Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
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Novel protein sources in diets for weaned piglets – effect on growth performance, gut function, and health

Nye proteinkilder til avvente smågris – effekt på vekst, tarmfunksjon og helse

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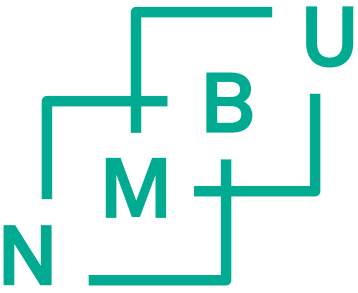

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Supervisors

Dr. Liv Torunn Mydland
Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences
P.O. Box 5003, NO-1432 Ås, Norway

Prof. Margareth Øverland
Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences
P.O. Box 5003, NO-1432 Ås, Norway

Dr. Jon Øvrum Hansen
Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences
P.O. Box 5003, NO-1432 Ås, Norway

Evaluation Committee

Prof. Mike D. Tokach
Department of Animal Sciences and Industry
Kansas State University
Manhattan, KS, USA

Assoc. Prof. Nuria Canibe
Department of Animal Science
Aarhus University
Tjele, Denmark

Committee coordinator

Assoc. Prof. Nils Petter Kjos
Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences
P.O. Box 5003, NO-1432 Ås, Norway

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Ingrid Marie Håkenåsen
Klæbu, June 2022

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List of papers

This thesis is based on the papers listed below.

Paper I: **Håkenåsen, I. M.**, Øverland, M., Ånestad, R., Åkesson, C. P., Sundaram, A. Y. M., Press, C. M. & Mydland, L. T. (2020). Gene expression and gastrointestinal function is altered in piglet small intestine by weaning and inclusion of *Cyberlindnera jadinii* yeast as a protein source. Journal of Functional Foods, 73: 104116. https://doi.org/10.1016/j.jff.2020.104118

Paper II: **Håkenåsen, I. M.,** Hedemann, M. S., Tornes, A. J. K., Øverland, M. & Mydland, L. T. (2022). Biochemical, immunological, and metabolic profiling of post-weaning piglets fed *Cyberlindnera jadinii* yeast as a protein source. (Manuscript)

Paper III: **Håkenåsen, I. M.**, Grepperud, G. H., Hansen, J. Ø., Øverland, M., Ånestad, R. M. & Mydland, L. T. (2021). Full-fat insect meal in pelleted diets for weaned piglets: effects on growth performance, nutrient digestibility, gastrointestinal function, and microbiota. Animal Feed Science and Technology, 281: 115086. https://doi.org/10.1016/j.anifeedsci.2021.115086

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Iakhno, S., Umu, Ö.C., Håkenåsen, I.M., Åkesson, C.P., Mydland, L.T., Press, C.M., Sørum, H. & Øverland, M., 2020. Effect of *Cyberlindnera jadinii* yeast as a protein source on intestinal microbiota and butyrate levels in post-weaning piglets. Animal Microbiome. 2, 1-14. https://doi.org/10.1186/s42523-020-00031-x

Iakhno, S., Delogu, F., Umu, Ö.C., Kjos, N.P., Håkenåsen, I.M., Mydland, L.T., Øverland, M. & Sørum, H., 2021. 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. bioRxiv. (preprint). https://doi.org/10.1101/2021.02.11.430725

Summary

The world is facing a growing food demand from an ever-increasing population. To meet this challenge, new resources must be utilized through innovative solutions. Meat is a valuable protein source in the human diet. However, in Norway, a northern country with limited agricultural land, we are today dependent on imports of protein-rich ingredients to feed our livestock. To increase self-sufficiency and ensure future food security, there is a need for alternative protein sources that do not occupy land areas suitable for human food production. The *Cyberlindnera jadinii* yeast and black soldier fly larvae (BSFL) are two promising novel feed ingredients that can be produced from renewable resources or side-streams, and thereby contribute to a circular bioeconomy. These two novel feed ingredients also contain several bioactive compounds which might be beneficial for the gastrointestinal function and health of the weaned piglet. It is well known that weaning causes changes in the gastrointestinal structure and function, and compromises health. The aim of this thesis was to evaluate *C. jadinii* yeast and BSFL as novel protein sources for weanling piglets and investigate if these ingredients could improve post-weaning (PW) challenges.

Two experiments were conducted. Experiment I (Paper I and II) investigated the effect of a high dietary inclusion of *C. jadinii* yeast (14.6%) on the early PW physiological changes in piglets. Results showed early PW changes in gastrointestinal function and health parameters, but changes were mainly time-dependent, not diet-dependent. However, the inclusion of *C. jadinii* yeast improved the apparent ileal digestibility of crude protein and affected the PW small intestinal gene expression. Weaning induced downregulation of several immune functions in the small intestine of the control piglets, whereas this

downregulation was not as evident in the yeast-fed piglets. Collective, the gastrointestinal function, transcriptional, and plasma immunological results indicated a less evident acute weaning phase in piglets fed yeast. In Experiment II (Paper III), the effect of increasing inclusion of full-fat BSFL meal (< 19.1%) on growth performance and gastrointestinal function in piglets was investigated for a four-week period PW. High inclusion of full-fat BSFL meal did not compromise growth performance, but the apparent total tract digestibility of crude protein was reduced by increased inclusion of BSFL, whereas both the apparent ileal and total tract digestibility of crude fat was improved. The inclusion of BSFL also affected the colon microbiome by lowering the relative abundance of *Lactobacillus*, although no effect was seen on microbial diversity indices.

The discussion of this thesis focused on comparing the two investigated feed ingredients. In conclusion, both *C. jadinii* yeast and BSFL meal are high-value protein sources and suitable feed ingredients for PW piglets, but future studies should investigate methods to improve the protein digestibility of the BSFL. Neither of the novel ingredients adversely affected the gastrointestinal function and health of the piglets. In conclusion, both *C. jadinii* yeast and BSFL could improve gut homeostasis PW, but especially *C. jadinii* yeast showed potential to improve PW gastrointestinal challenges, giving more robust piglets.

Sammendrag

Med en stadig økende verdensbefolkning, øker også behovet for mat. For å møte denne utfordringen må vi utnytte nye ressurser på innovative måter. Kjøtt er en verdifull proteinkilde i kostholdet, men i Norge er vi i dag avhengige av å importere proteinrike ingredienser for å fôre husdyrene våre. For å øke selvforsyning og sørge for framtidig matsikkerhet, er det behov for alternative proteinkilder som ikke opptar landarealer egnet til produksjon av menneskemat. Gjæren *Cyberlindnera jadinii* og larver av svarte soldatfluer (black soldier fly larvae - BSFL) er to alternative proteinkilder som kan produseres fra fornybare ressurser eller sidestrømmer, og dermed bidra til sirkulær bioøkonomi. Disse to fôringrediensene inneholder også bioaktive komponenter som kan være gunstige for smågrisens tarmfunksjon og helse etter avvenning. Det er kjent at avvenning forårsaker endringer i tarmstruktur og -funksjon, og dermed påvirker smågrisens helse negativt. Hensikten med denne avhandlingen var å evaluere *C. jadinii* og BSFL som to alternative proteinkilder i fôret til smågris etter avvenning, og undersøke om disse ingrediensene kan minske utfordringene som oppstår ved avvenning.

To forsøk ble gjennomført. Det første forsøket (Artikkel I og II) undersøkte effekten av et fôr med høy andel *C. jadinii* gjær på fysiologiske endringer hos smågris, kort tid etter avvenning. Resultatene viste endringer i tarmfunksjon og helse i perioden etter avvenning, men endringene var først og fremst tidsavhengige og ikke fôravhengige. Tilsetning av *C. jadinii* gjær i fôret økte imidlertid ilealfordøyelighet av råprotein, og påvirket genuttrykket i tynntarmen etter avvenning. Gener involvert i flere immunologiske funksjoner ble nedregulert etter avvenning, men dette var ikke like tydelig hos smågrisene som hadde spist gjær. Samlet sett antydet resultatene for

tarmfunksjon-, genuttrykk- og blod immunologiske analyser at smågrisene som spiste gjær hadde mindre tydelig akuttfase etter avvenning.

Det andre forsøket (Artikkel III) undersøkte effekten av økende tilsetning av fullfett BSFL-mel (< 19.1%) på vekstparametere og fordøyelsesfunksjon hos smågris, i en fire ukers periode etter avvenning. Høy tilsetning av BSFL hadde ikke betydelig effekt på vekstparametere, men reduserte fekalfordøyelighet av råprotein. Både ileal- og fekalfordøyelighet av råfett økte imidlertid ved økt tilsetning. Mikrobiomet i tykktarmen ble også påvirket. Tilsetning av BSFL reduserte den relative mengden av *Lactobacillus* i tykktarmen, men påvirket ikke det mikrobielle mangfoldet.

Diskusjonen i denne avhandlingen fokuserte på sammenligning av de to undersøkte fôringrediensene. Avhandlingen konkluderer med at både *C. jadinii* gjær og BSFL-mel er høyverdige proteinkilder og egnede fôringredienser for smågris etter avvenning, men fremtidige studier bør undersøke metoder for å forbedre proteinfordøyeligheten av BSFL. Ingen av de undersøkte ingrediensene hadde negativ innvirkning på tarmfunksjonen eller helsen til smågrisene. Både *C. jadinii* gjær og BSFL kan bidra til å forbedre tarmhomeostasen etter avvenning, men spesielt *C. jadinii* har potensial til å forbedre avvenningsutfordringer og gi mer robuste smågriser.

Abbreviations

ADG – Average daily gain

ADFI – Average daily feed intake

AGP – Antimicrobial growth promoters

AID – Apparent ileal digestibility

ALP – Alkaline phosphatase

AMP – Antimicrobial peptide

ATTD – Apparent total tract digestibility

AST – Aspartate aminotransferase

BSF – Black soldier fly

BSFL – Black soldier fly larvae

CD – Crypt depth

CF – Crude fat

CP – Crude protein

DEG – Differentially expressed gene

DM – Dry matter

EC - European Commission

ELISA - Enzyme-linked immunosorbent assay

ETEC - Enterotoxigenic *Escherichia coli*

GC-MS - Gas chromatography-mass spectrometry

G:F – Gain to feed ratio

IFNγ - Interferon gamma

IL – Interleukin

IL-1ra – Interleukin 1 receptor antagonist

IgA, IgG, IgM – Immunoglobulin A/G/M.

LAP - Leucine aminopeptidase

LC-MS - Liquid chromatography-mass spectrometry

MCFA – Medium-chain fatty acid

NPN – Non-protein nitrogen

PCA - Principal component analysis

PW – Post-weaning

SBM – Soybean meal

SCFA - Short-chain fatty acids

SID – Standardized ileal digestibility

TGF – Transforming growth factor

TID – True ileal digestibility

TNFα – Tumor necrosis factor alpha

VH – Villus height

1. Introduction

1.1 Why is there a need for novel protein ingredients?

The global population is constantly increasing and is expected to reach between 9.4 and 10.1 billion people in 2050 (United Nations 2019). To feed the increasing world population, the Food and Agriculture Organization of the United Nations (FAO) has estimated that the global agricultural food production must increase by 60% from 2005/07 to 2050. It is also estimated that the world's aggregated demand for meat will grow by 1.3% per year in this period (Alexandratos & Bruinsma 2012). Meat is a valuable protein source in the human diet. In Norway, meat accounts for 27% of the protein consumption, and 12% of the daily energy intake in the adult population (Totland et al. 2012). In 2019, 39% of the consumed meat in Norway was pork (Kjos et al. 2019). Norway is almost self-sufficient in meat products (Flaten & Hisano 2007), however, our livestock industry is heavily dependent on imports of protein-rich feed ingredients. In 2020, Norway imported 94.6% of the protein ingredients used for compound livestock feed, where soybean meal (SBM) and rapeseed cake accounted for 87% of the import (Kjos et al. 2021).

Soybeans are the most widely used protein ingredient in the world and are usually included in animal diets as the defatted SBM (Stein et al. 2016). The popularity of SBM as a protein source is due to its high protein content, high digestibility, and especially because of its richness in amino acids that are deficient in cereal grains, and therefore well complements the feed grains for monogastrics. In addition, it is highly accessible at a low cost (Cromwell 2017). On world basis, 75% of the produced SBM is fed to pigs or poultry (Stein et al. 2013). The largest producers of soybeans are Brazil and USA

(International Grains Council 2022; Landbruksdirektoratet 2021b). Norway only imports GMO-free (GMO; genetically modified organism) soybeans, and mainly from Brazil (Landbruksdirektoratet 2021a). The cold climatic conditions and short growing seasons in Norway make agriculture challenging. Therefore, no soybeans can be cultivated in Norway. Also, the topography with mountains and steep hills limits the area of agricultural land. Only 3% of the area in Norway is agricultural land, and a large part of this is only suitable for roughage production. Only 30% of the agricultural land is suitable for grain production and 3% for the production of vegetables, fruits, and berries (Ministry of Agriculture and Food 2016).

To increase Norway's self-sufficiency, we need to search for alternative protein sources which can be produced independently of the limited agricultural land area and cold climatic conditions. To meet the increasing food demand, it is crucial to produce feed ingredients that do not directly compete with human food production. There are also large concerns about the relationship between soybean production and deforestation in Brazil (Rajão et al. 2020). In addition, reliance on imports makes countries vulnerable to world crises. Climatic changes, increased food demand from an increasing world population, changes in the world's trade flows, legislative changes, conflicts, and recently a pandemic, are all factors influencing Norway and other countries' food security, which emphasize the need for new feed sources. Novel technologies, as well as a new use of existing resources, might be the solutions. Both insects and microbes (e.g., yeasts, microalgae, and bacteria) are promising novel feed ingredients that can be produced from renewable resources or side-streams that do not compete with human food production (Gasco et al. 2020; Reihani et al. 2019; Spalvins et al. 2018; Øverland & Skrede 2017). However, limited knowledge exists on the impact of using these novel protein sources in diets for piglets. Thus, this PhD thesis

aims to increase our knowledge on the impact of using some of these novel protein sources on key aspects of growth performance, health, and welfare in piglets.

2. Background and status of knowledge

2.1 Weaning challenges for piglets

2.1.1 Physiological challenges during weaning

In nature, weaning is a gradual process occurring from 14 to 17 weeks of age (Jensen 1986). However, in commercial production systems, weaning is abrupt, and usually occurs between the ages of 18 to 28 days worldwide (Edwards et al. 2020). The EU directive 2008/120/EEC determines that "*No piglets shall be weaned from the sow at less than 28 days of age…*". However, the directive allows weaning at 21 days of age if the piglets are moved to specialized disinfected housings. In Norway, weaning earlier than 28 days of age is prohibited, except for medical reasons (Forskrift om hold av svin 2003). The average weaning age in Norway is 33.4 days (Animalia & Norsvin 2021).

A well-functioning gastrointestinal tract (GIT) is important for the overall growth performance and health of pigs at all stages, but especially for the newly weaned piglets. At weaning the piglet is exposed to several stress causing life changes, including abrupt transition in diet, maternal separation, change in environment, increased exposure to pathogens and dietary or environmental antigens, litter mixing, and establishment of social hierarchy (Campbell et al. 2013; Lallès et al. 2007b; Weary et al. 2008). These stress factors are causing the newly weaned piglet to have a very low feed intake (Dong & Pluske 2007). Stress causes activation of physiological mechanisms to maintain body homeostasis (Jayaraman & Nyachoti 2017), and when combined with reduced energy intake, it may result in disruption of normal epithelial, immune, and enteric nervous system development (Moeser et al. 2017). It is well known that weaning causes changes in the GIT structure and function. Several authors have described post-weaning (PW) changes such as

villous atrophy and crypt hyperplasia (Cera et al. 1988; Degroote et al. 2020; Lallès et al. 2007b), changes in enzyme activity (Hampson & Smith 1986; Lindemann et al. 1986; Makkink et al. 1994), gut microbiota (Lallès et al. 2007b), and increased intestinal permeability (Moeser et al. 2007; Spreeuwenberg et al. 2001). As a consequence, PW piglets are highly susceptible to pathogenic enteric conditions (Pluske 2013), and PW colibacillosis caused by enterotoxigenic *Escherichia coli* (ETEC) is, therefore, common (Wellock et al. 2008).

The severity of these changes depends on the age at weaning (Moeser et al. 2007). Wellock et al. (2008) reported shorter ETEC infections in piglets weaned at six weeks of age compared with four weeks of age. In a study by Leibbrandt et al. (1975), increased weaning age from two to four weeks improved PW feed intake and reduced the PW weight gain depression, indicating that increased weaning age enhances the adaptability to the PW environment. Van der Meulen et al. (2010), also reported higher PW feed intake in piglets weaned at seven weeks compared with four weeks of age. Moreover, higher plasma cortisol levels were observed in the piglets weaned at four weeks compared with seven weeks of age.

Montagne et al. (2007) investigated the development of the PW physiological changes by performing a controlled experiment to mimic a weaning starving situation. Piglets were weaned at 21 days of age, fasted the first 48 hours PW, and then tube-fed. The results showed that the temporal changes in the GIT can be divided in two periods: an initial acute period immediately after weaning (< 5 days PW), with villus atrophy, reduced brush boarder enzyme activity, and increased epithelial paracellular permeability, followed by a

more progressive adaptive and maturational phase where the GIT adapts to the weaning diet.

It is well accepted that weaning is stressful for the immature digestive system of the piglet, but the gut is also the largest immune organ in the body. First at seven weeks of age, the immune system of the intestine reaches an adult-like structure (Zheng et al. 2021), and weaning is associated with a prolonged, but transient increase of pro-inflammatory cytokines in the intestine of piglets (de Groot et al. 2021; Stokes et al. 2004). The cytokine response in the gut can be divided in two periods, starting with an early acute response during the first two days PW, followed by a late long-lasting response from day two to eight PW (Pié et al. 2004). Microbial colonization is important in the development of the piglets immune system (Stokes et al. 2004). Their fermentation products, short-chain fatty acids (SCFA), are among others important for water absorption, pH control, and pathogen inhibition. However, fermentation products could also be toxic substances (Lallès et al. 2007a). The microbiota lives in symbiosis with the host, where the intestinal epithelial cells are cross talkers between the microbes and the host, and have a major role in maintenance of the mucosal homeostasis (Lallès & Montoya 2021). Thus, controlling the microbial community and intestinal inflammation are important strategies to improve PW gut health.

2.1.2 Feeding strategies to control post-weaning challenges

Several dietary adjustments can be made to improve gut development and health for the PW piglet. The use of antimicrobial growth promoters (AGP) became common in Europe around the 1960'ies, considered as a feed additive regulated separately from veterinary medicines (Wegener 2006). Inclusion of AGP in the feed improved growth performance and reduced mortality PW

(Stein 2002). However, the use of AGP in animal feed causes selection of antimicrobial resistant bacteria, which also increases the risk of transmission of resistant bacteria from animals to humans (Wegener 2006). Thus, the wide use of antibiotics has caused resistant bacteria to be an increased risk to public health. Therefore, the AGP was banned in the EU in 2006. However, several countries prohibited inclusion of AGP earlier. Sweden eliminated the use of AGP already in 1986 and Denmark in 1999 (Dibner & Richards 2005). In Norway, the use of AGP in animal feed was voluntary banned by the livestock industry in 1995 (Sundsfjord & Sunde 2008).

The ban of AGP has led to great research efforts in searching for alternative feed additives to improve gut health and growth of PW piglets. There is a continuous strive to find the optimal feed ingredients, additives, and dietary composition. Several feed additives are included in the PW diet today as alternatives to AGP. The feed additives are mainly aimed at enhancing the pig's immune response (e.g. immunoglobulins; ω-3 fatty acids, yeast-derived ß-glucans), reducing pathogen load in the pig's gut (e.g. organic and inorganic acids, high levels of zinc oxide, essential oils, herbs and spices, some types of prebiotics, bacteriophages, and anti-microbial peptides), stimulate the establishment of beneficial gut microbes (probiotics and some types of prebiotics), or stimulate digestive function (e.g. butyric acid, gluconic acid, lactic acid, glutamine, threonine, cysteine, and nucleotides) (De Lange et al. 2010). Combining several additives might give synergistic effects, such as combining pro- and prebiotics (Heo et al. 2012). However, there is less knowledge concerning the antagonistic effects of combining several different feed additives. Table 1 are listing some of the most common additives researched and used after the AGP ban, with advantages and disadvantages.

