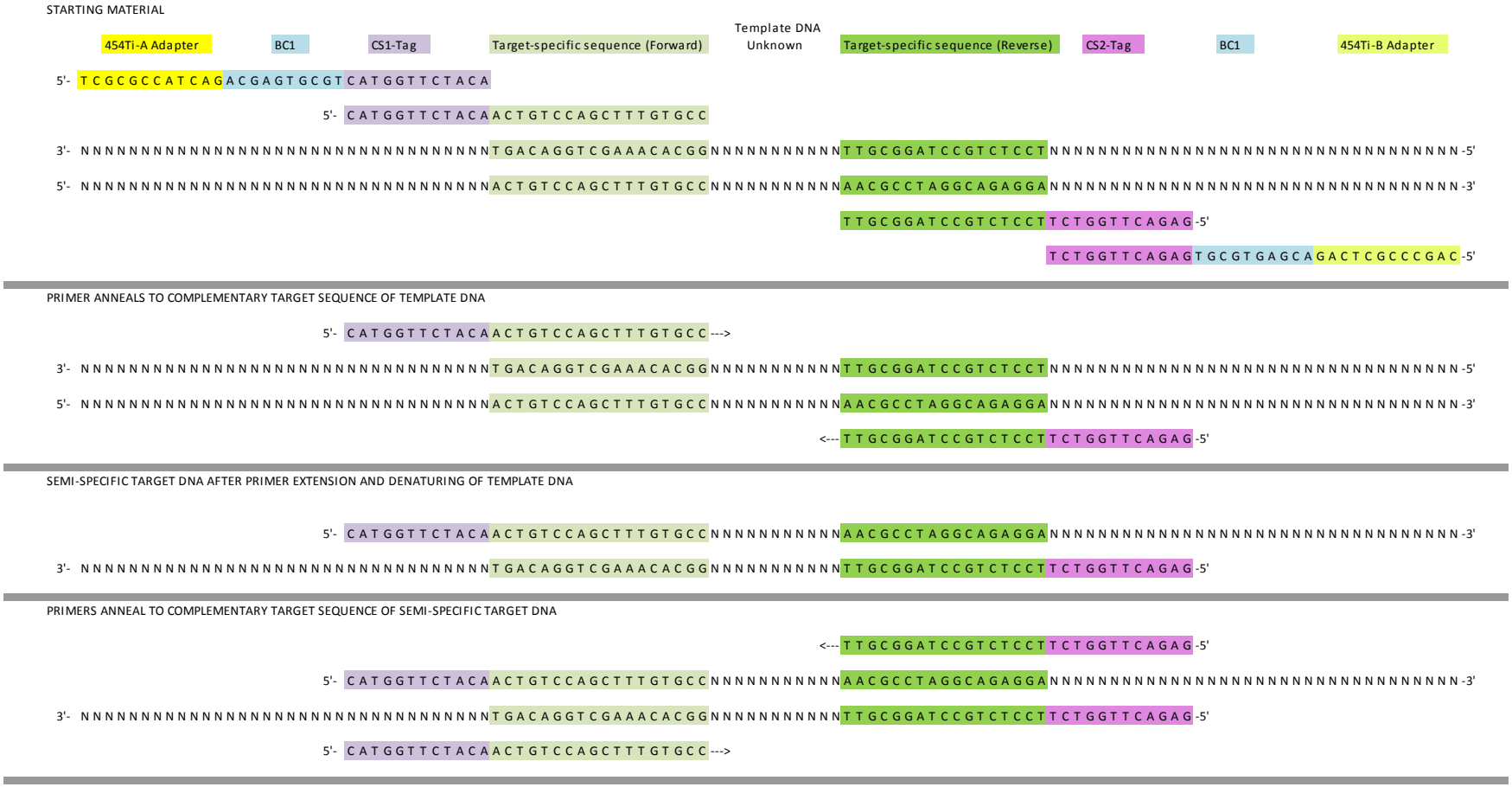
MRC1	AGAGCACACCTTCCTTTGGA	CAACACAATCGGCATCTTCA	113	98	106	Mannose Receptor, C Type 1
MUC1	GGATTTCTGAATTGTTTTTGCAG	ACTGTCTTGGAAGGCCAGAA	116	90*	110	Mucin 1
MUC2	GCACGTCTGCAACAAGGAC	CAAAGCCCTCCAGGCAGT	125	109	104	Mucin 2
NOD1	CTCGACCTGGACAACAACAA	TGAGTCTGATGACCGTGAGG	85	100	99	Nucleotide-binding oligomerization domain containing 1
NOD2	GAAAGTCCTGAAGCTGTCCAAC	CCAGACTTCCAGGATGGTGT	97	NA	90*	Nucleotide-binding oligomerization domain containing 2
OCLN	GACGAGCTGGAGGAAGACTG	GTACTCCTGCAGGCCACTGT	102	109	106	Occludin
PPIA	CAAGACTGAGTGGTTGGATGG	TGTCCACAGTCAGCAATGGT	138	104	109	Peptidylprolyl isomerase A (cyclophilin A)
RIG-I	TTGCTCAGTGCAATCTGGTC	CTTCCTCTGCCTCTGGTTTG	79	100	98	Retinoic Acid Inducible Gene I
RPL13A	ATTGTGGCCAAGCAGGTACT	AATTGCCAGAAATGTTGATGC	76	101	109	Ribosomal protein L13a
SAA	GCTAAAGTGATCAGCGATGC	AGTGGTTGGGGTCCTTGC	145	101	107	Serum amyloid A
TBP	ACGTTCGGTTTAGGTTGCAG	CAGGAACGCTCTGGAGTTCT	96	95	104	TATA box binding protein
TGFB	GCAAGGTCCTGGCTCTGTA	TAGTACACGATGGGCAGTGG	97	109	105	Transforming Growth Factor Beta
TLR2	CGGAGGTTGCATATTCCACAG	TGTGAAAGGGAACAGGGAAC	128	101	100	Toll like receptor 2
TLR3	ATTGTGCAAAAGATTCAAGGTG	TCTTCGCAAACAGAGTGCAT	130	100	101	Toll like receptor 3
TLR4	TGGTGTCCCAGCACTTCATA	CAACTTCTGCAGGACGATGA	116	96	105	Toll like receptor 4
TLR5	CGCTTGGACCTATCCAAAAA	GATCAATGGCCTTCAAGGAA	85	96	99	Toll like receptor 5
TLR6	TGGATGTTAGCTCGAATTCTTTG	GAACCTTGATCCTGGGAGGT	141	96	104	Toll like receptor 6
TLR7	GGAAATAGCATCAGCCAAGCTC	TTCCAGGTTGCGTAGCTCTT	132	98	99	Toll like receptor 7
TLR8	GCAAAGACCACCACCAACTT	ATCCGTCAGTCTGGGAATTG	129	94	104	Toll like receptor 8
TLR9	CCTGTTCTATGATGCCTTCGTG	GGTACCCAGTCTCGCTCCTC	144	90*	NA	Toll like receptor 9
TNFA	CACGTTGTAGCCAATGTCAAAG	GAGGTACAGCCCATCTGTCG	129	91	102	Tumor necrosis factor
TTF2	GCTGCTTCGACTCCCAAGT	CATGACGCACTCCTCAGACT	80	101	NA	Trefoil factor 2
TTF3	TGTTCTGGCTGCTAGTGGTG	CAGTCCACCCTGTCCTTGG	112	104	104	Trefoil factor 3
YWHAE	GCTGCTGGTGATGATAAGAAGG	AGTTAAGGGCCAGACCCAAT	124	98	105	Tyrosine 3-monooxygenase

* Default efficiency of 90 % used as r^2 of standard curve was below 0.95

Photo: author.

Methodology appendixes

Appendix A: Incorporation of the 454 barcode library



Schematic Illustration on how amplicons are tagged with the 454 barcode library (454BL) for 454 GS FLX Titanium sequencing. The Target-Specific primers are tagged: the forward primer with CS1-Tag and the reverse primer with CS2-Tag. The 454BL is added to the sample and master mix. The library is composed of three sequences: 1) A CS1- or CS2-Tag which is identical to the one on the forward or reverse primer respectively; 2) A unique barcode that identifies the sample; and 3) A 454TI-A or 454TI-B Adapter dependent on whether it is a the forward tagged primer or a reverse tagged primer. For convenience several of the sequences in the illustration has been cut short, for full sequences and further information reference is made to [152]. Appendix A continues on the next page.

UNTAGGET SPECIFIC TARGET DNA AFTER PRIMER EXTENSION AND DENATURATION OF SEMI-SPECIFIC TARGET DNA

3'- GTACCAAGATGTTGACAGGTCGAAACACGGNNNNNNNNNNNNTTGCGGATCCGTCTCCTTCTGGTTCAGAG-5'

5'- CATGGTTCTACAACTGTCCAGCTTTGTGCCNNNNNNNNNNNNAACGCCTAGGCAGAGGAAGACCAAGTCTC-3'

454BL ANNEALS TO COMPLEMENTARY TAG-SEQUNCE OF TAGGED PRIMERS

5'- TCGCGCCATCAGACGAGTGCGTCATGGTTCTACA--->

3'- GTACCAAGATGTTGACAGGTCGAAACACGGNNNNNNNNNNNNTTGCGGATCCGTCTCCTTCTGGTTCAGAG-5'

5'- CATGGTTCTACAACTGTCCAGCTTTGTGCCNNNNNNNNNNNNAACGCCTAGGCAGAGGAAGACCAAGTCTC-3'

<---TCTGGTTCAGAGTGCGTGAGCAGACTCGCCCGAC-5'

SEMI-INCORPORATED 454BL OF PRIMER SPECIFIC TARGET DNA

5'- TCGCGCCATCAGACGAGTGCGTCATGGTTCTACAACTGTCCAGCTTTGTGCCNNNNNNNNNNNNAACGCCTAGGCAGAGGAAGACCAAGTCTC-3'

3'- GTACCAAGATGTTGACAGGTCGAAACACGGNNNNNNNNNNNNTTGCGGATCCGTCTCCTTCTGGTTCAGAGTGCGTGAGCAGACTCGCCCGAC-5'

454BL ANNEALS TO COMPLEMENTARY TAG-SEQUNCE OF PRIMER SPECIFIC TARGET DNA WITH SEMI-INCORPORATED 454BL

<---TCTGGTTCAGAGTGCGTGAGCAGACTCGCCCGAC-5'

5'- TCGCGCCATCAGACGAGTGCGTCATGGTTCTACAACTGTCCAGCTTTGTGCCNNNNNNNNNNNNAACGCCTAGGCAGAGGAAGACCAAGTCTC-3'

3'- GTACCAAGATGTTGACAGGTCGAAACACGGNNNNNNNNNNNNTTGCGGATCCGTCTCCTTCTGGTTCAGAGTGCGTGAGCAGACTCGCCCGAC-5'

