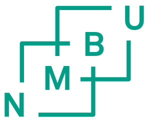

Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2021:31

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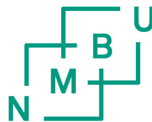

Thesis number 2021:31
ISSN 1894-6402
ISBN 978-82-575-1802-8

"The highest forms of understanding we can achieve are laughter and human compassion."

— Richard P. Feynman

Acknowledgements

This project would not have been possible without contributions from my colleagues, friends, and family.

I would like to thank my supervisor, Henning Sørum. I am grateful for choosing me as "the right man for the job". Henning, you and your colleagues have built a milieu driven by mutual respect, support, and team spirit. It has been an absolute privilege for me to join the team with the knowledge it was temporary, yet living it as if it would stay with me for long. And it will. I am deeply grateful for your genuine support, encouragement, and belief in my capacity. Your passionate commitment to what you do was inspiring. And it made it so much easier for me to endure throughout this project. That you keep reminding me that this project was a success story, means a lot to me .

I would like to acknowledge my co-supervisor, Özgün Umu, for all the help throughout this project. Since the very beginning, in October 2017, I was immediately able to immerse in our experimental activities. Your rigour and care in organizing those experiments granted me a seamless start. I am grateful for introducing me to the 16S *rRNA* gene sequencing business and for keeping my interest in the 16S matter alive by countless discussions. It is much appreciated.

I am grateful to Charles Press and his team for the fruitful collaboration during this project. Charles, I am thankful to you for our lengthy conversations and brainstorming much needed to get insights from the infection trial results. I thank Aleksandra, Randi, and Caroline for their professionalism and shoulder to shoulder contributions to the scientific work we produced.

I acknowledge Margaret Øverland for excellent management of the Foods of Norway project and providing the platform for my PhD project to exist. I thank my IHA colleagues, Liv Torunn, Leidy Lagos, Jon, Branka, and Ragnhild for their support and inputs to the project.

To my Foods of Norway PhD peers, Selina and Ingrid Marie. I was privileged to be on the same boat with you. Thanks for your support, help, and encouragement. It helped me a lot!

To Francesco Delogu, for showing me different perspectives of approaching the research process and for valuable contribution as a co-author.

To my dear colleagues and fellows at Linder. This miniature book would not have seen the light of day without the active involvement of the following squad: Alex, Ane, Anja, Aud Kari, Cristopher, Dhamo, Eiril, Elisabeth, Erik, Espen, Gabriela, Gaute, Grethe, Hege, Ingrid M, Ingrid O, Ingvild, Kamil, Karla, Leif, Mamata, Mari, Maria, Marius, Mette, Øystein, Preben, Ruchika, Sabrina, Salman, Silje, Simen, Solveig, Stine, Tatiana, and Turhan. A special thank goes to Håkon Kaspersen for his generous support with coding and design advice, and the thesis template.

The Lindern list cannot be complete without John Innacymuthu and Elin Pålerud. Their devotion provides the internal mechanism of the section to run smoothly.

To the Orion computer cluster team and Torfinn Nome for their computational support.

I thank Lars Terje Bogevik, Lars Johan Kalager, Anita Holm, Tina Viktoria Aas, Tore Engen, Veronika Stabell, Rory William Flack, and Linda Andreassen for their excellent assistance in and management of the practicalities of the project experiments.

To Kira, Camilla, and Øystein of Previwo AS, for reminding me that there is a life outside academia too, their humour, and the opportunity to try me in a fish tank. In a literal way…

I would like to thank my colleagues from Tromsø Mohammed Al-Haroni, Tordis Trovik, Julia Kloos, and Tracy Lunde for introducing me to the intricacies of academia and the microbial world.

To my Tromsø and "no-longer-Tromsø" friends: Sergey, Anna, Elizaveta, Vladimir, Natalja, Alexei, Polina, Denis, Connie, Joe, Oxana, Igor, Sara, Gonçalo, Kostas, Misha, Lena, Ilya, and Yana for their friendship and vital support.

I am grateful to Søren and Coralie, and Marie for letting me break away from academia at the times when it was needed.

To my dear teacher and lifelong friend Nadejda Davydova who supported and believed in me all the way through.

To my dear friend Kenneth Ayino for his caring friendship and support throughout these years.

I am indebted to Yury Kiselev for his friendship, wisdom, and support. Thank you so much for pulling my head out of the sand to show me a life beyond the routines of scientific work.

To my dear parents, Galina and Sergey, for their love, care, and respect for the decisions I have made. This work (hope not only that) proves that you have done an excellent job raising me. Thank you for looking after my children while I was busy with my work.

To Grigory, Evgeny, and Julia for their support and genuine interest in what I was involved in.

To my wife's family, Galina, Vladimir, Aleksandr, and Tatiana. Their selfless dedication to the well being of my little ones allowed me to stay focused on the subject of my studies.

Finally, I would like to express my endless appreciation of all the sacrifices that my wife, Irina, has made to let me pursue the PhD degree. Your help, care and support are shining from in between the lines of this work, but only I can see it. And I am gladly let others know. To my dear children, Alexander and Nicole, who were of no help at all but let me understand how time works. Love you all!

Disclosure

The work was carried out within the Foods of Norway project (The Research Council of Norway, Lysaker, Norway, grant number 237841/030). I was funded through a PhD fellowship from the Department of Food Safety and Infection Biology at Norwegian University of Life Sciences. Views presented are mine and do not represent those of funders. This thesis was prepared in RStudio with R version 4.1.0 (2021-05-18) [1] using the Bookdown package [2].

Summary (English)

The results of the PhD project have shown that high inclusion of *Cyberlindnera jadinii* yeast in pig feed exerts a prebiotic-like effect. Beneficial intestinal lactic acid-producing bacteria increase in numbers following feeding the novel diet. It seems that small intestine lactobacilli play a pivotal role in enabling yeast protein for pigs. Altogether, high levels of dietary yeast support animals. It does so by offering an extra layer of protection against enteric infections common in young pigs.

The porcine industry needs a source of protein to provide for the growing human population of the globe. The production of soybean, a conventional protein in livestock diets, contributes to the environmental deterioration. Protein derived from a sustainably produced *C. jadinii* yeast is an alternative to soy. The yeast feed fulfils the nutritional needs of pigs. But also, it can influence the composition of the gut microbiome in animals. The gut microbiome plays an important role in the well-being and robustness of animals. This role becomes even more relevant when animals are young and exposed to pathogens before specific immunity has developed.

The *C. jadinii* cell wall contains non-digestible carbohydrates that are not accessible to the animal. These carbohydrates are available for microbial fermentation in the microenvironment of the gut. The microbial metabolites such as short-chain fatty acids contribute to the gut homoeostasis. Previously it has been studied how dietary yeast can change the gut microbiome and health in pigs. However, little is known about the effects of yeast inclusions at the level that it can replace conventional protein sources.

In this PhD project, it was investigated how a diet with 40% of crude protein replaced by protein from *C. jadinii* influences the gut microbial composition. The health-related parameters linked to the gut microbial composition were studied.

The microbial compositions were explored by using cultivation and the 16S *rRNA* bacterial

gene sequencing techniques; the microbially-produced short-chain fatty acids were measured by gas chromatography. The histology techniques were used to elucidate the morphology of the gut.

It was found that the composition of the gut microbial community was reshaped by the novel diet owing to its rich content of beta-glucans, mannan-protein, and chitin. These changes were distinct for distinct gut segments. Host-associated lactobacilli were found to be enriched in the microbiomes of healthy yeast-fed piglets. The findings suggest that lactobacilli enable pigs to take up protein from yeast cell by disrupting the cell yeast cell envelope.

Then, the piglets were challenged with enterotoxigenic *Escherichia coli*. The microbial diversity of the small intestine was higher in the yeast-fed piglets compared with that of the control-fed piglets.

The caecum and colon microbiomes were less diverse and were predominated by *Prevotella*-affiliated taxa in the yeast-fed piglets compared with the control-fed piglets. Surprisingly, the distal part of the large intestine microbiome had the opposite trend. The colonic microbiota of the yeast-fed piglets had a lower population of butyrogenic bacteria, lower concentrations of butyrate, and shorter colonic crypts than those of the control-fed piglets. These differences in metabolites and morphology were associated with a healthy gut state. More, the yeast-fed piglets exhibited reduced feed intake after being exposed to *E. coli* infection. This suggests the development of an adaptation that secures animal survival following the severe enteric infection.

Our findings motivate an upscaling of this research framework to a field-wide level. By doing so, a realistic estimate for the economy and environment following the use of the novel feed can be obtained.

Summary (Norwegian)

Resultatene fra PhD-prosjektet har vist at høy inkludering av *Cyberlindnera jadinii* gjær i grisefôret utøver en prebiotika-lignende effekt i grisene. Godartede melkesyreproduserende bakterier i tarmen øker i antall etter fôring med den nye dietten. Det ser ut til at melkesyrebakteriene i tynntarmen spiller en nøkkelrolle i å tilgjengeliggjøre gjærproteinet for grisene. Samlet viser det at høye nivåer av gjær i dietten støtter dyrene. Det skjer ved at det blir dannet et ekstra nivå av beskyttelse mot tarminfeksjoner som er vanlig hos unge griser.

Svinenæringa trenger en proteinkilde for å hjelpe verdens voksende befolkning. Produksjonen av soyabønner, en konvensjonell proteinkilde i husdyrfôr, bidrar til ødeleggelse av miljøet. Protein fra en bærekraftig produsert *C. jadinii*-gjær er et alternativ til soya. Gjærfôret oppfyller næringsbehovet til grisene. Men det kan også påvirke sammensetningen av tarmfloraen hos dyrene. Tarm-mikrobiomet spiller en viktig rolle i velvære og robusthet hos dyrene. Denne rollen blir enda mer relevant når dyrene er unge og eksponert for patogener før den spesifikke immuniteten har utviklet seg.

C. jadinii-celleveggen inneholder ufordøyelige karbohydrater som ikke er tilgjengelige for dyret. Disse karbohydratene er tilgjengelige for mikrobiell fermentering i mikromiljøet i tarmen. De mikrobielle metabolittene slik som de kortkjedede fettsyrene bidrar til tarm-homeostasen. Tidligere har det vært studert hvordan gjær i fôret kan endre tarm-mikrobiomet og helsen hos griser. Imidlertid er lite kjent om effektene av inkludering av gjær på et nivå der det kan erstatte konvensjonelle proteinkilder.

I dette PhD-prosjektet ble det undersøkt hvordan en diett med 40% av rå-proteinet erstattet med protein fra *C. jadinii* påvirker tarmflora-sammensetningen. Hvis en slik erstatning påvirker mikrobiomet hvordan henger det sammen med helse-relaterte parametere hos grisunger. De mikrobielle sammensetningene ble utforsket ved å bruke kultivering og 16S *rRNA* bakterie-gensekvenseringsteknikker; mikrobielt produserte kortkjedede fettsyrer ble

målt med gasskromatografi. De histologiske teknikkene ble brukt for å belyse morfologien i tarmen.

Det har blitt funnet at sammensetningen av tarmens mikrobielle samfunn blir omstrukturert av det nye fôret på grunn av dets rike innhold av beta-glukaner, mannan-protein og chitin. Disse forandringene var tydelige for avgrensede tarmsegmenter. Verts-assosierte laktobasiller ble funnet i økende forekomst i mikrobiomene hos friske gjær-fôrede grisunger. Funnene indikerer at laktobasillene gjør grisene i stand til å ta opp protein fra gjærcellen gjennom ødeleggelse av gjærcellens cellemembran.

Deretter ble grisungene smittet med enterotoksigene *Escherichia coli*. Den mikrobielle diversitet i tynntarmen var høyere i gjær-fôrede grisunger sammenlignet med kontrollfôrede grisunger.

Mikrobiomene i blindtarmen og kolon var mindre diverse og ble dominert av *Prevotella*-affilierte taxa hos de gjærfôrede grisungene sammenlignet med kontrollfôrede grisunger. Den bakre delen av stortarmen hadde overaskende den motsatte trenden. Mikrobiota i kolon hos gjærfôrede grisunger hadde en mindre populasjon av smørsyreproduserende bakterier, lavere konsentrasjoner av butyrat og kortere kolonkrypter enn hos kontrollfôrede grisunger. Disse forskjellene i metabolitter og morfologi ble forbundet med en frisk tarmstatus. I tillegg viste de gjærfôrede grisungene redusert fôrinntak etter å ha blitt eksponert for *E. coli*-infeksjon. Dette indikerer en utvikling av en tilpasning som sikrer dyrets overlevelse etter en alvorlig tarminfeksjon. Våre funn motiverer til en oppskalering av rammene omkring denne forskningen mot et felt-nivå. Ved å gjøre det kan et realistisk estimat for økonomien og miljøet som følge av bruk av det nye fôret oppnås.

Abbreviations

Term	Abbreviation
Amplicon sequence variant	ASV
Analysis of compositions of microbiomes	ANCOM
Arabinoxylan	AX
Arabinoxylo-oligosaccharides	AXOS
Cell wall	CW
Dry matter	DM
Enterotoxigenic Escherichia coli	ETEC
Gastrointestinal tract	GIT
Immunoglobulin A	IgA
Intestinal epithelial cells	IECs
Lactic acid-producing bacteria	LAB
Lipopolysaccharide	LPS
Metagenomic assembly	MAG
Microbiota-directed food	MDF
Mixed linked beta-glucan utilisation locus	MLGUL
Mixed linked beta-glucans	MLG
Operational taxonomic unit	OTU
Polysaccharide utilisation locus	PUL
Post-infection	PI
Post-weaning	PW
Post-weaning diarrhoea	PWD
Resistant starch	RS
Short-chain fatty acid	SCFA
Soybean meal	SBM
Toll-like receptor	TLR

List of publications

Effect of *Cyberlindnera jadinii* yeast as a protein source on intestinal microbiota and butyrate levels in post-weaning piglets

Stanislav Iakhno, Özgün C. O. Umu, Ingrid M. Håkenåsen, Caroline P. Åkesson, Liv T. Mydland, Charles McL. Press, Henning Sørum and Margareth Øverland

Animal microbiome 2, 13 (2020)

Longitudinal analysis of the faecal microbiome in pigs fed *Cyberlindnera jadinii* yeast as a protein source during the weanling period followed by a rapeseed- and faba bean-based grower-finisher diet

Stanislav Iakhno, Francesco Delogu, Özgün C. O. Umu, Nils Peter Kjos, Ingrid M. Håkenåsen, Liv T. Mydland, Margareth Øverland and Henning Sørum

bioRxiv 2021.02.11.430725; doi: https://doi.org/10.1101/2021.02.11.430725

Small intestine lactobacilli growth promotion and immunomodulation in weaner pigs fed *Cyberlindnera jadinii* yeast high inclusion diet and exposed to enterotoxigenic *Escherichia coli* F4^{+}: O149

Stanislav Iakhno, Selina S. Hellestveit, Özgün C. O. Umu, Lars T. Bogevik, Caroline P. Åkesson, Aleksandra B. Göksu, Charles McL. Press, Liv T. Mydland, Margareth Øverland and Henning Sørum

bioRxiv 2021.02.11.430732; doi: https://doi.org/10.1101/2021.02.11.430732

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1 Introduction

1.1 Animal gut microbiome

Terminology

The word "microbiome" can take several meanings depending on the context of the scientific discourse it is applied. Throughout this manuscript, the term **"microbiome"** will be used to designate "the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions", as proposed by Marchesi and Ravel [3]. Since this work dealt with those bacteria residing in the porcine gut, the above definition was narrowed down to encompass the gastrointestinal tract (GIT), including bacteria, "their genomes (i.e., genes), and the surrounding environmental conditions". The term **"microbiota"** here will be used to designate bacterial assemblages that are present in a particular environment (i.e. mammalian gut) without a reference to the surrounding environmental conditions [3]. The term **"phylotype"** will be used to designate a bacterial entity recovered from any sequencing effort and classified according to a suitable method.

Porcine gut physiology

The GIT of pigs represents a diverse ecological niche for bacteria to live in. In simple terms, the GIT is a pipe with the mouth at the proximal part and anus at the distal part. The GIT is split into several compartments that are distinct anatomically, histologically, chemically, and functionally (Figure 1.1). Each GIT segment (e.g. stomach, small intestine, large intestine, etc.) is characterised by a set of parameters which define the way of bacterial habitation. Among the key parameters are the following: the anatomy of the gut segments,

digesta flow rate and its direction, availability of the nutrients necessary for microbial growth, presence of toxic or growth-inhibiting compounds, presence of attachment site for bacterial retention, pH, and the redox potential. For a young growing pig with a body length of 125 cm, the small and large intestines could be 23 meters long [4]. While the total length varies with age, the small intestine and the large intestine occupy around 78 and 22 % of the total intestinal length, respectively [5].

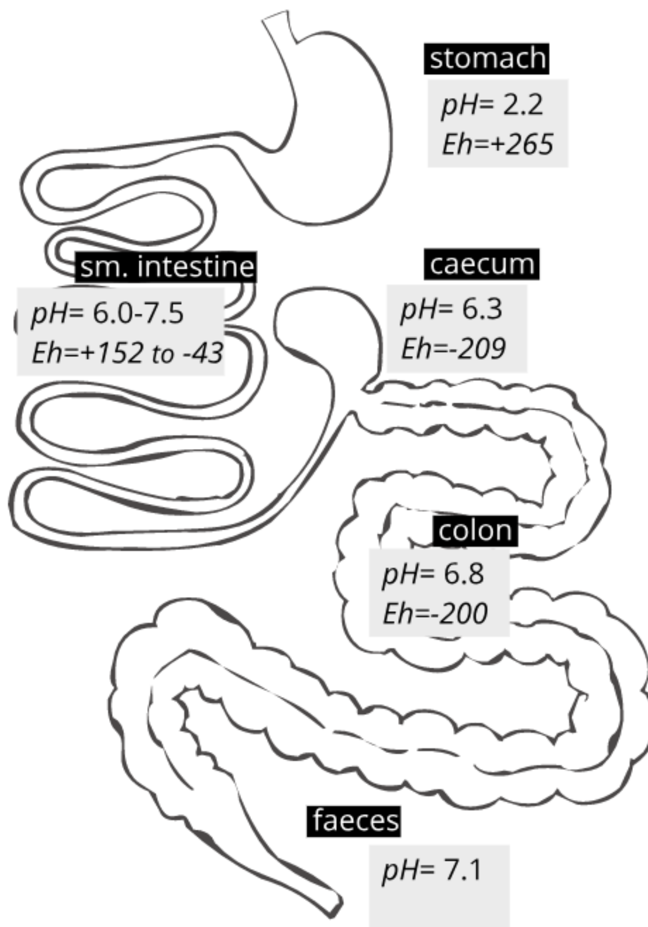

Figure 1.1: Gastrointestinal tract of pigs, scheme. The pH and redox potential gradients are given based on data from [6, 7]

Abiotic factors (e.g. oxygen, redox potential, *pH*, digesta flow), as seen from the microbial perspective, which affect intestinal bacterial populations, differ across the gut segments.

Bacterial relationship to **oxygen** is central to bacterial division into three categories relevant for the microbial ecology of the gut: aerotolerant anaerobes, facultative anaerobes, and obligate anaerobes. Hillman and coworkers reported that the concentrations of dissolved oxygen in the large intestine of pigs were comparable to those of the stomach and small intestine [8]. The presence of dissolved oxygen was reported by Scott et al. in the rumen of the cattle, sheep, and goats, wherein anaerobic fermenters are the major part of the microbial community [9].

In pigs, aerotolerant anaerobes (e.g. lactobacilli) prefer the small intestine as their habitat [10]. However, the concentrations of oxygen in the GIT can not alone explain why the obligate anaerobes dominate the large intestine of pigs. Vervaeke et al. showed that the number of total anaerobe bacteria count was inversely correlated with the oxidation and **reduction potential** (*Eh*) in the porcine GIT. In pigs, the *Eh* gradient ranges from 265 mV in the stomach to -200 mV in the colonic lumen [7] (Figure 1.1) serving a limiting factor for bacterial survival and proliferation [11].

Newborn and suckling piglets have a low acidic environment of the stomach reaching ***pH*** 6 during suckling colostrum and milk [12]. This allows bacteria acquired via familial transmission [13] to reach the small intestine of the animals. This situation changes for weaning and growing pigs. After the transition to solid feeds, the *pH* of the stomach becomes highly acidic with the *pH* as low as 2.2 at the posterior portion of the stomach [4, 6].

Low *pH* functions as a barrier to foreign microorganisms limiting their entrance to the microbial habitat of the intestines [12, 14]. For both, the small and large intestines, acidic-to-alkaline gradients were reported. Luminal *pH* of the small intestine gradually increases from *pH* 6.0 in the proximal part to *pH* 7.5 in the distal part. The large intestine is also characterised by an increase in *pH* levels from the caecum to the rectum, *pH* 6.3 - 7.1, respectively [6].

The intestinal **digesta flow rate** is another parameter of the GIT microbial ecology. Those

bacteria that evolved adaptation mechanisms of attachment to the intestinal wall can colonize and persist in the small intestine where the digesta flow rate is high. Also, such gut symbionts can exhibit host-specific adhesion to the intestinal epithelium followed by a biofilm formation. Frese and colleagues demonstrated that neither human nor pig nor chicken-originated intestinal *Lactobacillus reuteri* were able to attach and form biofilms on the epithelium of germ-free mice [15]. Lin et al. demonstrated that *L. reuteri* coexisted with the *L. johnsonii* cluster in several rodent species. Also, the authors proposed that *L. reuteri* had an evolutionarily earlier association with the GIT epithelium of rodents than *L. johnsonii* because of a wider host distribution of the former [13].

Porcine pathogens, e.g. enterotoxigenic *E. coli*, also have an affinity for the small intestine porcine receptors expressed in the young animals. Once successfully attached, the pathogen grows in numbers and mediates watery diarrhoea in piglets [16, 17].

In contrast to the small intestine, the digesta flow in the large intestine is slower [18]. This offers different opportunities for bacterial growth and microbe-microbe interactions (discussed below).

1.2 Microbe-microbe interaction

Microorganisms sharing the same ecological niche, i.e. the gut, exhibit various interaction patterns [19, 20]. It is not uncommon that the microbial metabolite end-products (e.g. short-chain fatty acids, SCFA) from one species become a substrate required for the growth of other bacterial species. This phenomenon is referred to as cross-feeding.
Belenguer et al. demonstrated that *Eubacterium hallii* and *Anaerostipes caccae* could utilise lactate produced by *Bifidobacterium adolescentis* cultured on starch. Without the *B. adolescentis* co-culture, *E. hallii* and *A. caccae* failed to grow [21]. A recent work by Kim et al. showed that *Faecalibacterium prausnitzii*, a renowned butyrate-producer, thrived in a co-culture with acetate-producing *B. adolescentis* in the mice gut [22]. The examples of antagonistic microbe-microbe interactions are mentioned in the next sections.
The volume of sequencing data obtained from various habitats increases with time [23]. Accordingly, the theoretical approaches for deciphering the microbe-microbe interaction are being developed [19, 20, 24–26]. One of the applications of these resources is a recovery of microbial co-occurrence networks from a particular habitat [24, 25]. In prinicple, the co-occurence network method alorithms search for those bacterial phylotypes that are quantitavely interdependent in a given ecological niche. So, if there is a linear relationship between the abundance of, for instance, phylotype A and B across several samples of a given microbial habitat, this will be shown as a link on the network graph [26].

This becomes a meaningful procedure if the research goal is to study microbial interactions in the context of varying microbial ecology parameters, e.g. nutrient availability and composition, temperature, *pH*, etc. The results of such an approach can inform about or propose potential microbe-microbe interactions that can be further tested under controlled lab conditions [27]. There have also been attempts to map the microbe-microbe interaction patterns to the host phenotype. In the porcine microbiome research, the microbial network data has been linked to animal performance and health-related parameters (e.g. scours) [28–30].

1.3 Host-microbiome interaction

The number of bacterial cells that inhabit the GIT is high. The bacterial population residing in the human GIT has once been compared to a 1.2 kg organ [31]. The gut microbiome, carries out a number of functions essential for animal homoeostasis. While thriving on the substrates available in the GIT of the host, the microbes offer a repertoire of unique functions that are essential for animal health, development, and growth.

Reliance of ruminants on the microbial fermentation of plant fibre from forages is one such example. It has been estimated that 60 to 85% of all amino acids that reach the small intestine of growing lambs represent those from the ruminal microbial biomass [32].
Another example is the degradation of dietary fibre in the large intestine of monogastric animals which results in the SCFA production. Butyrate, a microbially-produced SCFA, serves as a source of energy for the intestinal enterocytes. The enterocytes metabolise butyrate via β-oxidation [33]. Besides, SCFA contribute to the net energy pool in mammals (summarised in [34]). Recent findings have shown the involvement of microbially-produced SCFA in the mental health homoeostasis via so-called microbiota-gut-brain communication. For instance, Dalile and co-workers have shown that colonic SCFA can attenuate the cortisol response to psychosocial stress in healthy man [35].
Another aspect of the host-microbe relationship is the discrimination between beneficial and harmful microbes by the host. Recognition of harmful bacteria and mounting a defensive response are essential for the host survival.

Non-epithelial defence

Defence against pathogens starts in the oral cavity. Lysozyme is an enzyme secreted with saliva that can selectively inhibit or kill pathogenic bacteria entering GIT [36]. If an offender advances down to the stomach, a highly acidic treatment of the gastric environment along with endogenous proteolytic enzymes (e.g. pepsin) ensues [37]. In case the pathogen reaches the small intestine, other defensive mechanisms are deployed to prevent pathogen succession.

Small intestine indigenous microbiota, peristalsis, and the epithelial monolayer cooperate to expel pathogens from the GIT. For instance, some indigenous intestinal *Lactobacillus* spp. [13, 15, 38] exert a range of functions to prevent pathogenic *E. coli* attachment to the intestinal epithelium and growth [37, 39, 40]. Corr and colleagues studied interspecies interactions between host-associated *Lactobacillus* spp. and *Listeria monocytogenes*, an enteric pathogen. The authors' findings suggested that *Lactobacillus* spp. could interfere with the pathogen adhesion to the epithelial cells via producing acid and a proteinaceous molecule [41].
Sjogren and co-workers demonstrated the significance of intestinal motility as a response to enteric infections in rabbits [42]. The authors concluded that the adherence to the intestinal mucosa, but not the mere presence of pathogenic *E. coli* RDEC-1 caused alterations in the intestinal motility.

Epithelial defence

The role of the epithelial monolayer of the small intestine is unique. Its surface is located at the interface between the host and the environmental agents.
The two principal parts of the small intestine epithelium are villi, the appendages that are extruded into the lumen, and crypts of Lieberkühn, the invaginations into the intestinal wall (Figure 1.2). Progenitor, or stem, cells that are situated closer to the bottom part of the crypt migrate towards the tip of the villus along with differentiating to one of the three types of cells: epithelial, goblet, and enteroendocrine.
Continuous desquamation of the epithelial cells from villi to the lumen is a non-specific physiologic antimicrobial barrier that prevents epithelial establishment of pathogen colonies.
While enteroendocrine cells play a regulatory role, the goblet cells produce and secrete mucin granules into the lumen. The mucin granules coat the epithelial mono-layer with mucus hence creating a protective layer between the host tissues and microorganisms [43].
Paneth cells, located at the bottom of crypts, excrete defensin and lysozyme proteins with a broad range of bactericidal effects [44] (Figure 1.2).

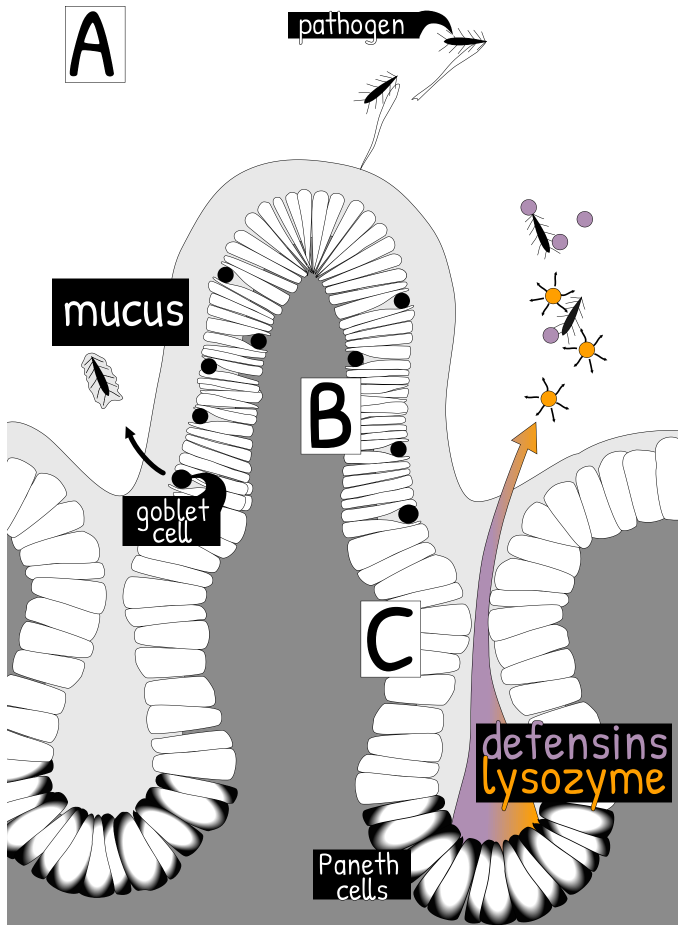

Figure 1.2: Small intestine mucosa, scheme. The principal components are denoted by letters (A, lumen; B, villus; C, crypts of Lieberkühn). Explanation in the text.

Secretory immunoglobulin A (IgA) The immunity component of the gut mucosa and gut surrounding tissues represents a distinct domain that regulates the balance between the host homoeostasis and the gut microbiota.

The gut lining (mucosa) and underlying gut tissues consist of the lymphoid tissues which account for a substantial proportion of the entire population of immune cells of the body [45]. The cellular component of the gut immune system is chiefly made up by lymphoid

folicles, Peyer's patches, intraepithelial lymphocytes, and lamina propria leukocytes [46]. A class of secretory antigens, immunoglobulin A (IgA), is pivotal for the maintenance of the gut integrity under the perpetual microbe-host cross-talk. While IgA deserves a broader account regarding its role in the gut mucosal immunity, here, it should be appreciated that IgAs originate in Peyer's patches [47, 48] and are released to the intestinal lumen [49] following an intricate chain of events (reviewed in [45, 50]). Briefly, the luminal antigens are picked up by special membranous cells that are overlying Peyer's patches facing the lumen. Then, the antigens are presented to T helper cells through antigen presenting cell. In response to T helper cells' signalling, B lymphocytes switch their synthesis to a specific IgA$^+$ class and migrate to lamina propria followed by the differentiation to plasma cells which release IgA to the lumen via transcytosis [45, 50]. Upon its release to the lumen, IgA can inhibit the invading pathogens in a highly specific manner [51].

Intestinal epithelial cells (IECs) are one of the key facilitators in a cross-talk between the luminal antigens and the host body. The fact that IECs carry out nutrient uptake and, at the same time, are a gatekeeper which detects luminal insults (e.g. pathogens, toxins), highlights the complexity of the functional organization of the epithelial monolayer. There is mounting evidence that the pivotal role in distinguishing between the offending bacteria and the gut commensals belongs to the toll-like receptors (TLR) of the IECs (reviewed in [52]. This group of receptors is specific in binding distinct bacterial compartments such as enterobacterial lipopolysaccharides (LPS), flagellin, bacterial CpG motifs, etc. (reviewed in [53]. As an example, TLR9, among other pattern recognition receptors, sense the bacterial CpG motifs and either induces homoeostatic response or initiates an inflammatory pathway (summarised in [54]. Lee et al. studied the role of the TLR9 localisation in relation to the IECs' polarity upon the TLR9 activation. They have demonstrated that activation of the TLR9 located apically, i.e. IECs part facing the lumen, triggers homoeostatic responses hence contributes to immune oral tolerance. If, however, the activation of the basolateral TLR9, i.e. the part of IECs that faces lamina propria, takes place, the inflammatory response pathway is triggered [55]. This balance holds only when IECs polarisation is preserved. In the case of breaches of the IECs integrity (e.g. damage), the inflammatory response similar to that due to basolateral TLR9 activation ensues [55]. For a comprehensive overview of

mounting the immune and tolerance responses in the gut, the readers are referred to the reviews in [56–58].

1.4 Enterotoxigenic *Escherichia coli* bacillosis

Adhesion

Enteric pathogens represent a health threat to human and farmed animals alike [59–61]. Meat production industry sustains economical losses due to, but not limited to, the bacterial infections [62]. Enterotoxigenic *Escherichia coli* (ETEC) affects calves, lambs and piglets [63]. The ETEC induced colibacillosis disease manifestations are dehydration, electrolyte imbalance, watery diarrhoea which may turn fatal [17, 64]. ETEC uses its proteinaceous appendages, fimbriae (F), to adhere to the intestinal mucus layer and IECs brush borders [65]. There are several distinct ETEC fimbriae variants (F4, F5, F6, F18, F41 , etc.) found in the isolates from diseased animals. The fimbrial variant F4 (K88) has been more often implicated in the porcine diarrhoea cases across Western countries [66–68]. The adhesion of ETEC F4$^+$ to the small intestine epithelial surface occurs in young piglets, from newborn to 4-5 weeks of age [69, 70]. Clinically relevant F4$^+$ fimbriae is present in three distinct antigenic forms, ab, ac, and ad [71, 72].

The three antigenic forms of ETEC fimbriae are specific in binding to the porcine adhesive receptors. Willemsen and de Graaf investigated the differences in affinity of various ETEC F4$^+$ variants to the porcine crude mucus and IECs' brush border receptors [65]. The authors found that F4ab^+ and F4ac^+ antigenic variants had a high affinity to 25, 35, and 60 kDa proteins of the crude porcine mucus, while the F4ad^+ variant only had a weak affinity to the mentioned porcine protein receptors.

Blomberg and Conway found that the number of the porcine mucus receptors increased from day 5 to day 26 post-natal, and then it decreased by day 47 post-natal compared to day 26 [73]. Further, Conway and co-workers identified 16-fold greater adhesion of *E. coli* 1107 to the ileum mucus of the 35-day old piglets compared to that of the newborns [70]. The authors attributed this dynamics of the porcine mucus receptor populations to the mucus defensive function in the protection of the underlying epithelial monolayer from the pathogen.

A high affinity of the F4ab^+ variant to 16 kDa, and a range of distinct proteins of 40-70 kDa

located at the IECs' brush border remained across all animals tested. The researchers noted a degree of variation in the binding affinity due to between-individual differences. Caloca et al. identified 6 distinct porcine IECs' brush border proteins ranging from 27 to 94 kDa that bound to F4ab^+ of ETEC K-12 W3110 [74].

Bijlsma and co-workers proposed a classification of animals into 5 phenotypes (A, B, C, D, and E) depending on susceptibility to different combinations of the ETEC antigenic variants [75].

The susceptibility of piglets to ETEC F4$^+$ is inherited in a Mendelian way whereby the animals with the dominant, "adhesive" allele (S), express the porcine adhesion receptor, whereas the animals with homozygous "non-adhesive" alleles (RR) lack the receptor [76]. To classify pigs into three F4ab^+ susceptibility groups, homozygous susceptible (SS), heterozygous susceptible (SR), and homozygous resistant (RR), Jørgensen and colleagues devised a DNA-based test [77]. The test is based on a single nucleotide polymorphism detection in the *MUC4* gene located on porcine chromosome 13 [77]. Several authors indicated other candidate loci involved in determining the resistance phenotype of the animals [78, 79].

In Norwegian pig herds, the homozygous resistant phenotype makes up around 6% out of all pig population (the National litter recording system, "Ingris"). This means that the majority of Norwegian pigs will be susceptible to the ETEC F4$^+$ to a degree which depends on yet unclear porcine genetic determinants.

The introduction of *E. coli* vaccines dramatically changed the course of the porcine colibacillosis. Maternal antibodies against ETEC F4$^+$ which are acquired via milk or colostrum protect piglets throughout the suckling period [69, 80]. Therefore, the piglets are the most vulnerable to the ETEC infection after weaning, when deprived of the maternal antibodies. The morbidity due to ETEC F4$^+$ gradual declines as the animals age [69].

The findings by Blomberg et al. and Conway et al. suggested that the decline in the porcine mucus receptors to F4$^+$ in the small intestine by the 47th day post-natal could be indicative of developing other complementary defensive mechanisms against ETEC [39, 70].

The role of the resident gut microbiota may be one such mechanism. A further work by Blomberg et al. has demonstrated that indigenous porcine intestinal lactobacilli can

suppress the growth of ETEC F4^{+} in the mucus of 35-day-old pigs [39].

Virulence

ETEC synthesise a number of virulence factors during the course of infection. Nagy and Fekete classified the enterotoxins into two distinct categories: (I) heat-stable toxins (ST), and (II) heat-labile toxins (LT) [16]. ST is stable at 100^{0}C and has lower molecular weight (2kDa) compared with LT which is heat-labile (88kDa). ST is further classified into STI, STII, and EAST1 varieties. A porcine specific STI (STI_p) is distinct from that of human type (STI_h).

STI_p, STII, and EAST1 are commonly implicated in post-weaning diarrhoea (PWD) in piglets [81]. There exist two biologically distinct LT variants, namely LTI and LTII. As with STI antigenic variants, there is a distinction between human (LTI_h) and porcine (LTI_p) antigenic variants of LTI. LTII, also, exists in 2 types: $LTII_a$ and $LTII_b$ (reviewed in [16, 81]). For the details of the bacterial enterotoxins' synthesis, the reader is referred to the review by Dubreuil et al. [17].

ETEC recruits a number of mechanisms to deliver the toxins into the host cell. The absolute requirements for this are the following: bacterial motility, contact with the host cell, and adhesion [82, 83]. The mechanism of the pathogen-to-host toxin delivery is an intricate cascade of molecular events [84, 85]. The bacterial type II secretion system and outer membrane vesicles mediate LT and ST translocation from the bacterial cell to the outer surface [82, 84]. Next, LT forms a 'bridge' between ETEC LPS and the GM1 host receptors of the brush border [17, 86]. This bond further stabilizes the host-pathogen adhesion [64, 86, 87]. Once bacterial toxins are internalized by the host cells, distinct pathogenic pathways are commenced by these toxins [17].

ETEC is known to possess several combinations of the enterotoxins which include, but not limited to, LT, ST_b, and EAST1. It has been indicated that a combination of LT, ST, F4 (K88), and EAST1 virulence factors is common for those ETEC strains that are associated with PWD in piglets [64, 68, 88].