Many pathogens preferentially ferment proteins (Heo et al. 2012). Fermentation of protein results in NH_3, branched-chain SCFA, and potentially toxic end products such as amines, volatile phenols, and indoles (Lallès et al. 2007a). Lowering the dietary protein level to reduce the amount of protein available for fermentation in the gut is, therefore, a common strategy to improve PW challenges. Wellock et al. (2008), reported firmer feces and decreased colonic digesta pH post-challenge by lowering the dietary crude protein (CP) level. The dietary protein source should also be considered. Legume-based diets, such as SBM, have, for instance, been reported to reduce digestive enzyme activity and increase immunoglobulin activity, compared with skim milk powder (Heo et al. 2012). In general, animal protein sources are more digestible and, therefore, have superior nutritional value for the PW piglets. However, processing might improve the nutritional value of the plant proteins, such as alcohol extraction of SBM to soy protein concentrate (Heo et al. 2012).

Table 1 Some of the additives commonly researched or used in diets to promote digestive function and health post-weaning.

Additive	Function	Benefits	Concerns	Comments	Reference
ZnO	Not fully understood, but anti-inflammatory and moderate antibacterial effect. Zn is a trace mineral which is a component of DNA and RNA synthetases, transferases, and several digestive enzymes.	Improves growth performance, digestive enzyme activity, nutrient digestibility, absorption, and intestinal morphology. Reduces intestinal permeability, modifies gut microbiota, and reduces PW diarrhea.	Environmental pollution, microbial resistance, and toxic effects on the animal in case of too high or prolonged administration.	Will be phased out after 2022 in the EU.	(Bonetti et al. 2021; Debski 2016; Heo et al. 2012; Lallès & Montoya 2021)
$CuSO_4$ and other Cu compounds	Not fully understood but modifies gut microbiota.	Improves growth performance.	Environmental pollution and microbial resistance.	The EU regulation No 349/2010 limits the dietary level to 150 mg/kg of Cu 4 weeks PW and 100 mg/kg 5-8 weeks PW. Will be further reduced to 25 mg/kg PW (EU 2018/1039).	(Debski 2016; European Commission 2018)
Short chained fatty acids (SCFA) (e.g., formic acid, lactic acid, butyric acid)	Reduces stomach pH and have antimicrobial effects.	Improves growth performance and protein digestibility, modulates gut microbial population, and reduces PW diarrhea.	No known concerns		(Heo et al. 2012; Kil et al. 2011; Liu 2015)

Table 2 Continues

Medium chained fatty acids (MCFA)	Quickly absorbed and provides energy for enterocytes. Antimicrobial effect.	Improves intestinal morphometry and gut microbiota.	No known concerns	(Liu 2015)
Prebiotics (e.g., Fructooligosaccharides, Non-starch polysaccharides)	Improves growth of beneficial microbes and increases microbial diversity.	Improves fecal consistency, growth, and small intestinal morphology.	No known concerns	(Heo et al. 2012)
Probiotics (e.g., Lactobacillus, Bifidobacterium, Yeast)	Colonizes in the intestine and competes for adhesion sites and organic substrates with pathogens. Produces anti-metabolites and stimulates the immune system.	Improves growth performance, reduced diarrhea, modulate immune system.	No known concerns	(Heo et al. 2012; Patil et al. 2015)

2.2 Methods to assess digestive function and health

This chapter will in brief describe some commonly used methods in nutritional experiments, with a focus on methods used in this thesis. More detailed description of methodology can be found later in the Materials and Methods section.

2.2.1 General

Effective gastrointestinal functionality is crucial for animal performance, health, and welfare (Celi et al. 2019). Maldigestion and malabsorption increase the nutrient load available for microbes in the distal intestinal segments and increase the chances of inappropriate fermentation. Moreover, ineffective digestion and absorption are symptoms of intestinal inflammation and reduced functionality (Celi et al. 2019). Therefore, effective digestion and absorption are not only important, for nutrient utilization of the feed, but also for the gut health of the animal.

What is a healthy gut?

Gut health is a popular scientific topic, but it is also a complex topic, depending on several factors and complex mechanisms. There is no single measurement that could define a healthy gut or optimal gastrointestinal functionality. The absence of clinical diseases has been used as a definition of a healthy gut, but it is also known that animal growth performance could be impaired without clinical signs (Celi et al. 2017). Celi and coworkers (2017), have identified some key components that the gastrointestinal functionality and health is depending on; a) the diet composition and form, b) an effective structure and function of the gastrointestinal barrier, c) the host interaction with the gastrointestinal microbiota, d) an effective digestion and absorption of the feed, and e) an effective immune status. They suggested this new definition of

a healthy gut: *"A steady state where the microbiome and the intestinal tract exist in symbiotic equilibrium and where the welfare and performance of the animal is not constrained by intestinal dysfunction"*.

How to measure gut health?

Diet modulations to enhance gut function and health in PW piglets were discussed in section 2.1.2. However, to this date, there is no single system to evaluate and compare diet effects on gut health. Celi et al. (2019), suggested there should be a diet scoring system evaluating the general inflammatory properties of diets for farm animals, similar to what has been developed for human nutrition (Shivappa et al. 2014). A panel of biomarkers can be used as a gastrointestinal functionality index (Celi et al. 2019). However, the challenge is to choose which biomarkers are most relevant and significant. Also, a lot of the analyses done in experimental conditions are costly, time-consuming, and cannot be done regularly on large scale to evaluate, for instance, the gut health situation at the farm level. Ideally, the biomarkers should be measured in blood, or most preferably in feces. However, the further information presented in this section will be on some of the common analyses used in nutritional experiments and which are used in this thesis.

Digestibility

Effective digestion and absorption of the feed is one of the key components identified by Celi et al. (2017) that affect gut health and functionality. There are several methods to measure the digestibility of nutrients *in vivo*. Digestibility is either estimated at the ileal or the total tract (feces) level. Total collection of feces or inclusion of an inert marker in the diet (index method) are two different methods used to estimate digestibility coefficients of a diet in pigs (Zhang & Adeola 2017). The total collection is laborious and requires

special facilities (Kavanagh et al. 2001). Pigs must be housed individually, and all feces have to be collected. A marker-to-marker method, which in brief is inclusion of a indigestive marker that color the feces, can be used to determine the beginning and the end of the collection period (Zhang & Adeola 2017).

Instead of total collection, an inert marker can be included in the feed for digestibility calculations. The marker should be homogeneously incorporated in the feed, easily and accurately analyzed even at low concentrations, non-toxic, indigestible, and non-absorbable, not affecting the animal's metabolism, passing through the GIT at the same rate as the dietary nutrients, and be hygienic and harmless to people and the environment (Austreng et al. 2000; Zhang & Adeola 2017). The most used inert marker is probably chromic oxide (Cr_2O_3), but oxides and salts of titanium, cobalt, yttrium, and other trivalent and tetravalent metals are also used. Internal markers such as indigestible acid detergent fiber and indigestible lignin can also be used for digestibility estimations (Jagger et al. 1992; Marais 2000). It is important that the marker recovery in feces is complete. If not, the marker-method may give lower estimated digestibility compared with the total collection (Zhang & Adeola 2017).

Ileal digestibility is considered more accurate for estimation of protein and amino acid digestibility than total tract digestibility (Sauer et al. 1981). Two methods are commonly used for estimating ileal digestibility; the slaughter technique, which involves euthanizing the experimental animals, or an ileal cannula which is operated into the animal. The slaughter technique gives a snapshot of the ileal digestibility, whereas using an ileal cannula provides the benefit of continuously sampling. However, cannulation requires that the pig undergo surgery and is, therefore, more stressful to the pig, whereas the

slaughter technique gives minimal disruption to the normal digestive function. Despite the snapshot sampling, studies have not shown any difference in data variability in nitrogen and amino acid digestibility estimates between the two methods (Donkoh et al. 1994).

The term "apparent digestibility" is used when deducting the total outflow from the dietary intake. The apparent ileal digestibility (AID) of e.g., amino acids represents the net disappearance of the amino acids from the first part of the digestive tract. However, the ileal digesta also contains unabsorbed amino acids of endogenous origin (Stein et al. 2007). These endogenous losses could be divided into basal endogenous losses, which are the amino acids that are lost from the animal regardless of the diet composition, and specific endogenous losses, which are losses induced by the specific feed ingredient characteristics, such as the concentration and type of fiber and anti-nutritional factors (Stein et al. 2007). Methods exist to measure the basal endogenous losses, such as feeding a nitrogen-free diet or using a regression method where the amount of ileal CP and amino acids are regressing on the amount of dry matter (DM) and extrapolating to zero intakes of CP, using a highly digestible protein source in the diet (Jansman et al. 2002). However, there exists no method to directly measure the specific endogenous losses (Stein et al. 2007). Some techniques can be used to provide estimates, but this will not be discussed further in this thesis.

Whereas the calculation of AID does not consider the endogenous losses, true ileal digestibility (TID) is calculated by deducting the endogenous losses from the ileal outflow. Although TID represents the true net disappearance of dietary amino acids, measuring the total endogenous losses is difficult, and the TID does not distinguish between feed ingredients that induce different

levels of specific endogenous losses (Stein et al. 2007). Therefore, standardized ileal digestibility (SID), where only the basal endogenous losses are subtracted from the ileal outflow, is a better option and is commonly used in feed formulation. In contrast to AID, SID values are additive and give a better prediction of the amino acid digestibility coefficients in mixed diets (Stein et al. 2005).

Intestinal mucosa

An effective structure and function of the gastrointestinal barrier is another of the key components for optimal gut function and health, as identified by Celi et al. (2017). The intestinal wall has a complex function. It should ensure absorption of nutrients, but on the other hand, also act as a barrier and protect the internal tissue against the constant exposure of pathogens from the luminal environment. The intestinal epithelial barrier forms a physical barrier through tight-junction complexes, mucus, antimicrobial proteins, and immunoglobulin A (IgA), and is the first line of defense to prevent a pathogen invasion (Sun et al. 2015). Selective intestinal permeability is maintained by the transcellular and paracellular pathways. Absorption of nutrients occurs through the transcellular pathways, mainly by special transportation or channels proteins in the epithelial cell wall. Whereas paracellular transportation of ions, solute, and water occurs in the intracellular space between adjacent epithelial cells and is regulated by thigh junction complexes (Sun et al. 2015). The functional integrity of the intestinal epithelial barrier relies on tight coordination of cell proliferation and migration (Parker et al. 2017). Potential gastrointestinal biomarkers of intestinal barrier function include intestinal fatty acid-binding proteins and diamine oxidase, which indicate intestinal mucosa damage (Celi et al. 2019; Niewold et al. 2004), gene expression of tight junction proteins, and the non-digestible and non-

metabolizable oligosaccharides lactulose, L-rhamnose, and mannitol, which are used in permeability assays (Celi et al. 2019).

The villi are microscopic projections that increase the intestinal surface, which is important for effective absorption of nutrients. Microscopic measurements of villus height (VH) and crypt depths (CD), therefore, also give valuable information about the gut function. The digestive enzymes in the small intestinal brush border are responsible for the final stage of luminal digestion prior to absorption (Hooton et al. 2015). Intestinal alkaline phosphatase (ALP) is one of the brush border enzymes and is considered a marker for mature enterocytes, as the activity is larger at the tip of the villus than in the crypt (Celi et al. 2019). Measuring brush border enzyme activities can, therefore, be seen as indicative of the maturity of the epithelial cells. Stem cells in the crypts proliferate and give rise to several epithelial cell phenotypes which migrate onto adjacent villi (Pacha 2000). The epithelial cell migration velocities along the villi are driven by the cell proliferation rate within the crypts (Parker et al. 2017). Elongation of the crypts indicates an increased rate of enterocyte production and migration, leading to more immature cells with lower digestive enzyme activities and nutrient uptake capacity. Thus, not only does a reduction in villi length reduce the intestinal surface area, but it also reduces the mature enterocyte population, hence affecting both the functional and absorptive capacity.

Immune status

The GIT is in direct contact with the environment and is constantly overloaded with external stimuli, including pathogens and toxic substances. The GIT, therefore, plays an important role in immunoregulation (Vighi et al. 2008), and biomarkers of gastrointestinal inflammation and immune function

provide important information about the GIT interaction with the environment (Celi et al. 2019). Gastrointestinal biomarkers of immune status include cytokines, secretory IgA, lactoferrin, eosinophils, calprotectin, myeloperoxidase, neopterin, and S100 proteins (Celi et al. 2019). An impairment in the tight junction barrier allows passage of noxious molecules and activates mucosal immune cells (Sun et al. 2015). Cytokines are involved in the response to disease or infection, and play a crucial role in the modulation of the inflammatory response in the gastrointestinal tract (Celi et al. 2019). Pro-inflammatory cytokines and growth factors such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-4, IL-6, and IL-13, increase the tight junction permeability, which might result in sustained inflammation and tissue damage, whereas anti-inflammatory cytokines and growth factors such as IL-10, IL-17, transforming growth factor (TGF)-α antibody, TGF-β, and epidermal growth factor decrease and restore the paracellular permeability (Suzuki 2013). Analyzing cytokines in blood or feces gives information about intestinal health, but cytokines are cleared from circulation within a few hours, which might limit their use in monitoring gastrointestinal functionality (Celi et al. 2019).

IgA is secreted from the intestinal epithelial cells as a part of the first-line defense, with levels increasing in response to pathogens (Celi et al. 2019). The secretory IgA is involved in the regulation of composition and function of the commensal microbiota and is important for the immune tolerance to commensal gut microbes as it adapts to the composition of the resident microbiota, thus allowing a dynamic host-microbiota interaction and protecting the epithelium against intestinal inflammation (Kaetzel 2014). The enzyme-linked immunosorbent assay (ELISA) is the most common way to detect immunoglobulins and cytokines. The ELISA is a simple method with high sensitivity, which exploits the specificity of antibodies and uses them to

capture and quantify the analyte of interest (Chiswick et al. 2012). Several analytes can be analyzed in a single sample by implementing a sequential or multiplex method (Chiswick et al. 2012).

Microbiota

To maintain intestinal homeostasis, there must be a balance between the immune response and tolerance to the intestinal microbiota (Sun et al. 2015). The host and the gastrointestinal microbes live in symbiosis with mutual benefits. For the host, the intestinal microbiome has several important functions such as being a source of vitamins, metabolizing various nitrogenous compounds, affecting energy metabolism, and acting as a barrier for pathogens (Celi et al. 2017; Davila et al. 2013). Early establishment of a favorable gut microbiota is important as it is affecting health and growth performance also later in life (Guevarra et al. 2019). However, several factors affect the establishment of the piglets' gut microbiome, including genes of the piglet, sow microbiota, diet, and administration of antibiotics, probiotics, and prebiotics (Guevarra et al. 2019; Nowland et al. 2021). Celi et al. (2019) suggested several biomarkers to indirectly characterize the gastrointestinal microbiota, which can be analyzed in blood, urine, digesta content, or feces. Biomarkers suggested were lactate and succinate, as indirect measurements of intestinal permeability, phenol, p-cresol, and indole, which are fermentation products of aromatic amino acids, and ammonia and hydrogen sulfide, which are associated with high levels of dietary protein, leading to excessive microbiota fermentation.

Traditionally, the microbiota was investigated by culturing, but the ability to grow the bacteria in laboratory conditions restricts the investigations (Ji & Nielsen 2015). Culturing is an adequate method to detect single pathogens

such as *E. coli*, which was the previous focus (Hiergeist et al. 2015), but gives limited information about the whole microbial community (Kim & Isaacson 2015). Today, next-generation sequencing provides a powerful tool to study the gut microbiome. Target amplicon sequencing of the 16S ribosomal RNA gene has become a common method to study the gut microbiome (Ranjan et al. 2016). The ribosomal RNA consists of two subunits, the large ribosomal subunit, and the small ribosomal subunit. In procaryotes, the 16S rRNA is found in the small ribosomal subunit and is about 1500 nucleotides long (Highlander 2012). The 16S rRNA genes are often used for phylogenetic studies because of the slow evolution of this gene region. However, the conserved domains are punctuated by nine variable regions, which are important for lower-ranking taxa determination. Amplicons covering two or more variable regions often give enough information to classify organisms at the genus level or higher (Highlander 2012). However, it is important to be aware that primers do not amplify all 16S rRNA genes at equal efficiency within a sample, which is a bias affecting the diversity result (Highlander 2012). Whole-genome shotgun sequencing has been shown to have advantages over the 16S rDNA amplicon sequencing, such as enhanced detection of bacterial species, increased detection of diversity, and increased prediction of genes (Ranjan et al. 2016).

Biological interpretation of the sequencing result is difficult as we have little knowledge about what is a normal or an optimal gastrointestinal microbial population (Celi et al. 2017). Diversity within the microbial community, differences between microbial communities, identification of members in the microbial community, and interrelation between the members are all research questions addressed with sequencing analysis. The presence or absence of specific microbes, or measuring the abundance, are often used to answer these questions (Highlander 2012). Diversity of the microbial

community is usually presented as alpha diversity, which is describing the diversity of bacteria within the microbial community (within-sample), and beta-diversity, which is describing the differences and similarities between two or more microbial communities (between samples). There are several methods to assess alpha- and beta diversity (Highlander 2012), which not will be described further. Other approaches include nutrigenomic technologies to understand the functionality of the microbiome. Genome-scale metabolic models can be used to predict the metabolic capacities of the gut microbiota, and to study diet-microbiome, microbe-microbe, and host-microbe interactions (Ankrah et al. 2021; Sen & Orešič 2019). However, these databases are biased towards human gut microbiota. Thus, when analyzing gut microbiota in other species, the predictive metabolic profiles need to be interpreted with caution.

2.2.2 Nutrigenomics

Omics technologies are novel tools that increase our understanding and knowledge about interactions between nutrition and biological processes (Zhang et al. 2008). Nutrigenomics could be defined as the study of the interaction of dietary components with the genome and the resulting proteomic and metabolomic changes (Subbiah 2007), but several definitions have been proposed for the term (Ordovas & Corella 2004). However, when talking about omics technologies, four main omics fields (Figure 1) affect the phenotype; genomics, which is the mapping and sequencing of all genes in the genome, transcriptomics is the study of all transcribed RNA products, proteomics is the analysis of all expressed proteins, while metabolomics is a comprehensive analysis of all metabolites (Zhang et al. 2008). Common for omics technologies is a comprehensive study of "all" (*"all transcribed RNA"; "all metabolites").* This generates large and complex multivariate data which require bioinformatical tools to analyze. Unlike traditional analytical

methods, omics technologies are often non-targeted and hypothesis-generating (Vailati-Riboni et al. 2017). In this thesis, the omics technologies transcriptomics and metabolomics have been used, and the further method description is, therefore, limited to these two omics methodologies.

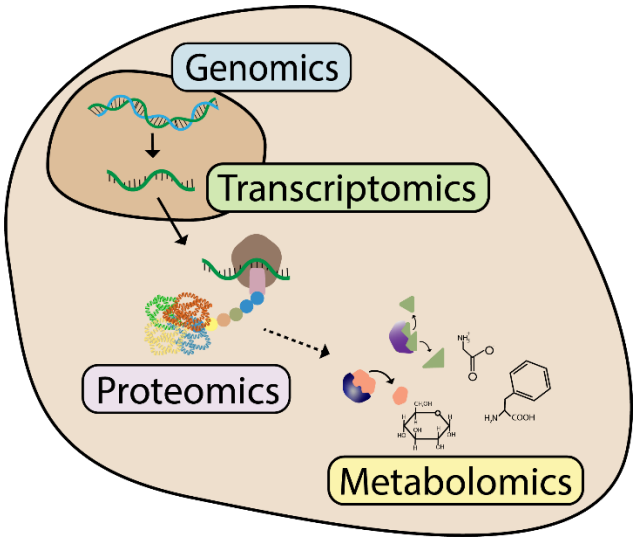

Figure 1 Illustration of the different omics levels

Transcriptomics

The transcriptome is a snapshot of the total RNA expressed by a cell or tissue (Vailati-Riboni et al. 2017). Unlike the genome, the gene expression is affected by environmental impacts, such as dietary intake (Zhang et al. 2012). The first method, which is still in use, to study the expression of many genes simultaneously was the microarray technique (Vailati-Riboni et al. 2017). In brief, RNA samples are converted to cDNA, labeled with different fluorescent colors, mixed, and added to a labeled DNA microarray slide, where the cDNA

samples bind to spots with known genes. The color intensity in each spot then describes the expression level of the specific gene in each sample and can be compared across samples. The huge development in next-generation sequencing in the last decades has introduced RNA sequencing as the new main tool for transcriptomic analysis. RNA sequencing can target specific genes of interest or be whole-transcriptome sequencing. Contrary to microarrays, the detection range of RNA sequencing is not limited to predetermined probes, which makes RNA sequencing able to detect new genes (Guo et al. 2013). On the other hand, there are also challenges to RNA sequencing. Greater coverage of complex transcriptomes requires more sequencing depth, which is more costly and potentially creates a trade-off between coverage and cost. Before sequencing, the long RNA is converted into a library of cDNA fragments. This library construction manipulation complicates the RNA sequencing analysis. In addition, there are bioinformatic challenges with developing efficient methods to store, retrieve and process the large amount of data from the sequencing (Wang et al. 2009). However, the technology is constantly evolving, creating cheaper and less time-consuming methods, which increases reproducibility and improves software for easier data processing and interpretation.