5'- TCGCGCCATCAGACGAGTGCGTCATGGTTCTACA--->

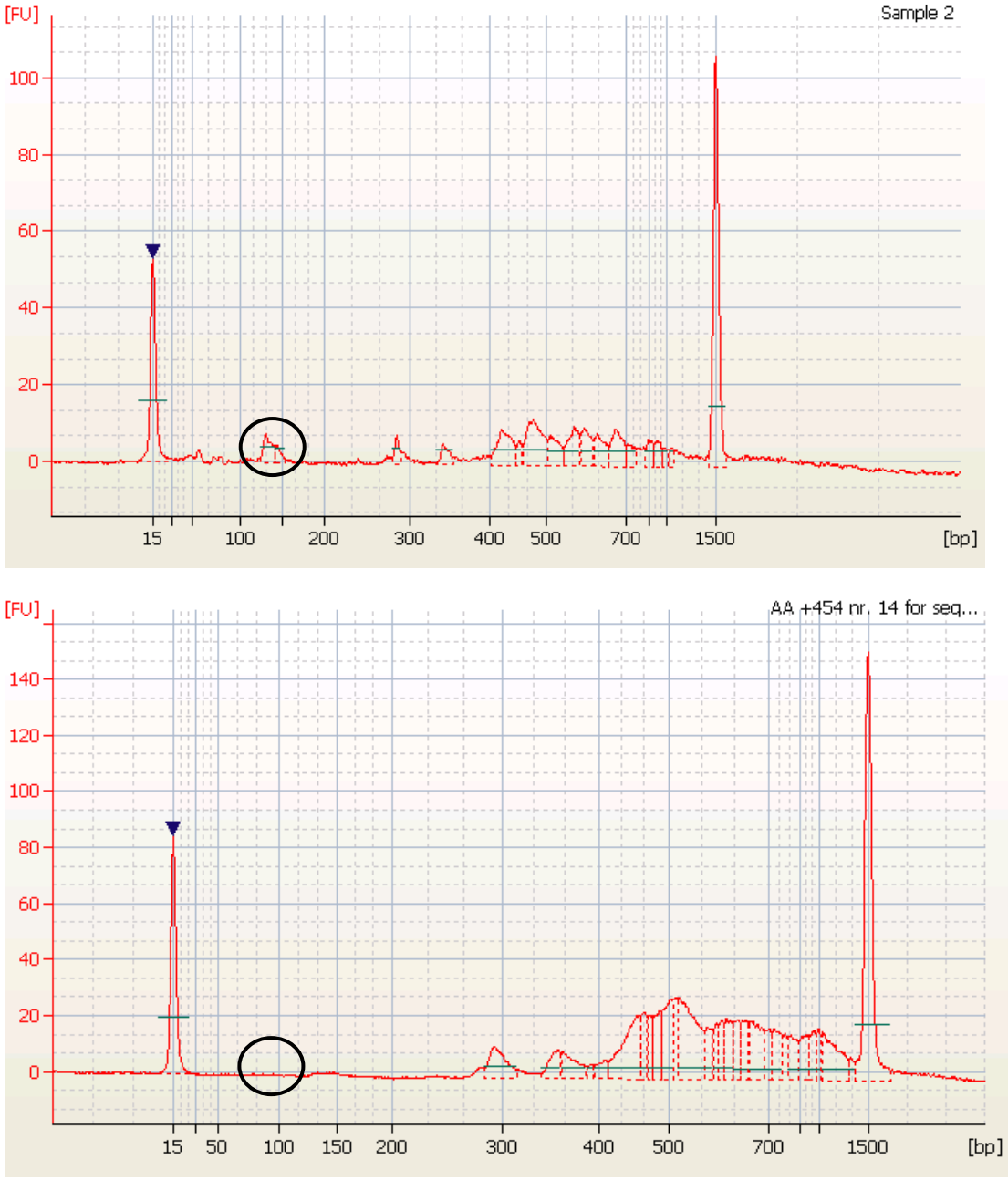
FULLY INCORPORATED 454BL OF PRIMER SPECIFIC TARGET DNA, READY FOR 454 SEQUENCING

3'- AGCGCGGTAGTCTGCTCACGCAGTACCAAGATGTTGACAGGTCGAAACACGGNNNNNNNNNNNNTTGCGGATCCGTCTCCTTCTGGTTCAGAGTGCGTGAGCAGACTCGCCCGAC-5'

5'- TCGCGCCATCAGACGAGTGCGTCATGGTTCTACAACTGTCCAGCTTTGTGCCNNNNNNNNNNNNAACGCCTAGGCAGAGGAAGACCAAGTCTCACGCACTCGTCTGAGCGGGCTG-3'

Appendix A continued.

Appendix B: PCR amplicon purification for 454-sequencing



DNA electropherogram of PCR amplicons harvested from the AA48.48. A) Harvested PCR product from DNA extracted from intestinal luminal content from one of the piglet included in Paper I before the purification process. The black ring encircles an unspecific peak which most likely represents PCR-bi-products. B) Pooled harvested PCR products from DNA extracted from intestinal luminal content from every piglet included in Paper I after removal of unwanted PCR-bi-products. The piglet samples were harvested individually, pooled in equal concentrations, run on an agarose gel, and the specific bands excised after which the DNA was extracted and measured once more.

Appendix C: Primer amplification efficiencies

Individual amplification efficiencies for the primer sets constituting the Gut Microbiotassay tested on 15 pure-cultured reference bacterial strains.

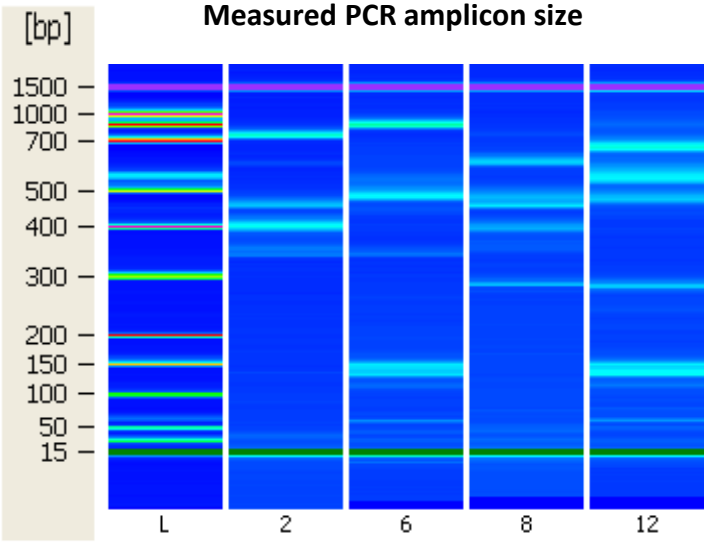
Primer set	Bacterial reference strain														
	Methanocorpusculum labreanum	*Brachyspira pilosicoli*	*Verrucomicrobium spinosum*	*Campylobacter fetus*	*Bilophila wadsworthia*	*Escherichia coli*	*Fusobacterium necrophorum*	*Bifidobacterium pseudolongum globosum*	*Bacteroides fragilis*	*Roseburia* sp.	*Faecalibacterium prausnitzii*	*Clostridium perfringens*	*Lactobacillus sakei*	*Streptococcus suis*	*Enterococcus faecalis*
Domain Bacteria A V2-V3		97	160	78	73	90	105	66	74	97	82	99	93	93	80
Domain Bacteria B V4-V5		75	106	89	82	85	93	75	88	87	89	80	100	83	88
Phylum Firmicutes										174	166	88	92	97	86
Class Bacilli													101	99	107
Genus *Enterococcus*															87
Genus *Streptococcus*														102	
Genus *Lactobacillus*													112		
Family Clostridium cluster I												96			
Species *Clostridium perfringens*												87			
Family Clostridium cluster IV											103				
Family Clostridium cluster XIV										96					
Phylum Bacteroidetes									91						
Genus *Bacteroides*									85						
Phylum Actinobacteria								92							
Family Bifidobacteriaceae								77							
Phylum Fusobacteria							85								
Class Beta- and Gamaproteobacteria						87									
Family Enterobacteriacea						84									
Species *Escherichia coli*						91									
Class deltaproteobacteria					91										
Class epsilonproteobacteria				86											
Phylum Verrucomicrobia			107												
Phylum Spirochaetes		80													
Domain Archaea	95														

Appendix D: Validation of successful incorporation of 454 barcode library

Expected PCR amplicon size

Lane number	2	6	8	12
Primer system	*E. coli* – 454	*E. coli* + 454	*Cl. per.* – 454	*Cl. per.* + 454
Domain Bacteria A V2-V3	380	494	380	494
Domain Bacteria B V4-V5	433	547	433	547
Phylum Firmicutes	-	-	473	587
Family Clostridium cluster I	-	-	582	696
Species *Clostridium perfringens*	-	-	258	258
Class β- and γ-proteobacteria	717	831	-	-
Family Enterobacteriacea	377	491	-	-
Species *Eschericha coli*	320	320	-	-

Measured PCR amplicon size



Expected and measured amplicon sizes (base pairs, bp) for *E. coli* and *Cl. perfringens* with (+454) and without (–454) 454 barcode library added. The table lists the expected amplicon sizes dependent on whether a 454 barcode library has been added or not. Gel picture shows PCR bands of amplicons harvested from the AA48.48. If the primers have been tagged the 454 barcode library will be incorporated. This is the case where the amplicon sizes are larger in lane 6 and 12 compared with lane 2 and 8, respectively. If the primers were not tagged, the 454 barcode library did not bind and the size of the amplicons remains the same. This is true for the species-specific primer sets targeting *E. coli* and *Cl. perfringens*. The other bands represent the PCR products from the higher taxonomy ranking primer sets also targeting *E. coli* and *Cl. perfringens.* However, from lane 6 and 12 it is also evident that the 454 barcode library causes some PCR-bi-products around 150 bp. L: ladder; 2: *E. coli* – 454 barcode library; 6: *E. coli* + 454 barcode library; 8: *Cl. perfringens* – 454 barcode library; 12: *Cl. perfringens* + 454 barcode library.

Appendix E: Sample preparations for different DNA extraction methods

For all DNA extraction methods reagents were enclosed with the kits unless stated otherwise.