The concerted action of the ETEC enterotoxins leads to the electrolyte imbalance in the IECs.

While chlorine and bicarbonate are excreted from IECs, the absorption of natrium cations from the lumen is inhibited [16, 17, 89–93]. The electrolyte imbalance elicits dehydration of IECs which results in watery diarrhoea in animals.

1.5 Porcine microbial compositions

Enteric infections in livestock animals require clinical management to maintain the production rates. Antimicrobials were banned from usage as growth promoters in livestock settings (EC Regulation No. 1831/2003). Targeted modulation of the animal gut microbial community became an appealing alternative to antibiotics. There is rapidly accumulating evidence of a connection between the animal gut microbial composition and the animal health, and zootechnical parameters [58]. The baseline information about the porcine microbiome is a valuable tool for evaluating the results of microbiome modulation attempts. The microbial compositions of the GIT is governed by the abiotic and biotic factors discussed above. Besides, such factors as animal age, affiliation to a certain geographic area, farm, or litter contribute to the biodiversity of the porcine gut microbiome [10, 94]. Further, technical aspects of sample processing and approaches of microbiota composition determination have an impact on the inference of the gut community structure [10].

There have been efforts to infer the composition of the core porcine gut microbiota on the basis of publicly available data. Here, the term "core microbiota" is used similarly to the way it was used in the Holman et al. work. The authors referred to the "core microbiota" as those bacteria that are present in $\geq$ 90% of samples in question [10]. Interestingly, the authors found that there was *no* 16S *rRNA* gene operational taxonomic units (OTUs) which were shared across all 939 GIT samples analysed in their study. Wylensek and co-workers arrived at the same conclusions when analysing 16S *rRNA* gene data from 1346 faecal porcine samples. The most frequently identified bacterial species identified across all the samples was *L. reuteri* (92% of all samples) [94].

Different approaches of the porcine gut microbiome study yield different results of bacterial biodiversity. A recent comprehensive work by Wylensek et al. compiled a porcine gut microbiome **cultivable** collection. This collection of porcine gut microbes spanned over nine phyla, 40 families, and 110 species [94]. In contrast, a meta-analysis of 16S *rRNA* gene sequences from 20 porcine datasets by Holman et al revealed as many as 35 phyla which made up 887 bacterial genera [10]. Despite substantial variation introduced by the methodology (differences of country of animal origin, study, GIT location,

age of animals, etc.), it is generally accepted that three bacterial phyla, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, dominate the porcine gut microbiome [10, 94, 95]. There is no agreement among the porcine cultivation-free microbiome studies on whether *Bacteroidetes* or *Firmicutes* dominate the GIT of pigs. Ke and co-workers demonstrated that the proportion of *Firmicutes* was slightly greater in the porcine faecal microbiomes than that of *Bacteroidetes* over the period of 25 days to 120 days of age [30]. Allen and colleagues estimated *Firmicutes* and *Bacteroidetes* to comprise 30% and 50% of the faecal microbiomes of pigs weaned at 14 days of age, respectively [96].

The microbial colonization of the porcine gut starts from the birth [97, 98]. The microbial community undergoes qualitative and quantitative changes as the animal grows [95, 99]. These changes may also occur throughout one day. For instance, diurnal rhythms have been linked to changes in the mucus microbial loads in animals [100–102]. While the changes due to the diurnal rhythms fluctuate within a day, there is a mounting evidence of a characteristic gut microbial community composition related to animal age.

After birth, there are certain decisive events during the animal life that affect the gut microbial communities. In fact, the birth itself marks the first encounter of the newborn animals with their resident gut microbiota inherited from the mother and acquired from the environment of the farrowing barn [98]. Lactic acid-producing bacteria (LAB), enterobacteria and streptococci colonise the intestine of suckling piglets soon after birth [103]. In Norwegian commercial pigs, weaning from mothers normally takes place around 28th day of age (https://lovdata.no/dokument/SF/forskrift/2003-02-18-175#KAPITTEL_4).

Weaning is one of the prime challenges for the piglet physiology. Milk cessation and the separation from mothers coincide with the distress caused by the animal rehousing, mixture of litters, and the introduction of solid feeds [104, 105]. This cascade of events has an impact on the animal physiology and the gut microbial community [97, 106]. Some major fluctuations of bacterial community members occur.

Arfken et al. have shown that the porcine faecal microbiome changes drastically as the animals transit from the suckling to the post-weaning period [99]. Yang et al. demonstrated that after weaning, *Proteobacteria* increased in the faecal microbiomes until it decreased

again by week 3 PW. The authors proposed that the increase in *Proteobacteria* during the first 3 weeks PW was at the cost of a reduction in both *Bacteroidetes* and *Firmicutes*. Weaning-associated perturbations of the small intestine microbiota were studied by Pieper et al. The authors showed that LAB decreased on d1 PW and restored only on d5 PW in the ileum of piglets [106].
The variation of the gut microbial composition is volatile right after weaning. One possible reason for that is the establishment of a new hierarchy in the pens with the mixed litters. This leads to unequal access to solid feed which, in turn, hampers the supply of nutrients needed for microbial growth. When the access to feed stabilizes, the gut bacterial community composition is influenced by the amount and the variety of the nutrients available for bacterial fermentation [107–110].

Carbohydrate microbial metabolism

Nutrients from the feed are primarily absorbed in the small intestine of monogastric animals. Monosacharides can be directly absorbed by the enterocytes of the small intestine. Host produced endogenous enzymes are required to break down disaccharides, starch, and glycogen to make them available for absorption. Those carbohydrates that are not digested by the host, termed dietary fibre (DF), advance down the intestine. Besides exogenous carbohydrate sources, mucus, desquamated cells, and peptides constitute an additional source of complex carbohydrates available for microbial fermentation [111–113].

The microbial community of the large intestine as a whole possesses numerous genes encoding enzymes degrading DF (discussed in [114]). For instance, some species within *Prevotella* genus can synthesize more than 200 enzymes that can degrade DF (http://www.cazy.org/). A recent study in pigs fed acetylated galactoglucomannan fibre has discovered that a certain *Prevotella* cluster employs various glycoside hydrolases, β-glucanases, and manosidases to ferment complex carbohydrates otherwise inaccessible to the host [115].

One of the main end-products of the microbial fermentation is short-chain fatty acid (SCFA). SCFA is a source of energy for colonic enterocytes [33]. A proportion of

microbially-produced SCFA reaches blood flow and is metabolized in the liver [34, 116, 117]. Butyrate is one of the microbially-produced SCFAs. This metabolite is an important molecule for the gut integrity and morphology. It has been shown that butyrate has different effects on the large intestine glands, i.e. intestinal crypts. On one hand, it supports proliferation of the crypt enterocytes which are located in the luminal compartment of the crypts [118, 119]. On the other hand, butyrate can diminish proliferation of the stem cells which are located in the stem cell compartment of the crypts [118–120] (Figure 1.2). Kaiko and colleagues proposed that the modification of crypt architecture might be a response to high butyrate concentrations to protect the stem cell of the crypt. That was supported by *in vitro* and *in vivo* findings by Wang et al. and Mentschel et al., respectively [119, 120]. The porcine gut morphology can be modulated by the feed tailored to promote certain bacterial groups. Michalak and co-workers have shown that a type of DF, acetylated galactoglucomannan, can select for butyrogenic bacterial populations in the large intestine of weaned piglets [115].

Biochemistry of porcine feeds

The porcine diet composition is one of the critical determinants that defines the microbial consortia residing in the gut. While designed to fulfil animal nutritional needs, feed ingredients provide gut microorganisms with the nutrients required for their existence. A typical formulation for weanling piglets is shown in Table 1.1. In general, it is based on various cereals (wheat, barley, oats), soybean meal (SBM), fish meal, potato protein concentrate, and rapeseed oil, among other ingredients. Pigs can utilise monosaccharides from feed via absorption by the small intestine enterocytes. Starch, disaccharides, and glycogen can be also partially absorbed following the release of monosaccharides due to the host endogenous enzyme activities. More complex carbohydrates are resorted to the microbial degradation. Some non-digestible carbohydrates are already accessible for microbial fermentation in the small intestine [121, 122]. Other non-digestible carbohydrates are fermented in the large intestine.

Table 1.1: Weanling piglet diets. Ingredient and chemical composition (g/kg) of diets based on soybean meal (Control) and *C. jadinii* (Yeast). *Premix : provided the following amounts per kilogram of feed: 120 mg of Zn (ZnO); 460 mg of Fe ($FeSO_4$. H_20); 60 mg of Mn (MnO); 26 mg of Cu (CuSO~4 x $5H_2O$); 0.60 mg of I ($Ca(IO_3)_2$; <1.0 mg of Se (Na_2SeO_3); 8000 IU of vitamin A; 1500 IU of cholecalciferol; 45 mg of dl-alpha-tocopheryl acetate; 105 mg of ascorbic acid; 4.64 mg of menadione; 5.63 mg of riboflavin, 3 mg of thiamine; 15 mg of d-pantothenic acid; 20 ug of cyanocobalamine; 45 mg of niacin. The table is adopted from Paper III.

Ingredients	Control piglet diet	Yeast piglet diet
Wheat	627.9	593.6
Barley	100	100
Oats	50	50
Yeast meal (C. jadinii) (47% CP)	0	146
Soybean meal (SBM) (45% CP)	80	19
Fish meal (68.4% CP)	20	4.8
Potato protein concentrate (72.5% CP)	33.8	9.1
Rapeseed meal (Mestilla) (35%CP)	20	4.9
Rapeseed oil	19.7	23.4
Limestone	9.2	9.4
Monocalcium phosphate	13.1	15.5
Sodium chloride (NaCl)	7.2	5.5
L-Lysine . HCl (98%)	6.5	5.7
L-Threonine	2.9	2.4
L-Methionine	2.1	2.9
L-Valine	1.4	1.2
L-Tryptophan	0.9	0.9
Premix*	5.3	5.5
Calculated contents	-	-
Net energy, MJ/kg	9.94	9.94
Crude protein from C. jadinii)	0	40
Analyzed content, g/kg	-	-
DM	869	885
Gross energy, MJ/kg	19	19
Crude protein	176	172
Crude fat	39	41
Ash	46	45
Neutral detergent fiber (NDF)	96	91
Starch	442	437

Resistant Starch

Because of its complex structure [123], starch partially escapes host digestion in the small intestine. The non-digested bulk is referred to as resistant starch (RS). The cereals of the porcine diet have variable proportions of RS. Barley and oats contain around 55 g per kg of dry matter (DM) of RS each compared to 4 g of that in wheat [23]. Pulse crops (e.g. faba beans, peas) are an alternative source of proteins and carbohydrates relevant for porcine diets. Faba beans contain 375 g of starch per kg of DM with 10% of that making up the RS fraction [23].
The bacterial amylolytic enzymes break down RS structural units, amylose and amylopectin, in a distinct way. *Alpha-amylase* hydrolyses 1,4-α linkages of glucose residues in amylose molecules. *Pullulanases* break down 1,6-α linkages between the amylose molecules and the amylose polymer backbone. *Amylopullulanase* is a hybrid of the two above enzymes capable of hydrolysing both 1,4- and 1,6-α linkages of the RS [124]. *Roseburia* spp., *Ruminococcus bromii*, and *Eubacterium rectale* are well studied amylolytic bacteria that are known to degrade RS in man [125, 126]. Genes encoding α-amylases, pullulanases, and amylopullulanases are widely distributed in the porcine gut microbiome. These genes are linked to *Prevotella* spp., *Blautia*, *Roseburia*, *Faecalibacterium*, and *Eubacterium* phylotypes [115].

Celullose

Cellulose is a building block of all plant cell walls. It is most abundant in hulls of protein crops (e.g. soybeans, fava beans, rapeseed) and cereals (e.g. oats, wheat, barley etc.) (summarized in [127]). Cellulose represents a linear sequence of d-glucopyranosyl residue homopolymers connected via β-1,4 bonds [127]. *Ruminococcus flavefaciens* is arguably the most studied model organism in the studies of cellulose degradation in the mammalian gut. An extracellular machinery, *cellulosome*, has been described in *R. flavefaciens* [128–130]. Cellulosome is a complex multimodular organelle that governs plant cell wall degradation. The organelle consists of the *scaffoldin* and *dockerin* subunits (the model is described in [131]). Scaffoldin is anchored to the bacterial cell surface on one end and is connected to

the enzymatic subunits via dockerin modules along the scaffoldin lenght. The structure of scaffoldins is thought to be species-specific which defines cellulolytic properties of the cellulosome (reviewed in [131, 132]). Two distinct metagenomic assemblies (MAGs) classified as Ruminococcaceae familiy had genes encoding scaffoldin as per a recent porcine gut metagenomic survey [115]. The presence of genes encoding for dockerins/cohesins in intestinal *Agathobacter*, *Blautia*, *Lachnospira* genera, and Muribaculaceae family MAGs in pigs may indicate other cellulosome candidates that are distinct from those that has been previously described in *R. flavefaciens* [131]. Bayer et al. has described free cellulolytic enzymes which are not part of the cellulosome. Those systems are ascribed to the cellulolytic activities of *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *R. albus*, *R. flavefaciens* [132]. Data from the porcine gut MAGs [115] have indicated that *Prevotella* spp., *Ruminococcus*, *Fibrobacter*, *Agathobacter* spp., *Blautia* spp., *Roseburia* spp., *Faecalibacterium*, *Eubacterium* spp., *Acetatifactor*, *Anaerovibrio*, *Treponema*, and unknown genera of the Muribaculaceae family carry the genes that encode for one or several free cellulolytic enzymes (Figure 1.3-1.4).

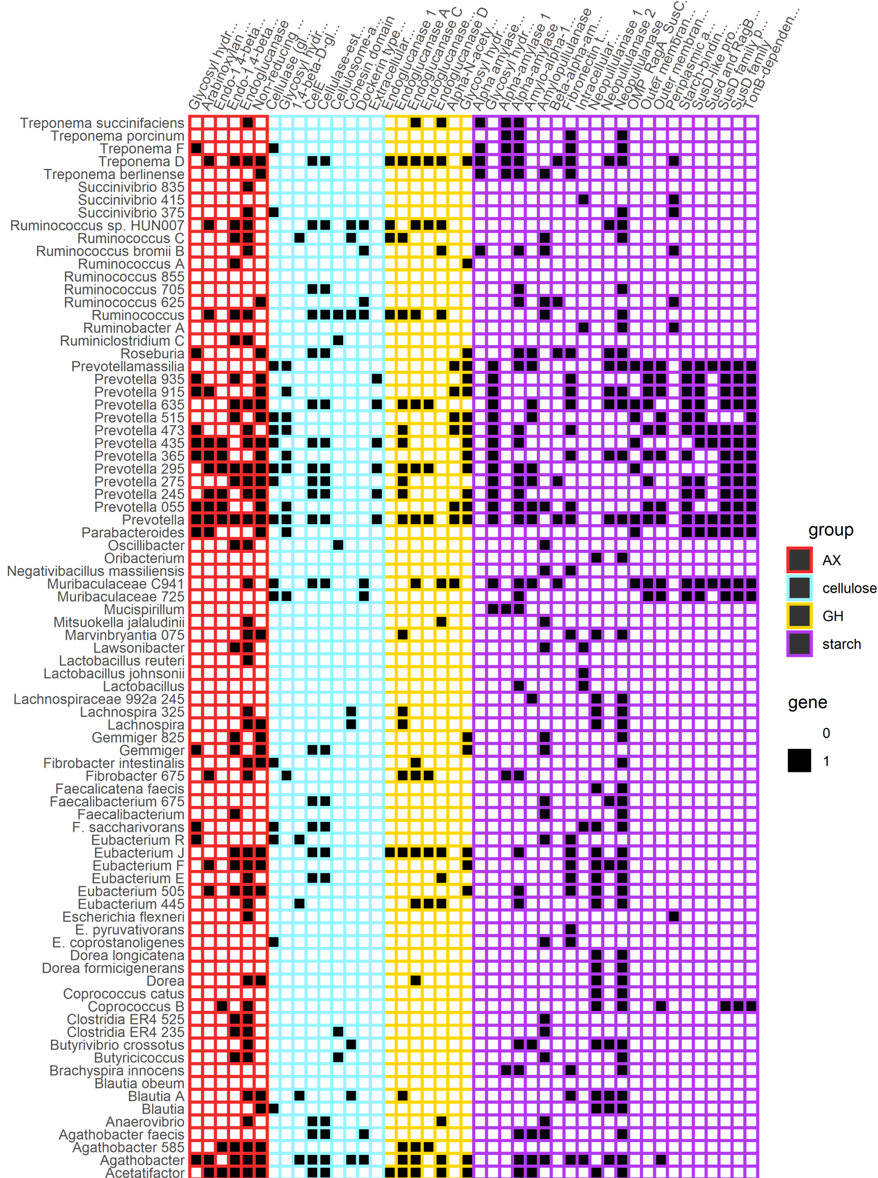

Figure 1.3: Porcine gut bacteria relevant in the dietary fibre (DF) degradation. The graph shows the presence of the genes which encode for the proteins related to specific DF degradation pathways across the selected porcine gut microbiota members. The enzyme names are given on the X-axis and truncated. Full names of enzymes can be accessed at https://github.com/stan-iakhno/PhD/tree/main/Figure1-2full. The enzymes are classified based on substrate/function and denoted as being present, “1”, or absent, “0” (legend). Bacterial phylotypes are given on the Y-axis ... (*next page*)

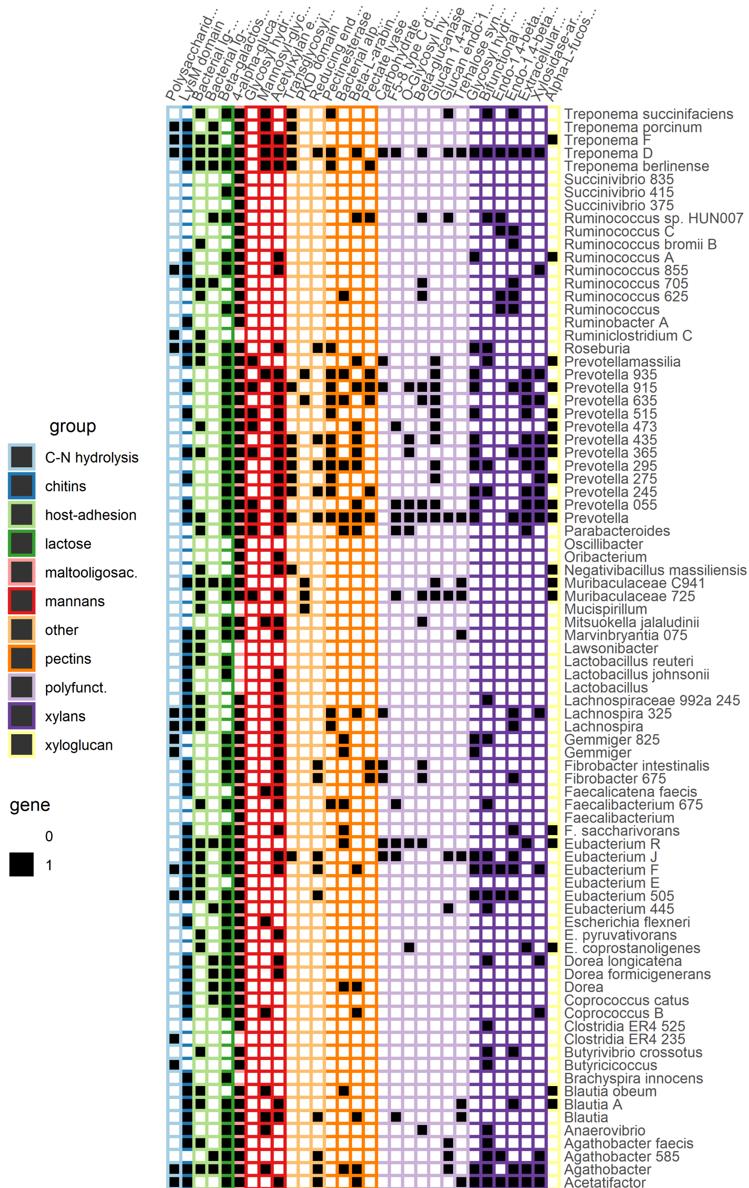

Figure 1.4: (*cont*)...and derived from GTDB taxonomy (https://gtdb.ecogenomic.org/) shortened for readability purposes. The data presented are the results of a manual search of a publicly available dataset [115] followed by an annotation using www.cazy.org and www.uniprot.org. For a more comprehensive account of dietary fibre degrading porcine gut microbiota, [115] and [133] are good choices. **AX**, arabinoxylan; **GH**, miscellaneous glycosyl hydrolases; **maltooligosac.**, transglycosylation of maltooligosaccharides; **poly**, multifunctional glycosyl hydrolases.

Arabinoxylans

Arabinoxylan (AX) is a noncellulosic polysaccharide that makes up the cell walls of cereals (wheat, rye, barley, oats, etc.). It represents a β-1,4-linked xylosyl back-bone decorated with arabinosyl side chains which is not accessible to host enzymes. AX can be attacked by several bacterial AX-degrading enzymes. The three main enzymes, endo-$\beta-$1,4-xylanase, β-D-xylosidase, α-L-arabinofuranosidase are associated with the bacterial cell wall and degrade AX to monomeric arabinose and xylose [134]. The hydrolysed AX products, arabinoxylo-oligosaccharides (AXOS) are transported by the dedicated transport systems to the periplasm for further degradation to arabinose and xylose. The latter pentoses are transferred to the cytoplasm by means of the transporter proteins specific for these sugars. Alternatively, AXOS are distributed to other members of gut microbiota that cannot hydrolyse AX on their own [134]. *Bacteroides* spp., *Roseburia* spp., *Bifidobacterium intestinalis* are reported to have AX degrading properties in the man gut [135, 136]. In the porcine large intestine, *Prevotella* spp., *Eubacterium* spp., and *Roseburia* spp. have the largest pool of genes encoding for AX degradation [115] (Figure 1.3-1.4).

Mixed linked beta-glucans

Mixed linked β-glucans (MLG) is another noncellulosic polysaccharide that makes up cereal grains cell walls. In comparison to AX, MLG comprises a minor fraction of noncellulosic polysaccharides [127]. The proportion of MLG is highest in barley (4% of DM), followed by oats (2.8%), and wheat (1%) (summarised in [23]). The MLG molecule is linear. It is formed by D-glucopyranosyl residues connected via a mixture of β-1,4 and β-1,3 bonds [137]. Cereal MLGs are known to increase viscosity of the lumen contents in the mammalian small intestine (reviewed in [138]). However, a work by Schop and co-workers showed that only stomach digesta viscosity was affected by oat MLG supplementation in growing pigs. That was not the case for the small and large intestine compartments [139]. A possibility of microbial degradation of MLGs in pig GIT was first reported by Jonsson and Hemmingson. They isolated MLG-degrading lactobacilli from the pig neonate faeces [140]. Further, Murphy et al. have demonstrated that lactobacilli thrive on MLGs in the ileum of growing

pigs. They compared lactobacilli viable counts between pigs fed either a barley-based diet or barley-based + enzymes diet [109]. The authors found that viable counts of lactobacilli were 1000-fold higher in the ileum of pigs fed a barley-based diet compared to those of barley-based + enzymes diet. The enzymes were a combination of endo-1,4-β-xylanase A and endo-1,3(4)-β-glucanase. The findings of that experiment suggested that lactobacilli of the small intestine could enzymatically degrade cereal β-glucans by either one or both of the enzymes. To the best of my knowledge, lactobacilli cannot produce endo-1,4-β-xylanases (according to cazy.org database). However, some lactobacilli (*L. acidophilus*, *L. murinus*) carry the genes that encode endo-1,3(4)-β-glucanase. Tamura et al. have demonstrated that MLG utilization locus (MLGUL) is essential for a human large intestine symbiont, *Bacteroides ovatus*, to break down MLGs [141]. The authors have indicated that other mammalian gut inhabitants, *B. xylanisolvens*, *B. cellulosilyticus*, *P. multiformis*, and *P. copri* possess MLGUL hence are potent MLG degraders [141]. Porcine metagenome data have indicated [115] that there may be additional microbial genes encoding enzymes which are operative in the MLG degradation (Figure 1.3-1.4).

Yeast cell wall components

Dried yeasts cells can be used as an alternative source of proteins in the porcine diets (discussed in the next section). The cell wall of yeasts (e.g. *Saccharomyces cerevisiae*) comprises up to 30% of the dry cell weight [142, 143]. The principal components of the wall are 1,3(6)-β-glucans, mannan-protein, and chitin accounting for 55- 65%, 35-40% and 1-7% of the cell wall mass, respectively [142]. The proportions of these components can vary substantially depending on the cultivation parameters (*pH*, temperature, growth media composition, etc.). For instance, chitin proportion of *S. cerevisiae* grown on the yeast peptone media can reach 6,2% of the cell wall dry mass. This proportion can be 3% when the yeast is grown on the yeast nitrogen base [144].

Beta-1,6-glucans connect the outer mannan-protein layer with the network of β-1,3-glucans and chitin of the inner layer of the cell wall [142]. The degradation of the yeast cell wall is critical for enabling yeast cell proteinaceous contents for animals to absorb. As with

other non-digestible carbohydrates, the yeast cell wall components are recalcitrant to the degradation by the host enzymes. Hansen et al. have demonstrated that fish cannot efficiently utilise nutrients from the intact dry inactivated *S. cerevisiae*. In contrast, physical disruption of the yeast cells can increase protein solubility and consequently lead to a better feed conversion ration [145].

Pigs, unlike fish, can utilise intact yeast cells [146, 147], although they lack the enzymes for the yeast cell wall degradation. Therefore, the microbial role in the degradation of dietary yeasts is crucial for animal nutrition and represents an example of a symbiotic relationship. A work by Cuskin and co-workers elucidated the mechanisms by which a human symbiont, *Bacteroides thetaiotamicron*, could degrade and utilise α-mannans from various yeasts strains. The authors have shown that not only *B. thetaiotaomicron* but also some phylogenetically close *Bacteroides*, and *Parabacteroides* possess three distinct α-mannan PULs (MAN-PULs) which are operative in the yeast cell wall mannan breakdown [148]. An analysis of the porcine large intestine metagenome has shown that *Bacteroidetes* including *Prevotella* spp., Rikenellaceae RC9 gut group, *Parabacteroides*, Porphyromonadaceae bacterium C941 carry the core elements of the 1-MAN-PUL, glycosyl hydrolase family 76, 92, 125 and *SusC*/*SusD* genes (Figure 1.3-1.4). In addition, Cherie J. Ziemer has been able to isolate *Bacteroidetes* from the porcine faeces that are positive for MAN-PULs (*B.ovatus*, *B. thetaiotaomicron*, *B. xylanisolvens*, *B. vulgatus*, and *Parabacteroides merdae*) [148–150].

While the role of mammalian *Bacteroidetes* phylotypes in the yeast cell wall degradation in the large intestine is well studied, the role of small intestine lactobacilli in degrading yeast remains unclear. Under laboratory conditions, *L. plantarum* displayed a chitinase-binding activity as shown by Sánchez et al. [151]. Both *L. johnsonii* and *L. reuteri* has a gene encoding LysM chitin-binding domain as per an *in silico* analysis (this work) of a published porcine metagenome data [115]. *In vitro* and *in vivo* findings by Charlet et al. have shown that *L. johnsonii* can directly attack and inhibit *Candida glabrata* yeast via a chitinase-like enzymatic activity [152]. Lactobacilli colonize the porcine GIT soon after birth [103], and after weaning, lactobacilli maintain its dominance in the ecosystem of the small intestine [106]. It has been proposed that their dominance is due to their ability to degrade

polysaccharides from the solid diets (e.g. MLG) [109, 153] Taken together, intestinal lactobacilli can be a good candidate for the yeast cell wall degradation in the small intestine of pigs, while in the large intestine, this duty is resorted to *Bacteroidetes*.

1.6 Soy and yeast in animal feeds

Soybean (*Glycine max*) meal is used as one of the main protein sources in the livestock feed. In a review by Coppock (1974), the author called soybeans "..the only expanding commercial source of protein able to meet the growing demand for a nutritionally balanced, high protein food suitable for both human and animal feeding." [154]. The only inaccuracy of this forecast was that the current demand for soybean is yet to be accommodated.

The production of soybeans is one of the major contributors to deforestation. This, in turn, brings about deterioration of the environment on several levels [155, 156]. Global warming and biodiversity loss represent some adverse events linked to the intensified crop production, soya included [155–158]. The use of soybean meal (SBM) in commercial livestock feeds reduces its availability for human needs. Those factors, along with the growing population [159], necessitates a search for sustainable alternatives to soybean products.

The idea of replacing the conventional proteins in livestock feeds is not new. In 1942, Macrae and co-workers investigated the nutritional value of *Cyberlindnera jadinii* (grown in a molasses medium) dried yeast as a protein source in 12 week-old pigs [160]. The authors noticed that the yeast inclusions provided sufficient nutritional levels and vitamins to support animal growth.

Upon further investigations [161–164], researchers concluded that inclusion of high levels of dried yeast to pig diets required also a supplementation of vitamin D and nicotinic acid to prevent rachitogenic effects of such diets. Russo and co-workers studied replacement of SBM or herring meal (HM) by the yeasts grown on n-paraffin. The authors found that the yeast replacement did not have detrimental health effects on the animals. The authors reported that zootechnical parameters of the yeast-fed pigs were comparable with those

fed either SBM or HM diets [165].

Currently, the technology for sustainable production of yeasts is available. *C. jadinii* yeast has shown to grow well on fermentation substrates based on renewable natural resources such as sugars derived from spruce trees, sugar and other nutrients derived from seaweed, as well as enriched nitrogen sources derived from locally produced poultry by-product [166, 167]. A particular strain of *C. jadiniii* yeast, LYCC-7549, from the Lallemand Yeast Culture Collection is being fermented with a combination of substrates and targets to obtain high yield and protein content from this particular strain.

C. jadinii yeast was used in the feeding trials as an alternative protein source because it has shown to support the best growth performance compared to other EU approved yeasts in diets for Atlantic salmon (*Saccharomyces cerevisiae*, *Kluveromyces marxianus*) as well as health beneficial effects, and modulation of the gut microbial composition [168].

A recent study by Cruz and colleagues has indicated that up to 40% of conventional proteins in a weaner pig diet can be replaced by those derived from heat-inactivated *C. jadinii* yeast [146].

There is a mounting body of knowledge about the effects of low inclusion levels of yeast (or yeast derivatives) to pig diets on the gut microbiota composition, metabolic contributions of the microbiota to the host homoeostasis, and the host immunity [169–175]. However, little is known about the effects of *high* yeast inclusion levels to the porcine diets on the above parameters.

2 The aims of the study

2.1 Main objective

This project was designed in a form of three feeding trials to gain insights into the effects of the diets with a high level of heat-inactivated *C. jadinii* on the gut microbiome of the weaner pigs and the microbial contribution to the host health-related parameters.

2.2 Specific objectives

A To describe changes in the gut microbiota in healthy weaner pigs fed a diet with 40% of crude protein (CP) from *C. jadinii* across different gut locations.

- This was accomplished by bacterial cultivation from the lumen content specimens collected across different GIT sites: jejunum, ileum, caecum, and colon (Paper I). Further characterisation was carried out by the 16S *rRNA* gene sequencing of the microbial communities of the ileum, caecum, colon, and rectum throughout the weaning and growing/finishing periods (Paper I, Paper III)

B To describe the contribution of the GIT microbiota to the morphology and short-chain acid pool of the large intestine in the healthy weaner pigs fed a diet with with 40% of CP from *C. jadinii*

- This was accomplished by a histological examination of the colon tissues and gas chromatography of the lumen content specimens collected from the caecum and colon (Paper I)

C To study the contribution of the novel diet to the response of the weaner pigs to experimentally induced enterotoxigenic *E. coli* colibacillosis.

- This was accomplished by the assessment of health-related parameters at the organismal level (scour incidence, body weight gain, appetite), at the cellular level (ileum *E. coli* F4ab^{+} colonisation, ileum intraepithelial lymphocyte populations), and the molecular level (characterisation of GIT microbial communities by 16S *rRNA* gene sequencing) in the weaner pigs challenged orally with the ETEC F4ab^{+}.

3 Summary of individual papers

3.1 Paper I

Effect of *Cyberlindnera jadinii* yeast as a protein source on intestinal microbiota and butyrate levels in post-weaning piglets

Stanislav Iakhno, Özgün C. O. Umu, Ingrid M. Håkenåsen, Caroline P. Åkesson, Liv T. Mydland, Charles McL. Press, Henning Sørum and Margareth Øverland

Animal microbiome 2, 13 (2020)

A response of the GIT (jejunum, ileum, caecum, and colon) microbiota, colon SCFAs, and colon histopathology to a diet with 40% of CP from *C. jadinii* was investigated in healthy weaner pigs. Bacterial cultivation results showed that there were more viable counts of lactic acid-producing bacteria (LAB) recovered from the jejunum (9.6 logCFU/g) and ileum (9.5 logCFU/g) on day 4 post-weaning (PW), ileum (10.0 logCFU/g), caecum (9.5 logCFU/g), and colon (9.8 logCFU/g) on day 7 PW, and colon day 14 PW (9.27 logCFU/g) in the yeast-fed piglets compared with those of the piglets fed the control diet (respectively, 7.3, 8.4, 8.6, 8.6, 8.9, and 8.4 logCFU/g).

The large intestine microbiomes (caecum, colon) were less diverse in the piglets fed the yeast diet at day 7 (Shannon diversity index on average between caecum and colon, meanSDi = 4.9) and day 14 PW (meanSDi = 4.8) compared with those of the control fed piglets (day 7 PW, meanSDi = 4.4 and day 14 PW, meanSDi = 4.1) as per the 16S *rRNA* gene sequencing analysis.

Next, the sequencing analysis revealed that the microbiota compositions of the large intestine were associated with the diet type (yeast or control) on day 7 and day 14 PW. The type of diet could explain around 24% variation in the Bray-Curtis dissimilarity.

The differential abundance test (ANCOM) indicated an over-representation of *Prevotella*, *Mitsuokella*, and *Selenomonas* phylotypes but lower relative abundances of *Faecalibacterium prausnitzii* in the large intestine microbiomes of the yeast-fed pigs compared with those of the pigs fed the control diet.

Microbially-produced SCFAs, butyrate and acetate, were found at lower concentrations in the colon digesta of the pigs fed the yeast diet (11.3 and 40.9 μmol/g, respectively) compared to those of the pigs fed the control diet (16.5 and 60.9 μmol/g, respectively).

The colonic crypt depth was greater on day 7 and day 14 PW in the control-fed piglets than that in the yeast-fed piglets. There were no differences in the histopathology scores of colon tissues between the two groups when compared at both day 7 and day 14 PW.

3.2 Paper II

Longitudinal analysis of the faecal microbiome in pigs fed *Cyberlindnera jadinii* yeast as a protein source during the weanling period followed by a rapeseed- and faba bean-based grower-finisher diet

Stanislav Iakhno, Francesco Delogu, Özgün C. O. Umu, Nils Peter Kjos, Ingrid M. Håkenåsen, Liv T. Mydland, Margareth Øverland and Henning Sørum

bioRxiv 2021.02.11.430725; doi: https://doi.org/10.1101/2021.02.11.430725

The faecal microbiomes had higher α-diversity in the yeast-fed piglets on day 8 PW (Shannon diversity index, SDi = 3.8) and day 22 PW (SDi = 4.5) compared with that in the pigs fed the control diet (SDi = 3.5 and SDi = 4.4, respectively). After the change of diets to the rapeseed- and faba bean-based diet, α-diversity in the porcine faecal microbiomes was less diverse on day 36 PW (SDi = 4.4) and day 57 PW (SDi = 4.4) and more diverse on day 87 PW (SDi = 4.9) in the yeast-fed piglets in comparison with that of the control-fed piglets (SDi = 4.5, 4.6, and 4.7, respectively).

Diet alone could explain 54% of the variance in the *weighted UniFrac* distances of the porcine faecal microbiomes on day 8 PW, while the diet could explain 15% of the variance in the *unweighted UniFrac* on day 8 PW.

Up to 25% of the variance in the *weighted UniFrac* distances of the faecal microbiomes were predicted by the diet on day 22 PW. After the change of the diet to the rapeseed- and faba bean-based diet, there was no effect of the diet on the β-diversity parameters (unweighted/weighted UniFrac) until day 87 PW where the diet and litter could explain 50% of the variance in the *weighted UniFrac* distance.

The major differences in the faecal microbiome composition were identified on day 8 PW. *Bacteroides*, *Blautia*, unclassified *Ruminococcus*, *R. bromii*, *Sphaerochaeta*, *Treponema*, and *Succiniclasticum* phylotypes were more predominant in the microbiomes of the yeast-fed piglets than those of the control-fed piglets. *R. bromii* remained differentially abundant on day 22 PW in the microbiomes of the yeast-fed piglets compared with that of the pigs fed the control diet. *Prevotella* affiliated phylotypes were more predominant in the microbiomes of the pigs fed control diet at day 8, 22, and 36 PW compared with that of the yeast-fed piglets.

The microbial network analysis indicated that there were more bacterial phylotypes (66 amplicon sequence variants, ASVs) found at all time points (except at weaning) in the microbiomes of the yeast-fed piglets (66 amplicon sequence variants, ASVs) compared with those of the pigs fed the control diet (55 ASVs). There were more stably connected (present in more than one consecutive networks) bacterial phylotype pairs in the microbiomes of teh yeast-fed piglets (3 ASV pairs) compared with those of the piglets fed the control diet (1 ASVs pair).