In assessing digestive function and health, gene expression has several applications, such as investigating host-microbiome interactions (Nichols & Davenport 2021), inflammatory disease development in the gut (Criado-Mesas et al. 2021; Hu et al. 2021), and effects of specific diet components on the intestinal tissue (Lee et al. 2021; Xue et al. 2022).

Metabolomics

Metabolomics is the comprehensive assessment of metabolites and the attempts to systematically identify and quantify these. Metabolites are low-weight molecules, including lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols, and carbohydrates (Zhang et al. 2012). Several biological fluids and tissues might be used for metabolic profiling (Vailati-Riboni et al. 2017), but the wide range of different molecules complicates both the analysis and identification of the metabolites (Zhang et al. 2012). In addition, factors such as diet, gender, time of day, age, health, and genetic background are all affecting the metabolome (Wishart 2013).

Several analytical techniques are used in metabolomics research, with different advantages and disadvantages. Nuclear magnetic resonance (NMR) spectroscopy is a widely used method for metabolomic studies. It is a fast, non-destructive, non-biased, easily quantifiable method, which requires little or no separation, no chemical derivatization, and permits the identification of novel compounds. However, it is the method with the lowest sensitivity, and the instrumentation is highly expensive and has a large instrumental footprint (Wishart 2013). Chromatographic techniques coupled to a mass spectrometer provide both higher sensitivity and selectivity and have therefore taken over as the most common methods (Lei et al. 2011). Direct mass spectrometric analysis can be done, but chromatographic separation prior to the mass spectrometric analyses reduces matrix effects and ionization suppression, separates isomers, provides additional data valuable for annotation, such as retention time, and allows for more accurate quantification of individual metabolites (Lei et al. 2011).

Gas chromatography-mass spectrometry (GC-MS) is a robust, relatively inexpensive technique that provides higher sensitivity than NMR spectrometry, excellent separation reproducibility, and can be used with relatively small sample volumes. Quantification of analytes can be done with calibration, and there is a large body of software and databases which can be used for identification of the detected compounds (Wishart 2013). However, GC-MS is limited to volatile, thermally stable, and energetically stable compounds (Lei et al. 2011), and novel compound identification is challenging (Wishart 2013). Another chromatography method is liquid chromatography-mass spectrometry (LC-MS). The LC-MS method can detect thousands of compounds, but can also be used for targeting specific metabolites. The LC-MS has the potential to detect the largest portion of the metabolome, as it has superb sensitivity even with minimal sample size. However, the method is slow and has poor separation resolution and reproducibility compared to GC-MS. The instrumentation is expensive, and it is not a very quantitative method. Also, similar to GC-MS, identification of novel compounds is challenging with the LC-MS (Wishart 2013), however, databases are improving. Also, integrating multiple analytical data from complementary instruments is an effective approach that increases both the chances and confidence of identification (Ghosh et al. 2021).

Multi-omics

The different omics technologies provide valuable information about biological functions, but a holistic approach integrating several omics technologies provides a more comprehensive biological insight and facilitates new discoveries (Song et al. 2020). As an example, combining sequencing of the microbiome with metagenomics, metatranscriptomics, and metabolomics gives a more comprehensive understanding of the gut microbiota and the host interaction (Celi et al. 2017; Ji & Nielsen 2015). Unfortunately, for several

reasons, missing data is often an inevitable limitation in multi-omics integrative studies, which complicates the bioinformatical analyses and hence interpretation of novel insight (Song et al. 2020). However, the technology is constantly evolving and will make data processing and interpretation of multi-omics data easier in the future.

2.3 Yeast as a feed ingredient

Yeasts are eukaryote single-cell organisms, belonging to the kingdom of fungi. Yeast can be found everywhere in our environment, including on cereal grains, in soil, and in water (Stone 1998). Since ancient times, yeasts have been used by humans to make bread, cheese, and fermented beverages such as beer and wine. There are about 1500 known yeast species, but only about 70-80 species have been investigated and given a potential value in biotechnology (Türker 2014). Whereas most species are neither known as harmful nor beneficial, a few genera of yeast are known to be pathogenic, and some species are known to provide beneficial effects. The *Saccharomyces cerevisiae* (Brewer's yeast) is best known, as it is the predominant species used in food, beverage, and ethanol production. Other commercially important yeast species include *Kluyveromyces marxianus* (whey yeast) and *Cyberlindnera jadinii* (Torula yeast, previously known as *Candida utilis*) (Shurson 2018).

Yeast can be produced on a large scale by submerged fermentation. The fermentation medium needs to contain carbon, as a source of energy, along with nitrogen, vitamins, minerals, and water. Traditionally, molasses was used as a carbon source of fermentable sugars for yeast production (Agboola et al. 2021b). However, the aim of developing novel protein sources is to transform non-edible biomass into food as part of sustainable production.

This can be achieved by utilizing by-products from forestry, agriculture, aquaculture, and the food industry in the production of yeast. Waste materials, such as beet pulp hydrolysate (Rajoka et al. 2012), virgin grape marc (Curto & Tripodo 2001), and fruit wastes (Adoki 2008; Mondal et al. 2012) are suitable carbon sources for yeast production. Lignocellulosic biomass from the agricultural and forestry sector is another non-food biomass that can be converted to high-value feed by yeast fermentation (Øverland & Skrede 2017). After breaking down the complex polysaccharides into fermentable sugars, lignocellulosic biomass represents a suitable and sustainable carbon source for the yeast (Agboola et al. 2021b), especially in Norway and the Nordic countries which have large forestry resources. Nitrogen is another part of the fermentation medium, important for the protein synthesis in the yeast. Yeast can utilize both organic and non-organic nitrogen sources (Türker 2014), but the source might affect the production yield (Reihani et al. 2019). Inorganic nitrogen sources include ammonia, ammonium salt, nitrate, and urea (Reihani et al. 2019). Suitable alternative organic nitrogen sources could be protein-rich hydrolysates from the meat and fish industry (Lapeña et al. 2018).

The nutritional composition of the yeast may depend on the species and the composition of the fermentation medium used in yeast production. In whole yeast, the CP content ranges from 40-60% in DM. The levels of indispensable amino acids are comparable to fish meal, except for the lower methionine level (Øverland & Skrede 2017). The CP and amino acid digestibility of yeast in pigs can also vary with yeast species and production, as well as the downstream processing procedure. However, the CP and amino acid digestibility of yeast meal and yeast products are in general high, and thus suitable for replacing protein sources such as high-quality fish meal, SBM, and spray-dried plasma (Cruz et al. 2019; Lagos & Stein 2020; Mateo & Stein 2007;

Wu et al. 2018). The lipid content in yeast is in general low (< 8% in DM) (Øverland & Skrede 2017), but for oleaginous yeast species, the total lipid content can be up to 80% of dry weight (Patel et al. 2020), dominated by oleic acid (18:1ω9) and saturated fatty acids (Sitepu et al. 2013). The nucleic acid content in yeast can be high (5-11.5%), and much higher than for plant feed ingredients (Øverland & Skrede 2017). For pigs, a high level of dietary nucleic acids should not cause any problems as the pig can utilize the nucleic acids, but high dietary levels can be problematic for other species such as broiler chickens (Cruz et al. 2020).

Yeast can be fed as live, active dry, dried, inactivated, or fractionated products (Shurson 2018). Active dry yeast is the most common form of viable yeast (Stone 1998). Different downstream processing methods might affect the nutrient availability and functionality of the yeast (Agboola et al. 2021a), however, processing also increases the costs of the product. Whole yeast is primarily a source of protein. However, the yeast cell wall consists of several polysaccharides which might have interesting functions as bioactive compounds (Figure 2). The yeast cell wall makes up 26-32% of the dry weight of the cell and consists of about 85-90% polysaccharides and 10-15% protein (Agboola et al. 2021b). The main polysaccharide is β-D-glucan, which constitutes 50-60% of the yeast cell wall, and is an important structural component of the cell wall (Garcia-Rubio et al. 2020; Kogan & Kocher 2007). The cell wall proteins occur in complexes with mannan polysaccharides known as mannoproteins (Agboola et al. 2021b). The cell wall proteins are involved in cell-to-cell recognition, interaction with the environment, and immunological specificity of the cell (Kogan & Kocher 2007).

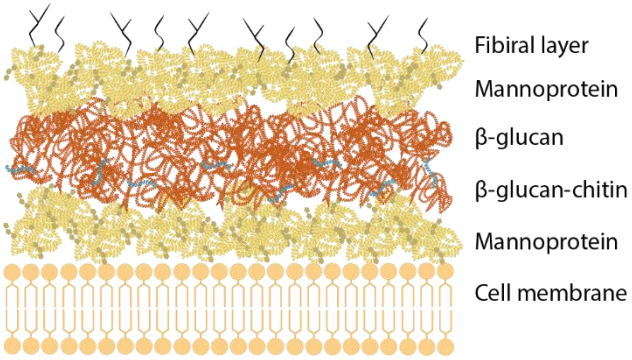

Figure 2 Composition of the yeast cell wall. Based on the illustration in Kogan & Kocher (2007).

2.3.1 Can feeding yeast reduce weaning challenges?

Since the ban of AGP, yeast and yeast products have been widely investigated as non-antibiotic functional products to improve PW gut function and health (Heo et al. 2012; Liu et al. 2018). In the following section, some of the dietary yeast products and their bioactive functions will be discussed.

Live yeast

Dietary live yeast can function as a probiotic by altering the gut microbiota (Shurson 2018). Inclusion of live *S. cerevisiae* in the diet has been reported to reduce the duration and severity of PW diarrhea in piglets (Trckova et al. 2014). Improved growth performance and improved intestinal health in PW piglets have also been reported by the inclusion of 2 g/kg of live *S. cerevisiae* (Bontempo et al. 2006) or 5 g/kg of a commercial yeast culture (Shen et al. 2009). In addition, Shen et al. (2009) reported increased jejunal VH and Bontempo et al. (2006) found improved intestinal adherent mucous layer in the PW piglets when feeding yeast. However, in another study, inclusion of 0.75% *S. cerevisiae* yeast culture did not affect growth performance or apparent digestibility in PW piglets (Kornegay et al. 1995).

Yeast cell wall components

Bioactive compounds present in the yeast cell and cell wall are also used to improve animal growth performance and health. Inclusion of 4% yeast-derived protein improved growth performance and partially enhanced anti-oxidative capability, as well as intestinal innate immunity, of piglets weaned at 26 days of age (Hu et al. 2014). Polysaccharide components found in the cell wall can stimulate the immune system and function as prebiotics (Shurson 2018). By binding to specific receptors, the yeast cell wall polysaccharide β-glucan can enhance the functional status of macrophages and neutrophils, modify immunosuppression, increase resistance to gram-negative bacteria, and stimulate the release of cytokines such as TNFα. In addition, it is shown that yeast β-glucans adsorb mycotoxins (Kogan & Kocher 2007). The cell wall of *S. cerevisiae* is capable of binding a wide range of toxins, but the capacity of adsorbing toxins depends on the yeast species and the glucan concentration in their cell wall (Moran 2004).

Adhesion of pathogens to the epithelium surface of the gut (colonization) is believed to be the first critical stage leading to infection (Moran 2004). Mannans can adsorb pathogenic bacteria, such as *E. coli* and *Salmonella* spp., by binding to their mannose-specific lectin-type receptors and thereby preventing them from binding to the epithelial surface (Kogan & Kocher 2007). Mannose, which are compounds of the mannan, is shown to largely decrease the number of adherent bacterial cells to an intestinal surface (Moran 2004). Inclusion of mannan oligosaccharides has, in a meta-study by Pettigrew (2001), shown to increase growth performance in weaning pigs (Moran 2004).

Nucleotides

As previously mentioned, the yeast cell can be rich in nucleotides, which are important in many biochemical processes in cells. During nutrient deficiency periods, dietary nucleotides may be important in tissues with a high turnover rate such as the intestinal mucosa (Sauer et al. 2011). Inclusion of pure nucleotides has been reported to increase plasma IgA concentration 20 days PW, in piglets weaned at 20 days of age (Sauer et al. 2012). However, in the study, nucleotide supplementation did not affect growth or gut morphology. Waititu et al. (2016), also reported no effect on growth performance by dietary inclusion of yeast-based nucleotides. However, increased inclusion (0.1% to 0.2%) of the nucleotide-rich yeast extract improved apparent total tract digestibility (ATTD) of DM, CP, and gross energy. The yeast extract used in the study also contained some yeast cell wall polysaccharides, which might have influenced the results.

Inactivated whole cell

Due to the numerous bioactive compounds, inclusion of inactivated whole yeast cells is believed to have several positive effects on the PW intestinal function and health. Partly replacing SBM with dried *Yarrowia lipolytica* yeast improved growth performance in weaned piglets (46 to 85 days of age) (Czech et al. 2016). Dietary inclusion of the *Kluyveromyces fragilis* yeast improved daily weight gain and N-metabolism in weaned piglets (Spark et al. 2005). Increasing (0-14.6%) inclusion of inactivated *C. jadinii* (previously named *Candida utilis*), increased fecal DM seven days PW. The highest inclusion level also improved ATTD of CP and ash in the diet, and increased VH and VH:CD ratio in jejunum compared with control, four weeks PW (Cruz et al. 2019). Inclusion of 14.6% *C. jadinii* also increased the abundance of lactic acid-producing bacteria in the intestine, two weeks PW (Iakhno et al. 2020). Contrary, Yang et al. (2016), reported increased incidences of diarrhea and

adverse effects on the intestinal morphology and barrier function when feeding a mixed yeast product (mixture of yeast culture, cell wall hydrolysates, and yeast extracts) to piglets weaned at 21 days of age. The authors discussed if the results could have been caused by an adverse effect of the yeast product mixture.

To conclude, there are several bioactive compounds in the yeast cell that can be used to improve piglets' intestinal function and health. Thus, feeding yeast to piglets can be a good strategy to reduce weaning challenges.

2.4 Insects as a feed ingredient

Insects are the largest animal group on the earth and constitute a huge amount of biomass. Insects have been eaten since ancient times, and over 1600 species are known to be consumed by humans all over the world (Ramos-Elorduy 2005). In the search for novel protein sources for animal feed, insects have been proposed as a high quality and sustainable protein source (Veldkamp et al. 2012) for pigs (DiGiacomo & Leury 2019), poultry (Chodová & Tůmová 2020; Józefiak et al. 2016), and aquaculture (Nogales-Mérida et al. 2019). Larvae or prepupae of black soldier fly (BSF; *Hermetia illucens*), house fly (*Musca domestica L*), and yellow mealworm (*Tenebrio molitor*) are identified as the most promising species for industrial production for monogastric diets (Veldkamp & Bosch 2015), but silkworm pupae, crickets, and grasshoppers are also suggested alternatives (Makkar et al. 2014).

Insects have a short life cycle, just over 40 days are needed for the black soldier fly larvae (BSFL) to develop into an adult fly, which then can produce around 300–600 eggs (Tomberlin et al. 2002). Most insect species go through

four stages of complete metamorphosis – egg, larva, pupa, and adult (Figure 3), whereas crickets and grasshoppers have a nymph stage instead (Truman & Riddiford 2019). The insects are often harvested as larvae or prepupae as the larvae are the most efficient to produce (Józefiak et al. 2016). They are also the most nutrient and energy-rich, as they accumulate protein and fat (DiGiacomo & Leury 2019). In 42 days, more than 180 kg of BSFL can be produced from 1m^2 (Józefiak et al. 2016). Because insects are cold-blooded, they do not use energy to maintain body temperature, making them highly effective feed converters (Chodová & Tůmová 2020). As insects also are very efficient in utilizing water, the feed can constitute the main and often only water source (Józefiak et al. 2016). For most insect species, the optimal rearing conditions are 27–30°C, and to ensure farm biosecurity, farming of insects for feed production should take place in a closed environment as an "all-in-all-out" system (Józefiak et al. 2016).

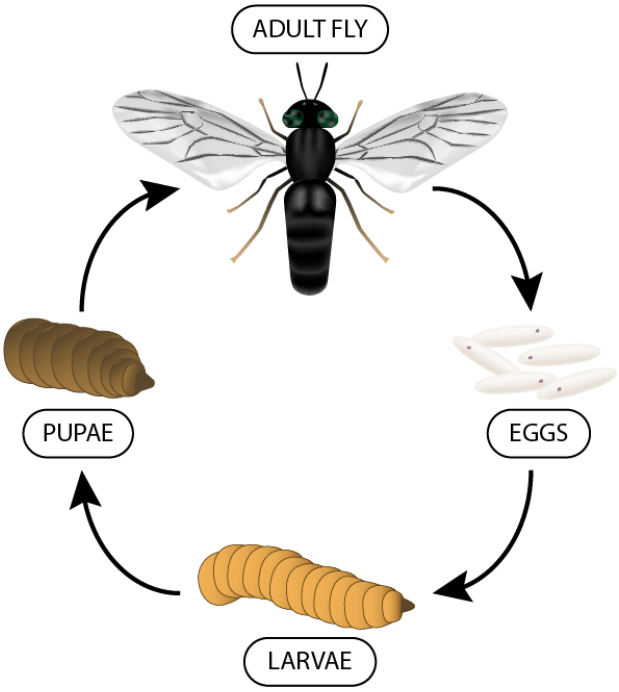

Figure 3 Illustration of the life cycle of the black soldier fly

The sustainability of insect production is highly dependent on what the insects are fed. Rearing insects on low-value food processing by-products and high-impacting waste are among the best strategies for sustainable production of insects as a protein resource (Smetana et al. 2016). Insects can even be used as a manure management tool (Newton et al. 2005; Roffeis et al. 2015) as the BSFL and housefly maggots are naturally found in manure (Veldkamp & Bosch 2015). Attributional life cycle assessment studies have shown that replacing SBM in animal diets with locally produced protein sources, such as waste-fed larvae meal, reduces land use and global warming potential (van Zanten et al. 2018). However, using a consequential life cycle assessment that accounts for indirect environmental consequences outside the production chain, van Zanten et al. (2018) also found that replacing SBM with waste-fed larvae meal reduces land use, but that the global warming potential and energy use actually increases. The reason was that the food waste included in the study was already utilized for biofuel production. Therefore, the need for fossil fuels and synthetic fertilizers increased by changing the application from biofuel production to waste-fed larvae. The whole production chain as well as indirect environmental consequences of the production should therefore be included when evaluating the sustainability of insect production for feed. Consequently, the sustainability of insect production for feed will differ between countries with different resources available and their current utilization.

Insects are most often included in the feed as a dried meal, but feeding a preserved paste (Weththasinghe et al. 2021a) or even live larvae (Tahamtani et al. 2021) are also alternatives. The nutrient composition of the feeder insects are depending on species and life stage, but also rearing and processing methods (DiGiacomo & Leury 2019). The three previously

mentioned most studied species, range from below 40 to over 60% CP in DM, which is close to SBM that has a CP content of 49 to 56% in DM (Veldkamp & Bosch 2015), but lower than fish meal which DM contains approximately 70% CP (Tran et al. 2015). Of the commonly studied insect species, BSF prepupae and yellow mealworm larvae are found to have the most preferable amino acid profile for growing pigs and broiler chickens (Veldkamp & Bosch 2015). In BSFL, the limiting amino acids are methionine and cystine, which is similar as for SBM (Veldkamp & Bosch 2015). Limited information exists about the amino acid digestibility of BSFL and houseflies, but a study recently published by Tan et al. (2020) reported that the SID coefficients in BSFL were between 0.767 and 1.177, and in houseflies, between 0.870 and 1.608. For houseflies, all SID coefficients were higher than what was reported for fish meal, and the SID values for methionine and cysteine were the only values lower than what was reported for SBM. For BSFL the SID coefficients were also overall higher than what was reported for SBM, except for methionine, alanine, cysteine, glycine, and proline.

Chitin is the dominant carbohydrate in insects. Chitin analysis in insect meal is not standardized, and often indirect analyses are performed for estimations. This causes variation in the reported chitin contents of insect meals, as they are depending on the analysis method, but also life stage as the chitin level increases with the life stage (Smets et al. 2020). The chitin polysaccharide is the major component of the insect cuticle and creates a strong skeleton together with minerals and proteins. The exoskeleton of insects differs from crustaceans by having more amino acids strongly bound to the chitin (Andersen 2010; Finke 2007). *In vitro*, the CP digestibility is found to negatively correlate with chitin content in BSFL (Marono et al. 2015). Because of the nitrogen-rich chitin, the Kjeldahl-N method overestimates the digestible protein content of insects (Jonas-Levi & Martinez 2017). Janssen et

al. (2017), suggested using a conversion factor of 5.6 to avoid overestimation of the protein content in BSFL. De-chitinization of the insect meal is used to increase the nutritional value.