D.1 QIAcube

Intestinal contents were stored at −80 °C, and 200 mg of intestinal content were suspended in 1.3 ml ASL buffer, followed by a short vortex and thoroughly homogenized by bead beating (Tissuelyser II, Qiagen, Hilden, Germany) with 0.1 mm-diameter zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK, USA) for 2 min. Suspensions were heated for 5 min at 70 °C, vortexed and centrifuged 1 min at 13 000 RPM, 20 °C. 1.2 ml of supernatant was transferred to new microcentrifuge tubes, an InhibitEX tablet was added and each sample was vortexed for 1 min followed by incubation for 1 min at room temperature. The samples were centrifuged at 13 000 RPM for 3 min at 20 °C. The supernatant was transferred to new 1.5 ml Eppendorf tubes and subsequently centrifuged for 3 min at 20 000 × *g* at 20 °C. After transferring 360 µl of supernatant to new 2 ml microcentrifuge tubes the samples were stored on ice until they were placed in the QIAcube (Qiagen) robot for DNA extraction using the QIAamp® DNA Stool Mini Kit (Qiagen) with the "Pathogen detection" protocol.

D.2 Maxwell

D.2.1 16 Blood DNA Purification Kit, AS1010 and 16 Cell LEV DNA Purification Kit, AS1140

Intestinal content samples were stored at −80 °C. 200 mg of intestinal content was suspended in 1300 µl PBS followed by vortexing. The supernatant was transferred to a microcentrifuge tube with 0.1 mm-diameter zirconia/silica beads (BioSpec Products Inc.) and homogenized thoroughly by bead beating (Tissuelyser II, Qiagen) for 2 min. Afterwards samples were centrifuged for 3 min at 13 000 RPM. Elution buffer (50 µl) was added to the bottom of the elution tubes, and 200 µl of the suspensions were transferred to the sample wells on the cartridge and DNA was extracted from intestinal content using either Maxwell® 16 Blood DNA Purification Kit, AS1010 (Promega) or 16 Cell LEV DNA Purification Kit, AS1140 (Promega) following the manufacturer's technical bulletin.

D.2.2 16 LEV Blood DNA Kit, AS1290

The samples were stored at −80 °C. 200 mg of intestinal content was suspended in 600 µl PBS followed by vortexing. The supernatant was transferred to a new tube and mixed with 350 µl lysisbuffer. The total volume was passed on to a microcentrifuge tube with a 5 mm steel bead (Qiagen) and homogenized thoroughly by bead beating (Tissuelyser II, Qiagen) for 2 min. Afterwards samples were centrifuged for 1 min at 1000 × *g*, followed by another transfer to new 2 ml tubes, and mixed with 30 µl Proteinase K. Then samples were incubated for 30 min at 56 °C, and then centrifuged at 13 000 × *g* for 1 min. All of the suspension was transferred to the sample inlet on the cartridge and 50 µl of elution buffer was added to

the bottom of the elution tubes and DNA was extracted from intestinal content using Maxwell® 16 LEV Blood DNA Kit, AS1290 (Promega) following manufacturer's instruction.

D.3 MagMAX

Intestinal contents were stored at −80 °C. 100 mg of intestinal content were suspended in 900 µl PBS buffer, followed by a short vortex and thoroughly homogenized by bead beating with a (Tissuelyser II, Qiagen) for 2.5 min at 20.0 hertz with a 5 mm steel bead (Qiagen). The suspension was centrifuged for 1 min at 1000 × *g*, and 50 µl of supernatant was transferred to the sample vessels, and DNA was extracted from intestinal content using MagMAX™-96 Viral RNA Isolation Kit, AM1836 (Applied Biosystems®) according to manufacturer's instruction.

Appendix F: NanoDrop measurements of DNA extracted by different methods

Comparable DNA measurements obtained with the NanoDrop® ND-1000 (NanoDrop Technologies Inc) spectrophotometer are listed together. Not all DNA extraction methods were applied to all samples, however, at least two methods are compared of which one of them is the method of choice. The colored rows represent DNA extraction using the Maxwell AS1290. This method was applied for all samples included in Paper II and Paper III.

Method	ID	Gut section	ng/µL	A260	260/280	260/230
QIAcube	16	ileum	2.68	0.054	1.93	0.85
Maxwell AS1290	16	ileum	116.3	2.326	1.96	1.58
QIAcube	16	colon	17.82	0.356	2.01	1.83
Maxwell AS1290	16	colon	169.01	3.38	1.93	1.63
Maxwell AS1010	27	colon	55.21	1,104	1.92	1.49
Maxwell AS1140	27	colon	41.32	0.826	1.9	1.96
Maxwell AS1290	27	colon	385.05	7.701	2.08	1.86
MagMAX	27	colon	59.4	1,188	2.13	1.57
QIAcube	30	ileum	13	0.26	1.98	2
Maxwell AS1290	30	ileum	646.93	12.939	1.94	2.09
Maxwell AS1010	30	colon	44.29	0.886	1.91	0.85
Maxwell AS1140	30	colon	53.04	1,061	1.96	1.96
Maxwell AS1290	30	colon	517.29	10.346	2.02	1.66
MagMAX	30	colon	94.52	1,890	2	0.52
QIAcube	35	colon	29.84	0.597	1.93	1.92
Maxwell AS1010	35	colon	8.59	0.172	1.47	0.19
Maxwell AS1290	35	colon	90.18	1.804	1.78	0.97
QIAcube	48	colon	22.82	0.456	1.91	2.06
Maxwell AS1010	48	colon	34.87	0.697	1.78	0.61
Maxwell AS1290	48	colon	595.66	11.913	1.94	1.45
QIAcube	56	colon	5.18	0.104	1.53	1.32
Maxwell AS1010	56	colon	12.09	0.242	1.53	0.41
Maxwell AS1290	56	colon	149.7	2.994	1.84	1.24
Maxwell AS1010	59	ileum	9.28	0.186	2.22	0.41
Maxwell AS1140	59	ileum	16.49	0.33	1.68	0.85
Maxwell AS1290	59	ileum	76.07	1.521	1.98	1.75
MagMAX	59	ileum	24.32	0.486	2.36	1.7
Maxwell AS1010	59	colon	27.04	0.541	1.96	0.63
Maxwell AS1140	59	colon	24.56	0.491	1.84	1.97
Maxwell AS1290	59	colon	372.33	7.447	2.13	2.1
MagMAX	59	colon	35.59	0.712	2.25	1.89
QIAcube	72	ileum	4.77	0.095	1.81	1.12
Maxwell AS1290	72	ileum	123.18	2.464	1.87	1.13

Maxwell AS1010	77	colon	40.31	0.806	1.96	1.27
Maxwell AS1140	77	colon	31.2	0.624	1.9	1.52
Maxwell AS1290	77	colon	208.24	4.165	1.99	1.84
MagMAX	77	colon	49.64	0.993	2.1	1.36
Maxwell AS1010	85	ileum	-322.38	-6,448	0.34	0.15
Maxwell AS1140	85	ileum	10.28	0.206	1.89	1.33
Maxwell AS1290	85	ileum	116.33	2.327	1.89	1.54
MagMAX	85	ileum	27.52	0.55	2.24	1.83
Maxwell AS1010	85	colon	24	0.48	1.98	0.95
Maxwell AS1140	85	colon	31.12	0.622	1.85	1.28
Maxwell AS1290	85	colon	155.12	3.102	1.92	1.4
MagMAX	85	colon	51.61	1,032	2.03	1.49
Maxwell AS1010	93	colon	55.53	1,111	1.89	1.28
Maxwell AS1140	93	colon	23.69	0.474	1.76	1.09
Maxwell AS1290	93	colon	123.52	2.47	1.89	0.98
MagMAX	93	colon	38.7	0.774	1.98	0.71
QIAcube	96	ileum	25.22	0.504	2.14	2.01
Maxwell AS1290	96	ileum	2238.17	44.763	2.11	2.15
QIAcube	96	colon	37.24	0.745	1.95	2
Maxwell AS1290	96	colon	155.88	3.118	1.86	1.26
Maxwell AS1010	123	ileum	9.53	0.191	1.83	0.41
Maxwell AS1140	123	ileum	21.39	0.428	1.94	1.56
Maxwell AS1290	123	ileum	1254.15	25.083	2.08	2.16
MagMAX	123	ileum	183.09	3,662	2.17	1.79
Maxwell AS1010	123	colon	23.58	0.472	1.88	1.09
Maxwell AS1140	123	colon	27.23	0.545	1.89	1.81
Maxwell AS1290	123	colon	103.44	2.069	1.9	1.39
MagMAX	123	colon	37.43	0.749	2.09	1.52
Maxwell AS1010	134	colon	66.06	1,321	1.92	1.69
Maxwell AS1140	134	colon	50.6	1,012	1.91	2.06
Maxwell AS1290	134	colon	561.22	11.224	1.91	1.58
MagMAX	134	colon	79.02	1,580	2.08	1.19
QIAcube	139	ileum	35.26	0.705	2.11	1.94
Maxwell AS1290	139	ileum	343.93	6.879	2.04	1.86
Maxwell AS1010	154	colon	6.08	0.122	1.76	0.21
Maxwell AS1140	154	colon	17.73	0.355	1.77	0.86
Maxwell AS1290	154	colon	642.91	12.858	1.95	1.61
MagMAX	154	colon	30.36	0.607	2.37	0.06
QIAcube	172	ileum	4.34	0.087	1.72	1.1
Maxwell AS1290	172	ileum	111.77	2.235	1.92	1.63
QIAcube	215	colon	16.24	0.325	1.93	1.79
Maxwell AS1290	215	colon	122.94	2.459	1.96	1.78

Discussion and conclusion

Discussion

15. The methods approaches

Through its various effects on gut maturation, stimulation of the immune system, defense against gut colonization by pathogens, metabolism of essential vitamins, and production of SCFAs the gut microbiota has an immense impact on the health and disease of the host [31,32,35,172-174]. In case of diarrhoea the microbiota is inevitable affected as a consequence of pathophysiological factors, such as: altered intestinal peristaltic; increased amount of intestinal luminal fluidics; and accelerated transit time [175]. Thus, whether or not gut microbial changes cause diarrhoea or diarrhoea induces gut microbial changes it can be expected that the gut microbiota from diarrhoeic piglets will differ from the gut microbiota from healthy piglets. Traditionally, culturing has been the gold standard approach used to study bacteria in the gut. However, modern molecular methods have gained a significant role when investigating bacterial diversity through the acknowledgement that the majority of the intestinal bacteria are still not culturable [173,176]. Several feasible molecular methods can be applied to characterize microbial communities, and which method to choose depends primarily on the type of information requested [162].

In Paper I the design, test, optimization, and verification of the Gut Microbiotassay was published. This approach offers deeper taxonomic resolution via the possibility of subsequent 454 next generation sequencing [120]. Unlike general sequencing approaches applied to study microbial ecology, which typically include the use of general primer sets to amplify 'all bacteria' by targeting conserved DNA genes followed by sequencing, the Gut Microbiotassay has some advantages: 1) it offers relative quantitative information of the abundance of some of the major bacterial groups inhabiting the gastrointestinal tract; 2) there is an increased likelihood of amplifying and detecting more diverse bacteria, because sequences are generated from use of 22 different primer pairs instead of just one; 3) the informative heatmap and the architecture of the AA48.48 make it possible to select interesting samples for sequencing, which reduce costs or improve the taxonomic information; and 4) the multi-target strategy that the Gut Microbiotassay applies in combination with sequencing enables search for unknown microorganisms indifferent to for example microarrays which only offer the possibility of getting information generated by probes targeting known microorganisms.