3.3 Paper III

Small intestine lactobacilli growth promotion and immunomodulation in weaner pigs fed *Cyberlindnera jadinii* yeast high inclusion diet and exposed to enterotoxigenic *Escherichia coli* F4^{+}: O149

Stanislav Iakhno, Selina S. Hellestveit, Özgün C. O. Umu, Lars T. Bogevik, Caroline P. Åkesson, Aleksandra B. Göksu, Charles McL. Press, Liv T. Mydland, Margareth Øverland and Henning Sørum

bioRxiv 2021.02.11.430732; doi: https://doi.org/10.1101/2021.02.11.430732

On day 7 after the ETEC challenge until the end of the experiment, the yeast-fed piglets had lower feed intake and average daily gain compared with those fed the control diet. There were no differences in the diarrhoea scores between the groups fed different diets. However, for those pigs from the farm without a history of post-weaning diarrhoea (PWD-naive), the scores were higher for the first three days after the challenge compared to the those in the pigs from the farm with a history of PWD (PWD-immune).
The colonisation of the ileum epithelium with the mucosal surface-associated ETEC F4^{+} on day 2 post-infection (PI) was 5% higher in the pigs fed the yeast-based diet compared

with the that fed the control diet. The distribution of intraepithelial lymphocytes (CD3) was not different between pigs fed either yeast-based or control diet. Notably, while there was no relationship between the ETEC F4$^+$ and CD3 in the ileum of the yeast-fed piglets, there was a negative correlation between the ETEC F4$^+$ and CD3 in the ileum of the pigs fed the control diet (*rho*= - 0.81).
The 16S *rRNA* gene sequencing analysis revealed *higher* α-diversity in the ileum and *lower* α-diversity in the caecum, and colon of the yeast-fed piglets on days 7 and 14 PI compared with those piglets fed the control diet. Next, β microbial diversity analysis of the 16S *rRNA* gene showed that the microbiota structure on day 2 PI was primarily associated with the litter (R^2 =38%) rather than the diet (R^2 =9%). The ileum microbial community structure on day 7 PI was still associated with the litter (R^2 =28%), while the diet was not. The association between the microbial composition of the caecum/colon and the diet was stronger on day 7 and day 14 PI (around R^2=14% for both). The intestinal microbiota compositions (ileum, caecum, and colon) were not associated with the litter on day 14 PI.
L. mucosae, *L. salivarius*, and *L. reuterii* on day 2 PI and *L. salivarius* on day 14 PI were differentially abundant in the ileum microbiomes of pigs fed the yeast-based diet compared with those fed the control diet. Actinobacilli, *E. coli*, *Str. luteciae*, *V. dispar*, and Pasteurellaceae phylotypes were differentially abundant in the ileum microbiomes of the yeast-fed piglets, while *Cl. perfringens* phylotype was differentially abundant in the ileum microbiomes of pigs fed the control diet.
Overall, on day 7 PI there were dozens more of the differentially abundant bacterial phylotypes in the large intestine (caecum, colon) of the control-fed pigs compared to those of the yeast-fed piglets. This trend changed on day 14 PI towards more equal number of differentially abundant phylotypes across the caecum microbiomes of pigs fed either the yeast-based or control diet. The colon microbiomes maintained more differentially abundant phylotypes on day 14 PI in the pigs fed the control diet compared with the pigs fed the yeast-fed diet.
The microbe-microbe interaction in the ileum was investigated by the network analysis. A pair of lactobacilli phylotypes, *L. johnsonii* and *L. reuteri*, was recovered from all ileum microbiomes throughout the experiment except on day 14 PI in the control-fed pigs. The interaction of small intestine lactobacilli pair was complemented by an additional connection with *L. mucosae* which was present on d2 PI and day 14 PI.

4 Discussion

4.1 General discussion

This project sought to close the gap in the knowledge of the porcine intestinal microbiome changes as a response to a diet with 40% of crude protein from *C. jadinii*. The microbiome-related contributions to the host homoeostasis were also explored.

An important consideration of introducing a novel diet is that it has to fulfil the nutritional needs of the animal. Dried yeast cells carry a wealth of nutrients such as proteins, amino acids, minerals, and vitamins. Intact yeast cell walls (CW) are a limiting factor for the nutrients to be accessible for absorption in the small intestine of some animals. It has been shown that physical or chemical disruption of the CW drastically improves nutrient digestibility in fish [145]. In pigs, such a pre-treatment of the yeast is not necessary. It has been demonstrated that the digestibility of the proteins from yeast is similar, or higher compared to that from the commercial feed proteins [146, 147, 176]. Microscopical analysis (data not shown) has demonstrated that intact yeast cells can be detected at varying levels in the digesta specimens collected across different GIT segments in the yeast-fed pigs. Much of the yeast cell is identifiable in the jejunal digesta, less so in the ileum, and it is virtually absent in the large intestine (caecum and colon). The yeast cell wall is made up of a complex matrix of 1,3(6)-β-glucans, mannan-protein, and chitin. Neither fish nor pigs have endogenous enzymes that break down the glycosidic linkages of those carbohydrates which stabilise the yeast CW. It suggests a pivotal role of the porcine gut microbiome in enabling the yeast cell nutrients for the host digestion.

One of the main findings of this project is that high inclusion of *C. jadinii* in a weaner diet has an impact on the porcine GIT microbiome. The changes in the microbiota compositions in the small intestine and large intestine were profoundly different. In fact, within the large

intestine, differences between distinct segments were found.

The **small intestine** microbiota is characterized by a dominance of few but ecologically adapted bacterial species. These commensals should not, under physiological conditions, elicit an inflammatory response. Instead, their contribution to the normal development of the immune response to pathogens is expected [177, 178]. In Paper I and III, we have demonstrated that the lactobacilli abundances were greater in the pigs fed the yeast-based diet compared with those fed the control diet. The internal validity of this finding was secured by the fact that the differences in lactobacilli were detected by using both cultivation and high-throughput sequencing methods.
The main difference in the diets was either presence or absence of the yeast. This, along with the randomisation of the animal allotment (blocked by weight and litter) to the diets, makes the differences in the gut microbiome to be attributable to the high levels of yeast inclusion (confounding is discussed in the next section).

Lactobacilli are known to be one of the first GIT colonizers [103]. The symbionts are also versatile carbohydrate degraders [109, 153]. The health benefits attributable to intestinal lactobacilli have been discussed elsewhere. Being able to produce biofilms [15] and adhere to the intestinal mucus [179, 180], lactobacilli switch to the fermentation of cereal mixed-linkage glucans soon after weaning [106, 109, 140, 153]. Interestingly, the predominance of enriched lactobacilli can be traced across all GIT segments [109, 140].

Charlet et al. have demonstrated that *Lactobacillus johnsonii* can directly attack and inhibit *Candida glabrata* yeast deploying a chitinase-like enzymatic machinery [152]. Porcine lactobacilli, *L. johnsonii* and *L. reuteri*, can encode a chitin-binding domain protein, LysM [115]. Another, unclassified species of *Lactobacillus* from the same dataset carried acetyl xylan esterase, an enzyme capable of hydrolysing relevant glycosidic bonds of the yeast CW polysaccharides [181, 182].

The existing knowledge about lactobacilli genomics, their microbe-host co-evolution record [13, 38], and the experimental evidence of fermentative functions of lactobacilli in the GIT, align well with the findings of this work: the enrichment of distinct host-associated species of genus *Lactobacillus* in the small intestine of pigs fed the yeast-based diet. This suggests

that high levels of *C. jadinii* in a weaner pig diet adds microbiota-directed food (MDF) properties to the feed [183]. In fact, dietary *C. jadinii* yeast could be classified as a prebiotic. But technically, it requires the documentation of both an augmentation of the beneficial species in the intestines and the health-related benefits of those species [183].
The characterisation of the intestinal lactobacilli activities was out of the scope of this work. However, we reported in Paper III a modulation of the immune response to the ETEC $F4^+$ infection due to high inclusion of *C. jadinii* yeast in the weaner pig diet.
Håkenåsen et al. have demonstrated an upregulation of the expression of genes involved in the immune signalling pathways (NF-kB, TLR1, TLR2, and TLR4) on day 7 PW in the jejunum of the yeast-fed piglets when compared with that in the control-fed piglets [147].
Lagos et al. have shown that the yeast-based diet is associated with an increase in the $CD3^-/CD8^+$ cell population in the lymphatic nodes of the distal jejunum on day 28 PW [184]. In neither of the two aforementioned experiments, high inclusion of *C. jadinii* to the weaner diet affected the feed intake and weight gain of the animals.
In Paper III, the effects of the yeast-based diet were found at the organismal level. More specifically, the yeast-fed piglets had a reduced appetite and subsequently a lower daily gain compared with the control-fed piglets. More, the population of intraepithelial T lymphocytes in the ileum tended to be less and not dependent on the degree of the ETEC colonisation in the ileum of the yeast-fed piglets compared with those fed the control diet (Paper III).
It is not clear whether the reduction in appetite has a positive or negative consequence on the animal well-being in the long term perspective. Previous works have demonstrated that anorexia during enteric infections is likely a life saving behavioural adaptation [185–187]. Also, it needs clarity whether the changes in appetite and the intraepithelial CD3 populations were due to a direct effect of the yeast components (mannans, and yeast β-glucans [172, 182, 188–191]) on the immune system or due to an increase in small intestine lactobacilli or both.

Large intestine microbial communities of the colon of the yeast-fed piglets were less diverse compared with those of the control-fed piglets (Paper I, III). However, when the comparison between the distal colon microbiota (faecal microbiota) of the yeast-fed piglets and that of

the control-fed piglets was made, the bacterial α-diversity was *higher* in the former than in the latter. The most abundant bacterial phylotype, affiliated to the *Prevotella* genus was more predominant in the *apex coli spiralis* microbiota of the yeast-fed piglets compared with that of the control-fed piglets (Paper I). Conversely, another most abundant bacterial phylotype, affiliated to the *Prevotella* genus was more predominant in the faecal microbiota of the piglets fed the control diet compared with that of the yeast-fed piglets on day 22 and day 36 PW (Paper II). Considering that the porcine *Prevotella* species possess an enzymatic repertoire that degrades multiple non-digestible carbohydrate sources (Figure 1.3-1.4), it is conceivable that the outgrowth of *Prevotella* affiliated phylotypes should be a function of the nutrient composition in the lumen.

One way of understanding the differences in the large intestine microbiota found during this project would be to look at the differences between the compositions of the yeast-based and the control diets. It may be suitable to relate those differences to the knowledge about the enzymatic potential of the gut symbionts.

Firstly, the inclusion of **wheat** differed between the diets with the control weaner diet containing 628 g/kg of wheat compared with 594 g/kg of that in the yeast-based diet (Paper I, II, III). Wheat contains a substantial proportion of DF of which cellulose, fructans, arabinoxylans [192] are of particular relevance to the large intestine microbial fermentation. For this subset of DF, one should expect to find the growth of *Roseburia* spp, *Blautia*, *Prevotella* spp., *Bacteroides*, *F. prausnitzii*, Ruminococceae family, Lachnospiraceae family, and other relevant bacterial phylotypes in the ecosystem of the large intestine [108, 115, 128, 135, 136].

Secondly, the proportion of **soybean** meal and **rapeseed meal** in the control diet was four-folds higher than that in the yeast-based diet. This may relate to the differences in availability of cellulose, arabinans, and arabinogalactans [23, 192] for microbial fermentation in the large intestine. Terpend et al. have demonstrated that arabinogalactans can be selective for the large intestine *F. prausnitzii* and *Bacteroides* spp. [193]. Further, *Prevotella* spp., *Roseburia* spp, *Dorea* spp., *Lachnospiraceae* family, *Porphyromonadaceae* family, and *Coprococcus* possess the genetic determinants for arabinan enzymatic

degradation (Figure 1.3-1.4) [115].

Lastly, the presence of high levels of *C. jadinii* heat-inactivated cells in the yeast-based weaner diet should enable yeast β-glucans, mannans, and chitin for the microbial degradation. Besides the potential of lactobacilli (discussed earlier) to engage in the yeast CW degradation, *B. thetaiotaomicron*, *Prevotella* spp., and *Parabacteriodes* [148, 152] can be instrumental in degrading the mannan fraction of the CW. Temple and coworkers have discovered a polysaccharide utilisation loci (PUL) of intestinal *Bacteroides* which is specific for the fungal 1,6-β-glucan degradation [149]. This $PU_{1,6\text{-}\beta\text{-glucan}}$ is syntenic to the PULs from the *Bacteroidetes* found in the porcine gut metagenomes. The majority of porcine *Prevotella* MAGs has an ample fermentative potential towards the yeast CW poly- and oligosaccharide degradation ([115], Figure 1.3-1.4).

This work illuminated the presence of two distinct microbiome types (enterotypes). The first enterotype (termed here "yeast-enterotype") featured the dominance of *Prevotella*, low microbial diversity, and low abundance of SCFA-producing DF-degrading bacteria in the caecum and colon. The "yeast-enterotype" microbial composition can be related to the high availability of yeast-derived fibre. The second enterotype was characterised by a diverse composition of potent SCFA-producing symbionts which can be attributed to a greater amount of DF from wheat, soybeans, and rapeseed in the control diet compared with the yeast-based diet ("plant-enterotype").

The colon microbiome (Paper I and III) had the features of the "yeast-enterotype". However, the faecal microbiome in Paper III had the "plant-enterotype" characteristics. To understand the reasons for this, it is vital to know which fibre and how much is available after the yeast CW degradation by the small intestine lactobacilli. With the premise of the findings by Charlet et al., that is *L. johnsonii* deploy chitinase-like fermentation of the yeast CW but not mannan utilizing PULs [152], it is conceivable that the remainder of the CW can find its way to the large intestine. It is worth noticing that the protein content of the *C. jadinii* is available for the host in the small intestine [146, 147].

If there are no other small intestine bacterial candidates to scavenge the residual β-glucans and mannans, the latter may become prey for large intestine *Bacteroidetes*. Large

intestine *Bacteroidetes*, including highly abundant *Prevotella* and *Paraprevotella*, are well-versed in degrading β-glucans and mannans [148, 149]. These gut symbionts deploy endo-α-1,6-mannanases and endo-1,6-β-glucanases. This allows them to "selfishly" degrade the yeast CW carbohydrate matrix [148, 149]. More diverse **faecal** microbiomes with low abundance of *Prevotella* in the yeast-fed pigs compared to that of the control fed pigs may be related to the *Prevotella* fermentative activity in the **proximal** part of the colon. The distal part microbial communities may utilise the fermentation end-products passing from the proximal colon.

Comparatively lower abundances of the **SCFA**-producing bacteria including *F. prausznitzii*, *Roseburia*, *Blautia*, *Dorea* (Paper I, III) resulted in lower concentrations of butyrate and acetate in the colon of the yeast-fed piglets compared with those of the control-fed piglets (Paper I). This finding is in line with the results by Nielsen et al. [108]. They found that AX dietary inclusion (enriched in wheat) resulted in a higher number of *F. prausnitzii*, *R. intestinalis* and *Blautia coccoides* in the faeces of growing pigs.

Next, the butyrate concentrations were associated with the depth of colonic crypts (Paper I). Knudsen et al. investigated the pathways of the colonic butyrate distribution [34]. The authors have demonstrated that the proportion of butyrate which is not metabolised by enterocytes and transferred to the liver via the portal vein is higher than it was previously suggested [33, 34].

In Paper I, a strong positive correlation between the colonic butyrate, and *F. prausnitzii* relative abundance, and the liver size of pigs were found. These findings support the results from Knudsen et al. [34]. More, our results (Paper I) suggest that there is a certain limit of the microbially-produced butyrate that *can* be utilised by the colon enterocytes. These findings may promote an interest in further characterisation of the MDF use as a health-promoting measure [120, 194, 195]. To support of this opinion, we have found no evidence of worse gut health in those animals with shorter colonic crypts and comparatively low butyrate concentrations (i.e. yeast-fed pigs) when examining 32 colon tissue sections histopathologically. In fact, it appeared quite the opposite (Paper I). In sum, the high levels of yeast inclusion in the weaner diet do change the microbial composition of the large

intestine promoting certain *Bacteroidetes* at the cost of a reduction in SCFA *Firmicutes* producers.

4.2 Methodological considerations

Cultivation and sequencing

The major part of this project dealt with detecting and comparing bacterial communities across the GIT of *the yeast-fed* and *control-fed* piglets.

For Paper I, we used both cultivation and 16S *rRNA* gene sequencing approaches. The LAB cultivation on MRS agar assay supported the findings derived from the DNA-based analyses. Paper III findings also indicated an outgrowth of lactobacilli in the ileum of the yeast-fed piglets. Complementary results obtained in both Papers demonstrated that the use of cultivation methods seems still to be a valuable microbiology tool in the era of the dominance of high-throughput sequencing approaches.

Recently, major advances in the area of the cultivable porcine gut microbiota have been made. Wylensek and colleagues cultivated 110 bacterial species from the porcine faeces [94]. A similar initiative was made for the ruminal gut microbiota [196]. The researchers used an exhaustive set of cultivation techniques which was made possible by an international collaboration involving many labs across the globe [94, 196]. This means that to obtain a detailed characterisation of a gut microbiota such as in our experiments, the cultivation effort may not be easily accessible. Cultivation-free, DNA-based, methods of microbiome characterisation have recently become a popular tool for identifying gut inhabitants whenever a specimen can be collected from an animal in question [197].

The context of this project requires a definition of two popular sequencing approaches, 16S *rRNA* bacterial gene sequencing and the metagenomic “shotgun” sequencing. The two sequencing approaches are similar in that they determine the bacterial composition of the microbial communities based on the extracted DNA. The former method uses a PCR-amplification of a fragment of the 16S *rRNA* bacterial gene. The latter method infers the community composition from the total DNA including host, environmental (feed components, soil), and microbial (bacterial, archaeal, viral, fungal, etc.) DNA.

It is important to keep in mind that current short-read sequencing technology has

constraints as to how much of the sequenced total DNA will be of the bacterial origin. While this is not of particular concern to handling large intestine luminal specimens where the bacteria constitute 24-78% of the luminal content [198], the specimens collected from gut epithelium can be dominated by the host-derived DNA. In the specimens laden with DNA from several sources, the identification and quantification of some bacterial species, especially rare ones, may become problematic [199, 200].

One drawback of the 16S *rRNA* gene sequencing, however, is that it lacks the resolution to reliably assign the amplicon sequence variants (ASV) down to the species taxonomy level. One example of this issue is that the SILVA [201] classification database has an "E. coli/Shigella" assignment. This means there will be occasions when a gut commensal will not be discriminated from an enteric pathogen (and vice versa) when using the 16S *rRNA* gene sequencing.
This project used the 16S *rRNA* gene sequencing approach to characterise the microbial communities in question. Since the prime task was to find **differences** between the microbiota that belonged to animals fed either yeast-based or a control diet, the results of such a comparison should hold despite all unknown flaws/biases accumulated during the sample collection, DNA extraction, hypervariable fragment of the 16S *rRNA* gene amplification, and other sequencing aspects. Given that all animals and collected samples are treated similarly, the results of the differences should reflect the effect of experimental conditions, i.e. the diet types.

Microbiome composition variation

One known concern to internal validity during sample collection (Paper I, III), was that the animals were sacrificed at different times of the day (from 8 am to 4 pm). Even though the running order of the animal sampling was random, some unavoidable variation of the gut microbial composition might have been introduced by this sampling procedure. For instance, it was noticed that some piglets had little to none digesta in their small intestines. That might have been related to the differences in the last meal-to-sampling time across the sampled animals.

Notably, since for some sampled animals there was a drastic difference in the time of the day (e.g. “morning” pigs vs “afternoon” pigs), diurnal rhythms might, too, have introduced some variation to the microbial compositions [100–102]. To the best of my knowledge, our research team accounted for the known and unknown biases by using randomization at the allotment, sampling, and evaluation of the results procedures. Whenever summarizing the data, it was done by one person in a blindfolded way.

In addition to the microbiome composition volatility linked to the diurnal rhythms, different sampling time points of the pig life cycle were of prime importance to the microbial composition inference. The GIT microbiome composition temporal change of mammals is thought to follow a certain pattern throughout the animal life cycle. This phenomenon is termed microbial succession and was reviewed by Conway P.L. in [202]. The course of the microbial succession can be appreciated in the context of the microbial ecology parameters which change continuously throughout the animal life cycle (e.g., immune system development)[203]. However, there are abrupt events throughout the animal life such as weaning, transportation, change of diets which have profound implications for the shifts of the gut microbiome and the microbial succession. Hornef and Torow proposed a temporal model of the mammalian immunity development system. According to their model, the weaning period is central to the development of the innate and adaptive immunity as well as the gut microbial community parameters [203].

In this work, the sampling time points for the screening of the gut microbial communities were chosen to cover the post-weaning period up to day 14, 87, and 21 PW as per Parer I, II, and III, respectively. It has become apparent to us that the gut microbial compositions are volatile right after weaning. Thus the results obtained during the first week PW may be problematic to generalise with enough confidence.

The major body of this work was focused on the luminal content specimens across the porcine intestine. The microbial compositions of the mucosal-associated micro-environment and also the microbiota of the gut segments upstream of the ileum were not explored. The microbial compositions of the porcine gut mucosa surface and the lumen differ qualitatively and quantitatively [10, 204, 205]. As an example, a work by Looft and co-workers has shown

that the mucosal-associated community species richness can measure as much as 300 OTUs compared to 13 OTUs recovered from the lumen of a corresponding ileum section of 3-month pigs [206]. While nearly all lumen bacterial OTUs were concomitantly identified in the mucosal-associated micro-environment of the porcine ileum, the authors detected around 30 bacterial OTUs that were differentially abundant on the mucosal surfaces of the gut when compared to the lumen bacterial composition [206]. Vast amount of immune tissues is located in the small intestine of pigs. Therefore Paper III was limited regarding investigating microbial communities at the very interface of host-bacteria interaction. In Paper III, an attempt to approach mucosal surface of the ileum by means of immunohistochemistry was made. Although a certain progress towards studying the interplay between the loads of the ileal challenge *E. coli* strain and the intestinal intraepithelial lymphocyte proportions, a deeper screening of the mucosal-associated microbial communities should benefit studies of health effects pertinent to dietary interventions.

Illumina v3 chemistry

In Paper I, the Illumina HiSeq sequencing resulted in the paired-end V1-V3 hypervariable region of the 16S *rRNA* gene amplicon sequencing data that were difficult to merge without introducing a bias. More specifically, a substantial proportion of the reads [https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA580284] had low quality of the 3' end of the reverse reads (Figure 4.1).

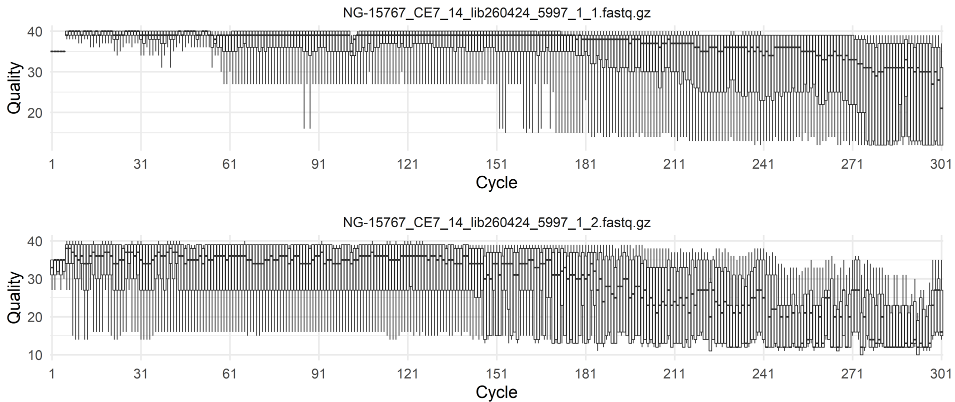

Figure 4.1: Per base quality control report of a representative pair of the forward (top graph) and reverse (bottom graph) fastq reads with quality issues. The horizontal axis shows the nucleotide base positions 1 to 300, from left to right. The vertical axis shows the quality scores where Q20 value corresponds to a probability of 1/100 of the nucleotide called wrong. The bold black line inside the boxes represents the median quality score; the box boundaries delineate 25 to 75% quantiles; and the whiskers represent the 10% of the lower and upper tails of the quality score distribution. The quality of the reverse reads sufficiently drops starting from 150th base with values around 10 seen from 230 nucleotide onwards (0.1 probability of an erroneous base call)

In practice, that meant many mismatches when it was attempted to merge the forward and reverse reads.

Since this technical issue affected the samples disproportionally, it was chosen to use only the single-reads which were informative of the V1-V2 hypervariable region after the quality trimming. Although, a fair comparison between the microbiomes in the pigs fed either yeast-based or control diet was possible, that probably resulted in a reduced taxonomic resolution of the inferred ASVs.

Table 4.1: Two mock communities (sample 1 and 2) which consist of ”a”-”e” species with the respective frequencies

species	sample 1	sample 2
a	5	50
b	10	100
c	5	50
d	3	30
e	10	100
f	10	100
Total	43	430

Library size

One of the core analyses applied in all three manuscripts was the detection of differentially abundant bacterial phylotypes. There are several statistical/analytical challenges inherent to the 16S *rRNA* gene amplicon datasets. Typically, when one wishes to compare the distributions of frequencies which represent a set of independent entities, the conditions of the independence and the normality of distribution would allow applying a *t-test* (Mann-Whitney test in case of not normal distributions). From an Illumina sequencer, a typical post-processed sample represents a frequency table comprised of all possible ASVs found in the habitat at certain frequencies.
Consider the following example. The Table 4.1 shows frequencies of a mock community which consists of 6 species (“a” - “f”). It is clear that each species in the sample 2 is 10-fold greater than those in the sample 1 (Table 4.1). This 10-fold difference in the absolute number of species indicates one of the common features of the amplicon sequencing studies, *uneven library sizes*.

In practical terms, it means that to enable a meaningful comparison between samples one has to somehow “normalize” the absolute abundances of species. One popular approach to deal with the unequal library sizes is to downsample all the sequencing data to an arbitrarily chosen value. This value typically represents an attempt to capture the majority of samples where a “true” diversity can be estimated. The true diversity here is referred to a state where no new (or very few) new species is discovered when more sequencing reads are recruited (Figure 4.2).

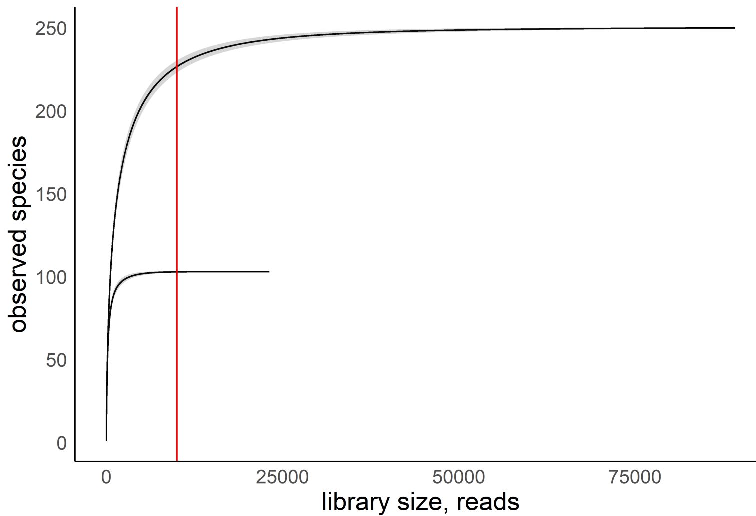

Figure 4.2: Rarefaction curves of two colon digesta samples: one having 100 unique species (the steep curve with a smaller sample (library) size, LS) and another having 250 unique species (the shallow curve with a bigger LS). If the rarefaction is made at 10,000 SS, new species will be lost for downstream analysis in the sample corresponding to shallow curve. The example is drawn from [207] dataset. The samples were agglomerated down to the species level of taxonomy. Generated using [208]

This strategy, termed rarefaction [209], has proponents [210] and critiques [211, 212]. McMurdie and Holmes (2014) and Willis (2019) provided a mathematical reasoning that rarefaction of the sequencing data, that already have measurement errors (undersampling, oversampling of microbial communities), may be problematic and introduce bias [211, 212]. In Paper I, the microbial communities were oversampled because of using the Illumina HiSeq protocol. The mean number of reads was 450,000 per sample which was much higher than that in Paper II and III where Illumina MiSeq sequencing was used (62,000 and 72,000 reads per sample on average, respectively).

Consequently, in Paper II and III, there were instances of low sequencing read samples which had to be accounted for. For instance, those samples with less than 40,000 reads per sample exhibited a positive relationship between the number of reads and α-diversity metrics, observed species and the Shannon diversity index (Figure 4.3).

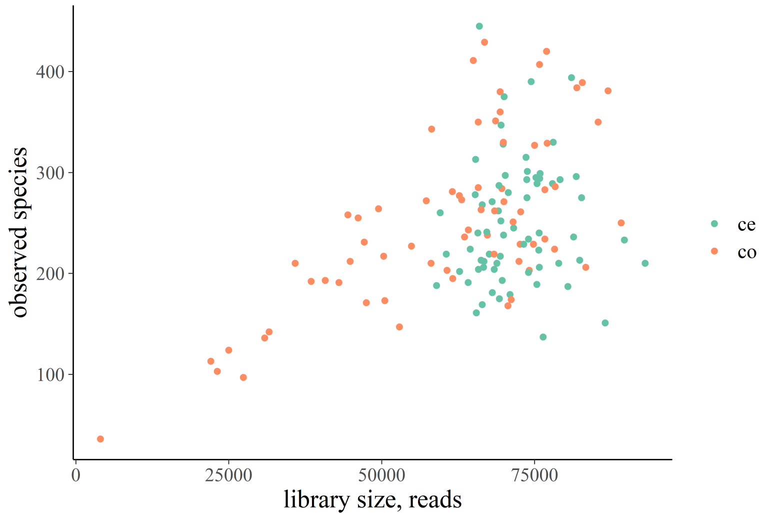

Figure 4.3: Relationship between the library size (LS) and the number of observed species per habitat (coloured orange and green for, respectively, the colon and caecum). There is a positive relationship (rho = 0.67, p=1.309e-10) between the number of observed species in the colon and the corresponding LS (especially for LS less than 40,000 reads). For the caecum samples, where the LS is well above 50,000 reads, there is no such correlation.

To hold the influence of the sequencing parameters out of the inference of biology, it was decided to adopt two strategies. To estimate α bacterial diversity, the DivNet algorithm was applied. The chosen method models unobserved species along with the measurement error estimation [213]. For estimating β-diversity, however, the samples with less than 40,000 reads were excluded from the analysis.

Excluding low read number samples inevitably reduces the power of the respective statistical procedures. With the reduction in power, the false-negative rate (type II error) increases [210]. The power issue makes studies of the gut microbiome in large animals (excluding those studies where faecal samples are to be collected) somewhat complicated. Practically, samples of only a limited number of animals can be collected at a given time to ensure their comparability. Other sources of power of study reduction are economic considerations and animal welfare principles.

Compositionality of microbiome data

Another common way of transformation applied for unequally sized sequencing libraries is to convert of the absolute frequencies to the proportions, or relative abundance. When applied, the compositions of the sample 1 and 2 look the same.

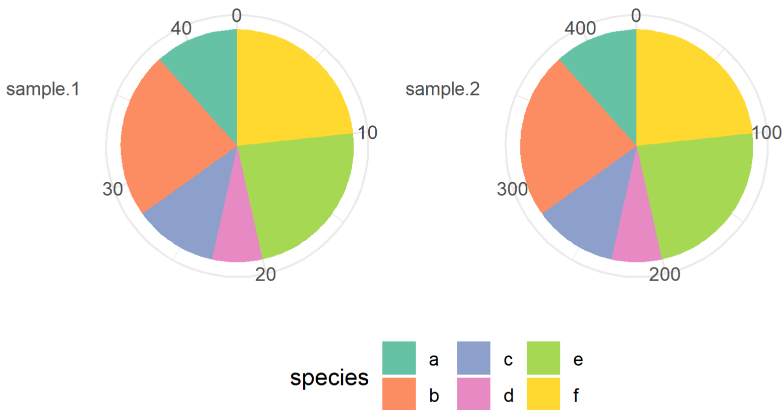

Figure 4.4: Two mock communities that are different in terms of absolute abundances but equal in terms of relative abundances. The frequencies of each species are given on the outer circles. See also Table 4.1

To demonstrate how the community composition changes in terms of the relative abundances, consider a modification of the above example. Suppose, species “e” in the sample 2 is reduced by a half (100 to 50) in terms of the absolute numbers. We now see that it is not obvious whether it is only “e” which reduces its relative abundance in the sample 2 compared with the sample 1 or this occur along with the increase of the other species relative abundance (Figure 4.4).

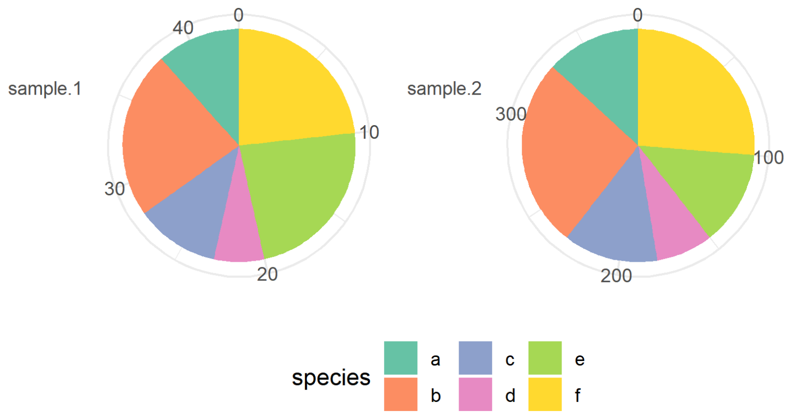

Figure 4.5: Two mock communities that are different in terms of the absolute abundances and different in terms of the relative abundances. A change in one species ("e") affects the relative abundances of other species in the sample 2. Explanation in the text

The knowledge about the true population of the microbial community is obscured. The inference on the community structure is made from the sequencing data of a sub-sample of the microbial community which is believed to be representative enough. The above example of species "e" manipulation indicates that the comparison of the communities based on the relative abundances alone may be misleading [214].

The quality of the multivariate data where a change of one component leads to changes in other components is referred to as compositionality [215]. Naturally, the use of the statistical procedures, where the assumption of independence has to be met, will lead to a high rate of false discoveries [210]. Weiss et al., ascribed as many as 40% false discoveries to the use of *t-test* on the relative abundance data, i.e. compositional data.

Mandal and co-workers devised a statistical procedure that accounts for the compositionality of the data [216]. In Paper I, their statistical procedure, termed ANCOM, was applied. While there was a certain correspondence between the ANCOM test results in Paper I and those in Paper III, a note of caution should be mentioned. Since the single-end data was used and the communities were sequenced at an excessive depth, it is possible that

the intra-species variability of the 16S *rRNA* gene was inflated by non-biological ASVs. Figure 4.6 demonstrates the variation within all lactobacilli ASVs inferred from the V1-V2 single-end data (Paper I) on the left ('A') compared with that from the V3-V4 pair-end data (Paper III) on the right ('B'). Accordingly, the number of distinct lactobacilli ASVs was 213 and 62 for the V1-V2 single-end and V3-V4 paired-end data, respectively.

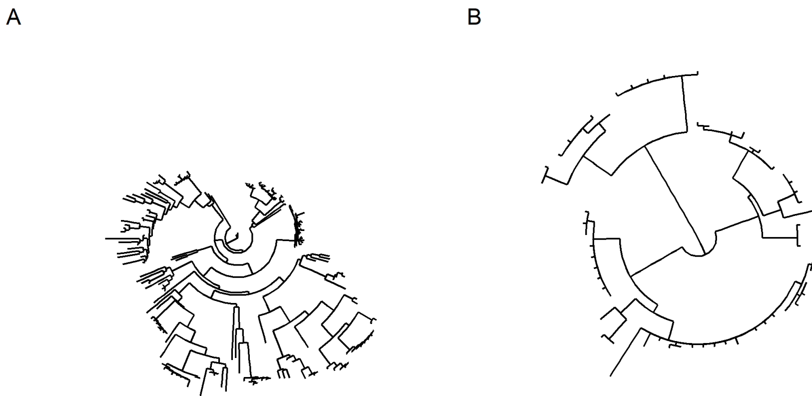

Figure 4.6: Phylogenetic trees of genus *Lactobacillus* retrieved from Paper I (panel A) and Paper III (panel B). Explanation in the text.

A high number of ASVs per habitat resulted in a high number of tests. For instance, there were approximately 7000 tests when analysing differentially abundant phylotypes in the colon samples (Paper I). Given that the Benjamini–Hochberg correction for multiple testing was applied (less strict than Bonferroni correction), it is possible that some findings from the Paper I were brought about by chance as per the ANCOM analysis. On the other hand, the correlation analyses which were performed in Paper I, yielded similar results using either the absolute or relative abundances. A drawback of the ANCOM test is that it is underpowered when less than 20 samples are analysed [210]. Provided there was a limit of animals per feeding group available for comparison (8-10 individuals), another algorithm for differential abundance testing, 'corncob', was used in Paper II and III. The 'corncob' fits the microbial data to the β-binomial regression model to detect the differentially abundant

species. As the authors pointed out, the 'corncob' power might be suboptimal too in case the sample size is less than 30 [217]. This means that differential abundance testing in the experiments similar to ours in design and implementation may be problematic due to an increased type II error rate.

Influential covariates

In Paper III, an estimation of the ileum ETEC colonisation was to be made. It was surprising that the litter was of greater importance (Figure 4.7) for the ETEC colonisation than other variables such as the *MUC4* gene polymorphism, diet, gender of animals, etc.

Piglets from the farm with a history of post-weaning diarrhoea tended to have a lower proportion of the mucosa-associated ETEC in the ileum, all known confounding factors controlled. However, the values of ETEC in the piglets from the 3288 litter suggest that some unknown genetic determinants can render piglets more robust/weak against ETEC colibacillosis irrespective of the environmental factors, diet included (Figure 4.7).

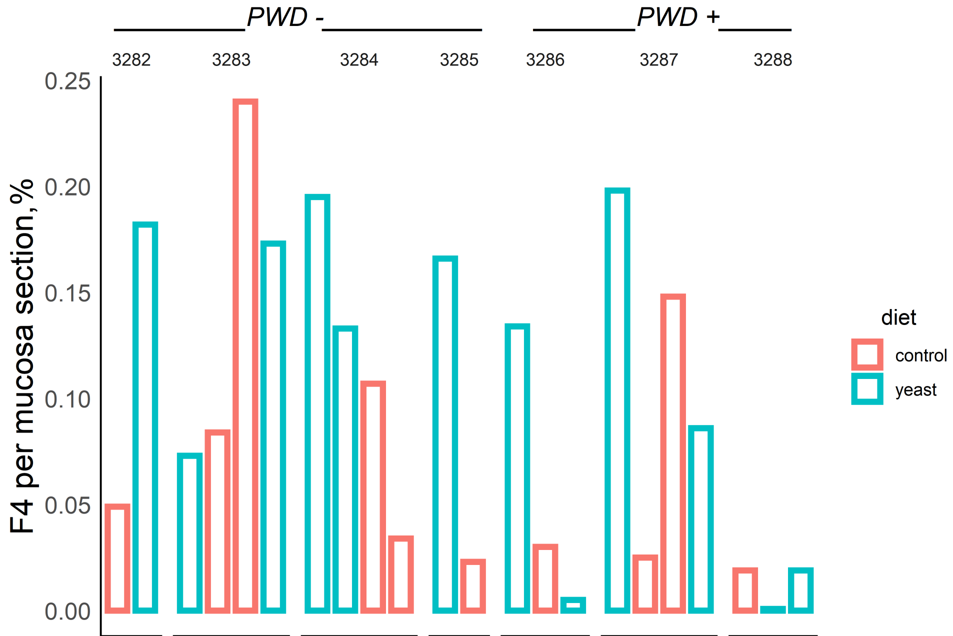

Figure 4.7: Distribution of mucosa-associated ETEC in the ileum of the ETEC challenged piglets on day 2 PI. The individual values taken from each animal are denoted by the litter (3282 - 3288), the herd (**PWD -**, the herd without a history of PWD; **PWD +**, the herd with a history of PWD) and coloured by diet (*red*, control; *blue*, yeast)

5 Main conclusions

High inclusion of heat-inactivated *C. jadinii* in a weaner diet affects the porcine GIT microbiome. The population of lactobacilli increases in the small intestine. The microbiota of the large intestine is dominated by a Prevotella-affiliated phylotype which decreases bacterial diversity of the habitat. The gut microbiome structure is likely shaped by the availability of dietary fibre that is specific to the yeast cell wall, among other relevant determinants (gut physiology and ecology, age of animals, litter, health state, etc.).