Insects also contain high amounts of fat. Between 10 to 30% of DM (Veldkamp & Bosch 2015). Often a defatting process is necessary to make the insect meal more suitable for inclusion in a diet formula. Defatting would also increase the protein content of the insect meal, and the insect oil can be used as a lipid ingredient (Heugten et al. 2019) replacing less sustainable vegetable oil sources (Veldkamp et al. 2021). However, comparing full-fat and defatted BSFL meal in diets for Atlantic salmon, Weththasinghe et al. (2021b) concluded that the full-fat BSFL meal was more optimal for the Atlantic salmon, also in terms of reducing additional processing costs. In BSFL, lauric acid (12:0) is the dominating fatty acid (Finke 2013), whereas the housefly maggot or prepupae and the mealworm are more dominated by palmitic acid (16:0) and oleic acid (18:1). The mealworm fat is also dominated by polyunsaturated linoleic acid (18:2) (Makkar et al. 2014). Insects are also rich in minerals, especially calcium content is high in the BSFL (Finke 2013).

2.4.1 Can feeding insects reduce weaning challenges?

Insects are mostly studied as protein and energy ingredients, but they also contain several compounds which are interesting in relation to piglet health. Reviews of the bioactive properties of insect products have recently been published by Gasco et al. (2021) and Veldkamp et al. (2021), and the reader is referred to these papers for a deeper understanding.

Chitin

Chitin can function as a prebiotic and immunostimulant (Song et al. 2014). Pigs and other mammals are not able to synthesize chitin, and it is therefore considered a potential target for recognition by their immune system (Komi et al. 2018). In piglets weaned at 16 days of age, inclusion of chito-oligosaccharides (derivates from chitosan, which is formed by partial deacetylation of chitin) for three weeks PW improved growth performance, increased ATTD of several main nutrients, improved small intestinal morphology, increased *Lactobacillus* counts in feces, and reduced the incidences and score of diarrhea (Liu et al. 2008). Moreover, in a study by Xiao et al. (2014), chitosan supplementation decreased the occurrence of diarrhea and alleviated intestinal inflammation in piglets weaned at 21 days of age and challenged with *E. coli*. Chitin-oligosaccharides from crab shells have also been shown to inhibit oxidative stress in human myeloid cells (Ngo et al. 2008). However, limited information is available about the actual effect of the unprocessed insect's chitin.

Lauric acid

Fatty acids with six to twelve carbon atoms, such as the lauric acid (C12:0) which is rich in BSFL, are categorized as medium-chain fatty acids (MCFA) (Veldkamp et al. 2021). The MCFAs are rapidly absorbed in the mucosa and can directly supply the enterocytes with energy, thereby improving morphological changes in periods of nutrient deficiency such as weaning (Zentek et al. 2011). Heugten et al. (2019), reported increased growth performance when including up to 6% BSFL oil in the diet (replacing corn oil) in piglets weaned at 21 days of age. The MCFAs are also known to have antimicrobial properties and have been found to be an effective alternative to AGP in piglets (Decuypere & Dierick 2003). Lauric acid is found to be one of the most antibacterial triglycerides among the MCFAs, and especially effective

against gram-positive bacteria (Zentek et al. 2011). Monolaurin, a monoester formed from lauric acid, has been shown to lower the viral load in human patients. Unlike antibiotics, it does not appear to have adverse effects on gut bacteria, and might even be effective against antibiotic-resistant bacteria (Lieberman et al. 2006). In a study by Yu et al. (2019), inclusion of 4% full-fat BSFL increased the abundance of *Lactobacillus* and several butyrate-producing bacteria and decreased the abundance of *Streptococcus* in the colon of grower-finishing pigs. Moreover, Yu and coworkers reported an increase in SCFA concentration, a decrease in metabolites involved in amino-acid metabolism, and a decrease in the expression of pro-inflammatory cytokines, whereas the expression of anti-inflammatory and intestinal barrier genes increased. Contrary, Spranghers et al. (2018) reported that BSFL fat suppressed growth the of both *lactobacilli* and *D-streptococci*, *in vitro*. However, the antibacterial effect against the *D-streptococci* was larger than for the *lactobacilli*. The effects were less evident *in vivo* when feeding BSFL meal to pigs.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are part of the insect immune system and are effective antimicrobial agents with a low risk of developing bacteria resistance (Lewies et al. 2019). The BSF is having one of the largest AMP repertoires reported in insects, by expressing over 50 genes encoding putative AMPs (Van Moll et al. 2022). The AMPs target lipids in the bacterial cell membrane, forming ion channels or transmembrane pores in the bacterial cell wall, causing leakage and disrupting cell homeostasis, thereby killing the bacterial cells (Veldkamp et al. 2021). They can also cover the cell membrane like a carpet, disrupting the bilayer curvature and causing dissolution of the cell membrane (Wang et al. 2016). Cecropins are some of the AMPs using this carpet strategy. The cecropin helices integrate into the

acidic cell membrane of bacteria and cause destruction (Veldkamp et al. 2021). Dietary supplementation of 400 mg/kg cecropin AD, isolated from the silkworm *Hyalophora cecropia*, improved growth performance, gut morphology, and immune status, whereas the PW diarrhea incidences decreased in PW piglets challenged with *E. coli* compared with a control (Wu et al. 2012). The results were comparable to feeding AGP. Cecropins from BSF have shown promising activity against gram-negative human pathogens (Van Moll et al. 2022). The AMPs have good potential as health promoters in pigs, even though there is limited information about the *in vivo* effects (Wang et al. 2016).

2.5 Regulatory constraints for novel feed ingredients

Introducing novel feed ingredients is not without precautions. The feed manufacturing industry is regulated through several regulations by the European Commission (EC), such as the regulation No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority, and laying down procedures in matters of food safety, the regulation No 6183/2005 laying down requirements for feed hygiene, the regulation NO 767/2009 on the placing on the marked and use of feed, the regulation No 1831/2003 on additives for use in animal nutrition, and the regulation 68/2013 on the catalogue of feed materials, which lists approved feed materials. The purposes of these regulations are to ensure that the feed is safe and not harmful for the animals eating it, but also in terms of the safety of human consumption of animal products and environmental impact. Norway follows these EC regulations, but we also have our own feed regulation (Forvareforskriften 2002).

Yeasts and yeast products are listed under point 12 “Products and by-products obtained by fermentation using micro-organisms inactivated resulting in absence of live micro-organisms” in the EC No 68/2013 (“Feed catalogue”). Among the approved species listed are several species of *Saccharomyces,* such as *S. cerevisiae* and *S. carlsbergensis*, but also *Cyberlindnera jadinii*, which is the strain investigated in this thesis. The requirements for the use of these yeasts are that they are grown on substrates mostly of plant origin and that they have been inactivated so they are not viable in the feed.

The regulations for use of insects in feed are more complicated. After an outbreak of bovine spongiform encephalopathy (BSE) in the UK in 1986, caused by feeding insufficiently processed meat and bone meal, the EC established several regulations to prevent prion infections by banning the use of processed animal proteins in the feed for farmed animals (Nesic & Zagon 2019). The Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control, and eradication of certain transmissible spongiform encephalopathies, generally prohibits the inclusion of processed animal proteins in the food chain. In 2017, the commission regulation (EU) 2017/893 amending was added to the 999/2001 regulation, allowing processed animal protein derived from seven common farmed insect species; BSF (*Hermetia illucens*), Common Housefly (*Musca domestica*), Yellow Mealworm (*Tenebrio molitor*), Lesser Mealworm (*Alphitobius diaperinus*), House cricket (*Acheta domesticus*), Banded cricket (*Gryllodes sigillatus*) and Field Cricket (*Gryllus assimilis*), to be fed to aquaculture animals. It is also allowed to use insects in pet food and for fur animals, as they are not part of the human food chain. The interest in insect products has been great in recent years in Europe, and in 2021 the commission regulation (EU) 2021/1372 authorized feeding processed animal

protein derived from farmed insects to pigs and poultry. One of the benefits of insect production is to utilize non-food products such as food waste, and even manure (Smetana et al. 2016). However, this is strictly regulated in section F Annex I, and only vegetable material and some animal by-products which are referred to as Category 3 material in the Regulation (EC) No 1069/2009, except those in Article 10(n), (o) and (p), can be used for insect production. The category 3 materials are mostly animal by-products that could have been fed to humans, but for some reasons, e.g. packaging defects or uncommonly consumed parts of the animal, are not used for human consumption. This excludes the use of household waste and manure. The insects must also be produced in approved processing plants exclusively dedicated to the production of farmed insects and in accordance with the specific processing and storage requirements for processed animal protein and other derived products laid down in Section 1 of Chapter II of Annex X to Regulation (EU) No 142/2011. An overview of which animals feed inclusion *C. jadinni* yeast and BSFL are approved for is shown in Figure 4.

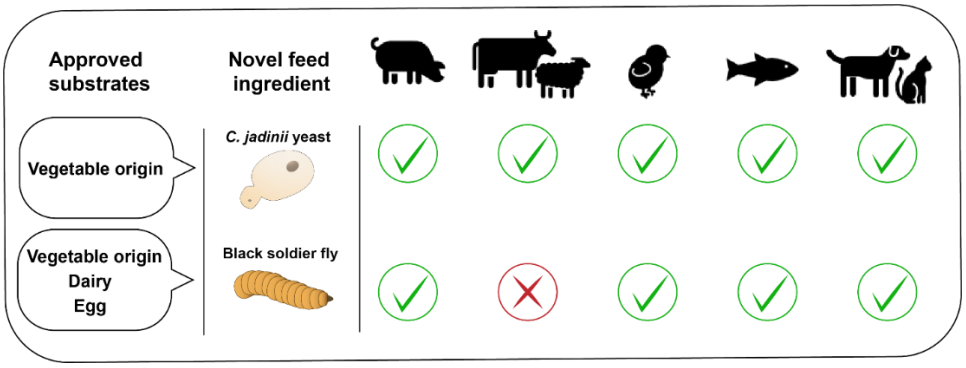

Figure 4 Approved substrates for production of *Cyberlindnera jadinii* yeast and black soldier fly larvae and which animals feed inclusion is approved for.

3. Aim of the thesis

The aim of this thesis was to evaluate two novel ingredients, *C. jadinii* yeast and BSFL meal, as protein sources for weanling piglets, and investigate if these ingredients could improve PW challenges. This was achieved by conducting two experiments with PW piglets fed a high level of *C. jadinii* yeast (Experiment I; Paper I and II) or increasing levels of BSFL meal (Experiment II; Paper III).

The main hypothesis was that novel, sustainable protein sources such as *C. jadinii* yeast meal and BSFL meal partially can replace conventional protein ingredients in piglet diets PW without compromising growth performance, and that such ingredients also can improve the gut homeostasis.

Experiment I

In Experiment I, the objectives were to examine early PW physiological changes in piglets and to investigate the effects of high dietary inclusion of *C. jadinii* yeast on the GIT function and health development during the two first PW weeks.

It was hypothesized that:

- Weaning causes changes in gut function and health.
- The *C. jadinii* yeast can be a highly digestible protein source for piglets PW.
- Dietary inclusion of the *C. jadinii* yeast would improve PW changes by
    - Reducing PW diarrhea
    - Reducing PW villus atrophy
    - Strengthening the immunity of the piglets

Experiment II

In Experiment II, the objective was to evaluate the effect of an increasing inclusion level of full-fat BSFL meal in a PW diet on growth performance, nutrient digestibility, gastrointestinal function, and microbiota.

It was hypothesized that:

- Full-fat BSFL meal can replace traditional dietary protein and lipid ingredients in a balanced diet without adverse effects on growth performance.
- Bioactive compounds in the BSFL meal, such as lauric acid, chitin, and AMPs, can contribute to improved intestinal morphology and beneficially alter the intestinal microbiota, thereby improving the PW gut function and health of piglets.

4. Materials and methods

4.1 Experimental design

Two experiments with weaning piglets were performed at the Center for livestock production (SHF), NMBU, Ås, Norway, which is an animal experiment unit approved by the National Animal Research Authority (permit no. 174). The first experiment was performed in November 2017 and the second experiment was performed in February 2019. All pigs were handled under the applicable laws and regulations controlling experiments with live animals in Norway regulated by the "Animal Welfare Act" and "The Norwegian Regulation on Animal Experimentation" derived from the "Directive 2010/63/EU on the protection of animals used for scientific purposes."

4.1.1 Experiment I (Paper I and II)

In Experiment I, a total of 64 crossbred ([Norwegian Landrace × Yorkshire z-line] × [Duroc] and [Norwegian Landrace] × [Duroc]) weaning piglets, selected from eight litters, were included in the experiment. The average weaning age was 27.4 ± 1.2 days, and the average weaning weight was 10.2 ± 1.6 kg. Piglets were allocated to dietary treatment and day of dissection based on litter origin and weaning weight. There were six pens per dietary treatment and five or six piglets in each pen. Pen design is shown in Figure 5.

Inactivated and drum-dried *C. jadinii* yeast (LYCC-7549; Lallemand Yeast Culture Collection) was obtained from Lallemand Inc. (Salutaguse, Estonia). The dietary treatments included a control diet based on wheat, barley, oats, SBM, fishmeal, potato protein concentrate, and rapeseed oil, and an experimental diet containing 14.6% inactivated *C. jadinii* yeast,

corresponding to a replacement of 40% of the protein in the control diet with yeast protein. Diets were formulated in collaboration with Felleskjøpet Fôrutvikling AS (Trondheim, Norway), using net energy and SID values (CVB 2016) to be isoenergetic, isonitrogenous, and to meet or exceed the requirements for pigs of this age (NRC 2012). Piglets had *ad-libitum* access to the experimental diets immediately PW. Eight piglets (littermates of piglets included in the experiment) were sampled on the day of weaning (day zero) to provide a baseline time point to facilitate detection and interpretation of changes due to weaning and potential yeast-induced changes. On days two, four, seven, and 14 PW eight piglets from each dietary treatment group were sacrificed and sampled. Figures 6 and 7 show an overview of the methods used in Paper I and II, respectively.

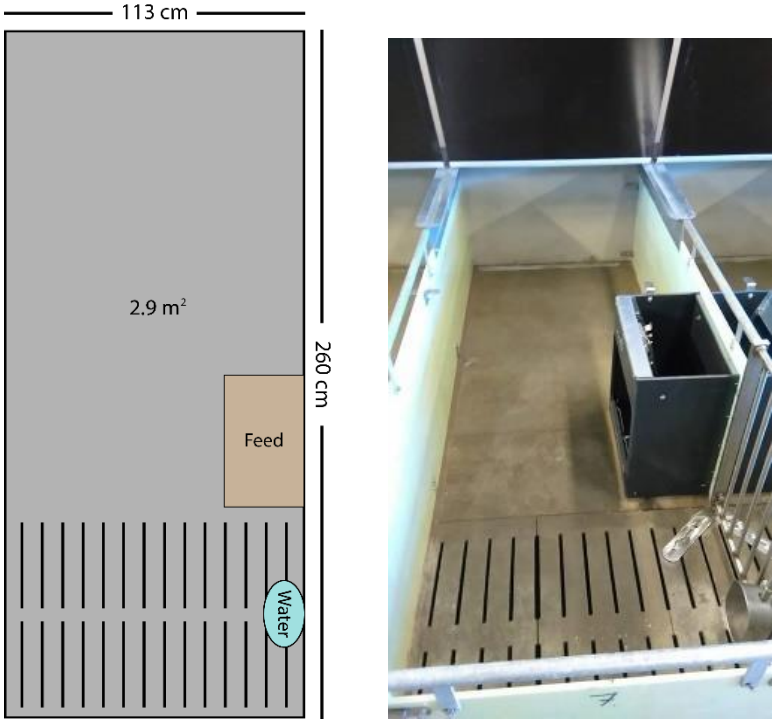

Figure 5 Illustrations of pens used in both experiments.

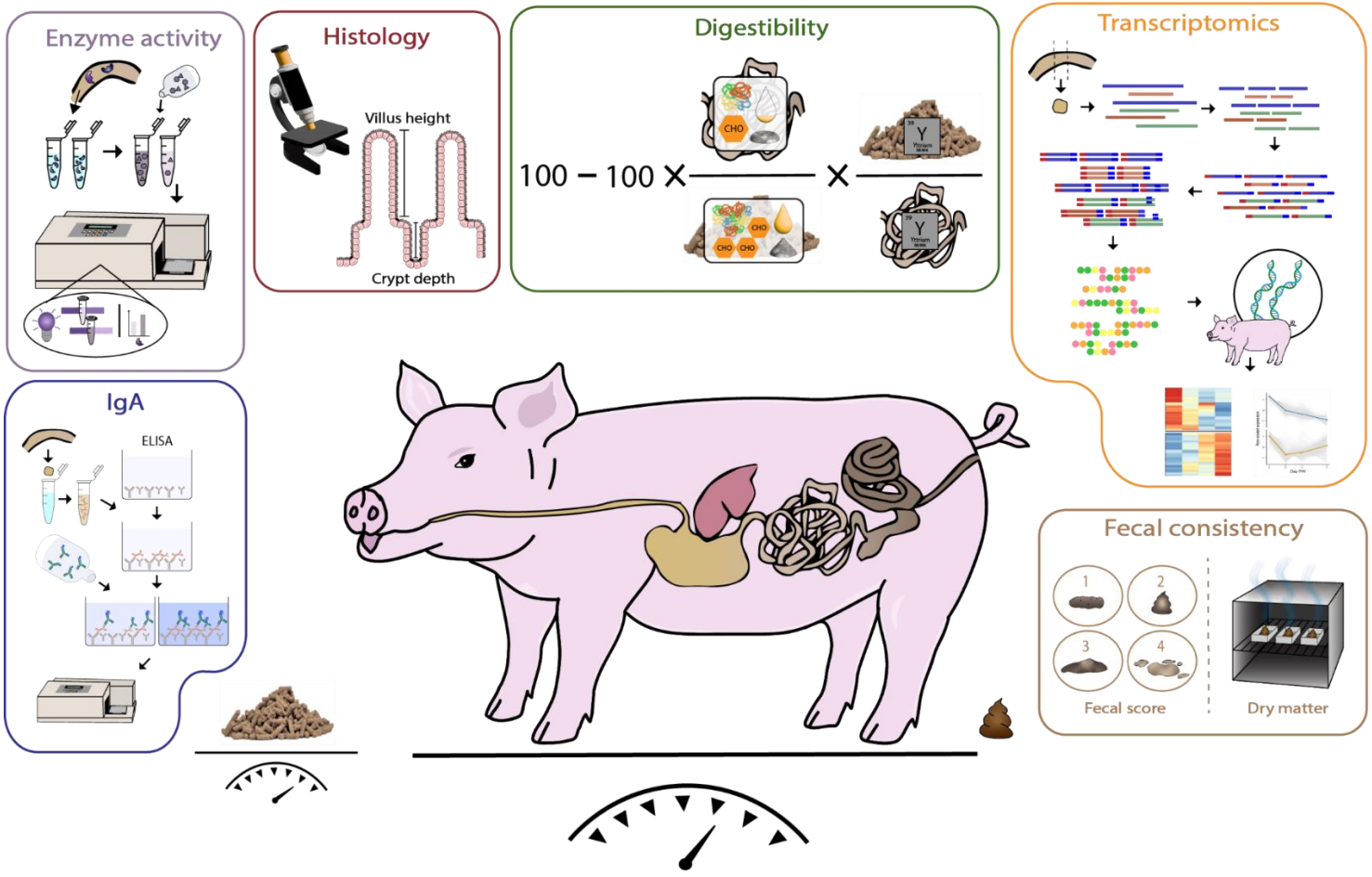

Figure 6 Overview of methods used in Experiment I, Paper I.