Compared with alternative high-throughput qPCR platforms (not launched by Fluidigm), the AA48.48 offers great flexibility, as primers are loaded manually, and though it provides a medium throughput it is still in the high end. Moreover, the possibility to harvest the PCR amplicons offers deeper insight into the contents

of the PCR amplicons (Table 5). Nonetheless, it does require the purchase of rather expensive equipment, though probably rentable in the long run.

Table 5: Comparison of three high-throughput qPCR platforms.

qPCR Platform	SlipChip	OpenArray™ Applied Biosystems	Access Array 48.48 Fluidigm
Sample throughput	2	48	48
Primer pair capacity	40 – 384	64	48
Reaction volume, nl	7 – 26	33	35
Comments	• Does not require expensive equipment • Possible to harvest amplicons after qPCR	• Primers are preloaded by the manufacturer • Samples cannot be harvested	• Amplicons can be harvested • Easy library preparation for NGS • Easy interpretable software
Reference	[177]	[178]	[120]

However, there are also some limitations to this approach which impact the final outcome and the results. As aforementioned, the AA48.48 is susceptible to the general PCR limitations. Some of the encountered complications are discussed in the following. The performance of the primers is influenced by numerous factors including: 3'-end specificity, amplicon size, melting temperature (T_m, the temperature at which double-stranded DNA melts into single-stranded DNA), and template base composition [153,179]. Though it would be optimal to test and verify the performance and efficiency of all primers against all bacteria, this is not practically possible. Hence, the primer sets constituting the Gut Microbiotassay were tested against 15 selected reference bacterial strains. Varying primer efficiencies are unavoidable since primer sets targeting bacterial groups have to cover a wide spectrum of diverse strains with different target sequences G+C-content. The higher the G+C-content, the higher the T_m. If primers are to anneal efficiently it is important that template DNA denatures completely [180]. Thus, higher G+C-contents increases the risk of these sequences being under-represented, as increment of the T_m enhances the risk of the DNA templates not being properly denatured [160]. The reference bacteria tested included species of different G+C-content, ranging from approximately 27 % for *Campylobacter fetus* to around 60 % for *Bifidobacterium pseudolongum* subsp. *globosum*, *Bilophila wadsworthia*, and *Verrucomicrobium spinosum* [181,182]. Consequently, the primer efficiencies for the different bacterial strains varied, and especially the general bacterial primer "Domain Bacteria A V2-V3" demonstrated the poorest performance for the three G+C-rich bacterial strains (Appendix C) [120]. Additionally, a broader coverage can compromise the 3'-end specificity of the primers which also contributes to varying primer efficiencies dependent on the composition of the bacterial strains present in the sample DNA. What complicates performance of the primer sets even further is that all of the primer sets are applied to one AA48.48, which means that they are all subject to the same running conditions.

The varying efficiencies complicate the subsequent quantification. One way to get around this issue is to run pooled sample calibration curves, and use the calculated primer efficiencies to adjust the Cq values. However, as shown in Paper I, the adjusted Cq values will never be able to reflect the real case scenario, as different bacterial strains will result in different primer efficiencies, and therefore depend on the bacterial composition of the sample [120]. Therefore an adjusted Cq value based on a pool of samples could reflect a consistent approximate average, however, it may as well be the result of a few samples with diverging bacterial communities pooling the Cq value. For that reason it can be discussed whether adjusted Cq values should be used or not. Paper I and II do not apply adjusted Cq values, whereas Paper III does. Interestingly, Paper II and Paper III are still able to detect much of the same gut microbial changes in piglets dependent on diarrhoeic status, which suggests that both adjusted and unadjusted Cq values can be applied.

Another parameter affecting the outcome is the bacterial 16S and 23S rRNA gene copy numbers which may differ by a factor 15 [159]. This means that bacteria with a high gene copy number by default are more numerous than bacteria with a low gene copy number and are more likely to generate low Cq values. Alternative, other genes can be targeted, as for instance the *cpn*60 which only exists in a single copy number. Nevertheless, this gene is not as commonly applied in microbial studies, and as a consequence related gene databases do not contain as much taxonomic information compared with 16S or 23S rRNA gene databases [162].

As a result of the above-mentioned issues qPCR data on microbial communities will always be semi-quantitative, though they will indeed be useful indicatives.

As for interpretation of reads generated by 454 next generation sequencing, this is highly dependent on the applied bioinformatics and level of similarities. In Paper I and II BION-meta was used for bioinformatics. This is an open source program developed by Dr Niels Larsen from Danish Genome Institute and customized to the Gut Microbiotassay as requested by the author. Default settings were used in the analysis of sequence data with similarities of 95 % to delineate species. This means that conclusions drawn from sequencing results should not be construed as final conclusions, but rather as possible suggestions of which bacterial genera, species or maybe even strains could be interesting to search for in future studies employing additional techniques. Also, due to nature's enormous variation, classification at species and particularly at strain level is connected with high uncertainty, as the databases, though continuously growing, will always be inferior to nature's diversity which they will never be able to reflect.

Moreover, the gel-purification step of the PCR amplicons preceding the 454-sequencing, may also introduce bias. The method is highly susceptible to correct excision of gel-bands. If too many are excised, this will result in loss of sequence information, as the higher number of amplicons will be limited to a

specific sequence capacity. Contrary, if too few are excised, this will also lead to loss of information, as information from excluded bands obviously are not acquired. The former statement is especially problematic if included products are smaller than the target-specific products (primer-dimers), as the 454-sequencer prefers smaller products over larger products and as result will sequence these rather than the target-specific products (personal communication, G. J. Nyakatura, LGC Genomics).

Finally, it must be mentioned that though the Gut Microbiotassay employs a multi-target strategy and covers many diverse gut bacteria, it does not cover *all* bacteria, and hence is not a reflection of the true gut microbial composition. Thus, there is a slight risk that NNPD-related bacteria might have been overlooked.

16. The gut microbiota and NNPD

The applied approached in all of the studies were able to distinguish the gut microbiota from diarrhoeic piglets from the gut microbiota of healthy control piglets. As expected the results revealed that NNPD was associated with a general disturbed composition of the gut microbiota, and findings across all studies were actually quite similar. The overall gut microbial changes significantly related to NNPD was the following: a depletion of bacteria belonging to the phyla Firmicutes, Actinobacteria, and Bacteroidetes, and an increased abundance of genus *Enterococcus* and species *E. coli.* This also account for the piglets examined in Paper I, which were excluded from the general field study investigating NNPD, due to varying inclusion criteria. However, it can be discussed whether these changes are truly related to NNPD, or if they are related to diarrhoea in general or other influencing factors. In fact, it has previously been demonstrated that non-infectious diarrhoea in humans leads to fecal gut microbial changes in the shape of decreased phylotype richness, and that diarrhoeic individuals were characterized by a decreased abundance of the phyla Firmicutes and Bacteroidetes, while Proteobacteria increased [175]. Accordingly, if disregarding the *homo sapiens-sus scrofa domestica* species difference as well as the intestinal origin of sample-material, but also Actinobacteria which are diminished in other intestinal disorders [183,184], these results suggest that the number one involved cause of NNPD shall be found within genus *Enterococcus.* This confirms with results published by a parallel Swedish research program that also investigates neonatal porcine diarrhoea (NPD) which appears to have much resemblance to NNPD [116].

Genus *Enterococcus* is comprised of Gram-positive, facultative anaerobic bacteria, which means that they are able to take advantage of the increased oxygen tension that is present in the intestinal atmosphere of newborns, and which they (and others) gradually utilize thereby creating a favorable environment for the strict anaerobic bacteria [185]. Members that constitute genus *Enterococcus* include bacteria reported to be potential pathogens involved in diarrhoea (for example *E. durans* and *E. hirae*) [58,115,116], but also beneficial gut commensal bacteria (for example *E. faecium and E. faecalis*) [117,186,187]. Some

Enterococcus strains express pili which contribute to their virulence and has been demonstrated to be up-regulated in the presence of bicarbonate which is commonly lost into the intestinal lumen as a consequence of diarrhoea resulting in increased luminal concentrations [188,189]. An important feature of genus *Enterococcus* is its high level of intrinsic but also acquired resistance to antibiotics [112,113]. This feature allows Enterococci to take advantage of the antibiotic-induced gut microbial disturbance, why it is often associated with nosocomial infections ("*nosos*": disease + "*komeion*": to take care of; hence, disease acquired while under medical care)[190]. However, recent research has demonstrated that the pili expressed by virulent Enterococci can be a target of immunotherapy, which is promising considering the prevalence of antibiotic resistance reported for this genus [191].

As mentioned in the introduction numerous factors can contribute to the development of diarrhoea including infectious agents, inadequate colostrum supply, wrong management, poor climate, and treatment with antibiotics. The conclusions drawn from the results generated in Paper II have undoubtedly been accompanied by some of these factors, though measures were taken to reduce many of them. However, the results generated in Paper III where NNPD was experimentally re-produced in healthy piglets under highly controlled conditions, thereby eliminating many of these factors, support the findings in Paper II. Concurrently, these results also suggest that bacteria belonging to the class Gammaproteobacteria including species *E. coli* might not be as important in the pathology of NNPD as proposed in Paper II. The findings related to *E. coli* were not as profound in Paper III compared with those in Paper II. Besides, Proteobacteria have been shown to increase as response to treatment with antibiotics [192], and approximately 50 % of the case piglets included in Paper II had been given antibiotics.

Paper II suffers from the lack of true control piglets from unaffected pig farms. Though the control piglets included in Paper II never suffered from diarrhoea, they were selected from the non-affected, or the least affected litters from the same farrowing unit, and could be potentially infected. Nonetheless, the supportive results found in Paper III, which included true control piglets collected from unaffected pig farms, implies that the control piglets included in Paper II were sufficient. The additional studies examining the same piglets by other means were also able to distinguish case and control piglets [1,2].

Paper III demonstrated that inoculating piglets orally with intestinal-material at an early age affected the gut microbial composition. Within the first days of life the microbial succession in the gastrointestinal tract forms the basis of the future gut microbial establishment, and thus this event has an immediate as well as a long term impact on animal health [193]. NNPD and diarrhoea is related with a depletion of the phyla Firmicutes, Actinobacteria, and Bacteroidetes. Therefore, it can be speculated if administering inoculum containing bacterial members from these phyla or inoculation with intestinal material from healthy piglets can have a preventative effect on the development of NNPD [194].