The novel diet changes the morphology of the large intestine. The colonic crypts are shorter in the yeast-fed piglets than in the control-fed piglets. These morphologic changes are associated with a shift of the resident microbiota towards a relative reduction in butyrogenic bacteria (and their products). The overall changes in the large intestine microecology due to the yeast inclusion are associated with a healthy gut.

High inclusion of heat-inactivated *C. jadinii* modulates the immune response in pigs challenged with enterotoxigenic *E. coli*. The effect of yeast inclusion extends to changes in behaviour (appetite reduction). This effect may be related to the promotion of small intestine lactobacilli. But the detail of this mechanism needs further investigation.

High inclusion of heat-ianctivated *C. jadinii* yeast in a porcine diet may serve two major purposes: 1) an improvement of the animal health via prebiotic-like properties of the yeasts; and 2) an alleviation of the soybean production-related environmental burden.

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Enclosed papers I - III

Paper 1

Iakhno *et al. Animal Microbiome* (2020) 2:13
https://doi.org/10.1186/s42523-020-00031-x

Animal Microbiome

RESEARCH ARTICLE Open Access

Effect of *Cyberlindnera jadinii* yeast as a protein source on intestinal microbiota and butyrate levels in post-weaning piglets

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Abstract

Background: Dietary yeast inclusions in a pig diet may drive changes both in gut bacterial composition and bacterial functional profile. This study investigated the effect of *Cyberlindnera jadinii* as a protein to replace 40% of the conventional proteins in a diet for weanling pigs on the microbiota in the small and large intestine, colonic short-chain fatty acid concentration, and colonic histopathology parameters. Seventy-two pigs weaned at 28 days of age were randomly assigned to either a control or a *C. jadinii*-based diet and followed for 2 weeks.

Results: Compared with the controls, higher numbers of cultivable lactic acid-producing bacteria in the small and large intestine were registered in the yeast group. Alpha and beta bacterial diversity were different between the diet groups with lower alpha-diversity and distinct bacterial composition in the large intestine in the yeast group compared with those of the controls. The large intestine microbiota in the yeast group had higher numbers of *Prevotella*, *Mitsuokella* and *Selenomonas* compared with those of the controls. The concentrations of colonic acetate and butyrate were higher in the controls compared with that of the yeast group. The colonic crypt depth was deeper in the control group. The gut histopathology of colonic tissues revealed no differences between the diets. The colonic crypt depth tended to be deeper with higher relative abundance of an unclassified Spirochetes, higher colonic butyrate concentration, and higher bacterial richness. The concentration of colonic butyrate was positively associated with the relative abundance of the *Faecalibacterium prausnitzii*, *Dialister*, and an unclassified amplicon of the Spirochaetaceae family in the colon.

Conclusions: The replacement of the conventional proteins by proteins from *Cyberlindnera jadinii* in a weanling pig diet reshaped the large intestine microbiota structure. The novel yeast diet appeared to be selective for *Lactobacillus* spp., which may represent an added value resulting from using the sustainably produced yeast protein ingredient as an alternative to conventional protein ingredients in animal diets. The large intestine bacterial composition and their metabolites may be involved in an adaptive alteration of the colonic crypts without pathological consequences.

Keywords: Pig microbiota, Yeast diet, *Cyberlindnera jadinii*, Gut, Butyrate, Crypt depth, 16S *rRNA* gene sequencing, *Lactobacillus* spp.

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Introduction

Up to 70% of globally produced soy is used to maintain livestock production [1]. Sustainable protein alternatives are needed to reduce the dependency on soy and other conventional proteins as ingredients in the feed for animal husbandry. As early as in the 1940's, researchers pursued the idea of replacing a substantial fraction of protein in animal feed with proteins from yeast derivatives [2, 3]. It soon became apparent that the lack of cost efficient methods for large scale yeast production would limit the use of yeast proteins [3]. Moreover, additional costs arose from the necessity of vitamin D and calcium supplementation to counteract the rachitogenic effect of yeast diets [4, 5]. Today, technology exists to produce yeast by industrial fermentation of *Picea abies* second-generation sugars as a carbon and energy source [6]. Our previous work has shown that up to 40% of conventional protein in a pig diet can be successfully replaced by proteins from a strain *Cyberlindnera jadinii* yeast [7]. The addition of yeast as a protein source supported high growth performance and improved gut health of the weanling pigs [7]. To date, a number of studies have investigated the effect of yeast supplementation on pig microbiota composition. Addition of live *Saccharomyces cerevisiae* to a diet promoted overgrowth of *Mitsuokella* bacterial genus in the large intestine microbiota in weanling piglets [8]. Inclusion of cider yeast probiotic to a diet shifted faecal microbiota towards higher numbers of *Selenomonas* and *Prevotella* in weanling piglets [9]. In addition, Upadrasta and co-workers reported reduction in *Faecalibacterium*, *Roseburia*, and *Eubacterium* in faeces of the yeast group. Impact of yeast derived components such as cell wall β-glucans and mannan-oligosaccharides on the gut microbiota in pigs has also been studied. Fouhse and co-workers reported high relative abundance of *Mitsuokella* and low relative abundance of *Coprococcus* and *Roseburia* in caecum of piglets supplemented with yeast derived mannan-rich fraction [10]. Nakashimada et al. studied changes in pig faecal bacterial composition using an in vitro intestinal model. These investigators found lower numbers of *Faecalibacterium* in the reactor system with addition of yeast cell wall components than without [11]. While supplementation of yeast ingredients does seem to promote distinct intestinal bacterial groups, the reduction in short-chain fatty acid (SCFA) producing bacteria may be another intrinsic feature of such diets. One of the major SCFAs produced by intestinal bacteria, butyrate, is an exogenous metabolite with a number of key functions related to gut homeostasis (reviewed in [12, 13]). While serving as fuel for colonocytes [14, 15], it is debatable whether high molarities of butyric acid are beneficial (reviewed in [16]). For instance, high colonic butyrate concentration is believed to modulate colonic crypt architecture [17], induce apoptosis in the stem cell compartment of crypts [18], and supress crypt stem cell proliferation [19]. Recent publications have been primarily focused on yeast as a feed additive and have investigated the effects of low levels of inclusion of yeast and its components on the gut microbiota in pigs [8, 9, 20]. However, little is known how inclusion of high levels of yeast affects microbial community of intestines. We hypothesize that the novel yeast diet can reshape intestinal microbiota composition in weanling piglets. The reason for featuring the post-weaning period in this study was because of the stress the animals experience during that period [21, 22], which may define the course of animal health development.

We used 16S *rRNA* bacterial gene sequencing and cultivation methods to compare the gut microbial consortia of yeast fed weanling piglets with that of the controls. Also, we investigated a possible role of individual bacterial groups in relation to the large intestine butyrate production and utilization.

Methods

Animals, housing, diet allocation

The trial was conducted at an experimental farm of the Norwegian University of Life Sciences (NMBU), Ås, Norway in the fall of 2017. A total of 72 crossbred [(Norwegian Landrace x Yorkshire z-line) x (Duroc) and (Norwegian Landrace) × (Duroc)] weanling piglets, selected from ten litters, was included in the experiment. The piglets were selected to enter the study based on their weight at the day of weaning, and after blocking by litter and body weight, the pigs were randomly allocated to either the control or the yeast diet. All animals were healthy during the nursery period and throughout the experiment. The animals were housed in environmentally controlled pens with a slatted floor at front and roofed resting area with a rubber mat. The animals were introduced to creep feed 2 weeks prior to weaning. The experiment was initiated when the piglets were weaned at 28 days of age (day 0 PW). Five to six piglets were grouped together in each pen and group-fed one of the allocated diets. All animals had ad libitum feeding and access to drinking water throughout the experiment. Diets were formulated to be isonitrogenous and isoenergetic based on the chemical composition of the ingredients and to meet, or exceed, the nutrient requirements of weanling pigs (Table 1). In the yeast diet, 40% of the crude protein derived from *Cyberlindnera jadinii* cells (LYCC 7549; Lallemand Yeast Culture Collection). The yeast cells were processed as described previously [6]. Briefly, after fermentation, the cells were washed, centrifuged, heat-inactivated, and dried. The diets were cereal-based (wheat, barley and oats), and the main protein ingredients in the control diet (soybean meal, potato

Table 1 Ingredients (g/kg as fed) and analysed chemical composition (g/kg DM, unless otherwise stated) of experimental diets

	Control diet	Yeast diet
Ingredients, g/kg as fed		
Wheat	627	594
Barley	100	100
Oats	50	50
Soybean meal	80	19
Potato protein concentrate	34	9
Fish meal	20	5
Rapeseed meal	20	5
Yeast - *Cyberlindnera jadinii*	–	146
Rapeseed oil	20	23
Minerals, vitamins and amino acids	49	49
Nutrients, g/kg of DM		
DM, g/kg	869	885
Crude protein	202	194
NDF	110	102
Starch	508	494
Crude fat	45.3	46.2
Ash	52.7	51.2
Phosphorus	8.01	9.08
Gross energy, MJ/kg	18.94	18.96

protein concentrate, fish meal, and rapeseed meal) were partly replaced by yeast meal in the yeast diet (Table 1). At the days 2, 4, 7, and 14 PW, eight animals from each of the two feeding groups were sacrificed followed by sampling (Fig. 1). In addition, eight littermates were sampled at day zero to provide a baseline point for the day of weaning.

Bacterial cultivation / DNA extraction / 16S *rRNA* gene amplicon sequencing

Luminal contents from *ileum distalis*, *apex ceci*, and *apex coli spiralis* were collected. Serial dilutions of 0.1 mg/ml of digesta in 0.9% saline were inoculated onto media. MacConkey, Tryptose Sulfite Cycloserine (TSC), de Man, Rogosa and Sharpe (MRS), and Slanetz and Bartley agar (Oxoid, Cambridge, UK) were used to recover and quantify coliforms, *Clostridium perfringens*, lactic acid bacteria (LAB), and enterococci, respectively. The dilution and incubation schemes were applied as described previously [23].

For 16S *rRNA* gene sequencing, digesta samples from *ileum distalis*, *apex ceci*, and *apex coli spiralis* were snap-frozen in liquid nitrogen and stored at -80 °C until DNA extraction. The DNA extraction was carried out on samples collected on days 0, 7 and 14 PW, according to a previously described protocol [24] with minor modifications. Briefly, 200 mg of thawed gut contents were added to 1 ml of InhibitEX Buffer (QIAGEN, GmbH, Hilden, Germany) following loading 500 mg of zirconia/silica beads (∅ = 0.1

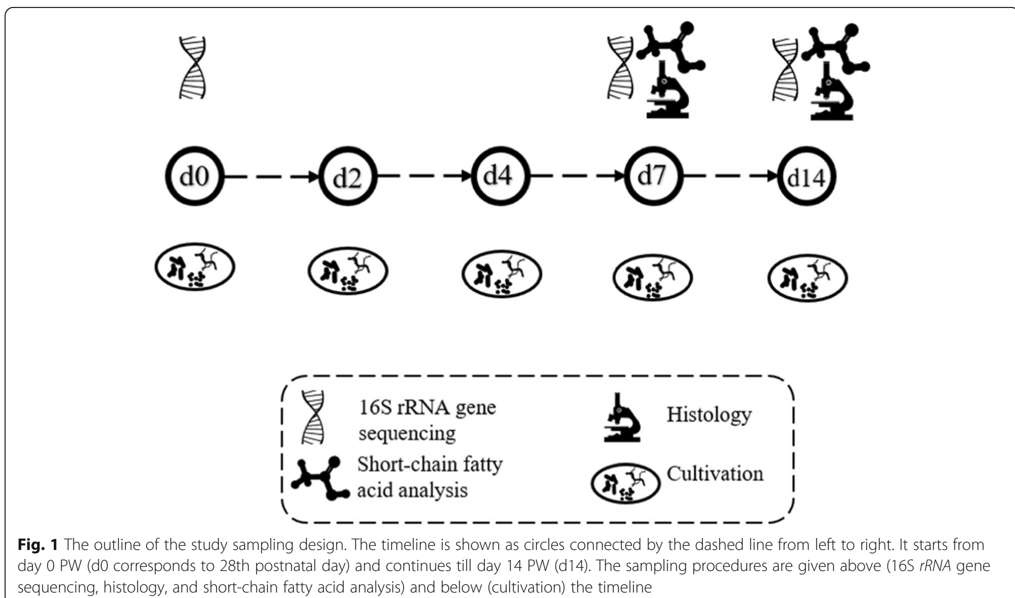

Fig. 1 The outline of the study sampling design. The timeline is shown as circles connected by the dashed line from left to right. It starts from day 0 PW (d0 corresponds to 28th postnatal day) and continues till day 14 PW (d14). The sampling procedures are given above (16S *rRNA* gene sequencing, histology, and short-chain fatty acid analysis) and below (cultivation) the timeline

mm, Carl Roth, Karlsruhe, Germany). The TissueLyser adaptors were cooled down at − 20 °C for 15 min prior to the bead-beating step. The bead-beating lasted for 1.5 min at 30 Hz in TissueLyser II (Qiagen, Retsch GmbH, Hannover, Germany). Proteins were digested with 30 μL of Proteinase K II (QIAGEN, GmbH, Hilden, Germany). DNA was bound to QIAamp spin column followed by washing with AW1 and AW2 buffers (QIAGEN, GmbH, Hilden, Germany). DNA was eluted with ATE buffer (QIAGEN, GmbH, Hilden, Germany). The yielded DNA purity was assessed by NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA) with subsequent quantification by Qubit fluorometric broad range assay (Invitrogen, Eugene, OR, USA).

The library preparation and amplicon sequencing of V1-V3 hypervariable region of bacterial 16S *rRNA* gene were performed at GATC Biotech AG (Konstanz, Germany) using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) primers. A microbiome standard, 20 Strain Staggered Mix Genomic Material (ATCC® MSA-1003™), was used as a positive control. The amplicon sequencing was run in three batches on an Illumina HiSeq 4000 sequencer. The resulting sequences were deposited in the SRA (PRJNA580284).

Illumina 16S *rRNA* gene amplicon data curation

Forward Illumina demultiplexed reads were taken to the analysis. The reads were analysed using DADA2 R package, version 1.8.0 [25]. The core DADA2 algorithm applied had the following setup: A) Quality filtering parameters: maxEE = 1, truncQ = 2 with forward primer clipping; B) Dereplication and denoising of the quality controlled reads; C) Resulting feature tables obtained from separate Illumina runs were merged with subsequent chimaera removal; D) Taxonomic assignment using RDP Naive Bayesian Classifier implemented in DADA2 R package (default settings). The GreenGenes database, version 13.8, [26] was used as a reference database for taxonomy assignment. The LULU post-clustering algorithm was applied to optimize diversity metrics [27].

Colonic SCFA, growth performance and liver index measurement

The colon digesta samples at day 7 PW and day 14 PW were the replicate samples of those used for the 16S *rRNA* gene sequencing analysis. The samples were thawed on ice. A mixture of 500 mg of gut contents sample and 500 μl of ice cold ddH_2O was sonicated for 5 min in cold water. Next, after mixing and centrifugation (15 min, 4 °C, 15000 g), the supernatant was transferred to a spin column (45 kDa). After another centrifugation step (15 min, 4 °C, 15000 g), the samples were spiked with internal standards. Short-chain fatty acids (SCFA) were measured by "TRACE 1300 Gas Chromatograph" with autosampler, "AS 1310" (Thermo Fischer Scientific, Milan, Italy). Parameters of the capillary column were as following: model – "Stabilwax – DA"; length – 30 m; inner diameter – 0.25 mm; film thickness – 0.25 μm (Restek corporation, Bellefonte, PA, USA). The column operating protocol was as follows: starting temperature – 90 °C (2 min); temperature increase - 10 °C/min until 150 °C, 50 °C/min until 250 °C (1 min). The rate of Helium flow was 3 mL/min. Concentrations of acetic, propionic acids, as well as butyric, valeric acids and their isomers, were reported in μmol per gram of intestinal contents. The average daily gain (ADG) in this study was calculated as: (slaughter day body weight – body weight at weaning) / number of days PW. The liver index was calculated as used previously in [7]: liver index = liver weight (kg)/ live body weight (kg).

Histology

Colon tissue samples were collected within 20 min of euthanasia and fixed in 10% formalin. The gut contents were emptied, and mucosal surface was rinsed gently with cold water prior to formalin fixation. After 48 h of fixation, the tissues were routinely processed, embedded in paraffin and 4 μm sections were mounted on glass slides. The sections were subsequently deparaffinized in xylene and rehydrated in graded alcohol before routine staining with haematoxylin and eosin. The colonic tissues were evaluated histopathologically and scored semi-quantitatively where no pathology was scored 0, very mild tissue changes received the score 1, mild changes 2, moderate changes 3, and severe changes 4. Formalin-fixed, paraffin-embedded tissue sections were also stained with high iron diamine and alcian blue (HID-AB). Digital images of the intestinal sections were captured using NanoZoomer (Hamamatsu Photonics). Morphometric measurements were performed using the software Aperio Image Scope v12.3.3.5048 (Copyright Leica Biosystems Pathology Imaging, 2003–2016). For crypt depth (CD) measurements, the ten longest and well oriented crypts were selected, and micrographs were captured at 10× magnification. CD was measured from the crypt opening at the mucosal surface to the deepest portion of the crypt adjacent to the *tunica muscularis mucosae*.

Statistical analysis

The sample-size estimation was based on our pilot study with the similar design and feed composition (unpublished). To compare bacterial average CFUs recovered from selective plates, the non-parametric Mann-Whitney-Wilcoxon (MWW) test was applied. The linear regression model was used to predict variance in LAB

colony-forming unit (CFU) with the diet as an explanatory variable.

The Shannon and Observed species alpha-diversity indices were calculated separately on the data at the ASV level and species level. To bin the ASVs to the species level, *tax_glom()* R function was applied [28]. Comparison of the resulting alpha-diversity figures between the diet groups was done using MWW test. The beta-diversity analysis was performed via principle coordinate analysis (PCoA) on Bray-Curtis dissimilarity matrix, and permutational multivariate analysis of variance (PERMANOVA) test for covariate significance using *adonis()* R function [29], 9999 permutations. The covariates included in the statistical model were the following: diet, sex, pen, and sow.

To screen for bacteria that appeared in higher numbers in one of the feeding groups compared with those of another group, or differentially abundant taxa, the analysis of composition of microbiomes (ANCOM) test was used (false discovery rate (FDR) = 0.05, multiple correction = 2). The test was performed at the phylum, family, and ASV levels [30].

To compare average concentrations of SCFA and to compare colonic CD in the colon between the diet groups, the MWW test was applied.

The loglinear analysis was applied to the histopathology parameter results for comparison between the diet groups. Multiple regression analysis was used to predict the colonic CD. The amplicon sequence variant (ASV) table was transformed to the relative abundances to derive individual bacterial ASV relative abundance figures.

To explore correlations between bacterial group relative abundance and metadata variables, the Pearson's correlation coefficient (reported as r) was applied. To aid the graphical representation of the multiple regression modelling, the numeric variables of the model equation were subjected to principle component analysis (PCA) in R using *prcomp()* function to be further displayed on a biplot. The statistical significance was declared at p-values < 0.05 for all tests.

Results

Cultivation results

All the piglets appeared healthy throughout the experiment. There was no mortality, and no difference in feed intake and growth rate between the dietary treatments.

Lactobacillus spp.

In the jejunum, LAB were found in higher numbers on average in the yeast group (9.57 logCFU/g) compared with the controls (7.30 logCFU/g) at day 4 PW ($p < 0.001$) (Additional file 1). The same pattern was observed in the ileum at days 4 and 7 PW (yeast = 9.48 vs control = 8.44 logCFU/g, and yeast = 10.0 vs control = 8.61 logCFU/g, respectively) (p < 0.001 for both tests). The variance in the LAB counts in the ileum at days 4, 7 and 14 PW was explained by diet ($R^2_{adj} = 0.45$, $p < 0.05$). Interestingly, when only day 7 was considered, the same linear model could explain 65% of the diet-related variance in the LAB counts. In the cecum and colon, LAB counts were also higher in the yeast group at day 7 PW, and day 14 PW ($p < 0.05$ for both), except in the colon at day 14 PW.

Enterococcus spp.

The counts of enterococci (8.98 logCFU/g) were found to be higher in the ileum of the yeast group at day 4 PW compared with those of the controls (8.09 logCFU/g) (p < 0.001). At day 7 PW, enterococci in the colon of the yeast group were higher than those of the control group (9.03 logCFU/g vs 8.20 logCFU/g) ($p < 0.001$).

Coliforms

Coliforms were at higher numbers (9.72 logCFU/g) in the cecum of the yeast group at day 7 PW compared with those of the controls (8.47 logCFU/g) ($p < 0.001$).

C. perfringens

No statistically significant difference was observed between the two feeding groups.

16S *rRNA* gene sequencing results

There were on average 449,177 (SD = 57,148) reads per sample after filtering, denoising, and chimeric amplicon removal. There were 2100, 3301, and 3485 ASVs detected in the ileum, cecum, and colon samples, respectively. At day 0 PW, there were 2645 ASVs identified for all sampled gut locations. Similarly, 3378 ASVs and 2994 ASVs were found at day 7 PW and day 14 PW, respectively. The results of sequencing of the positive controls (mock communities) are given in Additional file 2.

Alpha microbial diversity

The microbial communities in the large intestine of the yeast group were less diverse in comparison to those of the control pigs at day 7 and 14 PW (Fig. 2a, Additional file 3: Table S3) as measured by the Shannon diversity index at the ASV level. There were more distinct ASVs identified in the cecum of the controls than those of the yeast group at day 7 PW and day 14 PW (p < 0.05 for both). There was no difference in alpha microbial diversity when comparing ileum microbiotas between the two diets (Fig. 2a, Additional file 3). Interestingly, when compared at the species level, the Shannon diversity index was higher in cecum microbiota of the controls at day 7 PW only. Otherwise, alpha diversity analysis at the species level showed no difference between the diets (Additional file 3).

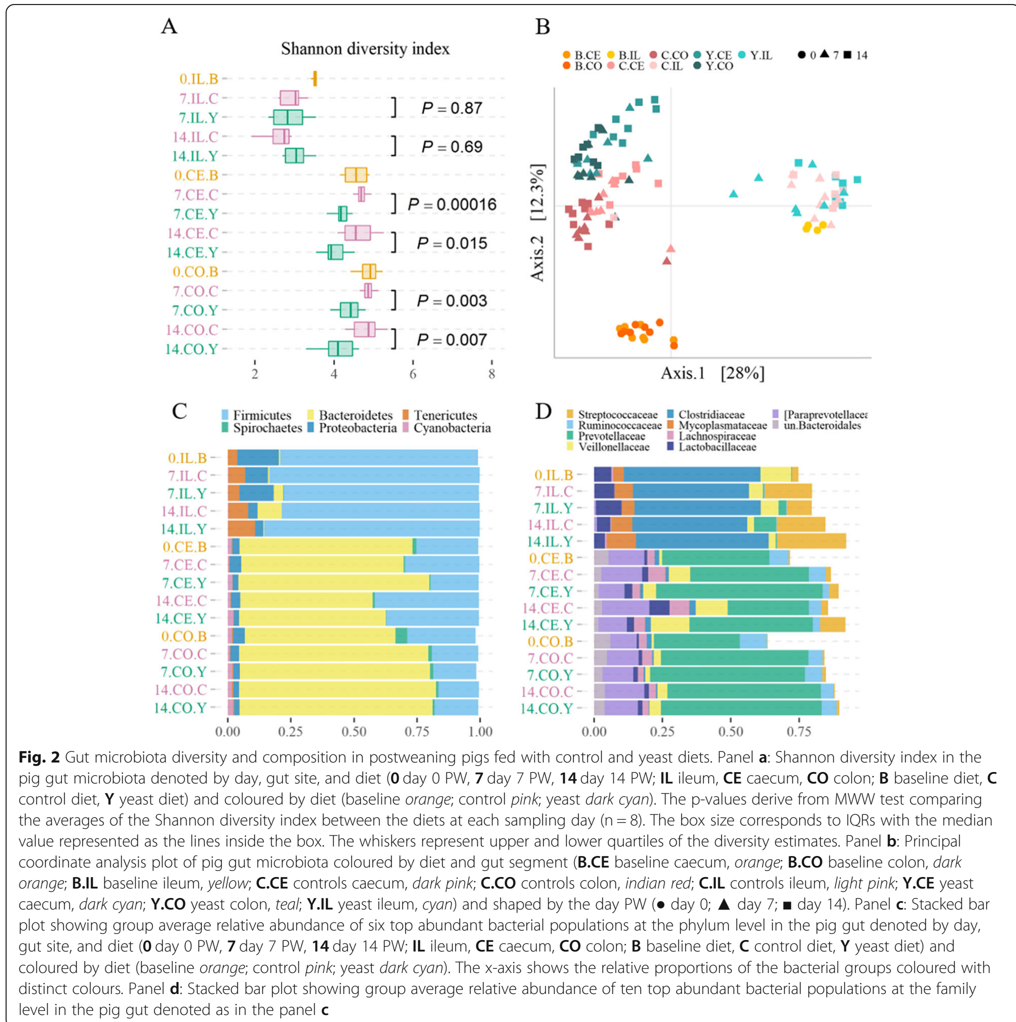

Fig. 2 Gut microbiota diversity and composition in postweaning pigs fed with control and yeast diets. Panel **a**: Shannon diversity index in the pig gut microbiota denoted by day, gut site, and diet (**0** day 0 PW, **7** day 7 PW, **14** day 14 PW; **IL** ileum, **CE** caecum, **CO** colon; **B** baseline diet, **C** control diet, **Y** yeast diet) and coloured by diet (baseline *orange*; control *pink*; yeast *dark cyan*). The p-values derive from MWW test comparing the averages of the Shannon diversity index between the diets at each sampling day (n = 8). The box size corresponds to IQRs with the median value represented as the lines inside the box. The whiskers represent upper and lower quartiles of the diversity estimates. Panel **b**: Principal coordinate analysis plot of pig gut microbiota coloured by diet and gut segment (**B.CE** baseline caecum, *orange*; **B.CO** baseline colon, *dark orange*; **B.IL** baseline ileum, *yellow*; **C.CE** controls caecum, *dark pink*; **C.CO** controls colon, *indian red*; **C.IL** controls ileum, *light pink*; **Y.CE** yeast caecum, *dark cyan*; **Y.CO** yeast colon, *teal*; **Y.IL** yeast ileum, *cyan*) and shaped by the day PW (● day 0; ▲ day 7; ■ day 14). Panel **c**: Stacked bar plot showing group average relative abundance of six top abundant bacterial populations at the phylum level in the pig gut denoted by day, gut site, and diet (**0** day 0 PW, **7** day 7 PW, **14** day 14 PW; **IL** ileum, **CE** caecum, **CO** colon; **B** baseline diet, **C** control diet, **Y** yeast diet) and coloured by diet (baseline *orange*; control *pink*; yeast *dark cyan*). The x-axis shows the relative proportions of the bacterial groups coloured with distinct colours. Panel **d**: Stacked bar plot showing group average relative abundance of ten top abundant bacterial populations at the family level in the pig gut denoted as in the panel **c**

Beta microbial diversity

The PCoA was conducted to compare the gut microbial compositions of individuals by visualizing Bray-Curtis dissimilarity matrices on the plot followed by PERMANOVA statistical test for significance of study covariates. The large intestine microbiotas tended to cluster together according to the diet type (Fig. 2b). At day 7 PW, the diet accounted for 23% of the variance in the large intestine microbial composition ($p < 0.001$). Even more of the explained variance in microbiota (26%) was attributed to the diet when the large intestine data at day 14 PW were analysed ($p < 0.001$). Notably, 12% of the variance in the large intestine microbiota was attributed to the sex of the animals at day 7 PW ($p < 0.05$).

The variance in the ileal microbiota composition was not explained by the dietary treatment, nor due to any other tested covariates (i.e. sex, pen, and sow).

Distribution of major taxa and differentially abundant taxa

Ileum

The ileal microbial consortia primarily consisted of *Firmicutes* (78%), *Proteobacteria* (9%), *Tenericutes* (7%),

and *Bacteroidetes* (1%) on average irrespective of the diet (Fig. 2c).

Phylum level There were no differentially abundant phyla identified when comparing the diet groups at any of the sampling days.

Family level The Porphyromonadaceae family was more abundant in the control group compared with that of the yeast group (Additional file 4B).

ASV level Clostridiaceae 02d06 ASV and family *S24* unclassified ASV of Bacteroidales order were found in higher numbers in the control group at day 7 PW compared with those of the yeast group (Additional file 4A). While *Prevotella* ASV9, *Prevotella copri* ASV23 and ASV33, and unclassified ASV of *Lactobacillus* genus were overrepresented in the control ileal microbiota day 14 PW, *Clostridium perfringens* ASV2 and ASV7, and *Lactobacillus salivarius* ASV5 were differentially abundant in the yeast group (Additional file 4B).

Caecum

The most abundant phyla in the cecum were the following: *Bacteroidetes* (64%), *Firmicutes* (30%), *Proteobacteria* (3%), and *Cyanobacteria* (1%) (Fig. 2c).

Phylum level There was a higher proportion of *Bacteroidetes* in the yeast group at day 7 PW compared with those of the control group (Fig. 3c, Additional file 4D). Low abundant *Spirochaetes* phylum was overrepresented in the control group at day 14 PW compared with that of the yeast group (Additional file 4E).

Family level An unclassified family of the *Tremblayales* order was found in higher numbers in the control group day 7 when compared with that of the yeast group (Additional file 5C). At day 14 PW, the family s24.7 of *Bacteroides* was more predominant in the control group, whereas an unclassified family of *Alphaproteobacteria* phylum (RF32 order) was overrepresented in the yeast group (Additional file 5D).

ASV level At day 7 PW, three *Prevotella* affiliated amplicons (ASV28, ASV33, ASV50), as well as

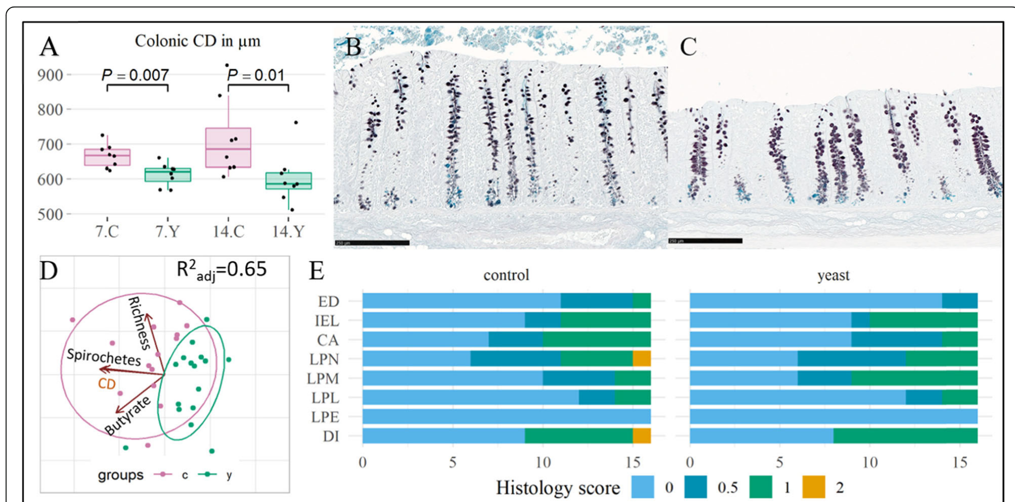

Fig. 3 Gut health parameters in postweaning pigs fed with control and yeast diets. Panel **a** Boxplot showing comparison of colonic crypt depth (CD) measurements between the control and yeast group at day 7 PW and day 14 PW coloured and denoted by the diet and day (**7.C** controls day 7 PW *pink*, **7.Y** yeast day 7 PW *dark cyan*, **14.C** controls day 14 PW *pink*, **14.Y** yeast day 14 PW *dark cyan*). MWW test p-values are provided above the boxes. Panel **b** Representative section of colon mucosal crypts from the control group at day 14 PW (HID-AB stain, scale bar 250 μm). Panel **c** Representative section of colon mucosal crypts from the yeast group at day 14 PW (HID-AB stain, scale bar 250 μm). Panel **d**: Principal coordinate analysis illustrating contribution of Spirochetes relative abundance, colonic butyrate concentration, and 16S *rRNA* gene richness to the colonic crypt depth. The individual observations and the correlation circles are coloured by the diet (controls *pink*, yeast *dark cyan*). The adjusted R^2 is given at the right top corner. Panel **e**: Histopathological assessment of the colon mucosa, evaluating epithelial damage (ED), number of intraepithelial lymphocytes (IELs), presence of crypt abscess (CA), and infiltration of leukocytes neutrophils (N), macrophages (M), lymphocytes (L), eosinophils (E), in addition the number of piglets that were diagnosed with a very mild or mild colitis (DI). The horizontal stacked bar plot shows the number of animals with none (0), very mild (1), mild (2), or moderate histopathological changes

Mitsuokella genus ASV17, were differentially abundant in the yeast group when compared with those of the control group. Conversely, *Faecalibacterium prausnitzii* ASV35 was found to be more abundant in the control group at the same time point (Additional file 5A). Seven different Veillonellaceae family ASVs, including *Selenomonas ruminantium*, *Bulleidia* p.1630.c5, *Parabacteroides*, and four other unclassified taxa were overrepresented in the yeast group at day 14 PW compared with those of the control group. Three variants of unclassified *Prevotella* ASV, *Selenomonas*, *Mitsuokella*, *Mucispirillum schaedleri*, and unclassified ASV of the Porphyromonadaceae family were overrepresented in the yeast group at day 14 PW when compared with those of the control group. An ASV classified as *Selenomonas ruminantium* and *Prevotella, Lactobacillus*, *Campylobacter*, and *Anaerovibrio* ASV33 genera were differentially abundant in the control group at day 14 PW in comparison with those of the yeast group (Additional file 5B).

Colon

The colon microbiota structure resembled that of the cecum with *Bacteroidetes* (73%), *Firmicutes* (19%), *Proteobacteria* (3%), *Spirochaetes* (1.8%) and *Cyanobacteria* (1.6%) representing the most dominant phyla (Fig. 2c).

Phylum level There was no statistically significant difference in bacterial phyla abundances between the diet groups at any of the sampling days.

Family level At day 7 PW, more Victivallaceae family ASVs were detected in the control group than in the yeast group (Additional file 6C). At day 14 PW, Succinivibrionaceae and *Bacteroidales* p.2534.18B5 families were found in higher numbers in the control group, whereas Oxalobacteraceae bacterial family was more abundant in the yeast group (Additional file 6D).

ASV level One *Mitsuokella* amplicon variant was more abundant in the yeast group, while an unclassified species of the *Bacteroidales* order (p.2534.18B5 family) was less abundant in the same group at day 7 PW. Two distinct *Prevotella* amplicon variants (ASV2 and ASV50) were differentially abundant in, respectively, control and yeast group. Notably, ASV50, also previously identified in the cecum of the same time point, ranked first on relative abundance, when all samples were considered (Additional file 6A). At day 14 PW, four *Prevotella* ASVs (ASV17, 50, 35, 41), two distinct ASVs classified as *Selenomonas ruminantium* (ASV10, 2), *Mitsuokella* ASV17, *Parabacteroides* ASV22 and ASV23, *Bulleidia* p.1630.c5 and an unclassified ASV of the Veillonellaceae family were found in higher amounts in the yeast group than those in the control group. Four different *Prevotella* amplicons (ASV18, ASV9, 67, 96), *Selenomonas ruminantium* ASV9, *Anaerovibrio* ASV16, and two unclassified ASVs of *Bacteroidales* and YS2 bacterial orders were more abundant in the control group than in the yeast group (Additional file 6B).

SCFA in the colon

The total colonic SCFA concentration did not differ between the diet groups when measured at day 7 PW ($p = 0.32$). However, at day 14 PW, the levels of total SCFA tended to be higher in the control group compared with those of the yeast group ($p = 0.065$). For day 14 PW, butyrate and acetate were found at higher concentration in the control group compared with those of the yeast group ($p < 0.05$) (Table 2). The concentrations of propionate, valerate, iso-butyrate, and iso-valerate did not differ between the two groups at the statistically significant level.

Histology

The colonic CD in the control group was on average deeper than that of the yeast group at both day 7 PW and day 14 PW (p-value = 0.007 and p-value = 0.01, respectively) (Fig. 3a, b, c). The colonic butyrate concentration positively correlated with the crypt depth irrespective of the diet ($r = 0.55$, p-value = 0.001). The prediction model of the colonic CD showed that the depth tended to be deeper with higher numbers of an unclassified Spirochaetaceae ASV ($\beta = 25{,}500$, $SE = 5920$, t-value = 4.3, $p = 0.0002$), higher colonic butyrate concentration ($\beta = 6.33$, $SE = 2.11$, t-value = 2.9, $p = 0.006$), and richer colon microbiota as calculated at ASV level ($\beta = 0.198$, $SE = 0.094$, t-value = 2.1, $p = 0.044$). Overall the statistical model could be predictive of 65% of variance in colonic CD (Fig. 3d). No statistically significant difference was found in histopathological parameters in the colon comparing the two feeding groups (Fig. 3e).