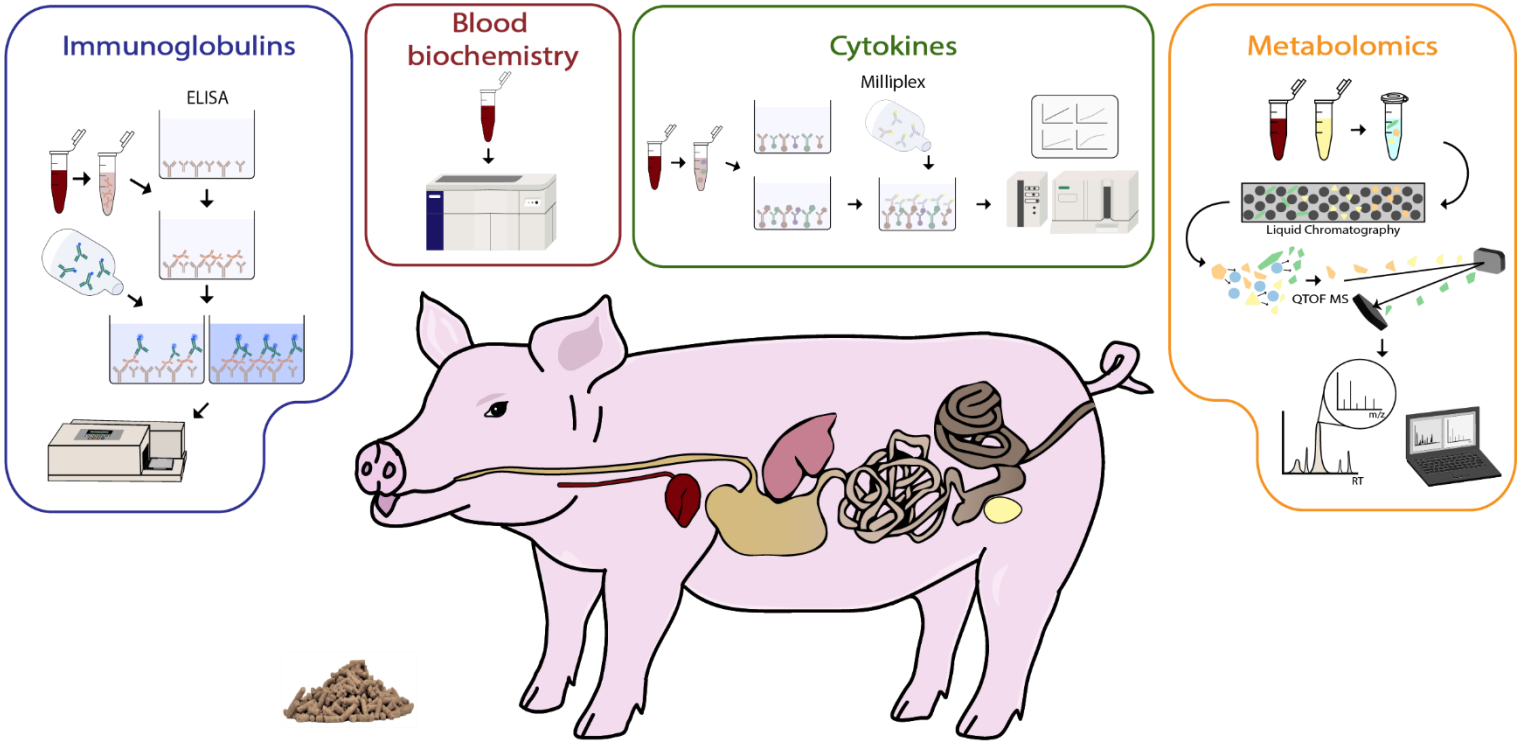

Figure 7 Overview of methods used in Experiment I, Paper II.

4.1.2 Experiment II (Paper III)

In Experiment II, 80 crossbred ([Norwegian Landrace x Yorkshire z-line] x Duroc) weaning piglets, selected from eleven litters, were included in the experiment. Piglets were selected based on their weaning weight to create a uniform group, and then equally distributed to the four dietary treatments based on litter, sex, and weight. The average weaning age was 32.8 ± 1.6 days, and the average weaning weight was 10.6 ± 0.8 kg. There were five pens per treatment with four piglets in each pen.

Full-fat meal of BSFL was produced at HiProMine S.A., Poznan, Poland. The dietary treatments included a control diet and three experimental diets with increasing inclusion of BSFL at 4.76% (BSFL5), 9.52% (BSFL10), and 19.06% (BSFL20). Diets were formulated in collaboration with Felleskjøpet Fôrutvikling AS (Trondheim, Norway), using net energy and SID values (CVB 2016) to be isoenergetic, isonitrogenous, and to meet or exceed the requirements for pigs of this age (NRC 2012). The piglets had *ad-libitum* access to the feed immediately PW. The experiment was terminated on day 28 PW and 12 piglets from each dietary treatment were sacrificed and sampled. Figure 8 shows an overview of the methods used in Paper III.

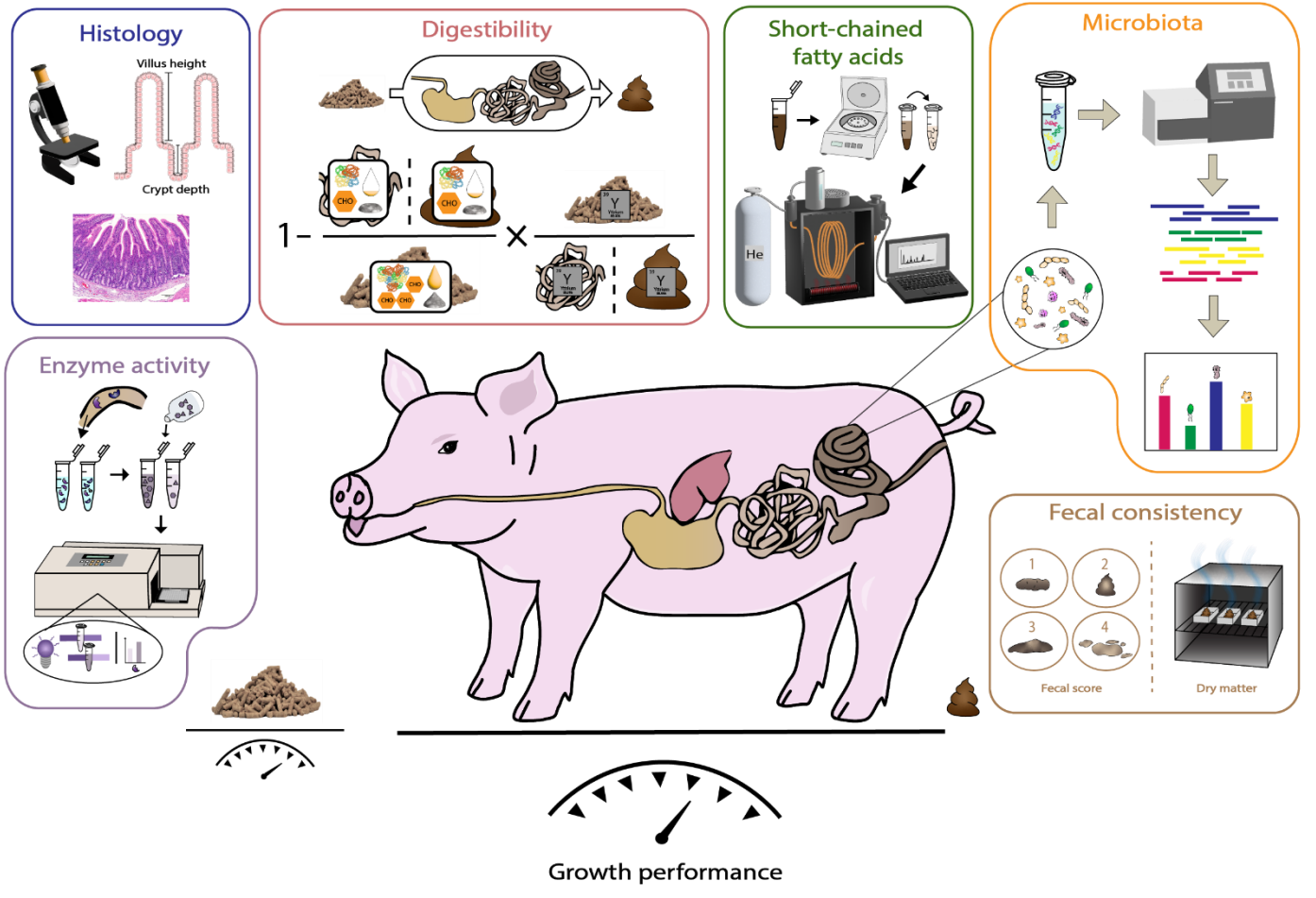

Figure 8 Overview of methods used in Experiment II, Paper III.

4.2 Growth performance and digestive function

Growth performance is an important economical measurement in pig production. In Experiment II, all piglets and feed residues were weighed weekly. Average daily gain (ADG), average daily feed intake (ADFI), and gain:feed ratio (G:F) were calculated per pen. Experiment I was not designed to investigate growth performance, as this was covered by a previous experiment (Cruz et al. 2019).

In both experiments, the effect of the dietary treatments on the digestive function of the piglets was investigated. In Experiment I (Paper I and II), the focus was on the PW development of the digestive function when feeding a diet with yeast or a control diet, whereas in Experiment II (Paper III) the digestive function was assessed to evaluate the inclusion level of insect meal. Methods to evaluate the digestive function included measuring pH, calculation of digestibility coefficients, and measuring enzyme activities. Complete descriptions of the different laboratory procedures are found in the material and methods sections of the respective papers.

Digestibility is an important parameter to assess the digestive function and nutritional value of the feed. In both experiments, we estimated the AID, and in Experiment II the ATTD was also calculated. An external inert marker (0.01% Yttrium(III)oxide: Y_2O_3) was included in the feed for digestibility calculations. Yttrium was chosen as the marker as it has been shown to give high accuracy in low concentrations (Austreng et al. 2000; Vhile et al. 2007). The ileal digestibility was assessed using the slaughter technique. At the sampling endpoint, piglets were euthanized, and intestinal content from the last two meters of the small intestine was collected and analyzed for nutrients and marker concentrations. For estimations of ATTD, feces were individually

collected for five days and the samples were pooled per pig. Marker and nutrient content were analyzed, and the digestibility of a given nutrient was calculated as described by Maynard and Loosli (1969), using the following equation:

Apparent digestibility of nutrient (%) =

$$100 - 100 \times \left(\frac{nutrient\ in\ ileal\ content\ or\ feces\ (\frac{g}{kg})}{nutrient\ in\ diet\ (\frac{g}{kg})} \times \frac{marker\ in\ diet\ (\frac{g}{kg})}{marker\ in\ ileal\ content\ or\ feces\ (\frac{g}{kg})} \right)$$

In Experiment II (Paper III), three of the pens per treatment were installed with rubber mats, but the remaining two had wood shavings as bedding material. The reason for this was an original plan to only investigate digestibility in the piglets that were going to be dissected at the end of the experiment, i.e., pens with rubber mats. The other pens were included to improve the power of growth performance investigations. However, feces samples were collected from all piglets, and we choose to include all samples in the analysis.

4.3 General gut health

As discussed in the introduction, digestive function and gut health are closely linked together. Many of the investigated parameters give information about both gastrointestinal function and health. In both experiments, the VH and CD were measured in the jejunum and ileum (Paper I and III). The gut morphometry is closely linked to intestinal function by affecting absorption surface and brush border enzyme activity but they are also important parameters for the intestinal health assessment. In Experiment II, the intestinal morphological characteristics were also more thoroughly evaluated

by visual inspection, and light microscopy, focusing on changes in epithelial cell and barrier morphology and integrity, crypt changes such as hyperplasia, dilation or abscessation, degenerative and inflammatory mucosal changes including increased numbers of intraepithelial lymphocytes, and infiltration by leucocytes. The different morphological characteristics were graded normal, mild, moderate, or severe.

Fecal consistency at the pen level was evaluated by daily fecal scoring (Paper I and III) and by weekly analyses of fecal DM content (Paper III). The fecal score was assessed based on the four consistency category scale by Pedersen and Toft (2011), with 0.25 intervals. A higher score indicated more watery feces. Scores one and two were considered normal while scoring three and four were considered diarrhea. In addition, every week an approximately equal amount of feces was collected from each pig and pooled for the pen before oven drying at 103°C for 24 h, for analysis of fecal DM. Fecal DM is an objective analysis compared to fecal scoring which is vulnerable to variation between observers (Pedersen & Toft 2011). Ideally, the same person should have done the fecal score classification every day throughout the experiments, but that was not possible.

4.4 Blood biochemistry and immunology

In Experiment I (Paper II), the health of the piglets was investigated more thoroughly. Blood samples were analyzed for biochemical parameters, cytokines, and immunoglobulins. The IgA was also measured in jejunal tissue in Experiment I (Paper I). Complete descriptions of the methods can be found in the respective papers.

4.5 Transcriptomics

Transcriptomics is the study of the complete set of RNA transcripts that are produced by the genome at a given time. Whereas the genome decides what can happen, the transcriptome represents what appears to be happening in the body. In Experiment I, we used transcriptomics to investigate the effect of weaning and feeding yeast on the gene expression in the small intestinal tissue. A detailed description of the method can be found in Paper I. In brief, RNA was extracted from both jejunal and ileal tissue samples. High-quality samples from day zero, two, four, and seven PW (42 piglets) were sequenced at the Norwegian Sequencing Centre, Oslo, Norway. Raw reads were cleaned and aligned to the *Sus scrofa* ENSEMBL genome Sscrofa 11.1 release 98. Differential expression between groups was analyzed independently for jejunum and ileum. The transcriptomic differences PW were investigated in separate heatmaps for each tissue and diet. Significantly differentially expressed genes (DEGs) between time points were included in the heatmaps. Genes with similar expression trends were grouped using hierarchical clustering. Genes from each cluster were then extracted and included in KEGG pathway enrichment analysis. Differences in the PW development of DEGs and KEGG pathway enrichment were used to discuss transcriptomic differences between the dietary treatments. KEGG pathway enrichment analysis was also performed for DEGs in jejunum between piglets fed the yeast and control diet on day seven PW.

4.6 Metabolomics

Metabolomics represents what has happened and is happening in the body. It is the study of small molecules and biological processes that have occurred within an individual. A detailed description of the method can be found in Paper II. In brief, urine and plasma samples were prepared and untargeted

analysis was performed in both positive and negative electrospray ionization mode using a Dionex UltiMate 3000 (Dionex, Sunnyvale, CA, USA) ultra-high pressure liquid chromatography system (UHPLC) coupled with an Impact HD Quadrupole Time-of-Flight (QTOF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Blank samples (H_2O + internal standard), blind samples (0.1% formic acid), and quality control samples (mixture of samples) were frequently injected between runs to ensure the quality of data. Raw spectral data were filtered, and peaks were identified and matched across samples. Retention times were corrected between samples and missing peak data was integrated. Features with retention time < 0.4 min, m/z > 800, features with higher mean values in blinds than quality control samples, detected isotopes, and adducts were discarded. Principal component analysis (PCA) was performed for separate ionization modes and sample days PW, to look for differences in the metabolic profiles between the dietary treatments on each sampling day. Metabolites were identified based on queries in online databases (METLIN, Human Metabolome Database, and LIPID MAPS).

4.7 Microbiota and SCFA

The gut microbiota consists of millions of both symbiotic and pathogenic microorganisms. We studied the microbiome, which is the collective genome of the micro-organisms in a particular environment (Valdes et al. 2018). 16S rRNA sequencing was used to investigate the colon microbiome in Experiment II. A detailed description of the method can be found in Paper III. In brief, content from the spiral colon was collected, and total DNA was extracted from the bacteria. DNA concentration was determined and normalized. Primers were used to amplify the V3-V4 regions of the bacterial 16S rRNA gene. After library preparation and indexing, samples were sequenced on the Miseq system (Illumina, San Diego, CA, USA). Raw sequences were cleaned, truncated, and quality controlled. Denoised read

pairs were merged and chimeras removed. For taxonomy assignment, the Silva v. 138 databases (Quast et al. 2013; Yilmaz et al. 2014) were used as a reference database. The individual microbial diversity within pigs was assessed by calculation of Shannon alpha-diversity indices. The Kruskal-Wallis Rank Sum Test was used to test for differences among dietary treatments. Beta-diversity, the variation in microbial communities among piglets, was assessed by principal coordinate analyses (PCoA) with the Bray-Curtis, unweighted, and weighted UniFrac distance matrices. Pairwise PERMANOVA tests, with multiple testing corrections, were performed to compare beta-diversity among dietary treatments. Differences in relative abundance at the phylum and genera level among dietary treatments were tested using the Kruskal-Wallis test with dietary treatment as the explanatory variable. If significant, a two-sample Wilcoxon test, corrected for multiple testing, was applied for pairwise comparisons. The concentration of SCFA in the colon content was determined by capillary gas chromatography.

5. Results

5.1 Experiment I (Paper I and II)

5.1.1 Post-weaning development of digestive function and health

The overall fecal score increased from day two PW, and PW diarrhea was seen between day four and eight PW (Paper I). Correspondingly, the ileal DM content decreased from day two to four PW before it increased on days seven and 14 ($P = 0.019$; Paper I). The digestive function was also affected by time PW (Paper I). In the jejunum, pH increased from 5.5 on day two, to 6.2 on day four, and 6.4 on day seven PW, before decreasing to 5.7 on day 14 PW ($P < 0.001$). Trypsin activity in the jejunal lumen increased with time PW ($P = 0.001$), whereas the jejunal lipase activity decreased during the first week PW, followed by an increase until day 14 PW ($P = 0.012$). The jejunal brush border enzymes, maltase, ALP, and leucine aminopeptidase (LAP), all showed some similarities in the PW activity patterns. Their activities increased during the first days PW, followed by a decrease in activities from day four to 14 PW ($P < 0.001$). The jejunal brush border enzyme activities correlated negatively with the jejunal CD (maltase: $r = -0.439$, $P < 0.001$; ALP: $r = -0.404$, $P < 0.001$; LAP: $r = -0.545$, $P < 0.001$). The jejunal CD first decreased PW compared with pre-weaning depth, before it increased from day four to day 14 PW ($P < 0.001$). The PW change in CD also caused decreased jejunal VH:CD ratio from day two to 14 PW. In ileum, both VH ($P = 0.008$) and CD ($P < 0.001$) increased with time PW.

Several parameters related to piglet health were measured in plasma (Paper II). Plasma concentrations of total protein ($P = 0.010$) and albumin ($P < 0.001$) decreased from day two PW, whereas plasma concentration of globulin

increased PW (P = 0.001). Plasma IgG concentration decreased until day 7 PW and remained at a low level (P < 0.001), whereas IgM plasma concentration increased PW (P = 0.027). The plasma IgA concentration decreased until day four PW, followed by an increase to above pre-weaning level on day 14 PW (P < 0.001). The PW development of plasma IgA levels correlated well with the IgA concentrations measured in proximal jejunal tissue (P < 0.001). Aspartate aminotransferase (AST), an enzyme used as an indicator of muscular inflammation and damage, increased the first week PW before it decreased on day 14 PW (P = 0.024). The inflammatory cytokine TNFα decreased from day four to 14 PW (P < 0.001). Similarly, the anti-inflammatory IL-10 increased numerically at weaning but then decreased from day two to seven PW (P = 0.001). The concentration of the pro-inflammatory cytokine IL-1β also decreased from day two to seven PW (P = 0.035). A total overview of the PW development of biochemical indices and cytokines can be found in Paper II.

5.1.2 Effect of yeast on the post-weaning digestive function and health

Some differences in the development of digestive function were found when including 14.6% inactivated *C. jadinii* yeast in the diet. Feeding yeast increased the overall DM content in the ileum compared with feeding the control diet (P = 0.007; Paper I). Inclusion of yeast also improved the AID of CP (P = 0.033) and tended to increase the AID of phosphorus PW (P = 0.089; Paper I). Moreover, compared with piglets fed the control diet, piglets fed yeast had higher ALP activity (P = 0.014) and tended to have higher maltase (P = 0.070) and LAP activity (P = 0.098) early PW in the jejunal tissue (Paper I).

In plasma, the PW development of ALP differed between the dietary treatments (P = 0.010; Paper II), but the PW pattern was different compared with the ALP enzyme activity measured in the jejunal tissue (Paper I). Whereas the ALP plasma concentrations in piglets fed yeast remained relatively stable PW, the concentrations in control piglets decreased from above yeast level on day two PW, to numerically below on day seven and 14 PW. The PW development of inorganic phosphate plasma concentrations also differed between yeast and control piglets (P = 0.037; Paper II). After a drop on day four PW, the inorganic phosphate plasma level increased PW in the piglets fed yeast. Whereas in the control piglets, the inorganic phosphate concentration, in general, was lower PW compared with the piglets fed yeast (P < 0.001) and remained relatively stable below the pre-weaning level. These results are in accordance with the higher AID of phosphorus of the yeast diet in Paper I.

Feeding yeast also affected some of the health parameters (Paper II). Piglets fed yeast had lower IgM plasma concentrations PW than the piglets fed control (P = 0.014). The piglets fed yeast and control also had different PW concentration patterns of the anti-inflammatory IL-1 receptor antagonist (IL-1ra) in plasma (P = 0.002). The IL-1ra concentration was 2.4 folds higher on day two PW in the control piglets compared with piglets fed yeast and 2.6-fold higher than the pre-weaning level. The level then decreased, and there were no significant differences in IL-1ra plasma levels between the dietary treatments on the other sampling days. Although not significant, this pattern was also observed for IL-1a and IL-1ß concentrations PW. Moreover, there was a large numerical difference in IL-10 plasma concentration on day 14 PW, where the plasma IL-10 concentration was 1.9 folds higher in the control piglets.