Finally, the gut microbial changes detected in Paper I-III may simply reflect the effect of suffering from NNPD caused by other infectious agents. A selection of case piglets included in Paper II were thoroughly tested for a range of viruses including viruses previously associated with enteric disorders in pigs or humans. The conclusion was that there was no evidence of virus being involved in NNPD (unpublished results; LE Larsen, National Veterinary Institute, Technical University of Denmark). Moreover, histopathological examinations did not reveal any parasites [1]. However, in Paper III a significant up-regulation of two viral RNA detecting intracellular receptors (MD5A and RIG-1) was found in case piglets inoculated with NNPD-material from the NNPD-field cases investigated in Paper II. Contrary to the former conclusion, this suggests that virus could be involved, and ongoing studies suggest that it might be rotavirus. Rotavirus was detected in 2 % [1] and 9 % (unpublished results; LE Larsen) of the tested NNPD-field cases, and intestinal NNPD-material was not sorted or excluded in the preparation of the inocula. This means that even though virus does not seem to be involved in NNPD in field case piglets, there is a risk that virus is involved in NNPD in the NNPD-infection model. Though the possible involvement of unknown viruses cannot be ruled out, the low detection of known viral agents in the field cases points towards that the gut microbiota plays a significant part in NNPD.

Conclusion and perspectives

The present thesis presents a new approach which can be applied in the search for unknown bacterial pathogens. In addition, this approach was used to characterize the gut microbiota of NNPD-affected piglets, NNPD-infected piglets, and healthy piglets. All of the papers included in this thesis arrive at the same conclusion: bacteria belonging to the phyla Firmicutes, Actinobacteria, and Bacteroidetes are depleted, while species *E. coli* and in particular genus *Enterococcus* are increased in case of NNPD. Some of these changes seem to be related intestinal disorders or to diarrhoea in general (Firmicutes, Actinobacteria, and Bacteroidetes), and others might reflect the effect of antibiotics (*E. coli*). Remaining is genus *Enterococcus* as a possible cause or contributor to NNPD, and a high abundance of this genus was associated with high expressions of host transcripts involved in intestinal barrier function. Though interpretation of sequencing results at species level are connected with some uncertainty, these revealed five *Enterococcus* species of which *Enterococcus hirae* was the highest represented. Considering the similar findings from the Swedish study on NPD (the Swedish parallel to NNPD) this could be an intriguing lead to follow.

With the benefits of hindsight and the experience acquired during this thesis it can be concluded that the Gut Microbiotassay is a useful approach in case of searching for unknown bacterial agents. In the future a specific 'NNPD-assay' should be developed including specific primer sets targeting taxonomic levels within the bacterial groups that are characteristic of NNPD. This would give further taxonomic information by

zooming in on the relevant bacteria. Due to the announcement of the shutdown of 454-sequencers, another sequencing technique should be implemented, however, at the time of developing the Gut Microbiotassay, 454 next generation sequencing was the optimal approach.

As part of the sampling procedure in Paper II daily rectal swabs were collected from all piglets included in the study. It would have been fascinating to analyze these samples in order to obtain continuous gut microbial profiles of the bacterial shifts happening over the sampling period. This would provide new information on the succession of the intestinal gut microbiota in both healthy and NNPD-affected piglets, and may contribute to elucidate the development of NNPD.

Reference list

1. Kongsted H, Jonach B, Haugegaard S, Angen O, Jorsal SE, Kokotovic B, Larsen LE, Jensen TK, Nielsen JP: **Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome.** *BMC Vet Res* 2013, **9:**206.

2. Jonach B, Boye M, Stockmarr A, Jensen TK: **Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea.** *BMC Vet Res* 2014, **10:**68.

3. Kongsted H, Stege H, Toft N, Nielsen JP: **The effect of New Neonatal Porcine Diarrhoea Syndrome (NNPDS) on average daily gain and mortality in 4 Danish pig herds.** *BMC Vet Res* 2014, **10:**90.

4. Dyce KM, Sack WO, Wensing CJG: **The Digestive Apparatus.** In *Textbook of Veterinary Anatomy*. 3rd edition. Philadelphia, Pennsylvania: Saunders; 2002:100-147.

5. Butler JE, Sinkora M: **The enigma of the lower gut-associated lymphoid tissue (GALT).** *J Leukoc Biol* 2013, **94:**259-270.

6. Willing BP, Van Kessel AG: **Host pathways for recognition: Establishing gastrointestinal microbiota as relevant in animal health and nutrition.** *Livest Sci* 2010, **133:**82-91.

7. Gelberg HB: **Alimentary System.** In *Pathologic Basis of Veterinary Disease*. 4th edition. Edited by McGavin DM, Zachary JF. St. Louis, Missouri: Mosby Elsevier; 2007:301-391.

8. Frappier BL: **Digestive System.** In *Textbook of Veterinary Histology*. 5th edition. Edited by Dellmann HD, Eurell JA. Baltimore, Maryland: Williams & Wilkins; 1998:164-202.

9. Cunningham JG: **Digestion and absorption: the nonfermentative processes.** In *Textbook of Veterinary Physiology*. 3rd edition. Edited by Cunningham JG. Philadelphia, Pennsylvania: W.B. Saunders Company; 2014:254-279.

10. Williams AE: **Immunology of the Gastrointestinal Tract.** In *Immunology: Mucosal and Body Defences*. 1st edition. Chichester, UK: John Wiley & Sons, Ltd; 2011:133-155.

11. McGlone J, Pond WG: ***Pig Production: Biological Principles and Applications.*** 1st edition. New York: Thomson, Delmar Learning; 2003.

12. Cunningham JG: **Regulation of gastrointestinal fuction.** In *Textbook of Veterinary Physiology*. 3rd edition. Edited by Cunningham JG. Philadelphia, Pennsylvania: W.B. Saunders Company; 2014:254-279.

13. Mestecky J, Mcghee JR: **Immunoglobulin A (IgA): Molecular and Cellular Interactions Involved in IgA Biosynthesis and Immune Response.** *Adv Immunol* 1987, **40:**153-245.

14. Kiyono H, Goto Y: **Epithelial barrier: An interface for the cross-communication between gut flora and immune system.** *Immunol Rev* 2012, **245:**147-163.

15. Sompayrac L: **The innate immune system.** In *How the Immune System Works*. 4th edition. Chichester, UK: Wiley-Blackwell; 2011:13-23.

16. Jullien D, Stenger S, Ernst WA, Modlin RL: **CD1 presentation of microbial nonpeptide antigens to T cells.** *J Clin Invest* 1997, **99:**2071-2074.

17. Butler JE, Weber P, Sinkora M, Baker D, Schoenherr A, Mayer B, Francis D: **Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-dependent antigens.** *J Immunol* 2002, **169:**6822-6830.

18. Pabst R, Rothkotter HJ: **Postnatal development of lymphocyte subsets in different compartments of the small intestine of piglets.** *Vet Immunol Immunopathol* 1999, **72:**167-173.

19. Kelly D, Coutts AGP: **Development of digestive and immunological function in neonates: role of early nutrition.** *Livest Prod Sci* 2000, **66:**161-167.

20. Butler JE, Sinkora M, Wertz N, Holtmeier W, Lemke CD: **Development of the neonatal B and T cell repertoire in swine: implications for comparative and veterinary immunology.** *Vet Res* 2006, **37:**417-441.

21. Grammaticos PC, Diamantis A: **Useful known and unknown views of the father of modern medicine, Hippocrates and his teacher Democritus.** *Hell J Nucl Med* 2008, **11:**2-4.

22. Bengmark S: **Prospects for new and rediscovered therapies: probiotics and phage.** In *Fighting infection in the 21st century*. 1st edition. Edited by Andrew PW, Oyston P, Smith GL, Stewart-Tull DE. Malden, Massachusetts: Wiley-Blackwell; 2005:97-132.

23. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA, Stobberingh EE: **Factors influencing the composition of the intestinal microbiota in early infancy.** *Pediatrics* 2006, **118:**511-521.

24. Morelli L: **Postnatal development of intestinal microflora as influenced by infant nutrition.** *J Nutr* 2008, **138:**1791S-1795S.

25. Swords WE, WU CC, Champlin FR, Buddington RK: **Postnatal changes in selected bacterial groups of the pig colonic microflora.** *Biol Neonate* 1993, **63:**191-200.

26. Willing BP, Jansson JK: **The Gut Microbiota: Ecology and Function.** In *The fecal bacteria*. Edited by Sadowsky MJ, Whitman RL. Washington, DC: ASM Press; 2011:1-xii, 315.

27. Neish AS: **Microbes in Gastrointestinal Health and Disease.** *Gastroenterology* 2009, **136:**65-80.

28. Hooper LV, Midtvedt T, Gordon JI: **How host-microbial interactions shape the nutrient environment of the mammalian intestine.** *Annu Rev Nutr* 2002, **22:**283-307.

29. Hardin G.: **The competitive exclusion principle.** *Science* 1960, **131:**1292-1297.

30. Berg RD: **The indigenous gastrointestinal microflora.** *Trends Microbiol* 1996, **4:**430-435.

31. Willing BP, Van Kessel AG: **Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig.** *J Anim Sci* 2007, **85:**3256-3266.

32. Pabst R, Geist M, Rothkötter HJ, Fritz FJ: **Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnobiotic pigs.** *Immunology* 1988, **64:**539-544.

33. Stappenbeck TS, Hooper LV, Gordon JI: **Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells.** *Proc Natl Acad Sci U S A* 2002, **99:**15451-15455.

34. Kenworthy R, Crabb WE: **The intestinal flora of young pigs, with reference to early weaning, *Escherichia coli* and scours.** *J Comp Pathol Therap* 1963, **73:**215-228.

35. Bauer E, Williams BA, Smidt H, Verstegen MWA, Mosenthin R: **Influence of the gastrointestinal microbiota on development of the immune system in young animals.** *Curr Issues Intest Microbiol* 2006, **7:**35-51.

36. Vallès Y, Artacho A, Pascual-García A, Loreto Ferrús M, Jose Gosalbes M, José Abellán J, Francino MP: **Microbial Succession in the Gut: Directional Trends of Taxonomic and Functional Change in a Birth Cohort of Spanish Infants.** *Plos Genetics* 2014, **10**.

37. Thompson CL, Wang B, Holmes AJ: **The immediate environment during postnatal development has long-term impact on gut community structure in pigs.** *ISME J* 2008, **2:**739-748.

38. Brook I, Barrett CT, III Brinkman CR, Martin WJ, Finegold SM: **Aerobic and anaerobic bacterial flora of the maternal cervix and newborn gastric fluid and conjunctiva: a prospective study.** *Pediatrics* 1979, **63:**451-455.

39. Smith HW, Jones JE: **Observations on the alimentary tract and its bacterial flora in healthy and diseased pigs.** *J Pathol Bacteriol* 1963, **86:**387-412.