Colonic bacteria – colonic butyrate association

The concentration of colonic butyrate positively correlated with the relative abundance of *F. prausnitzii* in the colon ($r = 0.73$, $p < 0.0001$) (Additional file 7A). The latter accounted for 52% variance in butyrate concentration as estimated by the linear model equation. Inclusion of an unclassified Spirochaetaceae family ASV and *Dialister* genus relative abundance to the existing linear model improved the model with 72% of variance in colonic butyrate production explained Additional file 7B). Notably, the *Oxalobacter* genus, a member of the differentially abundant Oxalobacteriaceae family in the yeast group, negatively correlated with the colon butyrate concentration ($r = -0.71$, $p = 0.002$).

Table 2 Colonic SCFA concentration comparison between the control and the yeast group. The concentration values are reported as µmol/g of colon digesta. Comparison pairs that correspond to p-values less than 0.05 (MWW test) are given in bold

	feed	day 7 PW		day 14 PW	
		mean (SD)	p-value	mean (SD)	*p*-value
Acetate, µmol/g	control	57.3 (13.2)	0.156	**60.9 (15)**	0.015
	yeast	46.6 (12.8)		**40.9 (7.07)**	
Propionate, µmol/g	control	24.7 (6.9)	0.528	26 (6.31)	0.959
	yeast	25.7 (6.54)		26.3 (9.49)	
Butyrate, µmol/g	control	12.08 (4.84)	0.235	**16.5 (4.8)**	0.038
	yeast	9.16 (1.96)		**11.3 (4.52)**	
Valerate, µmol/g	control	2.11 (1.0)	0.563	3.12 (1.43)	0.713
	yeast	2.16 (0.6)		3.23 (2.46)	
Iso-butyrate, µmol/g	control	0.87 (0.67)	0.558	0.68 (0.24)	0.364
	yeast	0.73 (0.44)		0.81 (0.25)	
Iso-valerate, µmol/g	control	0.75 (0.34)	0.242	0.71 (0.31)	0.791
	yeast	0.75 (0.71)		0.83 (0.4)	
Total SCFA, µmol/g	control	97.9 (21.8)	0.328	108 (22.3)	0.065
	yeast	85.2 (18.89)		83.5 (20.7)	

Butyrate – liver – colonic bacteria association

The linear model for predicting the liver index from individual bacterial groups, revealed that *F. prausnitzii* ASV and an unclassified Spirochaetaceae family ASV could explain 47% of variance in the liver index ($\beta = 43.4$, SE = 13.3, t-value = 3.2, p = 0.006 and $\beta = 49.7$, SE = 22.0, t-value = 2.26, p = 0.04, respectively) (Additional file 8A).

The colon concentration of butyrate positively correlated with the liver index when the two sampling days, day 7 and 14 PW, were considered ($r = 0.65$, $p < 0.0001$). However, when stratified by diet, the strength of association was different for the control group ($r = 0.9$, $p < 0.0001$) and the yeast group ($r = 0.66$, $p < 0.01$) (Additional file 8B, C).

ADG-butyrate association

At day 7 PW, the relative abundance of the *Proteobacteria* phylum in colon was negatively correlated with ADG ($r = -0.65$, p = 0.009). The same trend but of lesser magnitude was observed in the ileum and colon samples at both day 7 PW, and day 14 PW ($r = -0.37$, p = 0.04 and $r = -0.41$, p = 0.02, respectively). Notably, the relative abundance of the Succinivibrionaceae family was positively correlated with ADG ($r = 0.47$, p = 0.006). A positive correlation was found between the relative abundance of the Prevotellaceae family in the colon of the yeast group and ADG ($r = 0.53$, p = 0.04).

Discussion

We investigated the effect of a high level *Cyberlindnera jadinii* yeast diet on the gut bacterial compositions in weanling piglets. Protein from *C. jadinii* yeast was used to replace 40% of crude protein in a conventional Norwegian piglet diet. The growth performance and histopathology analysis of the gut tissues indicated that the data obtained was from equally healthy animals. To explore the bacterial composition, 16S *rRNA* gene sequencing and bacterial cultivation were used. The sensitivity of the V1-V3 16S *rRNA* gene amplification assay varied in relation to different bacterial groups. For instance, there was low sensitivity to *Escherichia coli* detection as revealed by the sequencing of a known community standard. With help of cultivation techniques, a fair comparison of coliform numbers between the control and yeast group was obtained. To compare the microbial composition between the diet groups, a phylogeny-agnostic method, permutational multivariate analysis of variance on Bray-Curtis dissimilarity matrix, was used. This decision was based on our observations that the single end sequencing data had provided a limited phylogenetic signal for phylogeny-informed beta diversity method estimates. In contrast, the use of Bray-Curtis dissimilarity matrix was discriminative enough for the comparison of the microbiotas that were presumably similar in their compositions. As expected, the microbiota of the ileum was structurally different from that of the large intestine. Conversely, there was similarity of microbiota compositions between the cecum and colon gut segments. The diets did shape the composition of the large intestine microbiota. The ileum microbiota composition, in contrast, was less affected by diet with only few differences in microbiota at the family and ASV level. This may due to several factors that the host exerts

on the bacterial succession in the small intestine. These factors, such as peristalsis, bile acids, pancreatic enzymes, hydrogen ion concentration, and local immunity seem to limit bacterial colonization of the small intestine to those bacterial species recognized by the host immune system [31, 32].

We detected a difference in the alpha bacterial diversity of the large intestine between the yeast and the control groups based on the Shannon index. The control group appeared more diverse at the amplicon level. However, when the bacterial richness and bacterial diversity were examined at the species level, the difference between the diets became less apparent. Only in cecum samples day 7 PW did the diversity figures differ at a statistically significant level. To explain this discrepancy between the analyses done at different resolution levels, namely, ASV and species level, we adopted the following logic; the calculation of bacterial community richness is affected by the number of ASVs inferred from DADA2 pipeline. The more 16S *rRNA* gene amplicon variants detected, the richer the community. On average, the number of the 16S *rRNA* gene copies per genome of the *Firmicutes* (F) tend to be twice as high as that of the *Bacteroidetes* (B) [33]. This estimate prompted us to revisit the difference in F:B ratio at day 7 PW. Indeed, the results of the ANCOM analysis at the phylum level supported that the F:B ratio was higher in the cecum samples of the control group at day 7 PW compared with that of the yeast group. Further, we attempted to verify that it was the intragenomic variability in the number of the 16S *rRNA* gene copies that contributed most to the Shannon diversity calculation. To achieve this, we compared the bacterial richness at the species level between the feeding groups. As expected, there was no difference in species richness between the feeding groups when ASVs were binned at the species level. Therefore, we conclude that the overrepresentation of *Bacteroidetes*-affiliated ASVs in the yeast large intestine resulted in a Shannon diversity that was lower compared with that of the control group. *Firmicutes* may appear in higher numbers in the microbiota of the control pigs because of differences in the diet formulation. In the yeast diet, yeast proteins mostly replaced the conventional protein sources used in the control diet, i.e. soybean meal, potato protein concentrate, fish meal, and rapeseed meal. The presence of various dietary fibres in a diet influences the gut microbiota composition by promoting the growth of *Firmicutes* (reviewed in [34]). It is known that up to 10% of soybean meal, and a considerable portion of rapeseed meal (> 15%) is neutral detergent fibre [7]. Thus, the higher level of soybean- and rapeseed meal in the control diet could account for the high presence of fibre-degrading *Firmicutes* in the large intestine of the control group.

According to our findings, a Prevotellaceae family-related amplicon, ASV50, was predominant in the large intestine of pigs in the yeast group. Even though the analysis of the microbiota composition was confined to sequencing of the 16S *rRNA* bacterial gene only, the overrepresentation of the Prevotellaceae family might be related to the availability of the non-digested parts of the yeast cells in the diet. It is conceivable that the method of yeast processing partially precluded its digestibility in the small intestine thus making the yeast cells available for microbial fermentation in the large intestine. The overgrowth of *Prevotella* in the yeast driven microbiota might also be attributed to the microbial peptidase and proteinase activities of this bacterial groups [35]. Previous studies by Mach et al. [36] and by Ramayo-Caldas et al. [37], showed that the enterotype dominated by *Prevotella* and *Mitsuokella* species is associated with lowered alpha diversity and improved growth performance. These findings are in line with our results on alpha bacterial diversity and ADG in the yeast group. The lower levels of butyrate and acetate in the colon of the yeast fed piglets may be due to the predominance of the *Prevotella-Mitsuokella*-affiliated groups and hence a suppression of certain SCFA-producers [37]. Higher abundance of *Mitsuokella* in the large intestine of yeast fed animals is consistent with the studies where yeast was supplemented [8]. Furthermore, in a study using similar dietary formulations as the present study, Cruz and co-workers [7] showed that the total tract digestibility of phosphorous was higher in the yeast group than in the control group. As *Mitsuokella* and *Selenomonas* genera are reported to release phosphorous from phytate [38, 39], it is tempting to ascribe this metabolic activity to these bacteria. The *Mitsuokella* and *Selenomonas* genera were found in higher numbers in the yeast group compared with the numbers in the control group. However, the resolution of the 16S *rRNA* gene method does not always provide enough confidence in assigning the PCR amplicons to the species level. To learn about the functional potential and contribution to host metabolism of the mentioned Selenomonadaceae and Prevotellaceae, use of anaerobic cultivation techniques may be necessary. It has previously been reported that in gut ecosystems supplied with low levels of yeast-derived components, the outgrowth of *Prevotella*, *Selenomonas*, and *Mitsuokella* commonly co-occurs with the reduction in SCFA producing bacteria [9–11]. The interpretation of the ANCOM analysis revealed more *F. prausnitzii* (97.5% identity to *F. prausnitzii* strain ATCC 27768, GenBank accession: NR_028961) in the caecum of the control group than that of the yeast group. To our knowledge, the ANCOM test we used for identification of differentially abundant taxa between the two dietary groups performs better than other tests with respect to

false discovery rate control. However, when applied to groups with less than twenty samples per group, the sensitivity of ANCOM decreases [40]. In our 16S *rRNA* gene sequencing setup, *F. prausnitzii* on average represented 0.9% of the caecum microbiota population and 0.3% of the colon microbiota population. It is possible that eight samples per group were not enough for ANCOM to detect differences in rare colonic *F. prausnitzii* between the two groups. However, irrespective of the dietary interventions, we found that *F. prausnitzii* was positively correlated with the colonic butyrate concentration. The opposite relationship was found for the *Oxalobacter* genus. *Oxalobacter* strain OxB, an oxalate degrader, was studied by Allison et al. [41]. Allison and co-workers showed that acetate is an essential nutrient for growth of the bacterium. Their findings suggest that a competition for the nutrient between *F. prausnitzii* and *Oxalobacter* is conceivable [41, 42]. There was a mutual exclusion relationship between the two bacteria at a statistically significant level when examined with the CoNet co-occurrence network analysis [43] (data not shown). In addition to the role of *F. prausnitzii* in butyrate production, our results suggest that *Dialister* and an unclassified member of the Spirochaetaceae family may contribute to the colonic butyrate pool.

Next, we have found an association between the concentration of colonic butyrate and the liver index. Similarly, the number of *F. prausnitzii* in the colon correlated with the liver index. The portal vein concentration of butyrate is known to reflect the production levels of butyrate in the colon [44]. Thus, it is likely that the liver index was related to the uptake and metabolism of butyrate in the liver in our study. It is intriguing to think that gut microbiota members may be involved in the butyrate metabolism to the extent where the size of the liver is affected. Reduced level of butyric acid has been shown to be associated with pathologic conditions in man [45]. Butyrate has been implicated to play a role in the integrity of the intestinal wall, serving as an energy source for colonocytes and as a regulatory molecule [46, 47]. However, it is unclear what concentration of butyrate is optimal to maintain gut integrity and homeostasis in weanling piglets. Furthermore, butyrate has been shown to impact actively on the colonic crypt stem cells [18, 19]. Wang and co-workers demonstrated that butyrate diminished the crypt cell proliferation in a dose-response manner in an in vitro human colon crypt array [17]. In the present study, we observed a difference in the colonic CD between the two diet groups that possibly could be attributed to the altered abundance of butyrate-producing bacteria. Similar findings but of a lesser magnitude were reported by Mentschel and Claus in a study where piglets were fed with resistant potato starch [18]. In the light of our findings and previous publications [17–19], there is a good reason to believe that crypt elongation is a compensatory change to protect the crypt stem cell compartment from butyrate toxicity. The correlation between the colonic butyrate and the liver size suggests that the colonocytes received butyrate levels exceeding their metabolic capacity, with the butyrate surplus being transported to the liver. Histopathological examination of intestinal tissues did not reveal any difference in gut health parameters between the two feeding groups. Thus, it is tempting to speculate that there is a saturation point in the butyrate microbial production beyond which butyrate is not required as a fuel for colonocytes.

The cultivation results demonstrated that overall the differences between the feeding groups in the counts of LAB, enterococci, and coliforms were detected from day 7 PW. Much of the inter-individual variation before day 7 PW may be attributed to the weaning event. The bacterial succession of the gut is governed by, but not limited to, the substrate availability, gut physiology, and immune status. Feed intake during the time of weaning, when the piglets shift from milk to solid feed, is a key factor for immune system maturation [22], and luminal wall development [21]. The weaning event entails an irregular and variable timing in the acceptance of the new type of diet. This, consequently, leads to a transient starvation in some animals. According to our observations, albeit non-systematic, this was the case in our experiment. To this end, it is to be expected that the major variability in bacterial succession occurs during the first two weeks PW. LAB were consistently found in higher numbers in the ileum and large intestine of the yeast group compared with intestines of the control group. This bacterial group has a range of bioactive properties known to benefit mammals (reviewed in [48]). Attempts have been made to graft LAB into GI tract to improve health or ameliorate disease [49]. The ileum has a very dynamic gut environment, where bacteria must overcome multiple factors (e.g. digesta flow, peristalsis, microbe-host interaction, and microbe-microbe interaction) if they are to colonize and persist in the intestinal segment [32]. It has been reported that LAB are capable of adhesion to the intestinal cell wall [48]. Russo et al. showed that adhesion to human enterocytes of some LAB strains in vitro was inducible by β-D-glucan extracted from *Pediococcus parvulus* [50]. Therefore, the higher abundance of intestinal LAB in the yeast group in the present study may be attributable to the presence of a β-glucan fraction from the yeast cell wall in the feed. It is also tempting to speculate that the presence of yeast cell wall glucans in the feed affects digesta viscosity in the lumen, which is a factor that

would favour LAB colonization. Snart and colleagues demonstrated that high-viscosity dietary fibre β-glucans supplementation was positively associated with higher numbers of lactobacilli in the caecum of rats [51]. Supplementation with the yeast cell wall was implicated in an increase in lactobacilli numbers in the ileal digesta of broilers in the studies by Liy et al. and Ghosh et al. [52, 53]. Their findings suggest that the yeast cell wall, or its components, may have selective properties towards LAB in a range of hosts. It is notable that *L. salivarius* 16S *rRNA* gene amplicon relative abundance was found in higher numbers in the ileum of the pigs fed yeast at day 14 PW compared with that of the control pigs. The concordance between the results obtained from culture-dependent and -independent methods strengthens the validity of our findings. The augmentation of intestinal LAB is a promising aspect of the yeast-derived diet in GI tract of pigs. However, further research is needed to elucidate whether it is the yeast wall β-glucans or other ingredients of the diet that favour the LAB increase.

Conclusions

The replacement of 40% of the crude protein from the main protein sources traditionally used in Norway with proteins from *Cyberlindnera jadinii* in a weanling piglet diet reshaped the large intestine microbiota structure. The microbiota of yeast fed piglets showed a dominance of *Prevotella*-, *Mitsuokella*- and *Selenomonas*-related taxa along with the decreased alpha-diversity. Larger numbers of viable LAB cells were recovered from both small and large intestines of the yeast fed piglets compared with the control piglets. Owing to the functional capacity of the above bacterial groups, we believe that *Cyberlindnera jadinii* yeast, in addition of being a high-quality protein source, promote growth of beneficial gut microbes.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42523-020-00031-x.

Additional file 1. Comparison of bacterial CFUs on the selective agar plates. The values are the group medians (IQR) of logCFUs per gram of lumen contents. The bold font indicates statistically significant level ($p < 0.05$) of MWW test.

Additional file 2. Relative abundance of bacterial genera in the sequenced mock community standards along with their expected abundance.

Additional file 3. Alpha bacterial diversity as measured by a) Observed species and b) Shannon diversity index. The average values between the control and yeast group are represented as mean values with standard deviation (SD) along with medians with inter-quartile ranges (IQR). Comparison pairs that correspond to p-values less than 0.05 (MWW test) are given in bold.

Additional file 4. Differentially abundant ASVs between the yeast and control diets (ileum, caecum). A ileum, d 7 PW, B ileum, d 14 PW, C ileum, d 14 PW (family level), D caecum, d 7 PW (phylum level), E caecum, d 14 PW (phylum level). All taxonomic entities appeared as differentially abundant at FDR = 0.05.

Additional file 5. Differentially abundant ASVs between the yeast and control diets (caecum). A, caecum, d 7 PW, B caecum, d 14 PW, C caecum, d 7 PW (family level), D caecum, d 14 PW (family level). All taxonomic entities appeared as differentially abundant at FDR = 0.05.

Additional file 6. Differentially abundant ASVs between the yeast and control diets (colon). A colon, d 7 PW, B colon, d 14 PW, C colon, d 7 PW (family level), D colon, d 14 PW (family level). All taxonomic entities appeared as differentially abundant at FDR = 0.05.

Additional file 7. Association of colonic butyrate concentration with individual bacterial groups. Panel A: Correlation plot of colonic butyrate concentration (measured in μM per gram of intestinal contents) against *F. prausnitzii* relative abundance measured at days 7 and 14 PW (n = 32). The dots are coloured by the diet (control *pink*; yeast *dark cyan*). Pearson's *rho* is reported above the regression line. Panel B: Principal component analysis performed on the relative abundance of Spirochaetaceae, Faecalibacterium, Dialister and molarities of butyrate in the colon of pigs measured at days 7 and 14 PW (n = 32 but 3 dots are not shown). The dots are coloured by the diet (control *pink*; yeast *dark cyan*). The vectors represent the degree of correlation between the bacterial groups data and the butyrate concentration data.

Additional file 8. Association of liver index with individual bacterial groups, and colonic butyrate concentration. Panel A: Principal component analysis performed on the relative abundance of Spirochaetaceae, Faecalibacterium and the liver index of pigs measured at days 7 and 14 PW (n = 32 but 2 dots are not shown). The dots are coloured by the diet (control *pink*; yeast *dark cyan*). The vectors represent the degree of correlation between the bacterial groups data and the liver index data. Panel B: Correlation plot of colonic butyrate concentration (measured in μM per gram of intestinal contents) against liver index measured in the control group pigs at days 7 and 14 PW (n = 16). The dots are coloured by the diet (control *pink*). Pearson's *rho* is reported above the regression line. Panel C: Correlation plot of colonic butyrate concentration (μM/gram of intestinal contents) against liver index measured in the yeast group pigs at days 7 and 14 PW (n = 16). The dots are coloured by the diet (yeast *dark cyan*). Pearson's *rho* is reported above the regression line.

Abbreviations

16S *rRNA*: 16S ribosomal ribonucleic acid; ADG: Average daily gain; ANCOM: Analysis of compositions of microbiomes; ASV: Amplicon sequence variant; CD: Crypt depth; CFU: Colony forming unit; DNA : Deoxyribonucleic acid; HID-AB: High iron diamine-alcian blue; LAB: Lactic acid-producing bacteria; MWW: Mann-Whitney-Wilcoxon; PERMANOVA: Permutational multivariate analysis of variance; PW: Post-weaning; PCoA: Principle coordinate analysis; PCA: Principle component analysis; SCFA: Short-chain fatty acid; SE: Standard error; SRA: Sequence Read Archive

Acknowledgements

We thank the Bacteriology group at the Department of Food Safety and Infection Biology at Norwegian University of Life Sciences for their support during sampling and in the culturing study. We are grateful to the Histology group at the Department of Basic Sciences and Aquatic Medicine Biology at Norwegian University of Life Sciences for their contribution during sampling and histopathology analysis. We also thank Mathieu Castex and Caroline Achard for critical comments on this manuscript.

Authors' contributions

CMP, LTM, and MØ contributed to the study conceptualization; CPA, CMP, HS, IMH, LTM, MØ, and OCOU contributed to the study design; CPA, CMP, OCOU, IMH, and SI contributed to data acquisition and data analysis; CPA, HS, IMH, LTM, MØ, OCOU, and SI were involved in data interpretation; SI wrote the original draft; all authors reviewed and edited the manuscript prior to submission; all authors read and approved the final version of the manuscript and approved publication.

Funding
SI was supported by a PhD grant from Department of Food Safety and Infection Biology at Norwegian University of Life Sciences. The authors thank Foods of Norway Centre for Research-based Innovation (The Research Council of Norway, Lysaker, Norway, grant number 237841/030) and the Centre's industrial partners for financial support.

Availability of data and materials
The raw sequencing reads are deposited in the SRA archive: PRJNA580284.

Ethics approval and consent to participate
All animal management procedures were in compliance with the Norwegian Animal Welfare Act 10/07/2009 and the EU Directive on the Protection of Animals used for Scientific Purposes (2010/63/EU).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 19 December 2019 Accepted: 7 April 2020
Published online: 05 May 2020

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Paper 2

Longitudinal analysis of the faecal microbiome in pigs fed *Cyberlindnera jadinii* yeast as a protein source during the weanling period followed by a rapeseed- and faba bean-based grower-finisher diet

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Abstract

The porcine gut microbiome is closely connected to diet and is central to animal health and growth. The gut microbiota composition in relation to *Cyberlindnera jadinii* yeast as a protein source in a weanling diet was studied previously. Also, there is a mounting body of knowledge regarding the porcine gut microbiome composition in response to the use of rapeseed (*Brassica napus* subsp. *napus*) meal, and faba beans (*Vicia faba*) as protein sources during the growing/finishing period. However, there is limited data on how the porcine gut microbiome respond to a combination of *C. jadinii* yeast in the weanling phase and rapeseed meal and faba beans in the growing/finishing phase. This work investigated how the porcine faecal microbiome was changing in response to a novel yeast diet with a high inclusion of yeast proteins (40% of crude protein) in a weanling diet followed by a diet based on rapeseed meal and faba beans during the growing/finishing period. The faecal microbiomes of the weanling pigs fed yeast were more diverse with higher relative abundance of *Firmicutes* over *Bacteroidetes* compared with those of soybean meal-based diet fed weanlings. Reduced numbers of *Prevotella* in the yeast fed faecal microbiomes remained a microbiome characteristic up until two weeks after the yeast diet was changed to the rapeseed/faba bean growing finishing diet. A number of differentially abundant bacterial phylotypes along with distinct co-occurrence patterns observed during the growing/finishing period indicated the presence of a "carry-over" effect of the yeast weanling diet onto the faecal microbiomes of the grower/finisher pigs.

Introduction 1

Soybean (*Glycine max*) meal (SBM) is a commonly used protein source in commercial 2
livestock diets in Europe. This leads to intensified crop production, which puts pressure 3
on land and water resources, and it reduces their availability as food for humans (reviewed 4
in [1]). Yeast proteins, or yeast-derived nutrients proved a potent alternative to the 5
soybean-based and other conventional protein sources in the feed for weanling piglets 6
[2–4]. In growing/finishing (G/F) pig diets, rapeseed (*Brassica napus* subsp. *napus*) 7

meal (RSM) based formulations are believed to offer the proteins required for animal growth along with the potential of prebiotic properties of RSM which are important for animal health [5, 6]. Gut bacterial consortia play a chief role in the large intestine carbohydrate fermentation whereby supplying the host the molecules valuable for the health and development (e.g. short-chain fatty acids) [7–9]. It has been shown that the replacement of the conventional proteins in weanling pig diets by those derived from yeast has both an impact on the large intestine bacterial composition [10] and positive effects on the pig immune system [11–13]. We previously characterised the compositional changes of the large intestine microbiota in weanling piglets fed *C. jadinii* yeast-based diet. Those changes featured lower alpha microbial diversity in the caecum and colon of the yeast group compared with those of the control group. *Prevotella*, *Mitsuokella* and *Selenomonas* affiliated taxa were more predominant in the yeast associated large intestine microbiomes compared with those of the controls [10]. Umu et al. showed that RSM-based diets during G/F period modulated the porcine gut microbiota favouring the microbial taxa that are linked to an improved gut health state. *Mucispirillum* in the ileum, as well as *Bulleidia*, *Eubacterium*, *Lachnospira*, and *Paraprevotella* in the large intestine, were differentially abundant in the RSM-based G/F pigs (aged 88 days) compared with those of the SBM-fed pigs [14].

While the effects of the SBM diet on the pig gut microbiota were studied separately for weaning period and for G/F period, there is a gap in knowledge on how the porcine gut microbiota respond to a combination of diets wherein the conventional proteins are replaced by the yeast-derived proteins during weaning followed by RSM-based diets during the G/F period. Furthermore, it is not clear whether the yeast diets at weaning have a “carry-over” effect on the pig gut microbiota of the G/F pigs, i.e. the microbiota composition changes due to the yeast diet remain in the G/F period.

To address these questions, we designed a longitudinal study of the porcine faecal microbiomes by using 16S *rRNA* gene metabarcoding sequencing. We characterized the faecal microbiome structure of pigs fed yeast-based weanling diet followed by the RSM-based diet during G/F period (YL group) contrasting it with those fed SBM-based weanling diet followed by the RSM-based diet during G/F period (CL group).

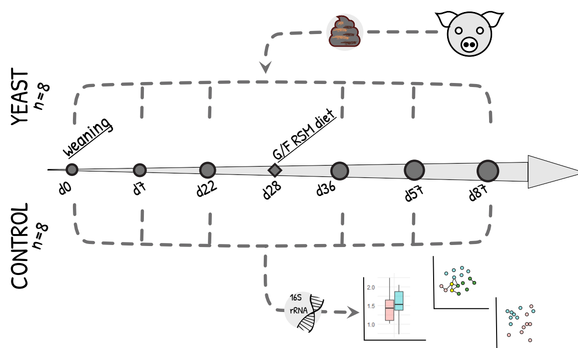


Figure 1. Overview of the experimental design. The timeline of the experiment is shown for two groups of animals in the experiment: YEAST (YL in the text) and CONTROL (CL in the text). Metabarcoding sequencing was done for the faecal samples collected at the days drawn as grey circles (d0, d8, d22, d36, d57, and d87 post-weaning)

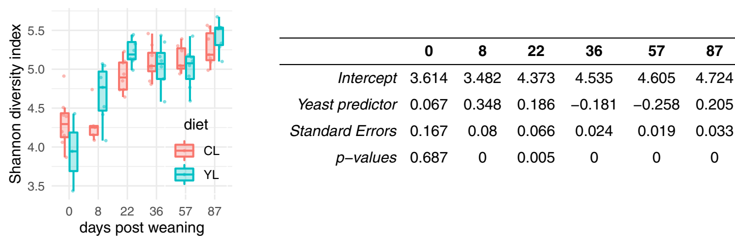
Results

The impact of *C. jadinii* yeast proteins on the faecal microbiome bacterial diversity

All animals were healthy during the time of the experiment. There were no major differences in zootechnical performance parameters between the pigs fed the SBM or the RSM diets (S1 Table). Profiling of the faecal microbial communities was performed in a time series way on 8 piglets from the YL group and 8 piglets from the CL group at the following time points: d0 (weaning), d8, and d22 post-weaning (PW). The sampling continued after the introduction of the animals to the grower-finisher RSM-based diet at day 28 PW. The YL and CL faecal samples were collected at d36, d57 and d87 PW. After filtering, denoising, and chimera removal with the DADA2 pipeline, there were on average 62472 (SD=16512) reads per sample available for downstream analyses (S1 Figure). The reads were demultiplexed into 3721 amplicon sequence variants (ASVs) representing the faecal microbiome at d0 (805 ASVs), d8 (1466 ASVs), d22 (2024 ASVs), d36 (1880 ASVs), d57 (2010 ASVs), and d87 PW (2050 ASVs) both feeding groups concerned.

Alpha diversity

Alpha diversity of the faecal microbiomes in both arms of the study increased between weaning (Shannon index mean = 4.16 (SD=0.38)) and d22 PW (Shannon index mean = 5.07 (SD = 0.24))(Figure 2). When pigs were allocated to the G/F diet, there was a less pronounced increase in the Shannon index of the faecal microbiomes in both arms of the study compared to that of the weaning period. It ranged from 5.07 (SD = 0.24) at d36 PW to 5.35 (SD=0.22) at d87 PW. The pairwise comparison of alpha diversity between the YL and CL groups was accomplished by using DivNet statistical procedure. There was no statistically significant difference in microbial alpha diversity between the CL and YL piglets at the baseline (p=0.69). The Shannon diversity index was higher in YL microbiomes than in the CL ones at d8, d22, and d57 PW, while that was opposite at d36, and d57 PW (Figure 2).



	0	8	22	36	57	87
Intercept	3.614	3.482	4.373	4.535	4.605	4.724
Yeast predictor	0.067	0.348	0.186	−0.181	−0.258	0.205
Standard Errors	0.167	0.08	0.066	0.024	0.019	0.033
p−values	0.687	0	0.005	0	0	0

Figure 2. Alpha microbial diversity. **Left** Distributions of the observed values of Shannon diversity index. **Right** The summary of the statistical inference for the alpha diversity measured by Shannon diversity index. The 'intercept' terms are the inferred estimates of the control group (CL) Shannon indices across d0-d87 PW. The 'Yeast predictor' terms are the inferred estimates of the yeast group (YL) Shannon indices across d0-d87 PW in relation to the 'intercept'.

Beta diversity

Beta diversity between the YL and CL pig faecal microbiomes was compared using weighted, and unweighted UniFrac distances as response variables for permutational multivariate analysis of variance (PERMANOVA) test (Figure 3, S2 Table). At the baseline (d0), the microbial communities did not differ, however, at day 8 PW, the diet was predictive of variance in faecal microbiome compositions estimated by UniFrac (F=1.17, R^2=14.6% , p=0.024) but not for weighted UniFrac (F=0.92, p=0.08). Notably, when "pen" variable was added to the model with the unweighted UniFrac distance as a response term, the prediction of variance in the beta-diversity metric increased to 46.4% (F=1.9, R^2=14.6%, p=0.007 and F=1.38, R^2=31.8, p=0.032 for 'diet' and 'pen' variables respectively). The variance in the weighted UniFrac distance could be predicted by the 'sow' variable for d8 PW microbiomes (F=2.03, R^2=53.6%, P=0.011). At day 22 PW, the diet could predict up to 24.8% of variance in weighted UniFrac distances of the faecal microbiomes (F=4.6, p=0.005) whilst the variance in unweighted UniFrac distance matrices could not be resolved by the diet (p>0.05). There was no difference in beta diversity metrics between the YL and CL pig microbiomes at d36, and d57 PW both distance matrices concerned. Of note, despite there was no effect of diet regimens during grower-finisher period (d36 - d87 PW), the results of PERMANOVA test showed that litter could be predictive of the faecal microbial composition structure. As much as 50.1% of the variance in the faecal microbiomes (weighted UniFrac distance) could be explained by the litter and diet (F=2.33, p=0.033).

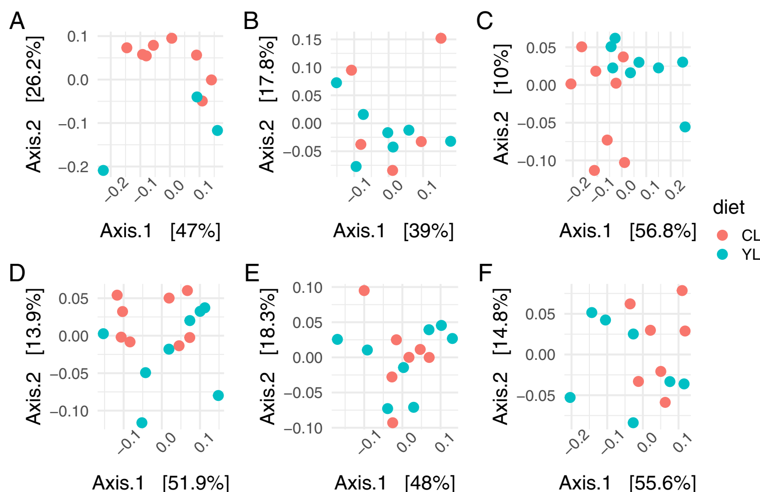


Figure 3. Beta microbial diversity. Principal coordinate analysis on weighted UniFrac distances of the faecal microbiomes coloured by diet at day 0 (panel **A**), 8 (panel **B**), 22 (panel **C**), 36 (panel **D**), 57 (panel **E**), and 87 (panel **F**) post-weaning

Relative abundance of bacterial phylotypes and differential abundance test

Two major bacterial phyla, *Bacteroidetes* and *Firmicutes*, constituted more than 85% of the faecal microbiomes in both feeding groups at all time points with 65.6% (SD =

6.8%) and 24.3% (SD = 4.46%) on average, respectively.

Weaning period At d22 PW, the yeast faecal microbiomes had higher relative abundance of *Firmicutes* (est=0.49 , p=0.004) and lower relative abundance of *Bacteroidetes* (est =-0.58, t=-3.81, p=0.002) than those of the SBM-based ones (S2 Figure). Also, for both phyla, *Firmicutes* and *Bacteroidetes*, the variability was lower in the yeast faecal microbiomes compared to those of the SBM-based ones (est =-4.19, t = -11.9, p= 2.31e-08; and est = -3.865, t= -11.03, p= 5.74e-08, respectively). We followed the two differentially abundant and variable phyla up to the class taxonomic level. These were of the *Bacteroidales* and *Clostridiales* orders. The major differences between the faecal microbiomes of YL and CL occurred on d8 PW when species agglomeration was applied (see methods). *Paraprevotellaceae*, *Desulfovibrionacea* ASVs, *Paludibacter, Prevotella stercorea*, and *Phascolarcobacterium* ASVs were more predominant in the CL faecal microbiomes at d8 PW compared with those of the YL (Figure 4). Unclassified *Bacteroides*, *Blautia*, unclassified *Ruminococcus*, *R. bromii*, *Sphaerochaeta*, *Treponema*, and *Succiniclasticum* ASVs were differentially abundant in the YL faecal microbiomes at d8 PW. At d22 PW, there were more *Fibrobacter* and *Prevotella*(ASV2) ASVs in the CL faecal microbiomes while *R. bromii* ASV was more abundant in the YL microbiomes (Figure 4).

G/F period At d36 PW, the relative abundance of the same as at d22 PW *Prevotella* (ASV2) was more prevalent in the CL faecal microbiomes (est = -0.33, t=-5.5, p= 0.0004) compared with those of YL diet (Figure 4). At d57 PW, two ASVs, RF32, manually reclassified (see methods) as *Novispirillum* sp., (*Alphaproteobacteria*) and *Butyricicoccus pullicaecorum* were observed at lower relative abundances in the YL faecal microbiomes compared with those of the CL (Figure 4). At d87 PW, *Campylobacter*, *Bacteroidales* order, and *Oscillospira* ASVs relative abundance was higher in the YL faecal microbiomes compared with that of the CL (Figure 4). A *Paraprevotellaceae* ASV was more predominant in the CL faecal microbiomes than those of YL on d87 PW (Figure 4).

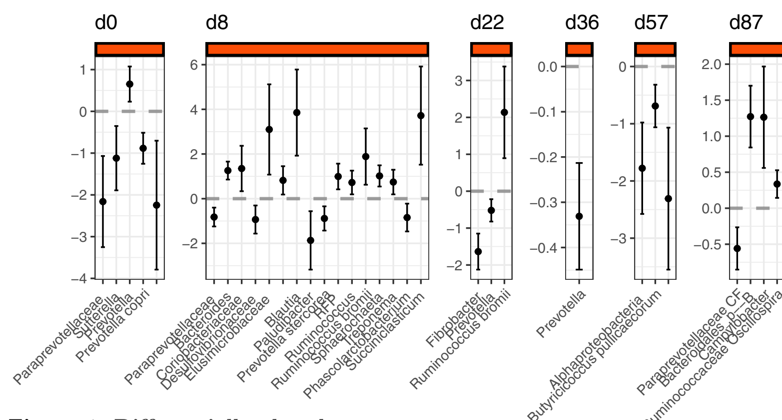


Figure 4. Differentially abundant taxa. The estimates of the beta-binomial regression on the porcine faecal microbiomes along with its standard errors across d0-d87 PW; the positive estimates (above the grey dashed line, "0") indicate the taxa that are more predominant in the yeast group (YL in the text) compared with those of the controls (CL). Some differentially abundant ASVs are not printed on the X-axis due to taxonomic ambiguity

Microbial network analysis

Next, we applied Sparse Inverse Covariance Estimation for Ecological Association Inference approach (SPIEC-EASI) to investigate networked microbial communities' patterns of the faecal microbiomes of the YL and CL pigs. The connectivity of the networks, i.e. the way the nodes are connected via edges, was sparse and increased moderately over the time; however, no evident difference was present in the two conditions (Figure 5). Moreover, in all the samples the majority of the nodes remained disconnected from the few connected components. Within those, we looked which ASVs (genus level) transited across the CL or YL microbiomes networks consecutively from one point of time to the next (Figure 5).

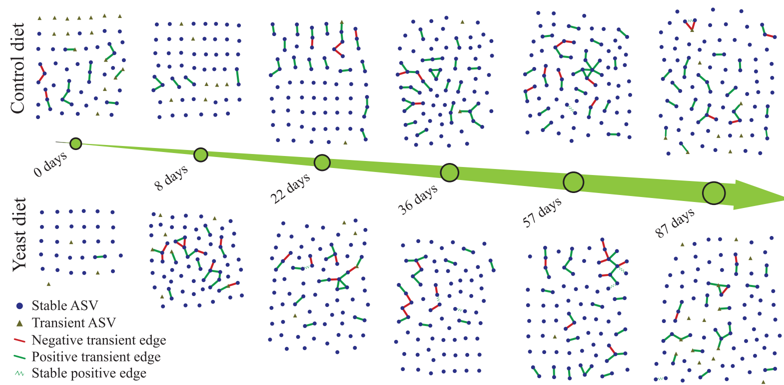


Figure 5. Development of faecal microbial networks across time and feeding groups. Stable ASVs are defined as those nodes which were present in at least two consecutive networks. **Transient ASVs** are defined as those nodes which were not present in consecutive networks. **Negative transient edges** are defined as the edges that are present in one network, but do not appear in the following network. The "negative" means the presence of an inverse proportional relationship between two nodes (ASVs). **Positive transient edges** are defined as the edges that are present in one network, but do not appear in the following network. The "positive" means the presence of a proportional relationship between two nodes (ASVs). **Stable positive edges** are defined as the edges that are present in one network and in the following network. The "positive" means the presence of a proportional relationship between two nodes (ASVs)

Vertex persistence There were 11 more ASVs in the YL microbial networks (66 ASVs) that transited throughout the whole experiment compared with those of the CL microbial networks (55 ASVs) (d0 excluded). Those 11 ASVs belonged to 8 different bacterial phyla: *Actinobacteria, Bacteroidetes, Firmicutes, Lentisphaerae, Proteobacteria, Synergistetes, Tenericutes*, and TM7. More specifically, a Coriobacteriaceae family ASV, *Bacteroides* ASV, *Turicibacter* ASV, Peptostreptococcaceae family ASV, *Eubacterium biforme* ASV, and R4-45B, Desulfovibrionaceae, Dethiosulfovibrionaceae, Mycoplasmataceae familiy ASVs transited across the microbial networks starting from d8 PW in the YL faecal microbiomes. In the CL microbiomes, in turn, the transition of a *Blautia* ASV was observed 4 times compared to 3 transitions of that in the YL faecal microbiomes.