The development of metabolic profiles in urine and plasma was assessed by separate PCA plots for each day PW (Paper II). Increased separation of the metabolic profiles between the two dietary treatments was observed in urine, according to day PW. In plasma, the separation was not that clear, but on day 14 PW the piglets fed yeast were closer grouped together. Betaine was identified as the most discriminating metabolite in both urine and plasma, with higher levels in piglets fed yeast.

5.1.3 Development of post-weaning gene expression in the small intestine

Gene expression in the small intestinal tissue was only investigated during the first week PW (Paper I). The major changes in gene expression by time PW were found in the jejunum of piglets fed the control diet. A total of 2963 genes were differentially expressed with time PW in the jejunum of the control piglets, whereas only 276 genes were differentially expressed with time PW in the jejunum of piglets fed yeast. In the ileum, 2067 and 80 genes in piglets fed the control and yeast diet, respectively, were differentially expressed with time PW. The DEGs in the jejunum of control piglets were divided into two cluster trends. A decreased relative expression with time PW was seen for DEGs in enriched pathways related to environmental information processing and immune system processes, whereas increased relative expression with time PW was found for DEGs in enriched pathways related to endocrine systems, metabolism, and others such as genetic information processing. The full list of enriched pathways can be found in Paper I. Increased jejunal functionality with time PW was also found in the yeast-fed piglets, where the relative expression of DEGs in the global metabolic pathway and the protein digestion and absorption pathway increased with time PW. In accordance with the control piglets, the relative expression of DEGs in the B cell receptor signaling pathway and the hematopoietic cell lineage, both related to the

immune system, decreased PW in the jejunum of the yeast-fed piglets. However, these were the only two identified pathways in that cluster.

The PW jejunal expressions for the DEGs in cluster 1 of the control group were also plotted for the yeast-fed piglets. The PW expression trend for these genes in the yeast-fed piglets differed from the control piglets. The expression trend declined to a large extent from day zero to day two but then increased from day two to seven. The largest difference in gene expression between the dietary treatments was in jejunum on day seven PW. Nine pathways related to the immune system were significantly upregulated in the yeast-fed piglets compared with control on day seven, and the most distinctly enriched pathway was the intestinal network for IgA production.

In the ileum of the control piglets, two opposing expression trends were identified. The first cluster included DEGs which had an increased expression trend from day zero to day two PW, followed by a decline to below day zero level on day seven PW. The enriched pathways were involved in genetic information processing, transport and catabolism (autophagy and mitophagy), along with the NOD-like and RIG-I-like receptor signaling pathways. The DEGs in the other cluster showed an opposite trend and included mainly signaling pathways related to environmental information processing and different pathways in organismal systems. Only a few genes were differentially expressed with time in the ileum of piglets fed yeast.

5.2 Experiment II (Paper III)

5.2.1 Diet

The analyzed crude fat (CF) content of the diets varied from 7% in the control diet, 8% in the intermediate diets (BSFL5 and BSFL10), to 9% in the diet containing the highest inclusion of insects (BSFL20). Due to the high concentration of saturated fatty acids in the insect meal, especially the C:12 lauric acid, there were also differences in the fatty acid composition of the diets. The analyzed sum of saturated fatty acids in the diets increased with increasing inclusion of BSFL, whereas the dietary sum of mono- and polyunsaturated fatty acids decreased with increased BSFL inclusion.

5.2.2 Growth performance and digestive function

Increasing levels of BSFL in the diets gave a negative cubic effect on the average ADG for the whole experimental period ($P = 0.031$). Piglets fed the BSFL5 had the lowest ADG (496 g/day), and piglets fed the control diet had the highest ADG (552 g/day). There were no significant differences in ADFI or G:F. All growth performance results and P-values can be found in Paper III.

The inclusion of BSFL affected the digestion of diets. The ATTD of CP decreased linearly with the increased inclusion of BSFL ($P = 0.011$). Lysine digestibility also decreased with the increasing inclusion of BSFL ($P = 0.020$). Whereas the AID ($P = 0.043$) and ATTD ($P < 0.001$) of CF increased linearly with increased inclusion of BSFL. Dietary treatment did not affect jejunal trypsin or lipase activity (Mean- and P-values can be found in Supplementary Table 2 of Paper III).

5.2.3 Gut health

The overall fecal score increased (>2) in the first week PW until day 12, but there were no differences in fecal score or analyzed fecal DM content among the dietary treatments. The inclusion of BSFL did not affect intestinal VH or CD, but some morphological changes in the intestine were observed (Figure 9). Enterocyte vacuolization was found in the jejunum of piglets fed diets with BSFL ($P < 0.001$). However, no other morphological changes observed in the small intestine were diet-induced. Mild to moderate inflammatory morphological changes were observed in the colon, but the changes were not influenced by dietary treatment.

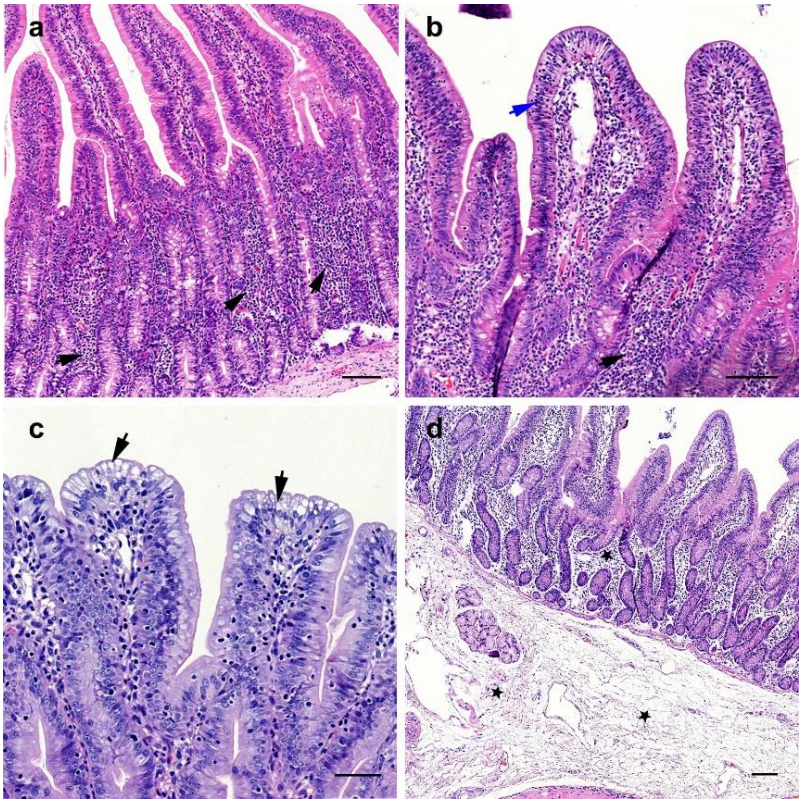

Figure 9 Representative images of the main jejunal morphological changes observed in Experiment II. In all images, the scalebar represents a length of 100 µm.

(a) Increased content of lymphocytes and plasma cells (black arrows) in the inter-crypt region and lamina propria compartment.

(b) Mild increase in the numbers of intra-epithelial lymphocytes in the villi epithelial barrier (blue arrow).

(c) Mild to moderate enterocyte supranuclear vacuolization (black arrows) at the villi tips of the jejunum.

(d) Moderate to marked oedema (black stars) of the submucosa and lamina propria of the jejunum.

5.2.4 Microbiota

The colon microbiota was investigated. However, due to an outbreak of edema disease, which was an ongoing problem at the farm, all piglets were treated with intramuscular antibiotic injections (Borgal vet., Ceva Sant´e Animale, Libourne, France) for three consecutive days (11–13 days PW). This was done after several piglets showed symptoms of edema disease and an experimental piglet suddenly was found dead. It is important to point out that the antibiotic treatment may have affected the microbiota results.

There were no differences in alpha (Shannon, P = 0.355) or beta (Bray-Curtis, unweighted and weighted UniFrac) diversity among the dietary treatments. Bacteroidota and Firmicutes were the dominating phyla in the colon microbiota. Almost half of the detected microbes were classified as Bacteroidota. The abundance differed with dietary treatment (P = 0.026), where the lowest abundance of Bacteroidota was found in the control group. In the piglets fed BSFL, the Bacteroidota abundance decreased with an increased inclusion level of BSFL. The most abundant genus was *Prevotella*, contributing 15.2%, 18.2%, 14.0% and 13.6% to the colon microbiome in piglets fed control, BSFL5, BSFL10, and BSFL20, respectively (P = 0.496). A dietary treatment effect was found for the relative abundance of *Lactobacillus* (P = 0.015). Piglets fed the control diet had a higher relative abundance of *Lactobacillus* compared with piglets fed the BSFL20 diet (P = 0.031).

6. Discussion

This thesis aims to evaluate the two novel feed ingredients *C. jadinii* yeast and BSFL as protein sources for weanling piglets. In addition, this thesis focuses on evaluating the effect of these two novel feed ingredients on the PW gut function and health, as there are indications that bioactive compounds in the ingredients may improve PW challenges. In Experiment I, Paper I and II, the effect on the PW development of gut function and health when including *C. jadinii* yeast as a protein source in diets for piglets were investigated. Piglets were fed a diet with 14.6% inclusion of whole inactivated *C. jadinii* yeast. The gut function and health were investigated through analyses of enzyme activity in the jejunal lumen and epithelial brush border, calculations of AID of nutrients, small intestinal morphometry (VH and CD), assessment of fecal consistency, gene expression of the small intestinal tissue, metabolic profile in urine and plasma, and plasma biochemistry and immunology. In Experiment II, Paper III, increasing inclusion of full-fat BSFL meal (< 19%) was fed to piglets for four weeks PW. The effects on growth performance, nutrient digestibility, gastrointestinal function (e.g., enzyme activity, intestinal morphology, and intestinal morphometry), and microbiota were investigated.

6.1 Yeast and insects as novel feed ingredients

In a northern country like Norway, where food production is challenging, looking for novel feed ingredients grown on alternative substrates is important for self-sufficiency. The *C. jadinii* yeast has been shown to grow well on lignocellulosic biomass from the forestry industry. However, the process from wood to yeast ingredient requires several steps, including pretreatment of the biomass to remove lignin and increase access to cellulose and

hemicellulose, enzymatic hydrolysis to convert the cellulose and hemicellulose into C6 and C5 sugars, yeast fermentation of sugars, nitrogen, phosphate, and other nutrients, and in the end downstream processing into yeast products (Øverland & Skrede 2017). The BSFL is another promising feed ingredient, which recently was approved for pig feed in the EU (European Commission 2021). Insects are highly efficient in converting organic material, and the BSFL thrives in a wide range of organic substrates (Dzepe et al. 2021; Siddiqui et al. 2022). However, even though the BSFL has shown promising results growing on manure (Siddiqui et al. 2022), only vegetable biomass and some animal byproducts are approved substrates for the production of BSFL for feed (European Commission 2021). Mixed wastes such as catering waste are not approved. Diets containing a high amount of mill by-products or vegetable waste has shown promising bioconversion rates by the BSFL. Mixing waste streams might also be beneficial to improve utilization and waste management (Siddiqui et al. 2022).

Both yeast and insects are high-value protein sources, containing about 40-60% CP in DM (Veldkamp & Bosch 2015; Øverland & Skrede 2017). The *C. jadinii* used in this study contained 48% CP on a DM basis, whereas the BSFL meal contained 42% CP on a DM basis. Supplementary Table 1 shows a comparison of the analyzed chemical compositions of the two novel feed ingredients investigated in this thesis. The CP content was determined using the Kjeldahl-N method. The Kjeldahl method converts the nitrogen compounds in the sample into ammonia, which then is quantified and converted into protein by multiplying with a nitrogen-to-protein conversion factor (Jonas-Levi & Martinez 2017). Based on the idea that proteins usually contain approximately 16% nitrogen, a conversion factor of 6.25 is often used and was used to calculate the CP contents in this thesis. However, the accuracy of this conversion factor depends on the protein source (Merrill & Watt 1955).

There was a discrepancy between the content of CP and total amino acids for both ingredients, summarized as non-protein nitrogen (NPN) in Supplementary Table 1. As previously discussed in the introduction, Janssen et al. (2017) suggested that a conversion factor of 5.6 would be more accurate for insects, due to the high content of NPN, such as chitin. Consequently, using the 5.6 conversion factor for insects, the CP content of the BSFL meal was reduced to 38% on a DM basis. It is, therefore, reasonable to think that several authors have overestimated the protein content of insect meals. Chitin is also present in the yeast but is only compromising 1-8% of the yeast cell wall (Aguilar-Uscanga & Francois 2003). However, the discrepancy between the CP and the total amino acid content was also distinct for the yeast. Yeast is rich in nucleic acids. Lapeña et al. (2020) reported a content of 4.4-4.9% nucleic acids in DM of *C. jadinii* yeast grown on different substrates. Agboola et al. (2022) recently reported that 40-44% of the CP in the *C. jadinii* can be made up of NPN, whereas the NPN content ranged from 14-20% of CP in different yeast species in the study by Lapeña et al. (2020). The difference in NPN content of the yeast might be affected by several factors such as strain, growth rate, growth stage, and fermentation conditions (Øverland & Skrede 2017), which makes it difficult to determine the appropriate nitrogen to protein conversion factor. However, Jacob et al. (2019) found a conversion factor of 5.5 to be suitable for yeast extracts, to prevent overestimation of the CP content. Using a conversion factor of 5.5 reduced the discrepancy between CP and total amino acid content of the studied *C. jadinii* yeast and reduced the CP content to 42% on a DM basis (Supplementary Table 1).

The amino acid compositions of the two studied novel protein sources are in general favorable for pigs. However, the sulfur-containing amino acids methionine and cysteine are the first limiting amino acids in both BSFL and yeast (Agboola et al. 2021b; Veldkamp & Bosch 2015; Øverland & Skrede

2017). The CF content was the biggest difference between the two studied protein ingredients. While the *C. jadinii* yeast is low in CF, the BSFL meal consisted of 32% CF and should, therefore, also be considered as a lipid and energy ingredient, in addition to being a protein source.

6.1.1 Dietary treatments

The novel protein sources replaced several of the protein ingredients in the experimental diets. The aim of the thesis was not to compare the novel ingredients with SBM but to investigate their effect ingredients in the feed. Therefore, the novel ingredients partly replaced all protein ingredients on a protein basis. However, when replacing feed ingredients, one should be aware that observed effects could be attributed to the absence of a feed ingredient rather than the inclusion of the novel ingredient. In Experiment I, *C. jadinii* yeast partially replaced SBM, potato protein concentrate, fish meal, and rapeseed meal, compared with the control diet. Experiment I was a follow up from the dose-response study by Cruz et al. (2019), which concluded that the *C. jadinii* yeast (known under its previous name *C. utilis* in the paper) could replace 40% of CP in diets for weaned piglets while maintaining growth and improving digestive function. The diet with the highest dose of yeast from the Cruz et al. (2019) study (14.6% inclusion on diet basis) was, therefore, selected for a more in-depth investigation looking into how the yeast can affect the gut function and health in the two most critical first weeks PW (Experiment I).

Experiment II was a dose-response study where BSFL inclusion increased from 0% to 19% in four diets. Increased inclusion of BSFL meal lowered the inclusion of SBM and reduced the inclusion of soy protein concentrate and fish meal to zero in the diet with the highest BSFL inclusion. In addition, since

the BSFL meal also was a lipid source, the lipid ingredients rapeseed oil and saturated vegetable fat also were reduced by increased inclusion of BSFL. In both studies, the aim was to make the experimental diets as similar as possible in the chemical composition of main nutrients and amino acids. However, chemical analyses of the dietary treatments showed some variations in the nutritional composition of the diets in Experiment II. The gross energy content increased from 19.5 MJ/kg in the control to 19.9 MJ/kg in the BSFL20. Moreover, in the diets containing BSFL, the level of CP increased with increased inclusion of BSFL, whereas the level of starch decreased with increased inclusion of BSFL. The decrease in starch content was caused by a reduction in wheat content when the BSFL inclusion increased.

The CF content increased by increasing the inclusion of BSFL. Because of the high CF level (29%) and the distinctive fatty acid composition of the BSFL, the fatty acid composition among the dietary treatments also varied distinctly (Table 4 in Paper III). The lipid feed ingredients were rapeseed oil, saturated vegetable fat, and the BSFL meal. The saturated vegetable fat, rich in palmitic acid (C16:0), was added as an attempt to make the ratio of saturated and unsaturated fatty acids in the diets more similar because of the high proportion of saturated fatty acids in the BSFL. However, similar fatty acid composition among dietary treatments was not the main focus during diet optimization. Rapeseed oil is low in saturated fatty acids and mostly consists of the monounsaturated fatty acid oleic acid (18:1; 60-90%). It also contains some amount of polyunsaturated fatty acids, mostly the ω-6 linoleic acid (18:2), but also the ω-3 α-linoleic acid (18:3), in a 2:1 relationship (Sakhno 2010). The BSFL is especially rich in saturated lauric acid (C12:0), but also contain considerable amounts of linoleic acid (C18:2; Table 4 in Paper III). Both the inclusion of saturated vegetable fat and rapeseed oil decreased when

the inclusion of BSFL increased, resulting in an increased content of saturated fatty acids and a decreased content of monounsaturated fatty acids by increased inclusion of BSFL. Coconut oil is a lipid source also containing a high amount of lauric acid (Dayrit 2015) and could have been an alternative lipid source to make the fatty acid profile of the diets more similar. However, coconut oil is not a common feed ingredient in Norway.

6.1.2 Growth performance

It was hypothesized that full-fat BSFL meal could replace traditional dietary protein and lipid sources in a balanced diet without adverse effects on growth performance. Some differences in growth performance were observed between the dietary treatments in Paper III. There was a negative cubic effect of increased BSFL inclusion level on the ADG. Piglets fed the control diet had the overall highest ADG, whereas piglets fed the lowest BSFL inclusion had the lowest ADG for the overall experimental period, differing with 56 g/day. Although not significant, the ADFI showed the same numerical trend (Table 5 in Paper III). The reason for reduced growth performance by the low inclusion level of BSFL is not clear, and contradictory to Yu et al. (2020). Yu and coworkers reported a linear improvement in both ADG and feed to gain ratio when increasing dietary BSFL inclusion from 0% to 4% (replacing fish meal), in the first two weeks PW, whereas no differences were found for the four-week feeding period. Moreover, in a study by Biasato et al. (2019), inclusion of up to 10% of partially defatted BSFL, replacing SBM in a corn and barley based diet, did not affect the growth performance in piglets for 61 days PW. Also, Ipema et al. (2021) reported that access to live BSFL PW did not influence growth performance even though pellet intake decreased.

The study design of Experiment I did not include growth performance. Therefore, it is not possible to compare growth performance results between the two experiments in this thesis. However, feeding the same yeast, Cruz et al. (2019) reported growth performance results for four weeks PW and concluded that growth performance was not affected by inclusion of up to 14.6% yeast. The reported growth performances by Cruz et al. (2019) were in general lower than the growth performances reported in Paper III when feeding BSFL. This was also the case when comparing the growth performance of piglets fed the control diets in the two studies. In Cruz et al. (2019), piglets were fed individually two to three times a day, whereas in Paper III piglets had *ad libitum* access to the feed throughout the whole experimental period. This resulted in a higher ADFI for the pigs in the BSLF study in this thesis compared with the ADFI reported by Cruz et al. (2019). Therefore, it is not possible to directly compare the growth performance of these two studies.

In another study, piglets were fed a yeast-based weaning diet followed by a rapeseed-based diet during the growing/finishing period (Iakhno et al. 2021). The yeast diet was the same as in Experiment I, Paper I and II, of this thesis, and piglets had *ad libitum* access to the dietary treatments. Growth performance results for the first four weeks PW of the study by Iakhno et al. (2021) are presented in Supplementary Table II. There were no differences in growth performance between the dietary treatments, and growth performance results were improved compared with Cruz et al. (2019), but still lower than in Paper III. However, in Paper III, the energy level of the diets was higher, probably due to the high fat content in the diets which was required to get high inclusion levels of the lipid-rich BSFL meal. Also, piglets in the study by Iakhno et al. (2021) were weaned at 28 days of age compared with 32 days of age in Paper III. Espinosa et al. (2020), reported that the torula

yeast could replace fish meal and plasma protein without affecting growth performance two weeks PW. Similar to Cruz et al. (2019) they also reported that increased dietary yeast inclusions improved G:F ratio in week three to four PW. In conclusion, both the *C. jadinii* yeast and BSFL meal seem to be suitable ingredients in a balanced diet for piglets which does not compromise the PW growth performance.