40. Smith HW: **The development of the flora of the alimentary tract in young animals.** *J Pathol Bacteriol* 1965, **90:**495-513.

41. Savage DC: **Microbial ecology of the gastrointestinal tract.** *Annu Rev Microbiol* 1977, **31:**107-133.

42. O'Hara AM, Shanahan F: **The gut flora as a forgotten organ.** *EMBO REPORTS* 2006, **7:**688-693.

43. Wang S, Liu G, Cai L, Ding J, Yin Y: **Development of micro-ecological system in small and large intestine of piglets.** In *Nutritional and physiological functions of amino acids in pigs*. 1st edition. Edited by Blachier F, Wu G, Yin Y. Wien: Springer; 2013:75-87.

44. Naito S, Hayashidani H, Kaneko K, Ogawa M, Benno Y: **Development of intestinal lactobacilli in normal piglets.** *J Appl Bacteriol* 1995, **79:**230-236.

45. Inoue R, Tsukahara T, Nakanishi N, Ushida K: **Development of the intestinal microbiota in the piglet.** *J Gen Appl Microbiol* 2005, **51:**257-265.

46. Konstantinov SR, Awati AA, Williams BA, Miller BG, Jones P, Stokes CR, Akkermans ADL, Smidt H, de Vos WM: **Post-natal development of the porcine microbiota composition and activities.** *Environ Microbiol* 2006, **8:**1191-1199.

47. Petri D, Hill JE, Van Kessel AG: **Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig.** *Livest Sci* 2010, **133:**107-109.

48. Avunduk C: **Diarrhea.** In *Manual of Gastroenterology: Diagnosis and Therapy*. 4th edition. Philadelphia, Pennsylvania: Lippincott Williams & Wilkins; 2008:182-194.

49. Wagstrom EA, Yoon KJ, Zimmerman JJ: **Immune components in porcine mammary secretions.** *Viral Immunol* 2000, **13:**383-397.

50. Leiser R, Kaufmann P: **Placental structure: in a comparative aspect.** *Exp Clin Endocrinol* 1994, **102:**122-134.

51. Rooke JA, Bland IM: **The acquisition of passive immunity in the new-born piglet.** *Livest Prod Sci* 2002, **78:**13-23.

52. Yao K, Sun Z, Liu Z, i Z, in Y: **Development of the gastrointestinal tract in pigs.** In *Nutritional and physiological functions of amino acids in pigs*. 1st edition. Edited by Blachier F, Wu G, Yin Y. Wien: Springer; 2013:75-87.

53. Wischner D, Kemper N, Krieter J: **Nest-building behaviour in sows and consequences for pig husbandry.** *Livest Sci* 2009, **124:**1-8.

54. Alonso-Spilsbury M, Ramírez-Necoechea R, González-Lozano M, Mota-Rojas D, Trujillo-Ortega ME: **Piglet survival in early lactation: A review.** *J Anim Vet Adv* 2007, **6:**76-86.

55. Berthon D, Herpin P, Ledividich J: **Shivering thermogenesis in the neonatal pig.** *J Therm Biol* 1994, **19:**413-418.

56. Corson AM, Litten JC, Clarke L: **The effect of abnormal intra-uterine growth on survival and growth performance of piglets.** *J Physol-London* 2000, **528P:**37P-38P.

57. Devillers N, Farmer C, Le Dividich J, Prunier A: **Variability of colostrum yield and colostrum intake in pigs.** *ANIMAL* 2007, **1:**1033-1041.

58. Gin T: **Diagnostic des nouveaux cas de diarrhées néonatales anzootiques du porcelet: évaluation de la prise colostrale.** *PhD Thesis.* Ecole Nationale Vétérinaire de Toulouse - ENVT; 2008.

59. Taylor DJ: ***Pig diseases.*** 8th edition. Lennoxtown, Glasgow: D.J. Taylor; 2006.

60. Thomson JR: **Diseases of the Digestive System.** In *Diseases of swine*. 9th edition. Edited by Straw BE. Ames, Iowa: Blackwell Publishing; 2006:37-56.

61. Lippke RT, Borowski SM, Marques SMT, Paesi SO, Almeida LL, Moreno AM, Corbellini LG, de Barcellos DESN: **Matched case-control study evaluating the frequency of the main agents associated with neonatal diarrhea in piglets.** *Pesq Vet Bras* 2011, **31:**505-510.

62. Yaeger M, Funk N, Hoffman L: **A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus.** *J Vet Diagn Invest* 2002, **14:**281-287.

63. Chan G, Farzan A, DeLay J, McEwen B, Prescott JF, Friendship RM: **A retrospective study on the etiological diagnoses of diarrhea in neonatal piglets in Ontario, Canada, between 2001 and 2010.** *Can J Vet Res* 2013, **77:**254-260.

64. Kauffmann F: **The serology of the coli group.** *J Immunol* 1947, **57:**71-100.

65. Nataro JP, Kaper JB: **Diarrheagenic *Escherichia coli*.** *Clin Microbiol Rev* 1998, **11:**142-201.

66. Batt CA, Tortorello M-L: ***Encyclopedia of Food Microbiology.*** 2nd edition. San Diego, California: Academic Press; 2014.

67. Gyles CL, Fairbrother JM: ***Escherichia coli*.** In *Pathogenesis of Bacterial Infections in Animals*. 4th edition. Edited by Gyles CL, Prescott JF, Songer JG, Thoen CO. Ames, Iowa: Wiley-Blackwell; 2010:267-308.

68. Songer JG, Uzal FA: **Clostridial enteric infections in pigs.** *J Vet Diagn Invest* 2005, **17:**528-536.

69. Songer JG: **Enteric Clostridia.** In *Pathogenesis of Bacterial Infections in Animals*. 4th edition. Edited by Gyles CL, Prescott JF, Songer JG, Thoen CO. Ames, Iowa: Wiley-Blackwell; 2010:211-229.

70. Leiting REED: **Dealing Out Piglet Diarrhea.** *National Hog Farmer* 2013, **58**.

71. Waters M, Savoie A, Garmory HS, Bueschel D, Popoff MR, Songer JG, Titball RW, McClane BA, Sarker MR: **Genotyping and phenotyping of beta2-toxigenic Clostridium perfringens fecal isolates associated with gastrointestinal diseases in piglets.** *J Clin Microbiol* 2003, **41:**3584-3591.

72. Lee KE, Lim SI, Shin SH, Kwon YK, Kim HY, Song JY, An DJ: **Distribution of *Clostridium perfringens* Isolates from Piglets in South Korea.** *J Vet Med Sci* 2014, **76:**745-749.

73. Farzan A, Kircanski J, DeLay J, Soltes G, Songer JG, Friendship R, Prescott JF: **An investigation into the association between cpb2-encoding *Clostridium perfringens* type A and diarrhea in neonatal piglets.** *Can J Vet Res* 2013, **77:**45-53.

74. Prescott JF, Chan G, Friendship R, Farzan A: **How do swine practitioners and veterinary pathologists arrive at a diagnosis of *Clostridium perfringens* type A enteritis in neonatal piglets?** *Can Vet J* 2013, **54:**504-506.

75. Gurtner C, Popescu F, Wyder M, Sutter E, Zeeh F, Frey J, von Schubert C, Posthaus H: **Rapid Cytopathic Effects of *Clostridium perfringens* Beta-Toxin on Porcine Endothelial Cells.** *Infect Immun* 2010, **78:**2966-2973.

76. Arbuckle JB: **The attachment of *Clostridium welchii* (*Cl. perfringens*) type C to intestinal villi of pigs.** *J Pathol* 1972, **106:**65-72.

77. Niilo L: ***Clostridium perfringens* Type C Enterotoxemia.** *Can Vet J* 1988, **29:**658-664.

78. Alvarez-Perez S, Blanco JL, Bouza E, Alba P, Gibert X, Maldonado J, Garcia ME: **Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets.** *Vet Microbiol* 2009, **137:**302-305.

79. Hopman NEM, Keessen EC, Harmanus C, Sanders IMJG, van Leengoed LAMG, Kuijper EJ, Lipman LJA: **Acquisition of *Clostridium difficile* by piglets.** *Vet Microbiol* 2011, **149:**186-192.

80. Songer JG, Anderson MA: ***Clostridium difficile*: An important pathogen of food animals.** *Anaerobe* 2006, **12:**1-4.

81. Poxton IR, McCoubrey J, Blair G: **The pathogenicity of *Clostridium difficile*.** *Clin Microbiol Infec* 2001, **7:**421-427.

82. Waters EH, Orr JP, Clark EG, Schaufele CM: **Typhlocolitis caused by *Clostridium difficile* in suckling piglets.** *J Vet Diagn Invest* 1998, **10:**104-108.

83. Larson DJ, Glock RD, Songer JG, Post KW, Jost BH: **Infection of neonatal swine with *Clostridium difficile*.** *J Swine Health Prod* 2000, **8:**185-189.

84. Schwegmann-Wessels C, Herrler G: **Sialic acids as receptor determinants for coronaviruses.** *Glycoconj J* 2006, **23:**51-58.

85. Murphy FA, Gibbs EPJ, Horzinek MC, Studdert MJ: **Coronaviridae.** In *Veterinary Virology*. 3rd edition. San Diego, California: Academic Publishers; 1999:495-508.

86. Hooper BE, Haelterman EO: **Lesions of the gastrointestinal tract of pigs infected with transmissible gastroenteritis.** *Can J Comp Med* 1969, **33:**29-36.

87. Estes MK, Palmer EL, Obijeski JF: **Rotaviruses: a review.** *Curr Top Microbiol Immunol* 1983, **105:**123-184.

88. Yuan L, Stevenson GW, Saif LJ: **Rotavirus and Reovirus.** In *Diseases of swine*. 9th edition. Edited by Straw BE. Ames, Iowa: Blackwell Publishing; 2006:435-454.

89. Barreiros MAB, Alfieri AA, Alfieri AF, Medici KC, Leite JPG: **An outbreak of diarrhoea in one-week-old piglets caused by group A rotavirus genotypes P[7],G3 and P[7],G5.** *Vet Res Commun* 2003, **27:**505-512.

90. Azevedo AS, Yuan L, Jeong KI, Gonzalez A, Nguyen TV, Pouly S, Gochnauer M, Zhang W, Azevedo A, Saif LJ: **Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus.** *J Virol* 2005, **79:**5428-5436.

91. Ward LA, Rosen BI, Yuan L, Saif LJ: **Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs.** *J Gen Virol* 1996, **77:**1431-1441.