Edge persistence When looking at which bacterial genera maintained the same microbe-microbe relationship in more than one consecutive network within diet groups (see methods), there were 3 pairs of ASVs which did so in the YL microbiomes in contrast to one ASV pair in the CL microbiomes. The latter pair of nodes, namely *Asteroleplasma anaerobium* and *Eubacterium biforme* ASVs, were connected at d57 and d87 PW. For the same time points another pair, *Dialister* and *Sutterella*, maintained their connection in the networks recovered from YL microbiomes. The connection between the nodes affiliated to *Prevotella copri* and *Faecalibacterium prausnitzii* ASVs in the YL microbial network was maintained at d22 and d36 PW, the period of transition from weaning to grower-finisher diet. A node representing an unknown ASV of *Clostridiales* order and the node affiliate to *Roseburia faecis* ASV were connected at both d36 and d57 PW.

Discussion

In this study we attempted to close the knowledge gap on how the gut microbiota develops over time in pigs fed diets in which the SBM/conventional proteins are replaced by yeast-derived proteins during weaning followed by an RSM-based diet during the G/F period. We specifically looked into the possible carry-over effects of the changes in the weaning period faecal microbiomes onto the G/F period microbiomes. As expected, we found differences in alpha and beta microbial diversity between the faecal microbiomes of the yeast-based and SBM-based weaning diets.

We found that the bacterial diversity was higher in the yeast-rapeseed meal (YL) group during the weaning period, which is interesting and contrasts with our previous observations[10] that revealed lower bacterial diversity in caecum and colon microbiomes of the pigs fed with the yeast weaning diet. There are a number of differences between the studies to explain this discrepancy such as: 1) sequencing platform (Illumina Miseq (this study) vs. Illumina Hiseq in [10]; 2) sequencing depth (here we set a threshold of 40000 sequencing reads per sample); 3) 16S *rRNA* gene amplified region (V3-V4 and V1-V3, respectively)[15]; and 4) gastrointestinal (GI) tract originating the samples[15]. In an unpublished study of ours, wherein the caecum and colon microbiomes of pigs challenged with an ETEC *E. coli*, we also observed lower figures of alpha diversity in the microbiomes of pig fed the yeast diet. There too the V3-V4 region of the 16S *rRNA* gene was amplified and sequenced using the Illumina Miseq platform. This suggests that the GI tract region was the factor that contributed the most to the faecal microbiome diversity of the YL piglets. The resident microbial community in caecum or spiral colon can have different structure compared with those of the rectal part because of differences in substrate availability[16]. The way of yeast cell processing used to formulate the yeast-based feed might have had a large impact on the substrate availability in the large intestine hence a distinct microbial community structure. Mannose polymers, the components of the yeast cell wall, cannot be digested by the host[17] and therefore are the substrate for microbial fermentation in the large intestine. *Prevotella* species and *Selenomonas* were shown to be able to degrade mannose among other substrates (summarized in [18]). Our previous results from a study with a nearly identical to this study design showed that the *Prevotella* and *Selenomonas* affiliated taxa were more abundant in the caecum and colon of the weanling pigs fed the yeast-based diet compared with those of the SBM diet[10]. This difference was not replicated in this study in which the faecal microbiomes were analysed. This may suggest that: 1) the microbiome of the proximal part of the large intestine is indeed different than that of the distal part (rectum) and; 2) the bacterial diversity in the yeast related faecal microbiomes is driven by the activity of the bacteria that degrade yeast cell which in turn facilitates the release of nutrients from yeast cells for further bacterial fermentation in the distal part of the colon. To test these hypotheses and to further investigate the microbiome changes

due to the yeast-based diets, new research that draws conclusions from metagenomics, transcriptomics, proteomics and other 'omics' data at once might be suitable.

Next, we studied the beta-microbial diversity using two methods: unweighted UniFrac[19] which incorporates the phylogenetic information of the microbial communities and weighted UniFrac[20] which incorporates the phylogenetic information of the microbial communities as well as the abundances of the members of the communities. We found that the beta-diversity changes associated with the yeast diet occurred during the weaning period up to d22 PW. It is interesting that the importance of rare microbial species (unweighted UniFrac method) was more pronounced by the end of first week PW than in the end of the weaning period (d22 PW). We hypothesize that the yeast proteins in the feed of weaning piglets is involved in shaping the faecal microbiomes in a two-stage mechanism. First, phylogenetically more distant microbial species establish themselves at low numbers in the yeast related faecal microbiomes by the end of the first week after introduction of the yeast feed. And second, as the yeast feeding lasts for three weeks after the feed introduction, those phylogenetically distinct species increase in numbers, hence making the yeast-influenced microbiomes to cluster apart from the control microbiomes as estimated by the abundance-sensitive weighted UniFrac method.

When we looked into the dynamics of the relative abundance changes of the major bacterial phyla, i.e. *Bacteroidetes* and *Firmicutes*, the results showed that the *Bacteroidetes* fraction decreased on average from 75% to 60% as well as the fraction of *Firmicutes* increased from 20 to 30% in the YL faecal microbiomes during the weaning period. In the CL microbiomes the relative abundance of *Bacteroidetes* and *Firmicutes* seemingly remained invariable at 70% and 20%, respectively, within the same period of the experimentation (shown in Figure 2C). The key finding of this study is that there were less *Bacteroidetes* and more *Firmicutes* by the end of the weaning period (measured at d22 PW) in the yeast group compared with the control group. Although these differences at the phylum level were not retained during the G/F period, when animals were on the RSM-based diet, there was still less *Prevotella* ASVs of *Bacteroides* phylum in the yeast group faecal microbiomes compared with those of the control group. Following up the differentially abundant *Prevotella* in the control group, we discovered a co-occurrence pattern between the *Prevotella* ASV and a *Desulfivibrio* ASV (data not shown). *Desulfivibrio* is a sulphate-reducing hydrogenotrophic species of the pig intestines that participates in hydrogen removing and fermentation[21, 22]. It is intriguing that such a co-occurrence pattern was discovered only in the microbiomes of the control group weanling pigs but not in the yeast piglets. At the later stages of the G/F period (d57, 87 PW) there were differences in low abundance taxa such as *Novispirillum* and *Campylobacter lanienae* between the YL and CL faecal microbiomes. Both bacterial phylotypes represented a small fraction of the faecal microbiomes amounting for less than 1% of all bacterial faecal microbiota. While the presence and function of *Novispirillum* in a pig gut microbiome is less clear, the *C. lanienae* was isolated from faeces of healthy pigs and considered a commensal[23]. Another interesting aspect of finding *C. lanienae* in the faecal microbiomes of pigs fed yeast-based diet during weaning period is that there was a link between *C. lanienae* and the RF3 family of the *Tenericutes* phylum when as per the inference of the respective microbial network.

In order to explore microbe-microbe interactions in the microbiomes of YL and CL, we conducted network analysis by recovering interactions between the ASVs with the SPIEC-EASI algorithm[24]. There were more taxa that were recovered at consecutive time points from the YL microbial networks than that of those of the CL. It means that the members of the YL microbiome, once established during the first week PW, were maintained in the microbial networks until the final phase of G/F period. This also suggests that a combination of yeast diet during weaning and RSM during the G/F period supports the expansion of the core faecal microbiota over those with the control

diet during weaning period. Here we apply the term "core microbiome" to designate those bacterial species that are recovered from the faecal samples throughout the whole experiment. Of note, the fraction of the core microbiome to its "non-core" part was 17-20% which suggests that the microbial communities changed dramatically over the period of the experiment. Since our experiment covered nearly the whole life-span of a slaughter pig, it is conceivable that in this study we observed the degree to which the gut microbiome co-evolves together with the host, as seen by the structural changes of the microbiome as a function of the weaning, age, diet, management etc. As stated earlier, the faecal microbial communities may be very different from those that reside in the colon and caecum in terms of their functions and cross-feeding patterns. Our findings, based on the microbial network analysis here, show that only a few taxa were connected at more than one consecutive time points: one pair in the CL microbiomes and three pairs in the YL microbiomes. This suggests that the bacterial interactions were volatile throughout the experiment. Also, the intervals between the sampling events were long enough for the faecal microbiomes to undergo compositional changes hence the possibility of changing the way the microbes interact with each other.

On the other hand, from an ecological perspective, it is within reason for the microbial communities that reside in the terminal part of GI tract where carbohydrate substrate availability is scarce, to switch from an active fermentation to a "hibernation" state on their way out of the habitat. This hypothesis can be tested by analysing microbial networks recovered from samples from both the proximal part of the large intestine (e.g. caecum, colon) and its distal part (rectum) collected post-mortem which was unattainable for this longitudinal study. Previous works investigating pig faecal microbiomes using graph theory methods [25, 26] relied on inferring microbial networks from 16S *rRNA* gene sequencing data using correlation-based approaches[27, 28]. For instance, Kiros and co-workers were able to recover hub bacterial genera having more than 10 connections to other genera of the network when investigating *Saccharomyces cerevisiae* yeast supplementation to weanling piglets, (e.g. *Lactobacillus, Roseburia, Faecalibacterium, Prevotella* etc.) using the CoNet tool for the microbial networks' recovery[26]. Wang et al., studying pig faecal microbial networks longitudinally by using the SparCC tool, identified more than 10 edges for *Prevotella copri, Blautia, Bacteroides*, and *Faecalibacterium*[29]. In contrast to the mentioned studies, we recovered microbial networks wherein the nodes had 1 connection, or edge, mostly with only few having 3-5 connections. The difference in methodology and possibly a small sample size in this study[24] might have been the non-biological explanation of why the recovered microbial networks were of lower complexity compared to the ones discussed in Kiros et al and Wang et al. Yet, an interesting finding derived from the network analysis was that some pairs of bacterial phylotypes (connected one to another nodes) were observed across several consecutive time points. For instance, in the CL microbiomes, a *Clostridiales/Roseburia faecis*, and *Dialister/Sutturella* bacterial phylotype pairs were recovered in pairs from d36-57 PW and d57-87 PW networks of the G/F period, respectively. Another phylotype pair, *Prevotella copri/Faecalibacterium prausnitzii* was seen connected in both d22 and d36 PW microbial networks. It is intriguing that this finding supports previously discussed carry-over of *Bacteroides* phylum (*Prevotella*) relative abundance from the weaning period onto the beginning of the G/F period. Only one bacterial phylotype pair, *Asteroleplasma anaerobium/Eubacterium biforme*, transited across several time points (d57-d87 PW). This difference in the number of bacterial phylotypes observed across several consecutive time points, can be interpreted as an element of stability that was observed more often in the microbiomes of yeast fed weanling pigs than in that of controls. Also, this type of information may be indicative of the presence of continuous diet-dependent microbe-microbe cross-feeding patterns that is stably expressed during the gut microbiome development.

In conclusion, upon the longitudinal analysis of the pig faecal microbiomes of pigs fed either yeast-based or SBM-based weanling diets, the major differences in the microbiome composition were observed during the second-to-third week post-weaning. Those changes attributed to the differences in the dietary regimes were carried over to the G/F period and primarily represented as a retention of lower relative abundance of *Prevotella* in the yeast microbiomes compared with the control ones; and in the form of the microbe-microbe interactions. To further gain insight into the details of the effect of the animal diets produced in a sustainable way on the gut microbiome of pigs, a study with the exploration of the full genetic context of the entirety of gut microorganisms, that is a collection of all non-host genes, would be of a potential interest. This study seems to support the possible beneficial effect of introducing yeast-based feed ingredients in weanling pigs coupled with the RSM-based feed in the G/F period. The combination of the two sustainably produced feed worked well together rendering a more optimal large intestinal microbiota.

Materials and Methods

Ethics statement The experiment was carried out at the Center for livestock production (SHF) (NMBU, Ås, Norway) approved by the National Animal Research Authority (permit no. 174). All animals were cared for according to laws and regulations controlling experiments with live animals in Norway (the Animal Protection Act of December 20th, 1974, and the Animal Protection Ordinance concerning experiments with animals of January 15th, 1996).

Animals, allotment, and housing A total of 48 Norwegian crossbreed pigs (Landrace x Yorkshire x Duroc) from 5 litters were used for the animal performance part of the experiment. Average initial weight and final weight in the piglet period was 10.4 kg and 22.8 kg, and average initial weight and final weight in the growing-finishing period was 22.8 kg and 109.0 kg, respectively. The experiment was conducted as a randomized complete block design. At the start of the piglet period the pigs were blocked by litter and sex and allotted by initial weight to four dietary treatments (below). Piglets were kept in pens with four pigs per pen, giving three replicates per treatment. Each pen had partially slatted floors, and a total area of 2.6 m^2 (2.6 $\times$ 1.0 m). The pens were equipped with heating lamp. A rubber mat of approximately 90 $\times$ 100 cm was used as a replacement for other bedding materials, to minimize interference with the measurements of microbiome. The room temperature was kept on average at 19.9^0C $\pm$ 1.05 SD, with 8 h of light and 16-h darkness cycles. The piglet period lasted 28 days. The piglets were fed *ad libitum* from automatic feeders and had free access to drinking water. After the piglet period, the pigs were moved from the nursery room to a growing-finishing room and re-grouped. The growing-finishing period lasted on average for 89.5 days. At each feeding, pigs were individually restrained in the feeding stall until the feed was consumed in order to obtain individual feed intake. Thus, each pig was one experimental unit. Pigs were housed in an environmentally controlled barn with partially slotted concrete floor. Twelve 8.2 m^2 pens designed for individual feeding were used. Average ambient daily temperature in the growing-finishing room was 18.5^0C $\pm$ 1.45 SD.

Diets and feeding The dietary treatments in the piglet period were: 1) a control diet based on soybean meal, fish meal, potato protein concentrate and rapeseed meal as protein sources (Control piglet diet), and 2) an experimental diet where 40% of the protein was replaced by protein from heat-inactivated, dried *C. jadinii* cells (Yeast piglet diet). After the piglet period, pigs were switched to growing-finishing diets consisting of: 1) a soybean meal based control diet (Control G/F-diet), and 2) a rapeseed meal

and field bean based experimental diet (Local G/F diet). The diets were designed to be isonitrogenous and isoenergetic and to contain equal levels of methionine + cysteine, and threonine. The diets were produced and pelleted to 3 mm diameter at a commercial feed factory (Felleskjøpet Kambo, Moss, Norway). The content of digestible lysine, threonine, methionine and cysteine of the ingredients was estimated using analyzed values, multiplied by the standardized ileal digestibility coefficients (SID) for nitrogen and amino acids[30]. All diets were formulated to meet or exceed the requirements for indispensable amino acids and all other nutrients[31]. A cumulative feed sample from each dietary treatment was taken for chemical analysis. Composition and nutrient contents of diets are shown in Table S3 (piglet diets) and Table S4 (G/F diets)[32]. When combining the piglet period and the G/F period, the following four treatments were obtained: 1) Piglet control diet + G/F control diet. (Control/Control, or CC) 2) Piglet control diet + G/F local diet. (Control/Local, or CL) 3) Piglet yeast diet + G/F control diet. (Yeast/Control, or YC) 4) Piglet yeast diet + G/F local diet (Yeast/Local, or YL)

In the piglet period the pigs were fed pen-wise according to appetite. All four pigs in each pen were given the same feed. The average weight gain and feed intake for each pen was measured weekly, and average daily gain (ADG), average daily feed intake (ADFI) and feed conversion rate (FCR) as kg feed divided on kg gain were calculated for each pen.

In the growing-finishing period (G/F period), all pigs were individually fed twice per day according to a semi-ad libitum feeding scale[33]. Feed refusals for each pig were recorded and subtracted from the total feed intake. All pigs were given free access to water from nipple drinkers. Feed consumption and individual pigs' weight were recorded weekly to determine average daily gain (ADG), average daily feed intake (ADFI) and FCR.

Chemical analyses Samples of the diets were analysed for crude protein (CP) by Kjeldahl-N x 6.25 (EC No 152/2009), crude fat using ASE® 350 Accelerated Solvent Extractor, dry matter (DM) by drying to constant weight at 104^0C (EC No 152/2009), ash by incineration at 550^0C (EC No 152/2009), acid detergent fiber (ADF) and neutral detergent fibre (NDF) using a fibre analyser system (Ankom200; ANKOM Technologies, Fairport, NY, USA) with filter bags (Ankom F58; ANKOM Technologies). Gross energy (GE) content was determined by a Parr 1281 Adiabatic Bomb Calorimeter (Parr Instruments, Moline, IL, USA) according to ISO (1998). Analysis of amino acids in the diets were carried out according to EC (2009) using Biochrom 30 Amino Acid Analyzer. Tryptophan in the diets was determined according to EC (2009) using high-performance liquid chromatography system (Dionex UltiMate 3000, Dionex Softron GmbH, Germering, Germany) and the fluorescent detector (Shimadzu RF-535; Shimadzu Corp., Kyoto, Japan).

Faecal sample handling For the faecal microbiota profiling, 8 pigs were randomly chosen from each of the groups CL (n=12) and YL (n=12) (S1 Table), respectively, and tracked individually over the entire experiment. The collection of faecal samples was carried out at d0, d8, d22, d36, d57, and d87 post-weaning (PW). On d87 PW there were 7 samples from each group. The samples were liquid nitrogen snap frozen and kept at -80^0C until the DNA isolation. The DNA extraction was according to a previously described protocol[34] with minor modifications. Briefly, 200 mg of thawed and mixed faecal samples were added to 1 ml of InhibitEX Buffer (QIAGEN, GmbH, Hilden, Germany) followed by the beat-beating step in TissueLyser II (Qiagen, Retsch GmbH, Hannover, Germany) with 500 mg of zirconia/silica beads (∅ = 0.1 mm, Carl Roth, Karlsruhe, Germany) (1.5 min at 30 Hz). Proteins were digested with 30 μL of Proteinase

K II (QIAGEN, GmbH, Hilden, Germany). DNA was washed with AW1 and AW2 buffers (QIAGEN, GmbH, Hilden, Germany) and eluted with ATE buffer (QIAGEN, GmbH, Hilden, Germany). The yielded DNA purity was assessed by NanoDrop (Thermo Fisher Scientific, Waltham, MA) and quantified with the Qubit fluorometric broad range assay (Invitrogen, Eugene, OR, USA). Library preparation was performed at the Norwegian Sequencing Centre (https://www.sequencing.uio.no/, Oslo, Norway) using universal prokaryotic primers 319F(5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACNVGGGTWTCTAAT-3') that target the V3-V4 regions of the 16S *rRNA* gene. Sequencing was performed on a MiSeq sequencer following the manufacturer's guidelines. The resulting sequences were deposited in the ENA (PRJEB41040). Metadata can be accessed through https://github.com/stan-iakhno/bioRxiv_02.

Bioinformatics analysis and statistics Demultiplexed paired-end Illumina reads were pre-filtered with bbduk version 37.48 (BBMap – Bushnell B., https://sourceforge.net/projects/bbmap/) by trimming right-end bases less than 15 Phred quality score, removing trimmed reads shorter than 250 bp or/and average Phred quality score less than 20. The resulting reads were further quality filtered by trimming left-end 20 bp and removing reads with maxEE more than 1 for forward and 2 for reverse reads, denoised, merged, and chimera removed with DADA2 R package ver 1.12.1[35] (Figure S1). The resulting ASV tables that derived from two separate Illumina sequencing runs were merged followed by taxonomy assignment using RDP Naive Bayesian Classifier implementation in DADA2 R package (default settings) with GreenGenes database version 13.8 [36] as the reference database. The phylogenetic tree was reconstructed under the Jukes-Cantor (JC) nucleotide model with gamma distribution (number of intervals of the discrete gamma distribution (k)=4, shape=1 with invariant sites (inv=0.2)) in R. The pipeline code is available through https://github.com/stan-iakhno/bioRxiv_02.

DivNet statistical procedure[37] was used to estimate the Shannon diversity index and to test for differences in Shannon diversity estimates in networked gut microbial communities stratified by the day of sampling with the diet as a covariate. The beta diversity analysis was performed via the analysis of multivariate homogeneity of group dispersions[38] followed by the permutation test[39], 9999 permutations and principle coordinate analysis (PCoA) on unweighted[19] and weighted[20] Unifrac distances, and permutational multivariate analysis of variance (PERMANOVA) test for covariate significance in R, 9999 permutations. The samples with read count less than 40000 were discarded from the alpha and beta diversity analyses. To calculate the relative abundance of bacterial phylotypes per feeding group and per sampling time point, the group means were taken from the respective groups. To detect differentially abundant bacterial phylotypes, 'corncob' algorithm[40] was run on the microbial feature tables (ASV counts per each sample) by fitting a beta-binomial regression model to microbial data stratified by the day of sampling with the diet and litter as covariates. The false discovery rate due to multiple testing was addressed by the Benjamini-Hochberg correction with the cut-off of 0.05. The test was run at each taxonomic level (phylum, class, order, family, species, and ASVs) discarding the samples with the read count less than 10000. Those ASVs that lacked genus/species taxonomic classification, were classified manually by using web-based nucleotide BLAST on the non-redundant nucleotide database where possible. Ambiguous hits were ignored.

Microbial network analysis The ASV counts were collapsed at the genus level and filtered for at least 3 counts per ASV in at least 20% of the samples and at least 50% of the sample per time point (0, 8, 22, 36, 57 and 87 days) and condition (yeast diet and control diet) using the R package phyloseq[41] version 1.26.1. For each time point and condition a network was computed with the package SpiecEasi[24] version 1.0.7.

For each condition the permanence of nodes (ASVs) and edges (their relationships) was checked at two consecutive time points.

Acknowledgments

We thank the staff at the Centre for livestock production (SHF), NMBU, Ås, for their excellence in managing the animals during this experiment. SI was funded by a PhD fellowship from the Department of Food Safety and Infection Biology at Norwegian University of Life Sciences. The authors thank Foods of Norway Centre for Research-based Innovation (The Research Council of Norway, Lysaker, Norway, grant number 237841/030) and the Centre's industrial partners for financial support.

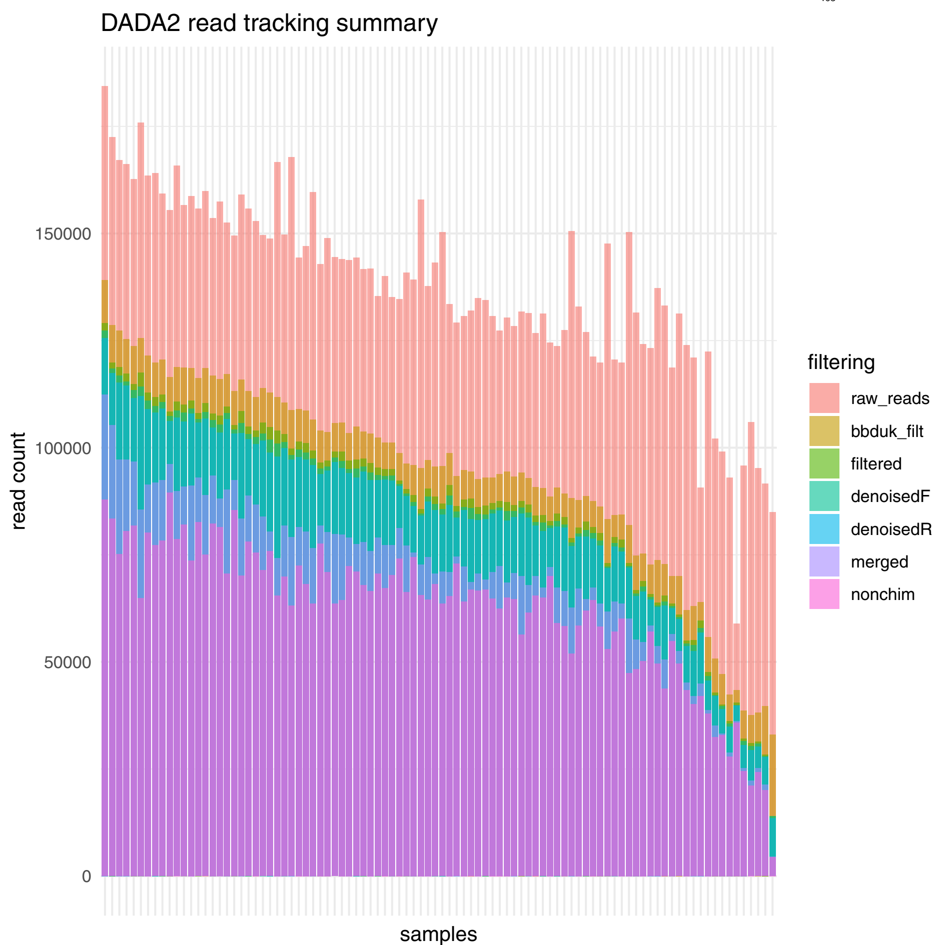
Supporting Information

S1 Table

	CC	CL	YC	YL	Std.err	P-value
No pens	3	3	3	3	-	-
Pig per treatment	12	12	12	12	-	-
Initial weight, kg	10.37	10.32	10.44	10.37	0.731	0.9995
Weight after 4 week	22.84	22.80	22.54	23.11	1.092	0.986
Final weight, kg	108.87	109.78	109.97	107.43	1.597	0.676
Overall ADG, g	841.4	837.5	853.7	829.8	13.62	0.671
Overall ADFI, g	1638.8	1653.8	1629.8	1652.5	29.23	0.924
Overall FCR	1.948ab	1.974b	1.909a	1.991b	0.016	0.032

Table S1. Performance results from weaning until slaughter. For the overall experimental period (from average live weight of 10.4 kg until slaughter) no significant differences among four treatments, control-control(CC), control-local(CL), yeast-control(YC),and yeast-local(YL), were found for average daily gain (ADG) ($P=0.671$) and average daily feed intake (ADFI) ($P=0.924$). Feed conversion rate (FCR) was influenced by treatment ($P=0.032$), and pigs given the control diet (CC and YC) in the growing-finishing (G/F) period in general had better FCR than the pigs fed the Local diet (CL and YL). In the piglet period (live weight 10.4 kg until 22.8 kg), FCR did not differ among treatments ($P=0.994$). Different letters indicate significant difference among treatments ($P < 0.05$). Average daily gain - ADG. Average daily feed intake – ADFI, and Feed conversion ratio – FCR

S1 Figure



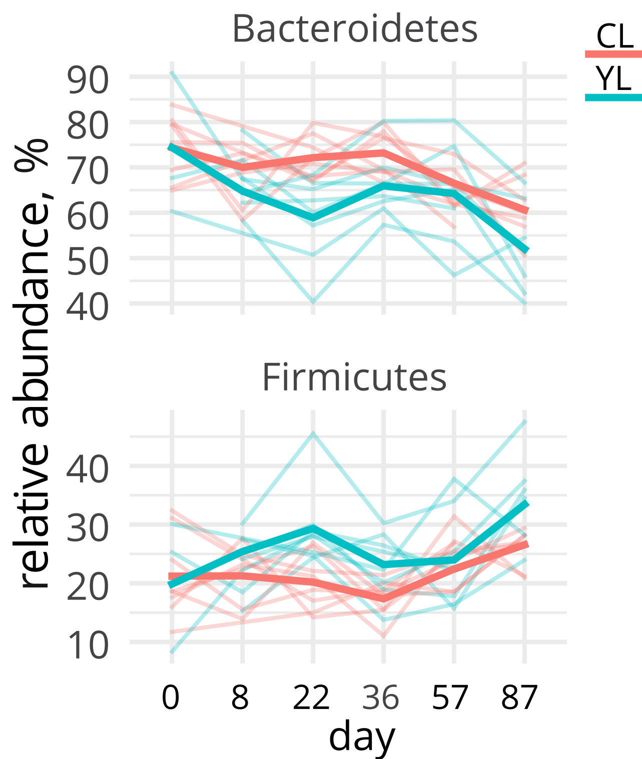
Read tracking summary. The bottom-most bars in the stack (**nonchim**) show the number of read that were the basis for making the feature count table (OTU/ASV-table). The bars above **nonchim** summarise the number of sequencing reads removed due to each procedure of the bioinformatics pipeline: a) filtered with the bbduk filtering algorithm (**bbduk filt**), b) filtered with the DADA2 algorithm (**filtered**), c) removed due to DADA2 denoising procedure (**denoisedR/F**), d) removed due to pair merging failures (**merged**). **raw reads** are raw demultiplexed reads derived from Illumina sequencer.

S2 Table

Unifrac						
day PW->	*0*	*8*	*22*	*36*	*57*	*87*
F	*1.508*	*1.704*	*1.165*	*0.844*	*1.033*	*1.26*
R^2	*0.143*	*0.146*	*0.077*	*0.057*	*0.074*	*0.095*
p-value	*0.125*	*0.024*	*0.212*	*0.704*	*0.359*	*0.122*
F	***0.002***	***0.444***	***0.113***	***2.57***	***2.133***	***0.049***
p-value	***0.976***	***0.527***	***0.748***	***0.122***	***0.168***	***0.83***
weighted Unifrac						
day PW->	*0*	*8*	*22*	*36*	*57*	*87*
F	*2.031*	*0.916*	*4.893*	*1.583*	*0.492*	*1.927*
R^2	*0.184*	*0.084*	*0.259*	*0.102*	*0.036*	*0.138*
p-value	*0.104*	*0.491*	*0.003*	*0.156*	*0.821*	*0.115*
F	***1.013***	***0.41***	***1.59***	***3.302***	***0.944***	***0.969***
p-value	***0.364***	***0.543***	***0.258***	***0.092***	***0.354***	***0.328***

Beta diversity PERMANOVA and permdisp test The tests were performed on the "Unifrac" and "weighed Unifrac" distances. The test statistics of the permutational multivariate analysis of variance (PERMANOVA) test are given in normal font, multivariate homogeneity of groups dispersions (permdisp) test are given in **bold**.

S2 Figure 481



482

Relative abundance of *Bacteroidetes* and *Firmicutes* phyla across d0-88 PW. 483
Individual observations are shown by the thin spaghetti lines, the average group values 484
are shown by the thick spaghetti lines 485

S3 Table

	Diet	
Ingredients	Control piglet diet	Yeast piglet diet
Wheat	627.9	593.6
Barley	100.0	100.0
Oats	50.0	50.0
Yeast meal (*C. jadinii*) (47% CP)	0	146.0
Soybean meal (SBM) (45% CP)	80.0	19.0
Fish meal (68.4% CP)	20.0	4.8
Potato protein concentrate (72.5% CP)	33.8	9.1
Rapeseed meal (Mestilla) (35%CP)	20.0	4.9
Rapeseed oil	19.7	23.4
Limestone	9.2	9.4
Monocalcium phosphate	13.1	15.5
Sodium chloride (NaCl)	7.2	5.5
L-Lysine HCl (98%)	6.5	5.7
L-Threonine	2.9	2.4
L-Methionine	2.1	2.9
L-Valine	1.4	1.2
L-Tryptophan	0.9	0.9
Premix[1]	5.3	5.5
Calculated contents		
Net energy, MJ kg^{-1} (FUn, kg^{-1})	9.94	9.94
Crude protein from *C. jadinii*)	0	40.0
Analysed content, g kg^{-1}		
DM	869	885
Gross energy, MJ/kg	19	19
Crude protein	176	172
Crude fat	39	41
Ash	46	45
Neutral detergent fibre (NDF)	96	91
Starch	442	437

[1] Provided the following amounts per kilogram of feed: 120 mg of Zn (ZnO); 460 mg of Fe ($FeSO_4$. H_20); 60 mg of Mn (MnO); 26 mg of Cu (CuSO4 x 5H2O); 0.60 mg of I ($Ca(IO_3)_2$; <1.0 mg of Se (Na_2SeO_3); 8000 IU of vitamin A; 1500 IU of cholecalciferol; 45 mg of dl-α-tocopheryl acetate; 105 mg of ascorbic acid; 4.64 mg of menadione; 5.63 mg of riboflavin, 3 mg of thiamine; 15 mg of d-pantothenic acid; 20 μg of cyanocobalamin; 45 mg of niacin.

Table S3: Piglet period. Ingredient and chemical composition (g kg^{-1}) of diets based on soybean meal (Control) and *C. jadinii* (Yeast). In the yeast diet, 40% of the crude protein was replaced by that from *C. jadinii* (LYCC-7549; Lallemand Yeast Culture Collection).

S4 Table 492

Ingredients	Diet	
	Control G/F diet	Local G/F diet
Barley	585.5	456.2
Oats	150.0	150.0
Soybean meal (SBM) (45% CP)	142.6	0.0
Rapeseed meal (RSM) (Mestilla)	60.0	180.0
Faba beans (Columbo)	0.0	161.12
Rendered fat	23.0	16.5
Molasses	10.0	10.0
Limestone	9.7	8.7
Monocalcium phosphate	3.2	1.4
Sodium chloride (NaCl)	6.3	6.4
L-Lysine HCl (98%)	3.3	3.0
L-Threonine	1.3	1.4
L-Methionine	1.0	1.0
L-Tryptophan	0.1	0.3
Premix[1]	4.0	4.0
Calculated contents		
Net energy, MJ kg^{-1} (FUn, kg^{-1})	9.42 (1.07)	9.42 (1.07)
Analyzed content, g kg^{-1}		
DM	868	870
Gross energy, MJ/kg	16.4	16.6
Crude protein	153	158
Crude fat	40	51
Ash	45	44
Neutral detergent fiber (NDF)	165	173
Starch	424	422

[1] Provided the following amounts per kilogram of feed: 20 mg of Axtra phytase, 72 mg of Zn (ZnO); 96 mg of Fe ($FeSO_4 \cdot H_2O$); 48 mg of Mn (MnO); 17 mg of Cu ($CuSO_4 \times 5H_2O$); 0.48 mg of I ($Ca(IO_3)_2$); 0.27 mg of Se (Na_2SeO_3); 6500 IU of vitamin A; 1500 IU of cholecalciferol; 75 mg of dl-α-tocopheryl acetate, 150 mg of Vitamin E (50%); 4.63 mg of menadione; 5.625 mg of riboflavin, 15 mg of d-pantothenic acid; 15 µg of cyanocobalamine; 45 mg of niacin; 0.30 mg of biotin; 1.69 mg of folic acid; Choline: 2300 mg (Control) and 1605 mg (Local).

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Table S4: Growing-finishing period. Ingredient and chemical composition (g kg^{-1}) 494
of diets based on soybean meal (Control) and locally produced protein ingredients (Local). 495

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Paper 3

Small intestine lactobacilli growth promotion and immunomodulation in weaner pigs fed inactivated *Cyberlindnera jadinii* yeast high inclusion diet and exposed to enterotoxigenic *Escherichia coli* F4^{+}: O149

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1 Abstract

Enterotoxigenic *Escherichia coli* (ETEC) F4^{+}: O149 is a causative agent for the development of post-weaning diarrhoea (PWD) in pigs that contributes to production losses. Yeast cell wall components used as a feed additive can modulate gut immunity and help protect animals from enteric infections. This work investigated how a novel yeast diet with high inclusion of yeast proteins (40% of crude protein) affected the course of ETEC mediated diarrhoea in weaner piglets from a farm with or without a history of post-weaning diarrhoea. We found that immune response to F4*ab* ETEC infection and appetite of the animals were altered by high inclusion *C. jadinii* yeast. The results indicate that the novel diet can support the diseased animals either directly through the effect of yeast beta-glucans and mannans or indirectly through the promotion of small intestine lactobacilli or both.

25 # 2 Introduction

26 Diarrhoea in neonatal and weaned piglets has been a concern to farmers due to the morbidity and
27 mortality [1, 2]. The introduction of *E. coli* fimbrial vaccines [3] shifted the peak of diarrhoea
28 from the neonatal and suckling period over to the weaning period where the mortality due
29 to diarrhoea is lower [4]. An enterotoxigenic *Escherichia coli* (ETEC) of the O149 serotype
30 has been incriminated in most of the post-weaning diarrhoea (PWD) cases contributing to
31 production losses [2, 5–7]. This enteric pathogen acts via (I) the adhesion to small intestine
32 enterocyte brush border with the help of receptor-specific fimbriae proteins F4 (K88) (*ab*,
33 *ac*, and *ad* variants) and (II) the production of toxins that induce enterocyte electrolyte/fluid
34 imbalance hence watery diarrhoea. However, not all piglets are equally susceptible to ETEC.
35 Some animals are immune to ETEC F4 *ab/ac* colonization due to an inherited trait that is
36 thought to be linked to chromosome 13 of the pig [8]. A 74-kDa glycoprotein (GP74) was found
37 to be key for ETEC adherence [9] but the genetic determinants encoding for this protein are
38 not fully investigated [8, 10, 11]. Polymorphism in the *muc*4 gene was used as a basis for a
39 DNA test to classify animals as either F4-adhesive or F4-non-adhesive [8]. Other candidate
40 genes have been proposed as genetic determinants for the non-adhesive porcine phenotype [11].
41 The receptors for F4 *ab* fimbriae are found in the small intestine of newborn and weaned
42 piglets [12] but not in older F4-adhesive animals [13]. While nursing piglets are protected from
43 ETEC by maternal transfer of antibodies from vaccinated dams [3, 14], there are currently no
44 measures available to protect piglets against ETEC-mediated diarrhoea after weaning (discussed
45 in [15–17]). Modulation of the immune response against ETEC may be one such solution. Yeast
46 cell wall components, mannans and beta-glucans proved potent immunomodulatory compounds.
47 Fouhse and co-workers demonstrated that supplementation of yeast-derived mannans to weaner
48 pigs positively affected jejunal villi architecture with corresponding changes in the gene expression
49 profile [18]. The findings of Che et. al suggested that yeast mannans in feed could reduce systemic
50 inflammation in pigs via suppression of lipopolysaccharide (LPS) induced TNF-alpha by alveolar
51 macrophages [19]. Stuyven and colleagues reported protective effects of *Saccharomyces cerevisiae*,
52 and *Sclerotium rolfsii* derived beta-glucans against ETEC F4^{+} with a reduction in pathogen
53 shedding and F4-specific serum antibodies in weaner pigs [20]. Our previous work showed that
54 feeding a strain of heat-inactivated *Cyberlindnera jadinii* yeast as a protein source changes the

intestinal microbiota composition in weaner piglets [21]. Using cultivation and 16S *rRNA* gene metabarcoding sequencing techniques, we have shown that the yeast diet promoted the growth of small intestine lactobacilli. Beneficial immunomodulatory properties of intestinal lactobacilli are well documented ([22]; reviewed in [23]). These findings indicate that targeting the lactobacilli populations through diets can have an indirect impact on the host immune response.