6.2 Can feeding *C. jadinii* yeast or black soldier fly larvae reduce weaning challenges?

6.2.1 Digestive function and development post-weaning

At the time of weaning in today's commercial production systems, the piglets feeding behavior, gut function, and immune system are still immature, and the physiological and dietary stress that weaning is adding is negatively affecting the piglets' GIT and their overall health (Lallès et al. 2004). As a result, PW diarrhea is common during the two first weeks PW (Madec et al. 1998). The aim of Experiment I was to examine early PW physiological changes in piglets, as well as to investigate how these changes were affected by dietary inclusion of *C. jadinii* yeast. Sampling occurred at several time points (days zero, two, four, seven, and 14 PW) in the two first weeks PW, to follow the development of the digestive system PW.

The fecal scores increased during the first week PW in both experiments presented in this thesis. A higher score indicates more watery feces, and the highest scores (>2) were seen around day four to eight PW before they decreased and stabilized in the second week PW. These results are consistent with the cohort study by Madec et al. (1998), involving 106 farms in France, which found diarrhea usually occurring three to four days PW, with a maximum prevalence around day seven to nine PW. Independent of dietary

treatment, more watery feces were observed in Experiment I than in Experiment II. In Experiment I, piglets were almost a week younger at weaning (27 days of age) than in Experiment II (33 days of age), and due to the removal of piglets for sampling the pen dynamic was constantly changed in the first week PW, which might have contributed to a higher stress level for the piglets in Experiment I. Interestingly, the development in ileum DM content also showed the same PW pattern as the fecal score (Paper I). Also, the PW development in ileal DM positively correlated with the PW development of AID of organic matter, CP, CF, and phosphorus, clearly indicating a disrupted digestive function in the first week PW.

Using easily digestible protein sources is especially important to avoid excessive fermentation of protein in the digestive tract of the newly weaned piglet. Both protein level and source are known to affect incidences of PW diarrhea (Heo et al. 2012). It was hypothesized that the *C. jadinii* yeast would be a highly digestible protein source for piglets. This was confirmed in Paper I, where the inclusion of *C. jadinii* yeast improved the AID of CP compared with the control diet. Improved digestibility of CP by inclusion of *C. jadinii* yeast was also reported in the study by Cruz et al. (2019). Moreover, the SID of amino acids in torula yeast have been reported to be greater than for fish meal (Lagos & Stein 2020). By contrast, the ATTD of CP decreased with increased inclusion of BSFL (Paper III). However, there was no such trend or difference in the AID of total amino acids, except for some of the individual amino acids including lysine whose digestibility decreased with increased inclusion of BSFL. The BSFL meal had lower lysine content than the *C. jadinii* yeast (Supplementary Table 2). Due to a lack of information on SID coefficients for lysine in BSFL when diets were made, the SID of lysine might have been overestimated, as discussed further in Paper III. More studies are needed to standardize the SID coefficients of the different amino acids in BSFL meal.

The CP digestibility of the BSFL diets, and especially the AID of CP reported in Paper III, was lower than the CP digestibility reported for the yeast diets in Cruz et al. (2019). The reason might be a high level of NPN in the BSFL diets, as discussed previously, but differences in feed intake caused by different feeding regimes might also have contributed to the different CP digestibility between the studies. Contrary, when feed intake increased in growing pigs, Moter and Stein (2004) found increased AID of CP and most amino acids and increased total daily basal ileal endogenous losses. However, reduced digestibility of CP by increased dietary inclusion of BSFL has also been observed in Atlantic salmon (Weththasinghe et al. 2021a). The authors discussed that the reduced protein digestibility probably could be explained by the poorly digestible chitin in BSFL and that the reduction in digestibility of some of the amino acids was because they were bonded to chitin, as insects use protein-chitin cross-linked fibers in their exoskeleton (Komi et al. 2018) and, therefore, are concealed for enzymatic digestion.

The availability of intestinal substrate affects the digestive enzyme secretion (Makkink et al. 1994). Abrupt weaning causes a change in available nutrient content, which the digestive system needs to adapt to. During the nursing period, lactose was an important energy source for the intestinal epithelial cells (Spreeuwenberg et al. 2001), whereas lactose often is absent in the weaning diet. Low feed intake during the first days PW is considered the main cause of several functional and structural changes occurring in the acute PW phase, including villus atrophy (Dong & Pluske 2007; Jayaraman & Nyachoti 2017; Makkink et al. 1994; Montagne et al. 2007). In Paper I, the PW VH:CD ratio was positively correlated with ileal DM, meaning that piglets having lower villi or deeper crypts had more watery content in ileum. A shortening of villus and deepening of crypts result in fewer absorptive and more

secretory cells, as correlated to the number of villus enterocytes and crypt cells (Nabuurs et al. 1993). There was also a negative correlation between the CD and the brush border enzymes maltase, ALP, and LAP. The intestinal cells are continuously renewed by differentiation along the crypt-villus axis, expanding from stem cells located near the base of the crypts (Yang et al. 2013), and deeper crypts imply increased rate of enterocyte production, resulting in immature cells with lower digestive enzyme activities.

Intestinal ALP activity PW was higher in piglets fed *C. jadinii* yeast early PW compared with control. Maltase and LAP activities also tended to be higher in piglets fed yeast, although the inclusion of yeast did not influence PW VH or CD (Paper I). Piglets fed yeast also had higher ileal DM content than piglets fed control (Paper I). It was hypothesized that the inclusion of *C. jadinii* would reduce incidences of PW diarrhea. Cruz et al. (2019), reported a linear increase in fecal DM on day 7 PW by increasing the inclusion of dietary yeast. However, in Experiment I, there was no difference in fecal score between the dietary treatments and, therefore, no basis to support this hypothesis. Even though, collectively the results indicate a local positive effect of feeding yeast on the development of digestive function early PW. Feed intake, hygiene, and management are all important factors affecting incidences and severity of PW diarrhea (Madec et al. 1998), and it might be that the effect of yeast would be more evident in a more challenging environment. Lee et al. (2021) found a tendency for lower diarrhea frequency in the two first weeks PW, when feeding a corn-based diet supplemented with 0.05% yeast cell wall products to piglets weaned at 28 days of age. The weaning age was similar to Experiment I, but piglets were approximately 8 kg at weaning, whereas piglets in Experiment I were 10.2 kg. Moreover, supplementation with live *S. cerevisiae* has been shown to reduce daily diarrhea score, diarrhea duration, and ETEC exertion, when challenged with an ETEC strain PW (Trckova et al.

2014; Trevisi et al. 2015). Whereas, in another study, inclusion of up to 10.8% torula yeast in a corn-based diet did not alter fecal score or diarrhea frequency PW (Espinosa et al. 2020).

To summarize this section, an early critical phase with a reduction in digestive function was observed PW. These results are in coherence with Montagne et al. (2007) who have described an initial acute phase in intestinal function < 5 days PW, followed by a maturational phase where the gut adapts to the weaning diet. The *C. jadinii* yeast appeared as a highly digestible protein source which seemed to improve the digestive function early PW. Moreover, further investigations are needed to determine the digestibility of insect CP to optimize piglet diets.

6.2.2 Gut homeostasis post-weaning

Homeostasis is the organism's maintenance of constant and stable physical-chemical conditions in the internal environment (Holck & Hauge 2020). The body will always strive to obtain homeostasis, a state of equilibrium, as it is crucial for optimal body functions, but different events and environmental factors might interrupt this state. Weaning is definitively an event affecting gut homeostasis. In the main hypothesis of this thesis, it was hypothesized that the investigated feed ingredients would improve gut homeostasis. This part of the hypothesis was based on the knowledge of bioactive compounds in the investigated novel ingredients. The yeast cell wall polysaccharides β-glucan and mannans are known to be prebiotic, stimulate the immune system, and adsorb pathogens and mycotoxins (Kogan & Kocher 2007; Shurson 2018). The chitin polysaccharide from the insect cuticle also has the potential to function as a prebiotic and immunostimulant (Song et al. 2014). Whereas the MCFA lauric acid, the dominant fatty acid of BSFL, is known to have

antimicrobial effects (Zentek et al. 2011). Moreover, the insect AMPs definitively have potential as health promoters in pigs, but more research is needed to investigate the *in vivo* effects of these when feed as insects (Wang et al. 2016).

Microbiota

A healthy gut was defined by Celi et al. (2017) as: "*A steady state where the microbiome and the intestinal tract exist in symbiotic equilibrium and where the welfare and performance of the animal is not constrained by intestinal dysfunction*". The gut microbiome is important for gut homeostasis, as it is involved in the nutritional, physiological, and immunological functions of the pig (Fouhse et al. 2016). Dietary composition, including dietary protein sources as well as fermentable carbohydrates, is heavily affecting the gut microbiome and the fermentation activities (Rist et al. 2013). The inclusion of BSFL meal reduced the relative abundance of *Lactobacillus* in the colon of the piglets (Paper III). The lactic acid-producing *Lactobacillus* is in general thought to be health-promoting bacteria and are commonly used as probiotics (Ohashi & Ushida 2009). The lauric acid-rich BSFL fat has been shown to inhibit the growth of *Lactobacillus*, *in vitro* (Spranghers et al. 2018), but contradictory results are found *in vivo* in pigs (Nekrasov et al. 2018; Spranghers et al. 2018; Yu et al. 2019). Iakhno et al. (2020) studied the microbiome of the piglets in Experiment I of this thesis and found higher *Lactobacillus* counts PW in all intestinal parts (jejunum, ileum, cecum, and colon) in piglets fed yeast compared with control. Increased *Lactobacillus* counts were also found in feces of piglets whose diet was supplemented with a combination of 3% *S. cerevisiae* and 3% blood plasma (Sampath et al. 2021). Whereas only inclusion of *S. cerevisiae* (6%) did not improve *Lactobacillus* counts in the study. Also, supplementation with live *S. cerevisiae* yeast

reduced the amount of *Lactobacillus* in feces of piglets weaned at 17 days of age, in comparison with a diet containing AGP (Van Heugten et al. 2003).

High gut microbial diversity is in general believed to be beneficial and a sign of good health (Patil et al. 2020). The inclusion of BSFL meal did not alter the alpha diversity in the colon of piglets (Paper III). On the other hand, feeding yeast gave piglets less diverse microbial communities in the large intestine on days seven, 14, and 28 PW, but did not affect the alpha diversity in the small intestine (Iakhno et al. 2020; Lagos et al. 2020). Iakhno et al. (2020) found that diet explained 26% of the microbial variance (beta diversity) in the large intestine of piglets fed yeast or control at 14 days PW. Lagos et al. (2020), also reported that inclusion of yeast affected beta diversity in the large intestine. Moreover, in another study with the same yeast, increased fecal diversity was observed in piglets fed yeast compared with control on eight and 22 days PW (Iakhno et al. 2021). By contrast, there were no differences in beta dispersion among the dietary treatments in the colon of piglets fed with increased inclusion of BSFL (Paper III). However, several authors have reported increased microbial diversity when replacing SBM with BSFL. In growing pigs, feeding BSFL as a single protein source increased alpha diversity in jejunum, compared with feeding SBM (Kar et al. 2021). Moreover, replacing SBM, the inclusion of 5 or 10% of partially defatted BSFL meal did not affect cecal alpha diversity, but increased beta diversity compared with control, 61 days PW (Biasato et al. 2020). Contrary, in Atlantic salmon, feeding 20% full-fat BSFL reduced alpha diversity, as measured by Shannon's index, when partly replacing wheat bran, SPC, fish meal, and fish oil (Weththasinghe et al. 2022).

The microbial composition of the diets might also differ and contribute to the result, and should, therefore, be considered when investigating microbiota.

Even though high temperature during feed processing eliminates most viral bacteria, their DNA might still be present and identified by sequencing. An overlap between feed and gut microbiota was observed in Atlantic salmon, where several of the high abundant taxa found in the BSFL feed also have been reported found in the gut of the BSFL (Weththasinghe et al. 2022). In summary, results are not consequent and suggest no clear conclusion of the two novel feed ingredients' effect on gut microbial diversity. More research is needed, and the results are probably affected by several factors, such as feed composition and replaced feed ingredients.

Immune response

The host interaction with the gastrointestinal microbiota is one key component affecting gastrointestinal functionality and health (Celi et al. 2017). A balance between immune response and tolerance to the intestinal microbiota is crucial in maintaining gut homeostasis (Sun et al. 2015). The GIT is the largest immune organ in the body, accounting for over 70% of the pigs' total immune cell population (Willing et al. 2013), and the gut microbiota plays an important role in the development of the gastrointestinal immune system post-partum (Stokes et al. 2004). The pigs' gastrointestinal immune system reaches an adult-like structure at seven weeks of age (Zheng et al. 2021) and is, therefore, not fully developed at weaning, while at the same time exposed to several new challenges.

It was hypothesized that feeding yeast would stimulate the immune system, mainly due to the cell wall polysaccharides. Stimulating the immune system is thought to be beneficial for the piglet to be prepared for pathogenic microorganisms in the PW environment. Feeding sows a diet supplemented with live (active dry) yeast, increased milk IgG concentration, and reduced

diarrhea incidences in piglets (Jurgens et al. 1997; Zanello et al. 2013). In Paper I, PW intestinal gene expression patterns differed between piglets fed yeast and control. In the control piglets, the expression of several genes in immune-related pathways declined with time PW, whereas in piglets fed yeast, the expression of the same genes declined from day zero to day two PW before increasing from day two to day seven PW. It has been suggested that immunosuppression is an optimal maturation strategy for the immune system during early GIT development, and that excessive immune stimulation might disrupt the development and long-term function of the gut immune system (Moeser et al. 2017). However, the immune system must react quickly in case of a rupture of the epithelial barrier, and complex regulation of the balance between control and reactivity is, therefore, required. The gene expression results in Paper I suggest a faster recovery of the PW immunosuppression in the piglets fed yeast, probably caused by immunostimulant effects of the yeast cell wall promoting an earlier maturation of the GIT immune system. On the other hand, there were few differences between dietary treatments in plasma cytokines and immunoglobulin levels reported in Paper II, suggesting the effect of yeast to be local rather than systemic. Yeast having a local rather than systemic effect was also suggested by Lagos et al. (2020). The authors reported no effect of yeast on hematological or biochemical parameters in blood PW, whereas feeding yeast increased the number of natural killer cells in the distal jejunal lymph nodes (Lagos et al. 2020). In Paper II, there were no differences in plasma biochemical parameters, except for the higher inorganic phosphate concentrations in piglets fed yeast, which corresponds to the increased digestibility of phosphorus by yeast inclusion, reported in Paper I and in Cruz et al. (2019). On the other hand, the plasma cytokine PW patterns in Paper II implied an early PW acute phase, as was also suggested by Pié et al. (2004). This acute phase seemed to be less evident in the yeast-fed piglets.

The PCA plot of plasma metabolomic profiles showed a grouping of piglets fed yeast on day 14 PW (Paper II), also implying that yeast has some systemic effect. However, the observed effect in plasma metabolic profile was related to change in metabolic substrates from digesting yeast, as among the identified metabolites most were fragments of fatty acids, amino acids, and yeast compounds. The separation in metabolomic profile was even more evident in urine, where the PCA plots showed a clear separation of the dietary treatments on day 14 PW (Paper II). Betaine was identified as the most discriminating metabolite in both plasma and urine, with considerably higher levels in the yeast-fed piglets. Betaine is a commonly used feed additive but was not included in the diets of Experiment I. However, betaine is known to be high in molasses (Fu et al. 2021), which was part of the cultivation substrate used for the studied *C. jadinii* yeast (Paper I). Betaine acts as a methyl group donor and osmoprotectant. Supplementation has shown to improve growth performance by reducing the pigs' maintenance-energy demands, increasing serum growth hormone and insulin-like growth factor-1 levels PW, maintaining an osmotic balance of the intestinal tissue, increasing water retention activity of the gut, and improving nutrient uptake by influencing intestinal cell structure, digestive enzyme activity, and intestinal microbiota (reviewed in Fu et al. 2021). Thus, the high level of betaine may partly explain some of the beneficial effects of the yeast, such as the increased ileal DM content reported in Paper I.

There are no studies similar to Experiment I which are investigating the effect of BSFL meal on PW gut development, and there is limited information about the effect of BSFL on the gut immune system. Yu et al. (2019) reported that BSFL inclusion may enhance mucosal immune homeostasis by altering the microbial composition and metabolic profile of the colon mucosa in finishing

pigs. They found BSFL inclusion to upregulate the expression of anti-inflammatory cytokines and intestinal barrier genes in the colonic mucosa, whereas the expression of pro-inflammatory cytokines was downregulated. The authors also reported a correlation between the changes in colonic microbiota and metabolites and the alterations of the epithelial gene expression. Moreover, other studies have reported that inclusion of up to 4% full-fat or 10% partially defatted BSFL meal does not negatively affect the health status of pigs (Biasato et al. 2019; Yu et al. 2020). Yu et al. (2020) found that including full-fat BSFL meal reduced the pro-inflammatory cytokine IFNγ and increased the level of the anti-inflammatory cytokine IL-10 and IgA in serum and suggested that BSFL meal has beneficial effects on piglet immune homeostasis. To summarize this section, the results indicate that *C. jadinii* yeast or BSFL in diets for PW piglets could improve gut homeostasis by altering the gut microbiome and immunological responses.

6.3 Future perspectives

This thesis has shown that both *C. jadinii* yeast and BSFL meal are suitable novel protein sources for piglets. However, several factors are influencing the future use of these novel ingredients in commercial pig feed, including research on feed technology and production optimization, the availability of substrates, regulations, and economic aspects. In the introduction of this thesis, the arguments supporting the need for novel protein sources were increased self-sufficiency and more sustainable pig production. The recently published Norwegian government platform (*Hurdalsplattformen 2021-2025*), emphasizes the goal of increased self-sufficiency and feed sustainability in Norway. Manufacturing yeast and insects for animal feed has the potential to help achieve this goal. However, for sustainable production of these feed ingredients, it is important to utilize biomasses that are not suitable for human consumption or that can be used directly as feed ingredients. Waste

streams, lignocellulosic biomass, and even manure have been suggested as sustainable substrates for yeast and insect production. However, the strict EU regulations are limiting the use of several biomasses. Future research should focus on identifying resources and side-streams in Norway suitable for yeast and insect production and develop regulations that enable the utilization of such resources while maintaining food safety. Research should also focus on how to optimize the production to be part of a circular bioeconomy. Organization of low-cost substrate streams, development of production facilities for large-scale production, and optimization of production performance are important factors for the novel ingredients to be produced at an affordable price. Moreover, downstream processing, such as drying, is a significant production cost, as it requires high energy input. Drying and other downstream processing methods also have the potential to change nutrient availability, in both directions. Research is needed to optimize the downstream processes to maintain or improve nutrient availability while reducing cost and energy use.

In the question of to which extent inclusion of *C. jadinii* yeast or BSFL meal improves PW challenges, the literature results are not consequent and are probably affected by different diet compositions, management, and health status of the piglets. It should be noted that neither inclusion of *C. jadinii* yeast or BSFL meal seems to increase the risk of PW challenges or negatively affect the health of the piglets. However, further investigations are needed to clarify the effect under different conditions, and at which dose the ingredients would be most effective with the goal to improve PW gut function and health. There are also no studies investigating how a combination of these two novel ingredients would affect the piglets.

In the end, some speculations about the different future potentials of the two investigated novel ingredients. Due to the long and complex process of producing yeast from lignocellulosic biomass, yeast probably has the greatest potential as an ingredient or additive in the PW period, where the inclusion of yeast has shown several beneficial health effects on the piglets. Whereas insects, with their wide range of substrates and rapid growth of biomass, have potential as a feed ingredient in the whole lifespan of pigs and other livestock.

7. Conclusions

This thesis has evaluated *C. jadinii* yeast and BSFL as two novel protein sources for piglets that can be produced on biomasses and side-streams unsuitable for human consumption, and thereby be part of the solution for increased self-sufficiency and sustainable feed production in Norway. The results indicate that both *C. jadinii* yeast and BSFL meal are suitable protein ingredients for PW piglets, but future studies should investigate methods to improve the protein digestibility of the BSFL. In addition, the effect of the two novel feed ingredients on PW challenges has been evaluated. An early acute phase was observed for digestive functions and immunological responses PW. The inclusion of 14.6% *C. jadinii* yeast seemed to reduce the severity of the early acute PW phase. Moreover, both *C. jadinii* yeast and BSFL have the potential to improve gut homeostasis by altering the gut microbiome and immunological responses. Consequently, the inclusion of *C. jadinii* yeast or BSFL meal as protein ingredients could give more robust piglets PW, but especially the *C. jadinii* yeast showed potential to improve gastrointestinal challenges PW.