92. Tzipori S, Williams IH: **Diarrhoea in piglets inoculated with rotavirus.** *Aust Vet J* 1978, **54:**188-192.

93. Ryan UM, Monis P, Enemark HL, Sulaiman I, Samarasinghe B, Read C, Buddle R, Robertson I, Zhou L, Thompson RCA, Xiao L: ***Cryptosporidium suis* n. sp (Apicomplexa : Cryptosporidiidae) in pigs (Sus scrofa).** *J Parasitol* 2004, **90:**769-773.

94. Fayer R, Ungar BLP: ***Cryptosporidium spp*. and cryptosporidiosis.** *Microbiol Rev* 1986, **50:**458-483.

95. Current WL: **Cryptosporidiosis.** *J Am Vet Med Assoc* 1985, **187:**1334-1338.

96. Moon HW, Bemrick WJ: **Fecal transmission of calf Cryptosporidia between calves and pigs.** *Vet Pathol* 1981, **18:**248-255.

97. Enemark HL, Bille-Hansen V, Lind P, Heegaard PMH, Vigre H, Ahrens P, Thamsborg SM: **Pathogenicity of *Cryptosporidium parvum*: Evaluation of an animal infection model.** *Vet Parasitol* 2003, **113:**35-57.

98. de Graaf DC, Vanopdenbosch E, Ortega-Mora LM, Abbassi H, Peeters JE: **A review of the importance of cryptosporidiosis in farm animals.** *Int J Parasitol* 1999, **29:**1269-1287.

99. Hamnes IS, Gjerde BK, Forberg T, Robertson LJ: **Occurrence of Cryptosporidium and Giardia in suckling piglets in Norway.** *Vet Parasitol* 2007, **144:**222-233.

100. Hamadejova K, Vitovec J: **Occurrence of the coccidium *Isospora suis* in piglets.** *Vet Med (Praha)* 2005, **50:**159-163.

101. Zhang WJ, Xu LH, Liu YY, Xiong BQ, Zhang QL, Li FC, Song QQ, Khan MK, Zhou YQ, Hu M, Zhao J: **Prevalence of coccidian infection in suckling piglets in China.** *Vet Parasitol* 2012, **190:**51-55.

102. Lindsay DS, Blagburn BL: **Biology of mammalian *Isospora*.** *Parasitol Today* 1994, **10:**214-220.

103. Harleman JH, Meyer RC: **Life cycle of *Isospora suis* in gnotobiotic and conventionalized piglets.** *Vet Parasitol* 1984, **17:**27-39.

104. Stuart BP, Lindsay DS, Ernst JV, Gosser HS: ***Isospora suis* enteritis in piglets.** *Vet Pathol* 1980, **17:**84-93.

105. Stuart BP, Gosser HS, Allen CB, Bedell DM: **Coccidiosis in swine: dose and age response to *Isospora suis*.** *Can J Comp Med* 1982, **46:**317-320.

106. Devriese LA, Vandekerckhove A, Kilpperbalz R, Schleifer KH: **Characterization and identification of *Enterococcus* species isolated from the intestines of animals.** *Int J Syst Bacteriol* 1987, **37:**257-259.

107. Švec P, Franz CMAP: **The genus *Enterococcus*.** In *Lactic Acid Bacteria*. 1st edition. Edited by Holzapfel WH, Wood BJB. Chichester: John Wiley & Sons, Ltd; 2014:175-211.

108. Jett BD, Huycke MM, Gilmore MS: **Virulence of Enterococci.** *Clin Microbiol Rev* 1994, **7:**462-478.

109. Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, Amorena B, Leiva J, Penadés JR: **The Enterococcal Surface Protein, Esp, Is Involved in *Enterococcus faecalis* Biofilm Formation.** *Appl Environ Microbiol* 2001, **67:**4538-4545.

110. Olmsted SB, Dunny GM, Erlandsen SL, Wells CL: **A plasmid-encoded surface protein on *Enterococcus faecalis* augments its internalized by cultured intestinal epithelial cells.** *J Infect Dis* 1994, **170:**1549-1556.

111. Nallapareddy SR, Singh KV, Sillanpaa J, Garsin DA, Hook M, Erlandsen SL, Murray BE: **Endocarditis and biofilm-associated pili of *Enterococcus faecalis*.** *J Clin Invest* 2006, **116:**2799-2807.

112. Ramos S, Igrejas G, Capelo-Martinez JL, Poeta P: **Antibiotic resistance and mechanisms implicated in fecal enterococci recovered from pigs, cattle and sheep in a Portuguese slaughterhouse.** *Ann Microbiol* 2012, **62:**1485-1494.

113. Ahmad A, Ghosh A, Zurek L, Schal C: **Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community.** *BMC Microbiol* 2011, **11**.

114. Cheon DS, Chae CH: **Outbreak of diarrhea associated with *Enterococcus durans* in piglets.** *J Vet Diagn Invest* 1996, **8:**123-124.

115. Johnson DD: **Enteric *Streptococcus durans*--An adhering *Streptococcus* as a cause of diarrhea in suckling piglets?** In *Twenty Fifth Annual George A. Young Conference*. Lincoln, Nebraska; 1984:1-8.

116. Larsson J, Lindberg R, Aspán A, Grandon R, Westergren E, Jacobson M: **Neonatal Piglet Diarrhoea Associated with Enteroadherent *Enterococcus hirae*.** *J Comp Pathol* 2014, **151:**137-147.

117. Taras D, Vahjen W, Macha M, Simon O: **Performance, diarrhea incidence, and occurrence of *Escherichia coli* virulence genes during long-term administration of a probiotic *Enterococcus faecium* strain to sows and piglets.** *J Anim Sci* 2006, **84:**608-617.

118. Bednorz C, Guenther S, Oelgeschlaeger K, Kinnemann B, Pieper R, Hartmann S, Tedin K, Semmler T, Neumann K, Schierack P, Bethe A, Wieler LH: **Feeding the Probiotic *Enterococcus faecium* Strain NCIMB 10415 to Piglets Specifically Reduces the Number of *Escherichia coli* Pathotypes That Adhere to the Gut Mucosa.** *Appl Environ Microbiol* 2013, **79:**7896-7904.

119. Mallo JJ, Rioperez J, Honrubia P: **The addition of *Enterococcus faecium* to diet improves piglet's intestinal microbiota and performance.** *Livest Sci* 2010, **133:**176-178.

120. Hermann-Bank ML, Skovgaard K, Stockmarr A, Larsen N, Mølbak L: **The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity.** *BMC Genomics* 2013, **14:**1-14.

121. Robinson IM, Whipp SC, Bucklin JA, Allison MJ: **Characterization of Predominant Bacteria from the Colons of Normal and Dysenteric Pigs.** *Appl Environ Microbiol* 1984, **48:**964-969.

122. Liu H, Zhang J, Zhang S, Yang F, Thacker PA, Zhang G, Qao S, Ma X: **Oral Administration of Lactobacillus fermentum I5007 Favors Intestinal Development and Alters the Intestinal Microbiota in Formula-Fed Piglets.** *J Agric Food Chem* 2014, **62:**860-866.

123. Ross R, Hall J, Orning A, Dale S: **Characterization of an *Actinobacillus* isolated from the sow vagina.** *Int J Syst Bacteriol* 1972, **22:**39-46.

124. MacInnes JI, Desrosiers R: **Agents of the "suis-ide diseases" of swine: *Actinobacillus suis*, *Haemophilus parasuis*, and *Streptococcus suis*.** *Can J Vet Res* 1999, **63:**83-89.

125. Wilson RJ, McOrist S: ***Actinobacillus suis* infection in pigs in Australia.** *Aust Vet J* 2000, **78:**317-319.

126. van der Wolf PJ, Vercammen TJM, Geene JJ, van Exsel ACA, Peperkamp NHMT, Voets MT, Zeeuwen AAPA: ***Salmonella typhimurium* DT104 septicaemia with meningitis in neonatal piglets.** *Vet Q* 2001, **23:**199-201.

127. Porter JR: **Antony van Leeuwenhoek: tercentenary of his discovery of bacteria.** *Bacteriol Rev* 1976, **40:**260-269.

128. Wieler LH, Ilieff A, Herbst W, Bauer C, Vieler E, Bauerfeind R, Failing K, Klos H, Wengert D, Baljer G, Zahner H: **Prevalence of enteropathogens in suckling and weaned piglets with diarrhoea in Southern Germany.** *J Vet Med B* 2001, **48:**151-159.

129. Nagy J, Bilkei G: **Neonatal piglet losses associated with Escherichia coli and Clostridium difficile infection in a Slovakian outdoor production unit.** *Vet J* 2003, **166:**98-100.

130. Yaeger MJ, Kinyon JM, Songer JG: **A prospective, case control study evaluating the association between Clostridium difficile toxins in the colon of neonatal swine and gross and microscopic lesions.** *J Vet Diagn Invest* 2007, **19:**52-59.

131. Cappuccio J, Quiroga M, Moredo F, Canigia L, Machuca M, Capponi O, Bianchini A, Zielinski G, Sarradell J, Ibar M, Vigo G, Giacoboni G, Perfumo C: **Neonatal piglets mesocolon edema and colitis due to *Clostridium difficile* infection: prevalence, clinical disease and pathological studies.** *Braz J Vet Pathol* 2009, **2:**35-40.

132. Morgan RL, Isaacson RE, Moon HW, Brinton CC, To CC: **Immunization of suckling pigs against enterotoxigenic *Escherichia coli*-induced diarrheal disease by vaccinating dams with purified 987**

or K99 pili: protection correlates with pilus homology of vaccine and challenge. *Infect Immun* 1978, **22:**771-777.

133. Riising HJ, Murmans M, Witvliet M: **Protection against neonatal *Escherichia coli* diarrhoea in pigs by vaccination of sows with a new vaccine that contains purified enterotoxic E-coli virulence factors F4ac, F4ab, F5 and F6 fimbrial antigens and heat-labile E-coli enterotoxin (LT) toxoid.** *J Vet Med B* 2005, **52:**296-300.

134. Springer S, Selbitz HJ: **The control of necrotic enteritis in sucking piglets by means of a *Clostridium perfringens* toxoid vaccine.** *FEMS Immunol Med Microbiol* 1999, **24:**333-336.

135. Gibbons RA, Sellwood R, Burrows M, Hunter PA: **Inheritance of resistance to neonatal *E. coli* diarrhoea in the pig: examination of the genetic system.** *Theor Appl Genet* 1977, **51:**65-70.

136. Ren J, Yan X, Ai H, Zhang Z, Huang X, Ouyang J, Yang M, Yang H, Han P, Zeng W, Chen Y, Guo Y, Xiao S, Ding N, Huang L: **Susceptibility towards Enterotoxigenic *Escherichia coli* F4ac Diarrhea Is Governed by the *MUC13* Gene in Pigs.** *Plos One* 2012, **7:**e44573.

137. Böhm R: **Disinfection and hygiene in the veterinary field and disinfection of animal houses and transport vehicles.** *Int Biodeter Biodegr* 1998, **41:**217-224.