Because beta-glucans and mannans are structural components of the yeast cell wall, and yeast replaced as much as 40% of the conventional proteins in the experimental diet, we hypothesized that *C. jadinii* yeast as a protein source can modulate the immune response towards ETEC F4$^+$ and hence affect the course of PWD in weaner piglets.

To test the ability of a *C. jadinii* yeast diet to modify the course of PWD, we recruited piglets from two herds (with and without a history of PWD), primed them with either control or yeast-based diets where 40% of the protein was replaced with yeast, and orally challenged weaned piglets with a haemolytic F4ab^+ O149 *E. coli* isolated previously from the herd with the history of PWD. To gain insights into the effects of yeast-derived feed, we compared gut microbial ecology metrics (diversity and composition), zootechnical performance, morphology and histology of gastrointestinal (GI) tract focusing on the ETEC F4$^+$ intestinal colonization between the control and the yeast-fed piglet groups.

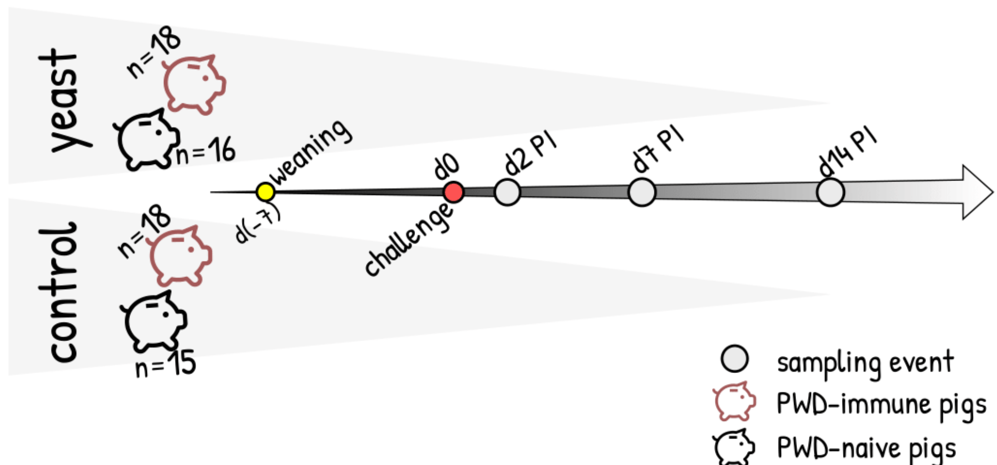


Figure 1: Overview of the experimental design

3 Results

3.1 General information

Post-weaning diarrhoea (PWD)

Of 68 piglets in the experiment, one animal from the control feeding group was euthanized *ad hoc* because of circulatory failure on d5 post-weaning (PW). There were no mortality cases due to the bacterial challenge throughout the experiment. Diarrhoea scores were higher for the first three days after the challenge in the piglets from the herd with no history of PWD (F4-naive herd) compared with those of the herd with the history of PWD (F4-immune herd) (Figure 2A).

Average daily gain (ADG)

Average daily gain (ADG) was analysed by fitting the multiple regression model where "day", "litter", and "diet" were the predictor terms (d2 PI was excluded). The analysis revealed that the pigs fed the yeast-based diet tended to gain 62 g/day less weight than those fed the control diet (Figure 2B). The litter contribution to ADG estimate was as follows: litter3283, and litter3286 pigs tended to gain 125 g/day less than litter 3282 ($p<0.00001$); litter 3284 was gaining 86 g/day less than litter3282 ($p=0.002$); and litter3287 had 57 g/day greater ADG compared with that of the litter3283 ($p=0.03$).

Feed intake

The feed intake pattern (pen level) diverged between the herds from d3 PI to d5 PI with the F4-immune herd piglets eating more than those of the F4-naive herd. Within the herds, feed intake pattern showed that the control piglets ate more than the yeast fed piglets. From day 8 PI onwards, the effect of herd was less pronounced and changes in feed intake were attributed to the diet with the control group eating more feed than the yeast group (Figure 2C).

3.2 Immunohistochemistry

F4 and CD3 in the ileum **d2 PI** The proportion of the mucosa-associated ETEC $F4^+$ per length of the ileum epithelium tended to be 5% greater in the pigs fed the yeast based diet than that of the pigs fed control diet (89% posterior probability)(Figure 3A). The piglets from the litter3288

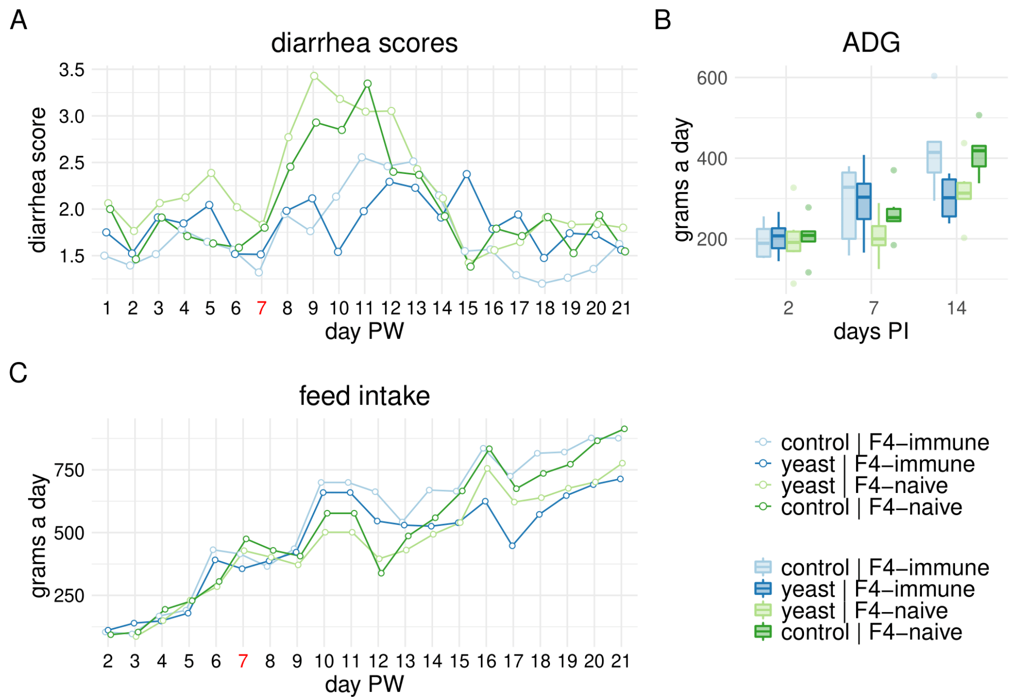


Figure 2: Diarrhoea scores and performance results. Panel A: Diarrhoea scores (pen level) across the experimental groups throughout the experiment. Day 7 post-weaning (coloured red) corresponds to the day the animals were orally challenged with ETEC F4$^+$. Panel B: Distribution of the average daily gain (ADG) across the experimental groups at d2, d7, and d14 post-infection. Panel C: Daily feed intake across the experimental groups throughout the experiment. Day 7 post-weaning (coloured red) corresponds to the day the animals were orally challenged with ETEC F4$^+$.

98 had 10% less mucosa-associated ETEC F4$^+$ per length of the ileum epithelium than that of the
99 litter3282 (89% posterior probability) (not shown).

100 At d7 PI, the prevalence of F4$^+$ *E. coli* was lower in the ileum of the piglets fed both diets than
101 that of d2 PI. Only two piglets in the yeast group had identifiable counts of F4$^+$ adjacent to the
102 epithelial surface compared with none of the control group. The remaining animals (n=16) were
103 negative for the presence of F4$^+$ *E. coli* in their ileum.

104 There was no clear relationship between neither the diet type, nor the litter, and the proportion
105 of IEL CD3$^+$ cells in the ileum epithelium of the pigs (Figure 3B). However, there was an inverse
106 correlation between the proportion of mucosa-associated F4 antigen and the proportion of IEL
107 CD3 populations in the ileum of the piglets fed the control diet at d2 PI (*rho*=-0.81, 95%CI
108 upper = -0.25, 95%CI lower = -0.94) (Figure 3B). This relationship was not found in the yeast
109 fed piglets (*rho*=0.1, 95%CI upper = 0.58, 95%CI lower = - 0.44) (Figure 3C).

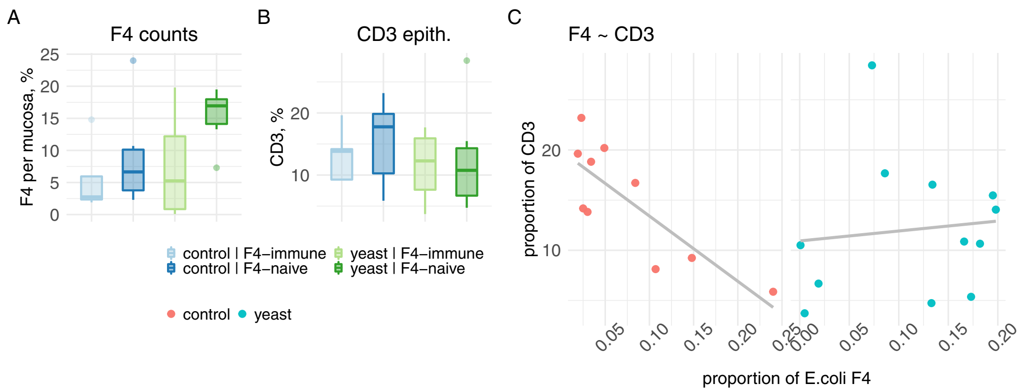


Figure 3: Immunohistochemistry results (d2 PI). Panel A: Distribution of the proportion of the mucosa-associated *E. coli* F4$^+$ per mucosa section (lumen conten excluded) across the experimental groups on d2 PI. Panel B: Distribution of the proportion of IEL CD3$^+$ cells in the epithelium across the experimental groups on d2 PI. Panel C: Correlation between the mucosa-associated F4$^+$ *E. coli* and IEL CD3$^+$ cells in the epithelium of control-fed (red dots) and yeast-fed (blue dots) piglets

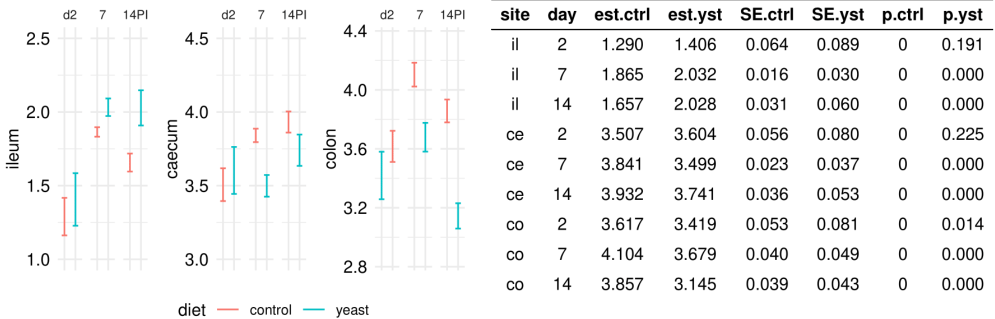
3.3 Microbial ecology

3.3.1 Sequencing results

Microbiota profiling was conducted on the ileum (n=63), caecum (n=67), and colon (n=66) digesta contents samples from pigs slaughtered on day 2, 7, and 14 PI (change to PW and same for the graph). Two sequencing runs produced a total of 58,045,034 raw reads. On average there were 71670 (SD=14239) reads per sample after filtering, denoising, and chimera removal (one sample with $< 10,000$ reads was deleted) (Supplementary Figure 10). Those reads were demultiplexed into 180, 856, and 906 unique amplicon sequence variants (ASVs) per ileum, caecum, and colon datasets, respectively (taxa not seen not more than once in 5% of a dataset were removed).

3.3.2 Alpha diversity

Alpha microbial diversity comparison was made between the diet groups on day 2, 7, and 14 PI using the DivNet method to infer on the Shannon index. The ileum gut microbial communities of the yeast fed pigs were similar on the modelled Shannon index at d2 PI to those of the control diet. On d7 PI the ileum microbiomes of the yeast fed pigs showed a higher diversity than those of the control diet (Figure 4). This difference became more pronounced on d14 PI (Figure 4). As with the ileum, the microbial communities in the caecum of the yeast fed pigs were not different than those of the control at d2 PI. However, the caecal communities of the control diet-fed piglets were more diverse compared with those of the yeast diet (Figure 4).



site	day	est.ctrl	est.yst	SE.ctrl	SE.yst	p.ctrl	p.yst
il	2	1.290	1.406	0.064	0.089	0	0.191
il	7	1.865	2.032	0.016	0.030	0	0.000
il	14	1.657	2.028	0.031	0.060	0	0.000
ce	2	3.507	3.604	0.056	0.080	0	0.225
ce	7	3.841	3.499	0.023	0.037	0	0.000
ce	14	3.932	3.741	0.036	0.053	0	0.000
co	2	3.617	3.419	0.053	0.081	0	0.014
co	7	4.104	3.679	0.040	0.049	0	0.000
co	14	3.857	3.145	0.039	0.043	0	0.000

Figure 4: Alpha microbial diversity. Left: Estimates of DivNet inferred Shannon diversity indices with its uncertainty across gut sites (ileum, caecum, and colon), diets (control, yeast), and time (d2, d7, and d14 PI). The diet coloured intervals represent 4 standard errors (SE) (+2SE and -2SE around the estimate). Right: Summary of the DivNet statistical test for differences in the inferred Shannon diversity indices between the control and yeast diets: *site* shows the gut site microbiomes were derived from, *day* indicates the day post-infection when the samples were collected, *est.ctrl* and *est.yst* show the estimates of the Shannon index inferred by the model for the microbiomes of the pigs fed either the control or the yeast diets, respectively, *SE.ctrl* and *SE.yst* show the standard errors of the estimates of the Shannon index inferred by the model for the microbiomes of the pigs fed either the control or yeast diets, respectively, *p.ctrl* and *p.yst*, show the p-values derived from testing the difference in the Shannon diversity indices between the control and yeast groups, respectively

3.3.3 Beta diversity

To study the impact of diets on beta microbial diversity in the intestines of ETEC challenged pigs, a multivariate model with permutations was fitted to the phylogeny-informed community data (see methods).

day 2 PI Although the **diet** was associated with the variance in the microbial communities on d2 PI across the **ileum, caecum, and colon** (R^2 =9%), the **litter** (parental genetics) was a much **stronger predictor** of the variance in the respective microbiomes (R^2=38%) (Figure 5, Supplementary Figure 11).

day 7 PI The litter could predict 27.9% of the variance in the microbial data from the **ileum** of pigs sampled on d7 PI, while the diet was not a statistically significant predictor of the variance. The proportion of the variance in the microbial data explained by **diet increased** for the large intestine microbiomes at d7 PI (R^2=14.7%) compared with d2 PI. Reciprocally, the **litter** accounted for **less variance** of the unweighted Unifrac distances of the respective microbiomes (**caecum, colon** d7 PI) (R^2=24.2%) than that of d2 PI.

day 14 PI About the same amount of variance in the unweighted Unifrac distances was accounted by the **diet** across the **ileum, caecum, and colon** at d14 PI (R^2=14.2%), whereas the **litter was not** a statistically significant **predictor** of the variance at that time point.

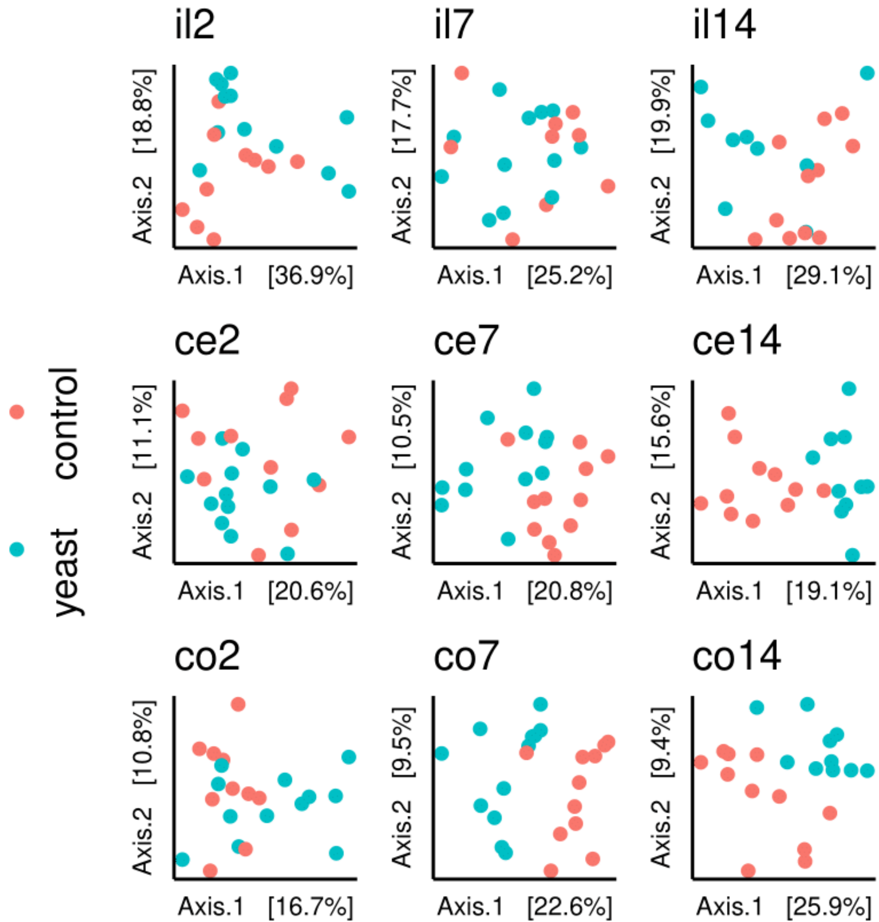


Figure 5: Beta microbial diversity. Principal coordinate analysis plot of the pig gut microbiotas coloured by diet (yeast, *blue*, control, *red*). The panel names designate distinct microbiomes across gut sites and time (ileum, *il*, caecum, *ce*, colon, *co* in combination with d2 PI, 2, d7 PI, 7, d14 PI, 14

146 ### 3.3.4 Differential abundance test

147 **3.3.4.1 Ileum** Two days after the challenge (d2 PI) there were more *Clostridia* class in the
148 ileum microbiome of the control piglets compared with that of the yeast piglets. *Bacilli*, in
149 contrast, were more predominant in the microbiome of the yeast fed piglets compared with
150 that of the control (Figure 6). At a higher taxonomic resolution, a *Lactobacillus* cluster (sp.
151 *reuteri*, *mucosae*, and *salivarius*) and *Streptococcus luteciae* were more predominant in the yeast
152 microbiomes, while *Sarcina* and *Clostridium* sp. G060 were more predominant in the microbiomes
153 of the control fed piglets.

154 At d7 PI, the differential abundance of *Clostridia* and *Bacilli* bacterial classes was similar to
155 the differential abundance at d2 PI (above). *Gammaproteobacteria* were more abundant in the
156 microbiomes of the ileum of yeast-fed piglets compared to those of the control-fed piglets (Figure
157 6). More specifically, *E. coli*, *Streptococcus luteciae*, *Veilonella dispar*, *Actinobacillus* unclassified.,
158 *Actinobacillus porcinus*, and Pasteurellaceae ASVs were differentially abundant in the yeast-fed
159 microbiomes of the ileum. Of note, *Clostridium perfringens* was more predominant in the ileum
160 of the control diet-fed piglets.

161 At d14 PI, there again were more *Clostridia* class and less *Proteobacteria*, *Actinobacteria*, and
162 *Gammaproteobacteria* bacterial classes in the control-fed ileum microbiomes compared with those
163 of the yeast-fed piglets (Figure 6). At the family level, there were more Enterobacteriaceae,
164 Streptococcaceae, Veillonellaceae, and Pasteurellaceae and less Clostridiaceae in the microbiomes
165 of the yeast-fed piglets than those of the control piglets.

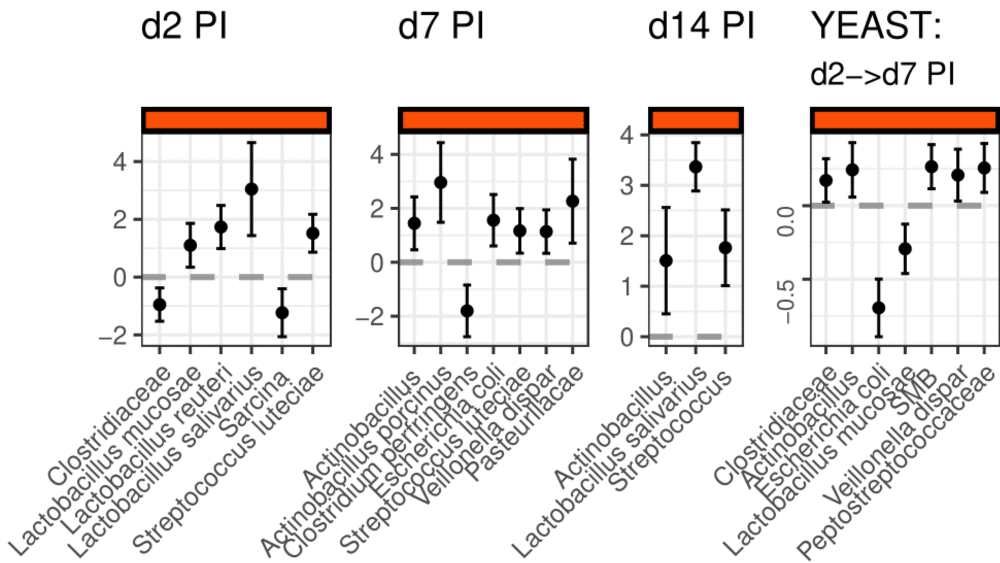


Figure 6: Differentially abundant taxa in the ileum (species level). The dots with the intervals represent the estimates of the beta-binomial regression model of the porcine faecal microbiomes along with its standard errors across d2-d14 PI; the positive estimates (above the grey dashed line, ”0”) indicate the taxa that are more predominant in microbiomes of the piglets fed the yeast diet compared with those fed the control diet. The $YEAST$ panel shows differentially abundant taxa between the microbiomes of the yeast fed piglets at d2 and d7 PI; the positive estimates (above the grey dashed line, ”0”) indicate the taxa that are more predominant in microbiomes of the pigs on d7 PI in comparison with abundance on d2 PI

166 **3.3.4.2 Caecum** At d2 PI there were more *Streptococcus luteciae*, Paraprevotellaceae
167 (CF231), and *Parabacteroides* taxa in the caecal microbiomes of yeast-fed piglets than in those
168 of the control diet (Figure 7). At d7 PI the relative abundance of *Proteobacteria*, *Firmicutes*,
169 *Deferribacteres*, *Actinobacteria*, and *Tenericutes* phyla were higher in the control fed piglet
170 caecum microbiomes compared with those of the yeast (Figure 7). The only phylum that
171 was more predominant in the yeast group caecum microbiomes than that of the control was
172 *Bacteroidetes*. As many as 36 taxa were more predominant in the control fed piglet caecum
173 microbiota compared with 2 taxa in that of the yeast (Figure 7).

174 At d14 PI, the relative abundance of bacterial classes *Deltaproteobacteria* and *Erysipelotrichi*
175 was differentially abundant in the yeast-fed piglet caecum microbiomes compared with those
176 of the control-fed piglets. In contrast, *Epsilonproteobacteria* relative abundance was higher in
177 the control-fed piglet caecum microbiomes compared with those of the yeast-fed piglets. At the
178 species taxonomic level, there were 10 differentially abundant taxa in the control-fed caecum
179 microbiomes compared with 11 of those in the yeast-fed piglets (Figure 7).

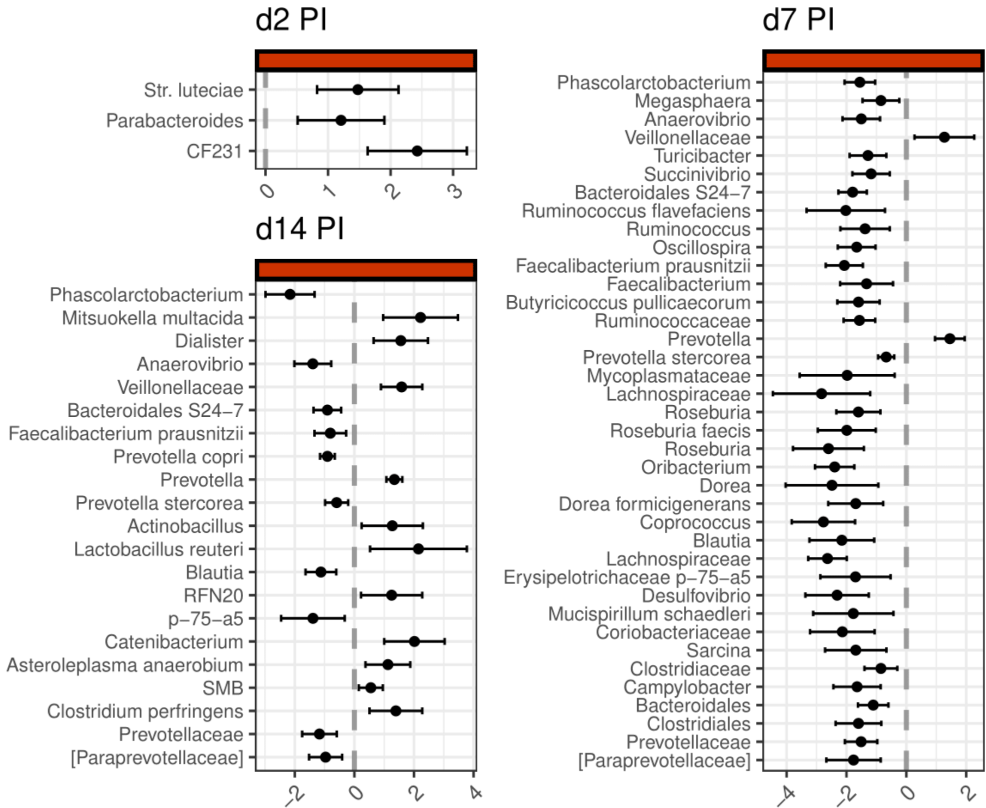


Figure 7: Differentially abundant taxa in the caecum (species level). The dots with the intervals represent the estimates of the beta-binomial regression model along with its standard errors across d2-d14 PI; the positive estimates (right of the grey dashed line) indicate the taxa that are more predominant in the microbiomes of yeast-fed piglets compared with those of the control-fed piglets

180 **3.3.4.3 Colon** At d2 PI, there more *Parabacteroides*, Paraprevotellaceae, Ruminococcaceae,
181 and *Novispirillum* affiliated ASVs in the yeast fed piglet colon microbiomes than in those of the
182 control-fed piglets. The relative abundances of *Campylobacter*, *Prevotella*, and *Desulfovibrio* were
183 higher in the colon microbiomes of the control fed piglets compared with those of the yeast-fed
184 piglets (Figure 8). At the species level of analysis, the relative abundances of *E. coli*, *L. johnsonii*,
185 and *P. copri* were differentially abundant in the colon of control-fed piglets compared with those
186 of the yeast-fed piglets (Figure 8).

187 At d7 PI, the relative abundance of *Proteobacteria*, *Firmicutes*, *Spirochaetes*, *Deferribacteres*,
188 *Actinobacteria*, and *Tenericutes* phyla was higher in the control fed piglet colon microbiomes
189 compared with those of the yeast-fed piglets. *Bacteroidetes* and *Elusimicrobia* phyla were
190 more predominant in the yeast-fed colon microbiomes than those of the control-fed piglets. At
191 the species level, there were 48 differentially abundant ASVs in the colon microbiomes of the
192 control-fed piglets and only 5 of those in the colon microbiomes of the yeast-fed piglets (Figure
193 8).

194 At d14 PI, the relative abundance of the bacterial phyla *Firmicutes* and *Tenericutes* was
195 differentially abundant in the control-fed piglet colon microbiomes compared with those of the
196 yeast-fed piglets. In contrast, *Bacteroidetes* phyla relative abundance was higher in the yeast-fed
197 piglet colon microbiomes compared to those of the control-fed piglets. At the species level, there
198 were 32 differentially abundant taxa in the control-fed piglet colon microbiomes compared with
199 5 of those in the yeast-fed piglet colon microbiomes (Figure 8).

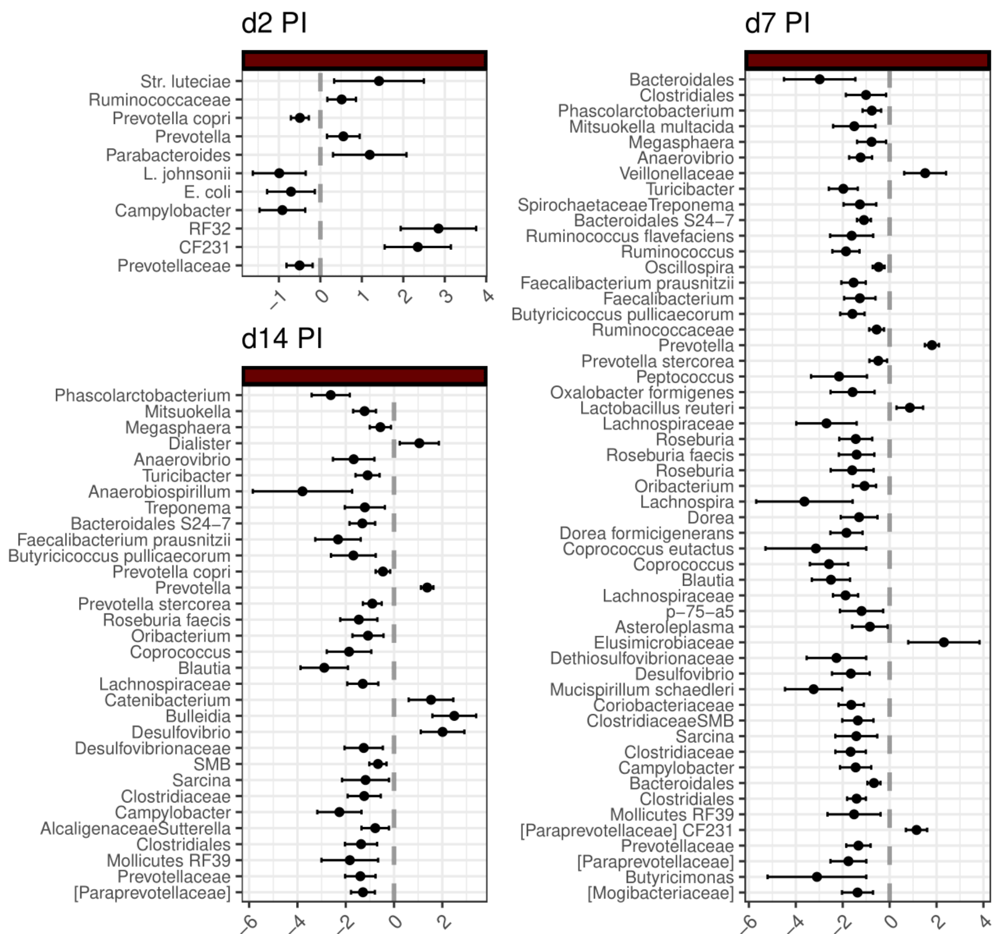


Figure 8: Differentially abundant taxa in the colon (species level)** The dots with the intervals represent the estimates of the beta-binomial regression model along with its standard errors across d2-d14 PI; the positive estimates (right of the grey dashed line) indicate the taxa that are more predominant in the microbiomes of yeast-fed piglets compared with those of the control-fed piglets

3.4 Microbial network analysis

To characterize further the microbial communities that reside in the small intestine, microbial networks were recovered with the Sparse Inverse Covariance Estimation for Ecological Association Inference approach (SPIEC-EASI) algorithm (see material and methods).

The connectivity in the microbial communities of the ileum of the challenged pigs was sparse irrespective of time. Among the connected nodes, lactobacilli formed cliques more often than other phylotypes. Three members of the yeast fed pig microbiome lactobacilli, *L. mucosae*, *L. reuteri*, and *L. johnsonii*, were connected on d2 PI and d14 PI (Figure 9). *L. mucosae* which decreased in numbers in the digesta of the yeast-fed piglets (Figure 6), became disconnected from the lactobacilli clique on d7 PI (Figure 9). Lactobacilli of the control fed pig microbiomes formed bipartite cliques on d2 and d7 PI which consisted of *L. reuteri* and *L. johnsonii*. On d 14 PI theses two species were not connected (Figure 9)

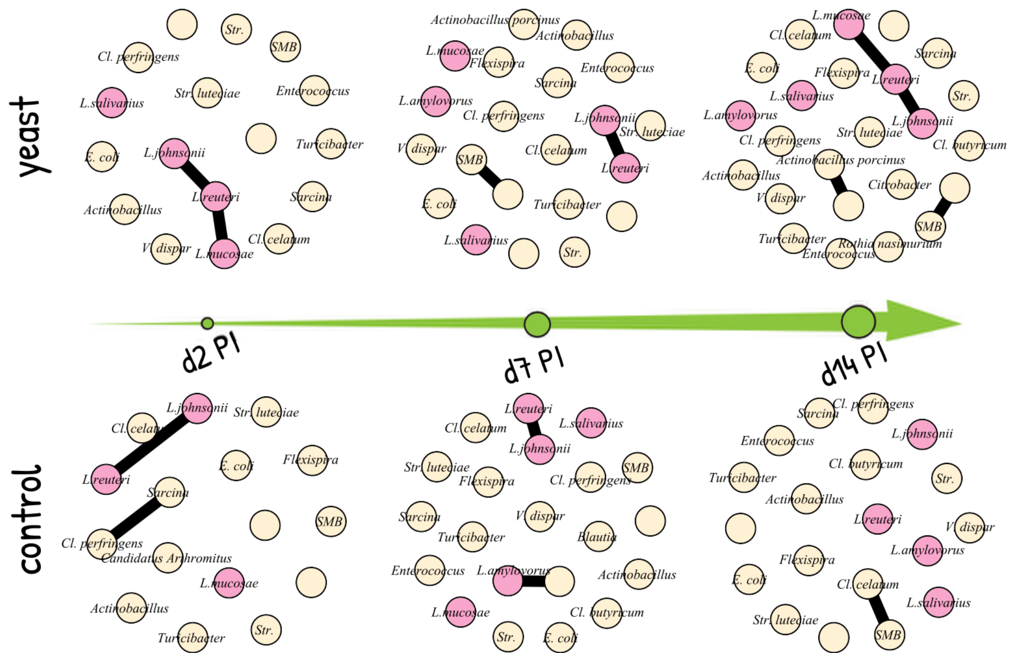


Figure 9: Microbial network of the ileum microbiomes across time and feeding groups. *Lactobacillus* genus is coloured pink, while other taxa are coloured in beige. The nodes (coloured circles) represent ASVs, while the black coloured lines represent connections between the nodes.

212 # 4 Discussion

213 This study investigated the impact of a novel yeast diet on weaner pig immunity assessed in
214 the context of the intestinal microbiome and health parameters. The yeast diet contained
215 beta-glucans and mannans as the structural components of yeast cell walls. Beta-glucans and
216 mannans are believed to possess immunomodulatory properties when supplied to human and
217 other mammals (reviewed in [24, 25]). In this study knowledge about the purity, quantity,
218 and bioavailability of these compounds is limited. The heat deactivated *C. jadinii* yeast cells
219 replaced 40% of crude proteins in the diet. The high dietary inclusion level suggests that large
220 amounts of the immunomodulatory compounds were readily available to the weaned piglets
221 through the experimental diet. A study by Håkenåsen et al. in healthy piglets fed a similar yeast
222 diet demonstrated changes in the immune response in the small intestines by utilizing RNA
223 sequencing analysis. Their findings featured an upregulation of immune signalling pathways,
224 NF-kB and Toll-like receptors, already at d7 PW in the yeast-fed animals [26]. Lagos and
225 co-workers showed that the *C. jadinii* yeast diet was associated with an increased CD3–/CD8+
226 cell population in distal jejunal lymph-nodes at d28 PW. However, the authors did not find this
227 association in the blood [27].

228 In contrast to the studies of Håkenåsen et al. and Lagos et al., the present study employed an *E.*
229 *coli* infection model to elicit changes in the immune response that are attributable to the yeast
230 diet and were not evident in the healthy animal experiments. The choice of the challenge strain
231 (F4ab) used in this study was guided by the relevance of that pathotype for the Norwegian and
232 European swine industry [2, 6, 28]. Once established in a pig farm, the pathogen can remain
233 in the environment for a long time and is hard to eradicate [1, 29]. Another important aspect
234 of this bacterium is that suckling piglets are mostly immune to the infection through colostrum
235 and milk from vaccinated mothers. Sow vaccination shifts the adhesive *E. coli* disease onset
236 to the post-weaning period where piglet mortality due to PWD is lower compared with that
237 of neonates [3, 4]. The reduced growth of the animals due to PWD, however, may be relevant
238 for the industry. From the experimental point of view, this infection model was an appropriate
239 replication of the field disease as the induced infection caused no mortality.