138. Curtis SE: **The environment in swine housing.** In *Pork industry handbook*. Edited by Teague H, Alhusen HD. Washington, D.C.: U.S. Dept. of Agriculture, Office of Government and Public Affairs; 1979:1-4.

139. Scheidt AB, Cline TR, Clark LK, Mayrose VB, Alstine WGV, Diekman MA, Singleton WL: **The effect of all-in-all-out growing-finishing on the health of pigs.** *J Swine Health Prod* 1995, **3:**202-205.

140. Ostovic M, Pavicic Z, Tofant A, Balenovic T, Ekert KA, Mencik S, Antunovic B, Markovic F: **Teat sanitation in lactating sows.** *Acta Vet (Beogr)* 2010, **60:**249-256.

141. Vansickle J: **Refining Feedback.** National Hog Farmer [http://nationalhogfarmer.com/health/refining-feedback], 15-8-2012.

142. VetStat, Ministeriet for Fødevarer, Landbrug og Fiskeri, and Fødevarestyrelsen: **Aktuelle antibiotikaopgørelser.** [http://www.foedevarestyrelsen.dk/Leksikon/Sider/VetStat.aspx], 5-11-2014.

143. Venes D: ***Taber's Cyclopedic Medical Dictionary.*** 22nd edition. Philadelphia: F. A. Davis Company; 2013.

144. Antharam VC, Li EC, Ishmael A, Sharma A, Mai V, Rand KH, Wang GP: **Intestinal Dysbiosis and Depletion of Butyrogenic Bacteria in *Clostridium difficile* Infection and Nosocomial Diarrhea.** *J Clin Microbiol* 2013, **51:**2884-2892.

145. Rajilic-Stojanovic M, Heilig HG, Molenaar D, Kajander K, Surakka A, Smidt H, de Vos WM: **Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults.** *Environ Microbiol* 2009, **11:**1736-1751.

146. Rajilic-Stojanovic M, Smidt H, de Vos WM: **Diversity of the human gastrointestinal tract microbiota revisited.** *Environ Microbiol* 2007, **9:**2125-2136.

147. Stahl M, Kokotovic B, Hjulsager CK, Breum SO, Angen O: **The use of quantitative PCR for identification and quantification of *Brachyspira pilosicoli*, *Lawsonia intracellularis* and *Escherichia coli* fimbrial types F4 and F18 in pig feces.** *Vet Microbiol* 2011, **151:**307-314.

148. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: **The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.** *Clin Chem* 2009, **55:**611-622.

149. Erlich HA, Gelfand D, Sninsky JJ: **Recent advances in the polymerase chain reaction.** *Science* 1991, **252:**1643-1651.

150. Mackay IM: **Real-time PCR in the microbiology laboratory.** *Clin Microbiol Infec* 2004, **10:**190-212.

151. Smith CJ, Osborn AM: **Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology.** *Fems Microbiol Ecol* 2009, **67:**6-20.

152. Fluidigm: ***Access Array System™ User Guide v3.*** 2010.

153. Lim J, Shin SG, Lee S, Hwang S: **Design and use of group-specific primers and probes for real-time quantitative PCR.** *Front Environ Sci En* 2011, **5:**28-39.

154. Clarridge JE: **Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases.** *Clin Microbiol Rev* 2004, **17:**840-862.

155. Liu ZZ, DeSantis TZ, Andersen GL, Knight R: **Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers.** *Nucleic Acids Res* 2008, **36:**e120.

156. Baker GC, Smith JJ, Cowan DA: **Review and re-analysis of domain-specific 16S primers.** *J Microbiol Methods* 2003, **55:**541-555.

157. Youssef N, Sheik CS, Krumholz LR, Najar FZ, Roe BA, Elshahed MS: **Comparison of Species Richness Estimates Obtained Using Nearly Complete Fragments and Simulated Pyrosequencing-Generated Fragments in 16S rRNA Gene-Based Environmental Surveys.** *Appl Environ Microbiol* 2009, **75:**5227-5236.

158. Jensen MA, Webster JA, Straus N: **Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms.** *Appl Environ Microbiol* 1993, **59:**945-952.

159. Case RJ, Boucher Y, Dahllof I, Holmstrom C, Doolittle WF, Kjelleberg S: **Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies.** *Appl Environ Microbiol* 2007, **73:**278-288.

160. Hill JE, Town JR, Hemmingsen SM: **Improved template representation in *cpn*60 polymerase chain reaction (PCR) product libraries generated from complex templates by application of a specific mixture of PCR primers.** *Environ Microbiol* 2006, **8:**741-746.

161. Yarza P, Yilmaz P, Pruesse E, Gloeckner FO, Ludwig W, Schleifer KH, Whitman WB, Euzeby J, Amann R, Rossello-Mora R: **Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences.** *Nat Rev Microbiol* 2014, **12:**635-645.

162. Inglis G, Thomas MC, Thomas DK, Kalmokoff ML, Brooks SP, Selinger L: **Molecular Methods to Measure Intestinal Bacteria: A Review.** *J AOAC Int* 2012, **95:**5-23.

163. Fluidigm: **Products » Chips and Kits.** [http://www.fluidigm.com/chips-kits.html], 7-11-2014.

164. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ: **Evaluation of Methods for the Extraction and Purification of DNA from the Human Microbiome.** *Plos One* 2012, **7**.

165. Jones WJ: **High-Throughput Sequencing and Metagenomics.** *Estuar Coast* 2010, **33:**944-952.

166. Sanger F, Nicklen S, Coulson AR: **DNA sequencing with chain-terminating inhibitors.** *Proc Natl Acad Sci U S A* 1977, **74:**5463-5467.

167. Siqueira JF, Fouad AF, Rôças IN: **Pyrosequencing as a tool for better understanding of human microbiomes.** *J Oral Microbiol* 2012, **4: 10743**.

168. Dames SA, Voelkerding KV, Durtschi JD: **Next-generation sequencing: From basic research to diagnostics.** *Biochimica Clinica* 2010, **34:**287-303.

169. van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C: **Ten years of next-generation sequencing technology.** *Trends Genet* 2014, **30:**418-426.

170. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL: **Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB.** *Appl Environ Microbiol* 2006, **72:**5069-5072.

171. Bio-IT World: **Six years after acquisition, Roche quietly shutters 454.** Bio-IT World Staff [http://www.bio-itworld.com/2013/10/16/six-years-after-acquisition-roche-quietly-shutters-454.html], 16-10-0013.

172. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI: **Evolution of mammals and their gut microbes.** *Science* 2008, **320:**1647-1651.

173. Guarner F, Malagelada JR: **Gut flora in health and disease.** *Lancet* 2003, **361:**512-519.

174. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE: **Succession of microbial consortia in the developing infant gut microbiome.** *Proc Natl Acad Sci U S A* 2011, **108:**4578-4585.

175. Gorkiewicz G, Thallinger GG, Trajanoski S, Lackner S, Stocker G, Hinterleitner T, Guelly C, Hoegenauer C: **Alterations in the Colonic Microbiota in Response to Osmotic Diarrhea.** *Plos One* 2013, **8**.

176. Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Møller K: **Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited.** *Appl Environ Microbiol* 2002, **68:**673-690.

177. Shen F, Du WB, Davydova EK, Karymov MA, Pandey J, Ismagilov RF: **Nanoliter Multiplex PCR Arrays on a SlipChip.** *Anal Chem* 2010, **82:**4606-4612.

178. van Doorn R, Szemes M, Bonants P, Kowalchuk GA, Salles JF, Ortenberg E, Schoen CD: **Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on OpenArrays (TM).** *BMC Genomics* 2007, **8**.

179. Dieffenbach CW, Lowe TMJ, Dveksler GS: **General concepts for PCR primer design.** *Genome Res* 1993, **3:**S30-S37.

180. Varadaraj K, Skinner DM: **Denaturants or cosolvents improve the specificity of PCR amplification of a G+C-rich DNA using genetically engineered DNA polymerases.** *Gene (Amsterdam)* 1994, **140:**1-5.

181. Lyons E, Freeling M: **How to usefully compare homologous plant genes and chromosomes as DNA sequences. [https://genomevolution.org/CoGe/OrganismView.pl].** *The Plant Journal* 2008, **53:**661-673.

182. Lee JH, O'Sullivan DJ: **Genomic Insights into Bifidobacteria.** *Microbiol Mol Biol Rev* 2010, **74**.

183. Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K, Palva A: **Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers.** *BMC Gastroenterology* 2009, **9**.

184. Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L: **The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease.** *Plos One* 2012, **7**.

185. Bezirtzoglou E: **The intestinal microflora during the first weeks of life.** *Anaerobe* 1997, **3:**173-177.

186. Caramia G, Silvi S: **Probiotics: From the Ancient Wisdom to the Actual Therapeutical and Nutraceutical Perspective.** In *Probiotic Bacteria and Enteric Infections: Cytoprotection by Probiotic Bacteria.* Edited by Malago JJ, Koninkx FJG, Marinsek-Logar R. New York, United States: Springer; 2011:3-37.

187. Du Toit M, Franz CMAP, Dicks LMT, Holzapfel WH: **Preliminary characterization of bacteriocins produced by Enterococcus faecium and Enterococcus faecalis isolated from pig faeces.** *J Appl Microbiol* 2000, **88:**482-494.

188. Bourgogne A, Thomson LC, Murray BE: **Bicarbonate enhances expression of the endocarditis and biofilm associated pilus locus, ebpR-ebpABC, in *Enterococcus faecalis*.** *BMC Microbiol* 2010, **10**.

189. Perex G, Oster J, Roger A: **Acid-base disturbances in gastrointestinal disease.** *Dig Dis Sci* 1987, **32:**1033-1043.

190. Nørby S: ***Klinisk ordbog.*** 16th edition. Copenhagen, Denmark: Munksgaard Danmark; 2005.

191. Pinkston KL, Singh KV, Gao P, Wilganowski N, Robinson H, Ghosh S, Azhdarinia A, Sevick-Muraca EM, Murray BE, Harvey BR: **Targeting pili in enterococcal pathogenesis.** *Infect Immun* 2014, **82:**1540-1547.

192. Pirker A, Stockenhuber A, Remely M, Harrant A, Hippe B, Kamhuber C, Adelmann K, Stockenhuber F, Haslberger AG: **Effects of antibiotic therapy on the gastrointestinal microbiota and the influence of *Lactobacillus casei*.** *Food Agric Immunol* 2013, **24:**315-330.

193. Tannock GW: **Commentary: Remembrance of microbes past.** *Int J Epidemiol* 2005, **34:**13-15.

194. Yin Y, Lei F, Zhu L, Li S, Wu Z, Zhang R, Gao GF, Zhu B, Wang X: **Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression.** *ISME J* 2010, **4:**367-376.