240 The degree of adhesiveness of F4^{+} *E.coli* to porcine enterocytes and subsequently the rate of
241 bacterial colonisation is determined by the genetic constitution of the pigs. One such genetic

242 determinants is an SNP located in the *muc*4 gene of porcine chromosome 13. Others have
243 suggested that additional SNP candidates are implicated in F4 susceptibility adhesion porcine
244 phenotypes [11]. To our knowledge, the only DNA based assay that can discriminate between the
245 adhesive and non-adhesive porcine phenotypes is the one developed by Jørgensen and colleagues
246 [8]. The present study involved two principally distinct herds: one with a history of PWD
247 (F4-immune) and another without a history of PWD (F4-naive). The F4-immune phenotype of
248 the pig herds was supported by DNA testing. There were 19 animals in the F4-immune herd
249 that had a mutant allele within the *muc*4 gene compared with none in the F4-naive herd. Our
250 observations of diarrhoea severity due to F4 *E. coli* supported the genotyping results related to
251 F4 susceptibility. The diarrhoea scores were higher in the F4 naive herd piglets from d1 PI to
252 d3 PI. This time-window corresponds to the classical development of PWD [20, 30]. The faecal
253 scores in the F4-immune herd were only slightly elevated post-infection. Feed intake figures also
254 highlighted a lower severity of PWD in the F4-immune herd than that in the F4-naive herd.
255 After the acute phase of the ETEC infection, on d4 PI, the F4-immune piglets were eating more
256 and gaining more weight compared with the F4-naive piglets. One of the key findings in the
257 present study was that the yeast-fed piglets were eating less and subsequently gaining less weight
258 d14 PI than the control-fed piglets. Unlike the figures at d7 PI, the effect of F4 susceptibility on
259 the feed intake and ADG was not pronounced. These findings contrast with previous studies in
260 healthy piglets where feed intake was comparable between yeast-fed and control-fed pigs [26, 31].

261 The implications of appetite loss in yeast-fed animals challenged with a pathogen are unclear. To
262 our knowledge, PWD-affected piglets recover well, and there was no production loss due to the
263 disease on the farm with a history of PWD (the National litter recording system, "Ingris"). It has
264 been proposed that reduced appetite is an adaptation trait which, in concert with the immune
265 response, helps mammals survive enteric infections [32]. Murray and colleagues demonstrated
266 that food avoidance in mice infected with *Listeria monocytogenes* resulted in 50% less mortality
267 compared with the infected force-fed mice [32]. Wang and co-workers [33] obtained similar results
268 by reproducing the experiment by Murray and colleagues [32]. The listeriosis and colibacillosis
269 infection models are not directly comparable concerning the mortality/morbidity rates. The
270 design of this study precludes us from making assumptions on how herds without a history
271 of PWD would fare after being exposed to PWD. However, here we can speculate that the
272 development of appetite loss in the yeast-fed piglets might render pigs more robust against

273 possible subsequent infectious stressor. A longitudinal study design, or a field trial, is essential
274 to address this research question.

275 While changes in appetite were observed towards the end of the experiment, changes in the
276 distribution of immune cell populations were already visible at d2 PI. There was an inverse
277 relationship between the intraepithelial CD3 populations located in the ileum and the degree of
278 $F4^+$ *E. coli* colonisation in the control-fed piglets. In contrast, this relationship was not present
279 in the yeast-fed piglets. This finding suggests that the yeast diet enabled intraepithelial T cell
280 populations to persist in the presence of high levels of mucosa-associated $F4^+$ *E. coli*.

281 Our results corroborate and elaborate on the findings of differences in the immune gene expression
282 in the porcine small intestine reported by Håkenåsen et al. [26]. These investigators demonstrated
283 that on day 7 after the introduction of yeast-based feed, several immune system pathways,
284 including Toll-like receptor and NF-kappaB signalling pathways, were enriched in the small
285 intestine of the animals. High inclusion levels of immunomodulatory yeast compounds in diets
286 likely stimulates small intestine immunity.

287 It is our speculation that the immune system was (I) modulated prior to the infection either
288 by the immunogenic compounds of the yeast cell walls or shifts in small intestine microbial
289 communities or both and then (II) exposed to antigenic stimuli due to the ETEC infection. This
290 speculation is encouraged by our observations of higher counts of $F4^+$ *E. coli* in the F4-naive
291 herd compared with those of F4-immune herd on the yeast diet. In other words, the growth of
292 intestinal ETEC was suppressed in the pigs from the herd with a history of PWD.

293 These findings indicate the presence of an effect of the yeast diet on the local immune
294 response and, later, on appetite. Hoytema van Konijnenburg et al. using a murine model
295 showed that intestinal intraepithelial lymphocytes (IELs) movements within the epithelium
296 are antigen-specific[34]. The authors demonstrated using live imaging that the IELs increased
297 their motility within the epithelial cell layer ("flossing") when exposed to *Salmonella enterica*
298 antigens. Also, they found that in the absence of pathogen (specific pathogen-free mice) in
299 the lumen the movement of IELs was reduced compared to that of the infected animals. It is
300 difficult to compare our immunohistochemical study to the live cell imaging reported in the
301 work of Hoytema van Konijnenburg and colleagues. While more $CD3^+$ cells were associated
302 with fewer $F4^+$ in the control diet-fed pigs and a similar association was not observed in the
303 yeast-fed pigs, a detailed investigation of the dynamics of IEL $CD3^+$ cells in the small intestine

304 during ETEC infection was not performed. It was also beyond the scope of this work to examine
305 the distribution of T cell subpopulations within in the epithelium. It would be interesting to
306 elaborate our preliminary findings to perform a more detailed characterisation of the IEL $CD3^{+}$
307 cells using this infection model.

308 The gut microbial ecology findings suggest that the pigs may have developed valuable traits after
309 the exposure to the yeast diet and the bacterial challenge. The divergence of gastrointestinal
310 microbiomes over the course of the ETEC infection was quite distinct for pigs fed either
311 the control or yeast diet. On the second day after the ETEC challenge, the small intestine
312 microbiomes of the yeast fed piglets were more diverse with a co-occurrence between *L. johnsonii*
313 and *L. reuteri*, and *L. reuteri* and *L. mucosae*. In addition, *L. reuteri*, *L. mucosae*, and *L.*
314 *salivarius* were differentially abundant in the yeast fed pig ileum microbiomes on the second
315 day after the ETEC challenge. No major differences in the large intestine microbiomes were
316 identified on the same day. An exception was higher relative abundance of *Str. luteciae* which
317 was present across the ileum, caecum, and colon microbiomes of piglets fed yeast compared with
318 that of the control-fed piglets. The data obtained by Yang and co-workers suggested that *Str.*
319 *luteciae* was one of the bacterial phylotypes that was more predominant in the healthy piglet
320 faecal microbiomes compared with those of the piglets with diarrhoea [35]. We could not test
321 this trend on our data since the diarrhoea scoring was performed at a group level.

322 The transition of the gut microbiomes of piglets fed the yeast diet from d2 to d14 PI was
323 characterized by an increase in alpha diversity of the small intestine microbiome compared with
324 those of the control fed piglets. While various phylotypes increased in numbers in the small
325 intestine, the caecum and colon microbiomes of pigs fed the yeast diet were distinct from those
326 of the control diet on d7 PI. A marked drop in a number of bacterial phylotypes, including
327 various dietary fibre degraders (Figure 7, Figure 8), on d7 PI in the yeast-fed piglet large intestine
328 microbiomes coincided with the loss of co-occurrence of *L. reuteri* and *L. mucosae* in the ileum
329 microbial networks. Interestingly, a decrease in *E. coli* coincided with a decrease on d 7 PI in *L.*
330 *mucosae* in the ileum of piglets fed yeast compared with that of d2 PI. This may suggest that
331 the clearance of the pathogen by the immune system also targeted *L. mucosae*. In contrast, the
332 populations of host-adapted *L. reuteri* and *L. johnsonii* [36, 37] were neither changed in size nor
333 was their co-occurrence pattern disturbed.

334 When the co-occurrence of *L. mucosae* and *L. reuteri* was re-instated on d14 PI, the caecum, but

335 not the colon, microbiomes of the piglets fed the yeast diet became more balanced in terms of the
336 differentially abundant phylotypes (Figure 7, Figure 8, Figure 9). The presence of the lactobacilli
337 co-occurrence cluster was another distinct feature of the ileum microbial communities of the
338 yeast-fed piglets. This sub-community was more pronounced in the yeast-fed piglet microbiomes.
339 This distinction in microbial communities may be attributed to the principal differences in the
340 feed formulation. Intact *C. jadinii* yeast cells were fed to animals that cannot enzymatically break
341 down the yeast cell wall components (chitin, mannan-proteins, and yeast beta-glucans). To our
342 knowledge, the ileal digestibility of the yeast feed proteins in weaner piglets is on a par, or higher
343 than that of the proteins from control diets [26, 31]. This means that yeast cell wall disruption
344 is necessary to make yeast intracellular nutrients available for host degradation/uptake. We
345 previously showed that there were more lactobacilli in the small intestine of the yeast-fed healthy
346 piglets compared with that of the control-fed piglets [21]. In the present study, we have also
347 found higher lactobacilli in the ileum and co-occurrence of *L. reuteri* and *L. johnsonii*, and *L.*
348 *johnsonii* and *L. mucosae* in the yeast-fed piglet gut microbiomes. This consistency in identifying
349 more lactobacilli in the small intestine of piglets fed yeast identifies these bacteria as suitable
350 candidates that are instrumental in degrading yeast cell walls. Tannock et al. demonstrated that
351 *L. johnsonii* and *L. reuteri* could co-exist *in vitro*, and in the mouse forestomach. Also, the
352 authors showed that the two strains could adapt nutrient utilization mechanisms depending on
353 whether the strains were alone or in a co-culture. These two lactobacilli strains can degrade mono-
354 and oligosaccharides via several alternative pathways [38, 39]. However, to degrade complex
355 carbohydrates, the bacteria may be obliged to act in concert to maximize nutrient utilization.
356 *In-silico* analysis of a published porcine gut metagenome database [40] shows that *L. johnsonii*
357 can produce mannan endo-1,4-beta-mannosidase, while *L. reuteri* seems to lack the gene. This
358 enzyme may be operative in the degradation of the yeast cell wall mannan-protein complex.

359 Charlet and co-workers demonstrated under laboratory conditions that *L. johnsonii* was able
360 to inhibit the growth of live *Candida glabrata* and *Candida albicans* by exerting a chitinase-like
361 activity [41]. The analysis of porcine metagenomic assemblies [40] revealed that both *L. johnsonii*
362 and *L. reuteri* had a gene encoding a LysM domain which is operative in chitin-binding (reviewed
363 in [42]). While both strains can theoretically bind to the yeast cell walls, only *L. johnsonii*
364 seemed to carry chitinase encoding determinants (GH 18). Based on the existing knowledge and
365 our findings, we argue that yeast cells in the feed undergo lactobacilli microbial degradation in

366 the small intestine. We were able to recover a stable connection between *L. johnsonii* and *L.*
367 *reuteri* from all ileal microbiomes except on d14 PI in the control group using the SPIEC-EASI
368 algorithm.

369 The two lactobacilli strains are known to be able to colonize non-secretory epithelia and co-exist
370 in biofilms in the alimentary tract of mammals [37, 39].

371 Based on co-occurrence patterns, our analysis suggests that a distinct lactobacilli phylotype, *L.*
372 *mucosae*, is the third member of the lactobacilli cluster. As all three strains adhere to surfaces
373 and form biofilms [37, 43], we speculate that these lactobacilli cooperate in degrading the yeast
374 cell wall. In support of this notion, *L. mucosae* was never connected to *L. johnsonii* in the
375 microbiomes of piglets fed diets that did not contain the yeast cell substrate. To pursue this
376 notion further, the microscopy of gastrointestinal tract digesta with lactobacillus species-specific
377 labelling may be useful. Our speculation on the possible role of lactobacillus species could
378 be relevant to animal welfare. Lactobacilli are generally thought to be beneficial bacteria of
379 gastrointestinal tract. Since the *C. jadinii* yeast-derived diet can both fulfil nutritional needs
380 of the animals and possibly augment lactobacilli group, the novel yeast diet could enhance the
381 immunity of the animals. In this study, we have demonstrated that yeast-fed piglets showed loss
382 of appetite. This is an evolutionary adaptation that helps animals withstand life-threatening
383 bacterial infections [32, 33].

384 Although it is beyond the scope of this work to study the mechanism of appetite loss, we do
385 not exclude possibility of a complementary effect of yeast immunomodulatory components and
386 intestinal lactobacilli to play a key role. A higher microbial diversity in the small intestine
387 may indicate higher tolerance levels of gut immunity. We also speculate that higher microbial
388 diversity of the ileal microbiomes and caecal microbiomes at d14 PI were linked. It is conceivable
389 that richer microbial communities at d14 PI in the ileum are a function of evolved immunologic
390 resilience boosted by the immunogenic properties of yeast. However, further studies are needed
391 to clarify this suggested interaction.

392 Previous studies have provided evidence that the novel yeast-based diet can support healthy
393 piglets. Irrespective of whether the immune modulation by the yeast diet occurs due to the
394 direct stimulation of the immune system by the yeast beta-glucans and mannans or the indirect
395 stimulation via small intestine lactobacilli growth promotion, or both, the present study shows

that the novel diet can improve the health of diseased piglets in herds with a PWD history. However, the response to such diets on the farm is not always comparable to that under controlled experimental conditions. Furthermore, the immunomodulatory properties of yeast are dependent on the species of yeast and down-stream processing conditions of the yeast [44]. Future work should investigate the effect of yeast strain and down-stream processing on nutritional value and health beneficial effects of yeast, and also assess the performance of novel yeast diets under field conditions.

5 Ethics statement

The animal study was conducted in compliance with the Norwegian Animal Welfare Act 10/07/2009 and the EU Directive on the Protection of Animals used for Scientific Purposes (2010/63/EU). Norwegian Food Safety Authority approved the use of animals under FOTS ID 16510 protocol.

6 Acknowledgements

We thank the Bacteriology group at the Department of Food Safety and Infection Biology at Norwegian University of Life Sciences for their support during sampling. We are grateful to the Histology group at the Department of Basic Sciences and Aquatic Medicine at Norwegian University of Life Sciences for their contribution during sampling and histology analysis. We also thank the staff at the Centre for livestock production (SHF) (NMBU, Ås) and Faculty of Veterinary Medicine, the Department of Production Animal Clinical Sciences (PRODMED) for their excellence in managing the animals during this experiment. Lars Johan Kalager is acknowledged for his help and valuable contributions to this study.

417 # 7 Methods

418 ## 7.1 Isolation and characterisation of the challenge *E. coli*

419 The bacterial strain was isolated from a diarrhoea sample of a 31 day old weaner (2 days
420 post-weaning) piglet from a farm with a history of post-weaning diarrhoea (PWD) (described
421 below). The isolate was cultured on blood agar followed by a morphological examination. The
422 bacterial strain was identified as a haemolytic *Escherichia coli* positive for F4 fimbrial antigen as
423 per result of F4(K88) F monovalent rabbit antiserum assay (Statens serum institut, Copenhagen,
424 Denmark). Neo-Sensitabs disc-diffusion antimicrobial susceptibility testing assay(A/S Rosco
425 Diagnostica, Taastrup, Denmark) categorized the strain as being resistant to penicillin, fusidic
426 acid, and streptomycin.

427 The isolate was propagated on blood agar plate at 37^0C for 24 hours. DNA was extracted
428 using a phenol-chloroform method (https://www.pacb.com/wp-content/uploads/2015/09/
429 SharedProtocol-Extracting-DNA-usinig-Phenol-Chloroform.pdf). The short-read sequencing
430 data were obtained from the Norwegian Veterinary Institute Sequencing unit (SEQ-TECH,
431 VI) (Nextera Flex library prep protocol, Illumina MiSeq 300 bp pair-end sequencing). The
432 long-read data were obtained from Nanopore MinION platform (SQK-RAD004 library
433 prep protocol). Short and long sequencing reads were quality filtered using bbduk version
434 37.48 (BBMap – Bushnell B., 395 https://sourceforge.net/projects/bbmap/) and Filtlong
435 v0.2.0 (https://github.com/rrwick/Filtlong), respectively. A hybrid (short and long reads)
436 whole-genome assembly was obtained with Unicycler v0.4.8 [45]. The sequenced *E. coli*
437 shared 93.21% genome with *E. coli* UMNK88 NC 017641 (99.81 average nucleotide identity)
438 as per the analysis of the assembled genome using MiGA web-server [46]. Virulence
439 genes of the sequenced *E. coli* were identified using VirulenceFinder 2.0 web-server [47]
440 (https://cge.cbs.dtu.dk/services/VirulenceFinder/). Briefly, the isolate carried genes encoding
441 following virulence determinants: K88/F4, EAST1, heat-labile enterotoxin, and heat-stabile
442 enterotoxin II. The assembled genome was deposited in ENA (ERS5259532).

443 ## 7.2 Experimental design

444 In total, 68 pure Landrace piglets were used in the study. The animals originated from two
445 farms: a) one with a history of recurrent post-weaning diarrhoea (PWD-immune herd, n = 32)
446 and b) one free of PWD problems (PWD-naive, n = 36). Multiparous sows were given "Porcilis
447 Porcoli Diluvac Forte vet." and "Porcilis Ery Parvo vet." (MSD Animal Health, both) before
448 farrowing as a routine vaccination procedure. At day 2 postnatal, piglet oral mucosal swabs
449 were collected followed by DNA extraction using QIAamp DNA Mini Kit (QIAGEN, GmbH,
450 Hilden, Germany). The animals were genotypically classified as being either homozygous
451 (n=48) or heterozygous (n=19) susceptible to F4ac bacterial fimbria adhesion to enterocytes
452 by a *muc*4 gene polymorphism test described previously [8]. Briefly, a DNA fragment of the
453 porcine *muc*4 gene was PCR-amplified (primers: 5'-GTGCCTTGGGTGAGAGGTTA-3' and
454 5'-CACTCTGCCGTTCTCTTTCC-3'), cleaned (NucleoSpin, Macherey-Nagel), and digested
455 with *Xba*I restriction enzyme. The susceptible allele was considered if 151 and 216 bp digestion
456 fragments were obtained. No digestion indicated the resistant allele. The piglets were weaned on
457 day 28 $\pm$ 2 postnatal (average weight of 8.9$\pm$1.5 kg) and transported to the research facility unit
458 where the experiment took place. At weaning, piglets were randomly allocated to either yeast
459 weaner diet or control weaner diet blocking by weight and litter. The resulting four groups,
460 Yeast/PWD-immune, Yeast/PWD-naive, Control/PWD-immune, and Control/PWD-naive,
461 were housed in 4 environmentally controlled pens with dry spruce wood chip bedding (1 pen per
462 each group). The bedding material was renewed twice a day. Feed and water were accessible ad
463 libitum at all times. The yeast diet contained 40% of the crude protein from heat-inactivated
464 and dried *C. jadinii* (LYCC 7549; Lallemand Yeast Culture Collection). The technology of
465 yeast processing was described previously [48]. The diet ingredients and chemical composition
466 are given in the supplementary data (Table 1). Piglets were weaned at 28 days of age. After
467 priming to the weaner diets for one week, all piglets were orally inoculated with 10^9 CFU/ml (in
468 2 ml of Lysogeny broth) of F4-positive enterotoxigenic *E. coli*. Faecal swab samples were taken
469 and cultured on blood agar plates to control for the shedding of the challenge strain before
470 and after the inoculation. The animals were sacrificed on day 2, 7, and 14 post-infection (PI)
471 followed by sampling.

472 ## 7.3 Sample collection

473 Faecal score measurements were taken twice a day throughout the experiment. The faecal scoring
474 system was adopted from [49] which ranged from 1 (firm and shaped) to 4 (watery). The faecal
475 scores were calculated as a mean score per pen per day. Feed leftovers were weighted once a day
476 prior to adding a new portion of the feed. Feed intake was calculated as follows:

477 $\frac{(F- L)}{n}$

478 ,where F is the total weight of feed in the feed dispenser on the day before (g), L is the weight
479 of leftovers on the current day, (g), and n is the number of pigs per pen. Due to the pen level
480 of both faecal scores and feed intake measurements, no statistical procedure was attempted,
481 and the figures were compared directly. Piglets' body weight was taken at weaning, one-week
482 post-weaning (PW), and at each sampling day for those animals who were euthanised to calculate
483 average daily gain (ADG). ADG was calculated as follows:

484 $ADG = \frac{(Ms- Mw)}{D}$

485 ,where Ms is weight at sacrifice (kg), Mw is weight at weaning (kg), and D is the number of days
486 weaning-to-sacrifice (days).

487 ## 7.4 Immunohistochemistry

488 Formalin-fixed, paraffin-embedded (FFPE) tissues were cut into 4-micron thick sections and
489 mounted on glass slides (SuperFrost Plus, Thermo Scientific™, Braunschweig, Germany) and
490 stored at 4°C until staining. The slides were then incubated at 58°C for 30 min, deparaffinized in
491 xylene and rehydrated in graded alcohols to distilled water. Before the labelling with the primary
492 antibodies, heat-induced antigen retrieval was performed. For immunolabelling with CD3
493 antibody, the slides were heated in a microwave in Tris-EDTA pH 9.1 buffer with the following
494 steps, first heated to and held at 92°C for 5 min, thereafter the slides were kept in the heated
495 buffer for 5 min. This cycle was repeated with change in the last step where the slides were kept
496 in the heated buffer for 15 min. For immunolabelling with F4 antibody, the slides were heated in
497 an autoclave at 121°C for 10 min in 0.01M, pH6 citrate buffer. Endogenous peroxidase activity
498 was inhibited with 3% H_2O_2 in methanol for 10 min. Non-specific binding of primary antibody
499 to tissue or Fc receptors was blocked by incubating the slides for 30 min in normal porcine serum
500 at 1:100 in 5% bovine serum albumin (BSA) for CD3 staining and at 1:50 for 20 min for F4

staining. For labelling of T lymphocytes and Fimbrial adhesin F4, monoclonal anti-porcine CD3 clone PPT3 (catalogue number 4510-01, Southern Biotechnology, Birmingham, USA) at 1:1200 and polyclonal rabbit anti F4 (catalogue number 51172, Statens serum institut, Copenhagen, Denmark) at 1:400 were used. The slides were incubated at RT for 1 h, followed by 30 min incubation with secondary antibody. Sections labelled for F4 were incubated with secondary antibody from kit polymer-HRP anti-rabbit (Dako En Vision+ System-HRP, Dako, Glostrup, Denmark) while sections labelled for CD3 were incubated with anti-mouse biotinylated secondary antibody (catalogue number BA-2000-1.5 Vector Laboratories, California, United States) at 1:50 with 1% BSA and thereafter incubated with Vectastain Elite ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories). Detection of peroxidase activity in the F4 and CD3 slides was detected with AEC + substrate from Dako En Vision+ System-HRP and ImmPACT® AEC Substrate, Peroxidase (HRP) (Vector Laboratories), respectively. For counterstaining, hematoxylin was used and as mounting media Aquatex (Merck, Darmstadt, Germany) was used. For enumeration of F4 and CD3 targes, QuPath, v0.2.3 was used (Bankhead2017). The region of interest (ROI) area was determined for F4 and CD3 and used as a reference for quantification: mucosa and the epithelium of four well-oriented villi, respectively. The detection of positive labelling was performed with the following parameters: Gaussian sigma = 2 um, hematoxylin threshold = 0.4, eosin threshold = 0.3. There were three parameters estimated: 1) "F4 counts", that is the proportion of the total number of mucosal surface-associated F4$^+$ *E. coli* positive staining to the mucosa ROI, 2) "F4 size", that is the average size of the F4$^+$ *E. coli* positive staining areas, or colonies, per the whole area of the section, and 3) "IEL CD3", that is the proportion of CD3 positive staining per respective epithelial ROI.

7.5 Microbial DNA sample handling

At each of the sampling days, 5±1 pigs per pen (12±1 per diet) were euthanised by captive bolt stunning and pithing to allow the collection of gut contents for microbial ecology studies. Digesta from the ileum, caecum, and colon were snap-frozen in liquid nitrogen and stored at -80^{0}C until DNA extraction. Total genomic DNA was extracted from 350 mg of ileum digesta by using QIAamp PowerFecal Pro DNA Kit according to the manufacturer's instructions, except the samples were homogenized using a bead-beating step with zirconia/silica beads (=

0.1 mm, Carl Roth, Karlsruhe, Germany) in TissueLyser II (Qiagen, Retsch GmbH, Hannover, Germany) with the following parameters: 1) pre-cooling of the TissueLyser adaptors down to 0^0C 2) bead-beating 1.5 min at 30 Hz. Total genomic DNA was extracted from 300 mg of the caecum and colon digesta by using QIAamp Fast DNA Stool Mini Kit according to the manufacturer's instructions, except the bead-beating step described above and digesting proteins with 30 L of Proteinase K II instead of 15-25 L suggested in the manufacturer's protocol. The purity of extracted DNA was quality controlled by NanoDrop (Thermo Fisher Scientific, Waltham, MA) followed by quantification by Qubit fluorometric broad range assay (Invitrogen, Eugene, OR, USA). Library preparation was performed at the Norwegian Sequencing Centre (https://www.sequencing.uio.no/, Oslo, Norway) using universal prokaryotic primers 319F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') that amplify the V3-V4 hypervariable region of the 16S *rRNA* gene. Sequencing was performed on a MiSeq sequencer following the manufacturer's's guidelines. The resulting demultiplexed raw sequences were deposited in the ENA (PRJEB41033).

7.6 Bioinformatics analyses

Demultiplexed paired-end Illumina reads were pre-filtered with bbduk version 37.48 (https://sourceforge.net/projects/bbmap/) by trimming right-end bases less than 15 Phred quality score, removing trimmed reads shorter than 250 bp or/and average Phred quality score less than 20. The resulting reads were further quality filtered by trimming left-end 20 bp and removing reads with maxEE more than 1 for forward and 2 for reverse reads, denoised, merged, and chimera removed with DADA2 R package ver 1.12.1 [50] (Supplementary Figure 10). The resulting ASV tables that derived from two separate Illumina sequencing runs were merged followed by taxonomy assignment using RDP Naive Bayesian Classifier implementation in DADA2 R package (default settings) with GreenGenes database version 13.8, [51] as a reference database. The phylogenetic tree was reconstructed using phangorn R package ver. 2.5.3 [52] under the Jukes-Cantor (JC) nucleotide model with a gamma distribution (k=4, shape=1) with invariant sites (inv=0.2).

DivNet R package [53] was used to estimate Shannon diversity and to test for differences in Shannon diversity estimates in networked gut microbial communities stratified by the day the samples were collected, the gut segment the samples were taken from, with the diet and

litter as covariates. Shannon entropy estimator of Phyloseq R package was used to calculate Shannon diversity point estimates. To estimate the Shannon diversity index and to compare it across the microbiomes of the pigs fed distinct diets, DivNet statistical procedure was used for each time point. The beta diversity analysis was performed via the analysis of multivariate homogeneity of group dispersions followed by the permutation test (9999 permutations) on unweighted Unifrac distances and principal coordinate analysis (PCoA) on unweighted Unifrac distances, and permutational multivariate analysis of variance (PERMANOVA) test in R, 9999 permutations. The samples with the read count less than 40000 were discarded from the alpha and beta diversity analyses.

To calculate the relative abundance of bacterial phylotypes in the microbiomes of pigs across diets and time points, group means were taken from the respective groups. To detect differentially abundant bacterial phylotypes, 'corncob' algorithm [54] was run on the microbial feature tables (ASV counts per each sample) by fitting a beta-binomial regression model to microbial data for each time point with the diet and litter as covariates. Benjamini-Hochberg correction (cut-off of 0.05) was used to deal with the false discovery rate due to multiple testing. The test was run at each taxonomic level (phylum, class, order, family, species, and ASVs) discarding the samples with the read count less than 10000. Those ASVs lacking genus/species RDP-derived classifications were attempted to be classified manually by using web-based nucleotide BLAST on the non-redundant nucleotide database, where possible. Ambiguous hits were ignored.

7.7 Microbial network analysis, ileum

The ASV counts were agglomerated at the genus level and filtered for a minimum of 3 counts per ASV in at least 20% of the samples and at least 50% of the sample per time point (2, 7, 14 days PI) and diet (yeast diet and control diet) using the R package phyloseq version 1.26.1[55]. For each time point and diet, a network was computed on the ileum microbial data with SpiecEasi R package version 1.0.7 [56]. For each recovered network, the edges and nodes were inspected manually.

585 ## 7.8 Statistical analysis

586 Except otherwise specified, the Bayesian generalized linear models with weakly informative priors
587 were fitted through either bayestesteR v0.7.5 [57] or rstanarm v2.21.1 [58]. The results of the
588 statistical analysis were given as a level of certainty of a certain even to be true given the model
589 and available evidence.

590 # 8 Supplementary information

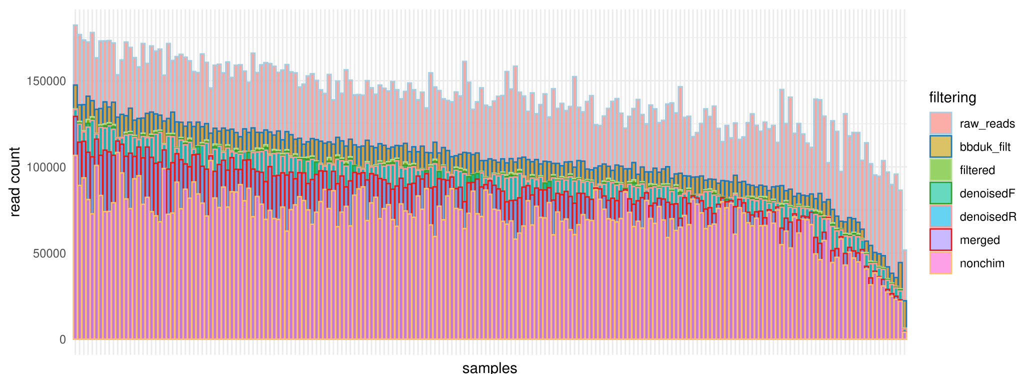


Figure 10: Summary of sequence processing pipeline. The bottom-most bar in the stack (nonchim) shows the number of read that were the basis for making the feature count table (OTU/ASV-table).The bars above nonchim summarise the number of sequencing reads removed at each bioinformatics pipeline step: a) filtered with the bbduk filtering algorithm ($bbdukfilt$), b) filtered with the DADA2 algorithm ($filtered$), c) removed after DADA2 denoising step ($denoisedR/F$), d) removed due to pair merging failures ($merged$). $rawreads$ are raw demultiplexed reads derived from Illumina sequencer.

Table 1: Piglet period. Ingredient and chemical composition (g/kg) of diets based on soybean meal (Control) and C. jadinii (Yeast). * Premix : provided the following amounts per kilogram of feed: 120 mg of Zn (ZnO); 460 mg of Fe ($FeSO_4$. H_20); 60 mg of Mn (MnO); 26 mg of Cu (CuSO~4 x $5H_2O$); 0.60 mg of I ($Ca(IO_3)_2$; <1.0 mg of Se (Na_2SeO_3); 8000 IU of vitamin A; 1500 IU of cholecalciferol; 45 mg of dl-alpha-tocopheryl acetate; 105 mg of ascorbic acid; 4.64 mg of menadione; 5.63 mg of riboflavin, 3 mg of thiamine; 15 mg of d-pantothenic acid; 20 ug of cyanocobalamine; 45 mg of niacin.

Ingredients	Control piglet diet	Yeast piglet diet
Wheat	627.9	593.6
Barley	100	100
Oats	50	50
Yeast meal (C. jadinii) (47% CP)	0	146
Soybean meal (SBM) (45% CP)	80	19
Fish meal (68.4% CP)	20	4.8
Potato protein concentrate (72.5% CP)	33.8	9.1
Rapeseed meal (Mestilla) (35%CP)	20	4.9
Rapeseed oil	19.7	23.4
Limestone	9.2	9.4
Monocalcium phosphate	13.1	15.5
Sodium chloride (NaCl)	7.2	5.5
L-Lysine . HCl (98%)	6.5	5.7
L-Threonine	2.9	2.4
L-Methionine	2.1	2.9
L-Valine	1.4	1.2
L-Tryptophan	0.9	0.9
Premix*	5.3	5.5
Calculated contents	-	-
Net energy, MJ/kg	9.94	9.94
Crude protein from C. jadinii)	0	40
Analyzed content, g/kg	-	-
DM	869	885
Gross energy, MJ/kg	19	19
Crude protein	176	172
Crude fat	39	41
Ash	46	45
Neutral detergent fiber (NDF)	96	91
Starch	442	437

cov	site	day	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
diet	il	2	0.206	0.206	3.369	0.107	0.0048
litter	il	2	0.855	0.142	2.333	0.446	0.0011
diet	il	7	0.121	0.121	1.113	0.050	0.3306
litter	il	7	0.677	0.169	1.558	0.279	0.0228
diet	il	14	0.248	0.248	2.361	0.116	0.0203
litter	il	14	0.635	0.106	1.009	0.297	0.4588
diet	ce	2	0.208	0.208	1.907	0.075	0.0139
litter	ce	2	0.932	0.155	1.427	0.336	0.0049
diet	ce	7	0.321	0.321	3.336	0.124	1e−04
litter	ce	7	0.622	0.155	1.616	0.241	0.0016
diet	ce	14	0.304	0.304	3.412	0.155	1e−04
litter	ce	14	0.497	0.083	0.930	0.254	0.6815
diet	co	2	0.228	0.228	2.171	0.087	0.0011
litter	co	2	0.934	0.156	1.482	0.355	6e−04
diet	co	7	0.397	0.397	4.873	0.169	1e−04
litter	co	7	0.574	0.143	1.759	0.243	8e−04
diet	co	14	0.319	0.319	3.504	0.156	5e−04
litter	co	14	0.546	0.091	1.000	0.267	0.4686

Figure 11: Summary of permutational multivariate analysis of variance (PERMANOVA) test. Each model build on the data across day and gut site is separated by the grey fill.

Virulence factor	Identity	Query / Template length	Protein function	Accession number
K88ab	100	852 / 852	K88/F4 protein subunit	M25302
K88ab	100	852 / 852	K88/F4 protein subunit	M25302
astA	100	117 / 117	EAST-1 heat-stable toxin	AB042002
astA	100	117 / 117	EAST-1 heat-stable toxin	AB042002
astA	100	117 / 117	EAST-1 heat-stable toxin	AB042005
astA	100	117 / 117	EAST-1 heat-stable toxin	AB042005
capU	100	1089 / 1089	Hexosyltransferase homolog	CP002729
cba	100	1536 / 1536	Colicin B	FJ664724
cba	100	1536 / 1536	Colicin B	FJ664724
cma	100	816 / 816	Colicin M	FJ664737
cma	100	816 / 816	Colicin M	FJ664737
gad	100	1401 / 1401	Glutamate decarboxylase	U00096
gad	99.86	1401 / 1401	Glutamate decarboxylase	U00096
iha	100	2091 / 2091	Adherence protein	AE005174
ltcA	100	777 / 777	Heat-labile enterotoxin A subunit	EU113243
ltcA	100	777 / 777	Heat-labile enterotoxin A subunit	EU113243
stb	100	216 / 216	Heat-stabile enterotoxin II	AY028790
stb	100	216 / 216	Heat-stabile enterotoxin II	AY028790
terC	100	1041 / 1041	Tellurium ion resistance protein	CP006262
terC	99.3	714 / 714	Tellurium ion resistance protein	CP007491
terC	99.37	959 / 966	Tellurium ion resistance protein	MG591698
traT	100	423 / 423	Outer membrane protein complement resistance	AKKX01000148
traT	100	129 / 129	Outer membrane protein complement resistance	AMTE01000156
traT	100	732 / 732	Outer membrane protein complement resistance	CXZR01000026
traT	100	732 / 732	Outer membrane protein complement resistance	CXZR01000026

Figure 12: Summary of virulence genes of the *E. coli* challenge strain

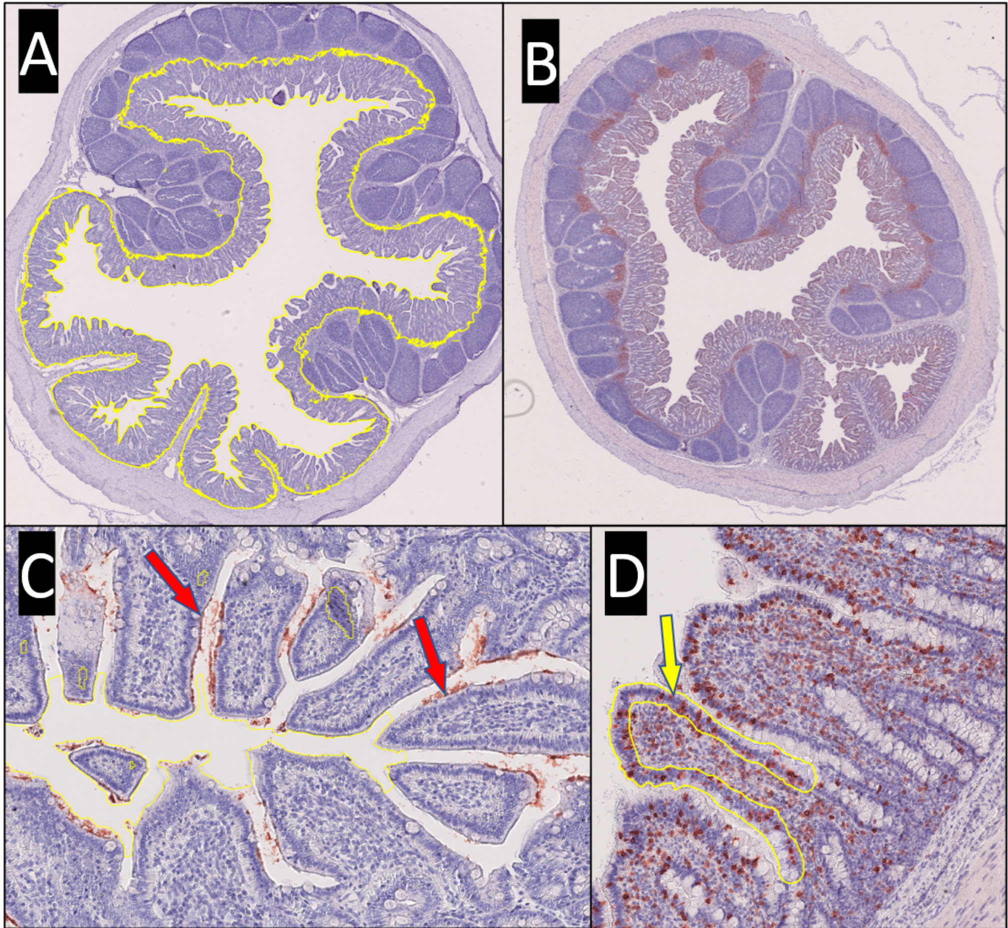


Figure 13: Immunohistochemistry quantification strategy. Panel A: Ileum section with the traced ROI for $F4^{+}$ quantification. Panel B: Ileum section labelled for $CD3^{+}$ quantification (hematoxylin). Panel C: Magnification of an ileal section with red arrows pointing at representative F4 colonies that were counted. Panel D: Magnification of an ileal section with the traced ROI for $CD3^{+}$ quantification.

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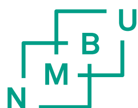
ISBN: 978-82-575-1802-8
ISSN: 1894-6402



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