8. Supplementary information

Supplementary Table 1 Comparison of the analyzed chemical composition of the two investigated ingredients

Composition, g/kg DM	*Cyberlindnera jadinii* yeast	Black soldier fly larvae meal
Main nutrients		
DM, g/kg	970	905
Crude protein[a]	484 (426)	420 (376)
Crude fat	16	320
Ash	80	93
Calcium	NA	19.2
Total phosphorus	0.97	8.6
Indispensable AA		
Arginine	25.2	18.4
Histidine	8.8	10.0
Isoleucine	22.3	18.2
Leucine	32.6	33.6
Lysine	31.5	24.2
Methionine	5.3	7.2
Phenylalanine	19.0	16.6
Threonine	26.3	16.7
Tryptophan	6.4	NA
Valine	26.6	23.8
Dispensable AA		
Alanine	29.1	30.7
Aspartic acid	43.2	36.7
Cysteine	4.1	3.0
Glutamic acid	69.1	48.4
Glycine	20.0	20.2
Proline	17.1	22.7
Serine	24.7	15.7
Tyrosine	13.9	22.4
Total amino acids	425.2	368.6
Non-protein nitrogen[b]	59.3 (1.1)	51.4 (7.7)

[a]Kjeldahl-N conversion factor: 6.25. Numbers in parentheses are crude protein calculated with a conversion factor of 5.5 for yeast, as in Jacob et al. (2019), and 5.6 for insect meal, as suggested by Janssen et al. (2017).

[b]Calculated as: crude protein – total amino acids. Note that total amino acids for insect meal does not include tryptophan. Numbers in parentheses are calculated with the 5.5 and 5.6 Kjeldahl-N conversion factors for yeast and insects, respectively.

Supplementary Table 2 Unpublished growth performance results from the first period of an experiment with yeast in PW period followed by a rapeseed diet in the growing/finishing period (Iakhno et al. 2021)

Growth performance[a] (0-28 days PW)	Control	Yeast
ADG, g/day	445	443
ADFI, g/day	644	642
G:F	0.69	0.69

[a]ADG: average daily gain; ADFI: average daily feed intake: G:F: gain to feed ratio

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Gene expression and gastrointestinal function is altered in piglet small intestine by weaning and inclusion of *Cyberlindnera jadinii* yeast as a protein source

Ingrid Marie Håkenåsen[a], Margareth Øverland[a], Ragnhild Ånestad[a], Caroline Piercey Åkesson[b], Arvind Y.M. Sundaram[c], Charles McLean Press[b], Liv Torunn Mydland[a,*]

[a] Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Aas, Norway
[b] Department of Preclinical Sciences and Pathology, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway
[c] Department of Medical Genetics, Oslo University Hospital, Oslo, Norway

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ABSTRACT

This study investigated the effect of feeding *Cyberlindnera jadinii* yeast on the development of gastrointestinal function and health in piglets during the first two challenging weeks after weaning. Changes in gastrointestinal function were mainly attributed to weaning, and not to dietary treatment. The post-weaning (PW) transcriptome profiles differed between dietary treatments showing an overall higher number of differentially expressed genes (DEGs) in control piglets than in yeast-fed piglets. DEGs in jejunum and ileum were compared between sampling timepoints within each feeding group and divided into clusters with similar expression trends. Pathway enrichment analysis was run on each cluster to reveal PW physiological changes. Weaning induced downregulation of several immune functions in the control piglets, which was not as evident in the yeast fed piglets. The results indicate that feeding *C. jadinii* yeast can improve PW gut homeostasis and give more robust piglets.

1. Introduction

Weaning is a critical time for piglets because major changes occur, including abrupt transition in diet, maternal separation, change in environment, increased exposure to pathogens and dietary or environmental antigens (Campbell, Crenshaw, & Polo, 2013; Lallès, Bosi, Smidt, & Stokes, 2007), stress associated with litter mixing and establishment of a social hierarchy. High stress causes activation of physiological mechanisms to maintain body homeostasis (Jayaraman & Nyachoti, 2017), and when combined with reduced energy intake, may result in disruption of normal epithelial, immune and enteric nervous system development (Moeser, Pohl, & Rajput, 2017). Therefore, weaning is often associated with a post-weaning (PW) decline in growth performance and a higher frequency of diarrhea.

Several authors have described PW morphological changes such as villous atrophy and crypt hyperplasia (Cera, Mahan, Cross, Reinhart, & Whitmoyer, 1988; Degroote et al., 2020; Lallès et al., 2007), changes in enzyme activity (Hampson & Smith, 1986; Lindemann, Cornelius, El Kandelgy, Moser, & Pettigrew, 1986; Makkink, Negulescu, Guixin, & Verstegen, 1994) and increased intestinal permeability (Moeser et al., 2017; Spreeuwenberg, Verdonk, Gaskins, & Verstegen, 2001). The changes induced in the gastrointestinal tract (GIT) by weaning are temporary and can be divided into two periods: an initial acute period immediately after weaning, followed by a more progressive adaptative and maturational phase (Montagne et al., 2007). However, the severity is dependent by the piglets age at weaning (Moeser, Ryan, Nighot, & Blikslager, 2007). Epithelial atrophy implies a reduction of intestinal absorptive capacity. Wang et al. (2008), compared jejunal gene expression in weaned piglets and age-matched suckling piglets and found a PW reduction in gene expression associated with oxidative defense capacity, intestinal transport and utilization of dietary nutrients, immune response, synthesis of glycoproteins and proliferation and differentiation of intestinal epithelial cells, whereas water permeation across the intestinal wall was enhanced. The adverse intestinal morphological and functional change PW is attributed to low feed intake, which causes limited energy supply to the gut epithelium (Dong & Pluske, 2007; Jayaraman & Nyachoti, 2017). The withdrawal of milk from the sow does not only mean a change from liquid to solid feed, but also a change in the main energy source from milk fat and lactose to starch (Spreeuwenberg et al., 2001). The withdrawal of milk also stops

* Corresponding author at: Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway.
E-mail address: liv.mydland@nmbu.no (L.T. Mydland).

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the supply of maternal antibodies (IgA) and other bioactive compounds, thus increasing the risk of enteric diseases in piglets (Heo et al., 2013).

After the antimicrobial growth promoter ban in the EU in 2006, yeast or yeast extracts have been investigated as non-antibiotic functional products to improve gut function and health of piglets PW (Heo et al., 2013; Liu et al., 2018). The yeast cell wall consists of different polysaccharides, such as mannan oligosaccharides and β-glucans (Shurson, 2018; Øverland & Skrede, 2017). Mannan oligosaccharides are known to suppress the toxic activity of mycotoxins and reduce pathogenic intestinal colonization by blocking the binding of pathogenic bacteria to the intestinal wall (Spring, Wenk, Connolly, & Kiers, 2015). β-glucans are known to stimulate the gut immune system (Vetvicka & Oliveira, 2014). In the nursing period, piglets receive passive immunity through the sow's milk, which gradually declines as the piglets develop their own immune functions until natural weaning. Under commercial conditions, weaning occurs when the adaptive immune system of the piglets is immature (Moeser et al., 2017), and stimulation of the piglet's immune system is therefore thought beneficial. Yeast also contains about 6–10% nucleic acids in a dried inactivated state (Øverland & Skrede, 2017). In nutrient deficiency periods, dietary nucleotides may be important in tissues with a high turnover rate such as the intestinal mucosa (Sauer, Mosenthin, & Bauer, 2011). Supplementation of the diets of PW piglets with yeast or yeast extracts has been shown to increase villus height (VH) (Bontempo, Di Giancamillo, Savoini, Dell'Orto, & Domeneghini, 2006; Cruz et al., 2019; Van der Peet-Schwering, Jansman, Smidt, & Yoon, 2007), improve the main nutrient total tract digestibility (Shen et al., 2009) and reduce PW diarrhea. Cruz et al. (2019) investigated piglets after a four-week period PW and showed promising growth performance and health results of weanling piglets fed diets with *Cyberlindnera jaidinii* (previous name: *Candida utilis*) as a protein source.

The objective of this study was to investigate the effect of high dietary inclusion of *C. jadinii* yeast on GIT function and health development during the two first weeks PW, as assessed by fecal score, apparent ileal digestibility (AID) of nutrients, enzyme activity, gut morphology, and gene expression profiles.

2. Materials and methods

The experiment was performed between 30th of October and 13th of November 2017 at the Center for livestock production (SHF), NMBU, Ås, Norway, which is an animal experiment unit approved by the National Animal Research Authority (permit no. 174). All piglets were handled in accordance with laws and regulations controlling experiments with live animals in Norway (regulated by the "Animal Welfare Act" and "The Norwegian Regulation on Animal Experimentation" derived from the "Directive 2010/63/EU on the protection of animals used for scientific purposes").

2.1. Animals and housing

A total of 64 crossbred [(Norwegian Landrace × Yorkshire z-line) × (Duroc) and (Norwegian Landrace) × (Duroc)] weanling piglets, selected from eight litters, were included in the experiment. Average weaning age was 27.4 ± 1.15 days and the average weaning weight 10.24 ± 1.56 kg. Piglets were allocated to dietary treatment and day of dissection based on litter origin and weaning weight. There were six pens per diet and five or six piglets per pen. Piglets were housed in an environmentally controlled room, with temperature average of 19.9 ± 1.05 °C. Temperature was logged every morning. Pen size was 1 m × 1.58 m, with a 0.8 m^2 area of slatted concrete floor in the front of the pen. In the rear, low roofing, covering 1 m^2, constituted a sheltered resting area. A rubber mat was provided in the resting area.

Table 1
Dietary composition of experimental diets, calculated total crude protein (CP) content in diets and calculated crude protein content from yeast in diets.

	Dietary treatments	
Ingredient, g/kg as fed	Control	Yeast
Wheat	627.8	593.5
Barley	100.0	100.0
Oats	50.0	50.0
Soybean meal[1]	80.0	19.2
Potato protein conc.[2]	33.8	9.1
Fish meal[3]	20.0	4.8
Rapeseed meal[4]	20.0	4.9
Yeast - *Cyberlindnera jadinii*[5]	–	146.0
Rapeseed oil	19.7	23.4
Monocalcium phosphate	13.1	15.5
Limestone	9.2	9.4
Sodium chloride	7.2	5.5
Selenium premix	0.7	0.9
Iron(II) fumarate	0.4	0.4
Micro-mineral premix[6]	2.0	2.0
Vitamins[7]	2.2	2.2
L-Lysine	6.5	5.7
L-Methionine	2.1	2.9
L-Threonine	2.9	2.4
L-Valine	1.4	1.2
L-Tryptophan	0.9	0.9
Yttrium (III) oxide	0.1	0.1
Calculated CP content (%)	17.1	17.3
Ratio CP from yeast (% of total CP)	0.0	40.0

[1] Non-GMO soybean meal, Denofa AS, Fredrikstad, Norway
[2] Cargill, Denmark
[3] Nordsildmel AS, Egersund, Norway
[4] Expeller-pressed rapeseed cake, Mestilla, UAB, Klaipeda Lithuania
[5] Yeast meal (*C. jadinii*): 970 g DM/kg, 78 g ash/kg, 470 g crude protein/kg, 16 g ether extract/kg, 19.9 MJ/kg.
[6] "Mikro-Svin"; provided per kilogram of diet: 475 mg Ca; 3.4 mg Mg; 13.2 mg S; 120 mg Fe; 60 mg Mn; 120 mg Zn; 26 mg Cu; 0.6 mg I.
[7] Provided per kilogram of diet: 0.8 g Vitamin A; 0.3 g Vitamin E; 0.8 g Vitamin ADKB mix; 0.3 g Vitamin C (Stay C 35%).

2.2. Diets and feeding

C. jadinii yeast (LYCC-7549; Lallemand Yeast Culture Collection), previously classified as *Candida utilis* (torula yeast), was grown on sugars derived from lignocellulosic biomass from Norwegian spruce trees and beet molasses (1:1), as described in Øverland and Skrede (2017). The yeast was inactivated and drum dried.

The dietary treatments included: 1) control diet based on wheat, barley, oats, soybean meal (SBM), fishmeal (FM), potato protein concentrate and rapseed oil, and 2) experimental diet containing 14.6% inactivated *C. jadinii* yeast where the yeast replaced 40% of the protein ingredients in the control diet. Diets correspond to the Control and CU40 diets in Cruz et al. (2019). Dietary composition is shown in Table 1 and chemical composition in Table 2. Both diets contained an inert marker (0.01% Yttrium(III)oxide: Y_2O_3). Diets were formulated on net energy and standardized ileal digestibility (SID) values to be isoenergetic and isonitrogenous, and to meet or exceed the requirements for indispensable amino acids and all other nutrients for pigs of this age (NRC, 2012). The formulation of the diets was done in collaboration with Felleskjøpet Fôrutvikling AS, Trondheim, Norway, using their optimization least-cost program. Diets were produced by the Center for Feed Technology (ForTek, NMBU, Ås, Norway), and pelleted with a 3 mm diameter.

In the nursery period, piglets had access to the sow's diet as creep feed. In the experimental period, piglets had *ad-libitum* access to the experimental diets immediately after weaning from an automatic feeder (FRH-2 Domino A/S, Tørring, Denmark). Total feeding space was 43 cm (x 15 cm) with a metal bar dividing the feeding space in two. New feed

Table 2
Analyzed chemical composition of experimental diets.

	Dietary treatments	
Nutrients, g/kg of DM	Control	Yeast
DM, g/kg	869.17	885.02
Crude protein	201.96	193.92
NDF	110.02	102.35
Starch	507.99	494.31
Crude fat	45.31	46.15
Ash	52.73	51.16
Phosphorus	8.01	9.08
Gross energy, MJ/kg	18.94	18.96
Indispensable AA, g/16 g N		
Arginine	5.28	5.04
Histidine	2.13	2.02
Isoleucine	3.82	3.90
Leucine	6.99	6.86
Lysine	7.15	7.01
Methionine	2.40	2.73
Phenylalanine	4.39	4.26
Threonine	4.97	5.23
Tryptophan	1.49	1.61
Valine	5.24	5.54
Dispensable AA, g/16 g N		
Alanine	3.62	4.18
Aspartic acid	7.83	7.30
Cysteine	1.45	1.36
Glutamic acid	20.14	21.35
Glycine	4.14	4.13
Proline	6.86	6.94
Serine	4.81	4.99
Tyrosine	2.63	2.64

was provided every morning, and average daily feed intake (ADFI) for the pen calculated. Piglets had *ad-libitum* access to clean drinking water from a drinking nipple located in the front of the pen next to the feeder.

Fecal consistency was assessed every day during the experimental period using a scoring system from 1 to 4 developed by Pedersen and Toft (2011), where fecal consistency scores one and two were considered normal while scoring three and four were considered as diarrhea. The score was given as a pen average with 0.25 intervals.

2.3. Sampling

Eight piglets (littermates of piglets included in the experiment) were sampled at the day of weaning (day zero) to provide a baseline time point to facilitate detection and interpretation of changes due to weaning and potential yeast-induced changes. At day two, four, seven and 14 PW eight piglets of each diet were sampled (one or two per pen). Live body weight was registered before sampling. All animals were euthanized using a captive bolt pistol, followed by exsanguination.

The abdominal cavity was opened immediately after exsanguination and the entire GIT removed. pH was measured in stomach and duodenal content. The small intestine was sampled at two sites: proximal jejunum (1 m aboral to the right lobe of pancreas) and ileum. Approximately 25 cm tissue of each segment were isolated, pH measured and digesta collected before it was washed with phosphate-buffered saline (PBS). Small pieces of tissue (5 × 5 mm) were collected from the two segments and mucosa was sampled from the jejunum segment by scraping with a glass slide. All samples were immediately frozen in liquid nitrogen, stored on dry ice during sampling, and then transferred to storage at −80 °C until analysis. For RNA sequencing, tissue samples of approximately 5x5 mm were stored in RNAlater (Merck, Germany) at −80 °C. Liver was weighted before samples were collected. Contents from the last two meters of the small intestine were collected for determination of AID. Samples were immediately frozen at −20 °C. Ileal content was later freeze dried, homogenized using a batch mill (A11 basic Analytical mill, IKA®, England), and chemically analyzed.

A pooled feed sample collected during the experiment was ground at 1 mm for chemical analysis of main nutrients and 0.5 mm for analysis of starch and yttrium, using a Fritsch Pulverisette 19 cutting mill (Fritsch, Idar-Oberstein, Germany).

2.4. Chemical analyses

Chemical analysis (Dry matter (DM), ash, crude protein (CP), starch, gross energy, neutral detergent fiber (NDF) and amino acids) of main ingredients and diets in triplicates, and singular analysis of ileal content were performed as described in Cruz et al. (2019) by the LabTek group at the Department of Animal and Aquacultural Science, NMBU, Ås, Norway. Organic matter was calculated by subtracting ash content. Crude fat was analyzed by Eurofins Food & Feed Testing Norway AS, Moss, Norway, using acid hydrolysis method with co-extraction with hydrochloric acid followed by extraction with petroleum ether. Marker (^{89}Y) and total phosphorus in feed and ileal samples were conducted at the Department of Environmental Sciences, NMBU. Samples were completely digested in concentrated nitric acid (HNO_3) in an UltraCLAVE III (Milestone, Sorisole, Italy) at 260 °C for 15 min, and diluted with deionized water before analysis of Y and P by inductively coupled plasma mass spectroscopy using an Agilent 8800 Triple Quadrupole ICP-MS/MS (Agilent Technologies Inc., Santa Clara, USA) in oxygen reaction mode.

2.5. Enzyme activities, IgA concentration and total protein

Amylase, trypsin and lipase activity were measured in intestinal content from the proximal jejunum, whereas alkaline phosphatase (ALP), leucine aminopeptidase (LAP) and maltase activities were measured in epithelium from the same intestinal segment. Samples were prepared as described by de Nanclares et al. (2017) and stored at −80 °C until further analyses.

The amylase, trypsin, ALP, LAP and lipase activity were determined using commercial kits (Abcam: ab102523, ab102531, ab83369, ab234627; Sigma-Aldrich: MAK048) according to the manufacturer's protocols. The maltase activity was determined following a modified protocol described by Dahlqvist (1968). In the final step of the protocol, glucose concentration was measured using a commercial kit (EIAGLUC, ThermoFisher). Total protein concentration was measured according to the Quick Start™ Bradford Protein Assay protocol (Bio-Rad Laboratories). Absorbance was measured using a SpectraMax M2e Microplate Reader (Molecular Devices).

Quantitative measurement of immunoglobulin A (IgA) in jejunal tissue were conducted using Abcam's IgA Pig enzyme-linked immunosorbent assay (ELISA) kit (Pig IgA, Colorimetric, Abcam, USA, ab190536). Approximately 70 mg of tissue were homogenized using a bead mill homogenizer with a lysis buffer. Supernatant was collected and stored at −80 °C after centrifugation. Total protein was determined as described above, and samples were normalized for protein concentration prior to IgA analysis, which followed the manufacture's protocol for the assay.

2.6. Morphology and morphometry

Intestinal tissues from jejunum and ileum were collected, processed and analyzed as described in detail in Cruz et al. [24]. Briefly, digital images were captured from tissue sections routinely stained with hematoxylin and eosin. VH was measured by from the tip of the villus to the villus-crypt-junction. Crypt depth (CD) was measured from the villus-crypt junction to the deepest portion of the crypt, adjacent to the *tunica muscularis mucosae*. Between four and seventeen villus and crypt complexes were measured in each intestinal segment from each piglet. For each villus-crypt-complex, a VH:CD ratio was calculated.

2.7. RNA extraction, library construction and RNA sequencing

Total RNA was extracted from proximal jejunal and ileal tissue from 56 piglets (day zero, two, four and seven PW) following the RNeasy Plus Universal Kit's protocol (Qiagen). Total RNA concentration and quality were determined using NanoDrop TM 8000 spectrophotometer (Thermo Fisher Scientific), and Agilent 2100 Bioanalyzer (Agilent Technologies). High quality samples (RIN ≥ 7) were sent for sequencing at the Norwegian Sequencing Centre (http://www.sequencing.uio.no). 84 samples (42 from ileum and 42 from jejunum: six from day zero, and six from each dietary treatment on day two, four and seven PW) were used for RNA-seq library preparation. TruSeq™ Stranded total RNA-seq (Illumina) library kit was used for library preparation following manufacturer's protocols targeting the mRNAs using their polyA tail. Libraries were pooled together and sequenced over 7 lanes of HiSeq 4000 (Illumina) employing 150 bp, paired end (Supplementary data 1).

2.8. Data analysis of RNA sequencing data

Raw reads was cleaned using BBDuk v34.56 (Bushnell, 2014) to trim/remove low quality reads, adapter sequences and PhiX (Illumina spike-in) using the following parameters: ktrim = r, k = 23, mink = 11, hdist = 1, tbo, tpe, qtrim = r, trimq = 15, maq = 15, minlen = 36, forcetrimright = 149. Cleaned reads were aligned to the *Sus scrofa* ENSEMBL genome Sscrofa 11.1 release 98 using HISAT v2.1.0 (Kim, Langmead, & Salzberg, 2015) using default parameters. Fragments mapping to the known genes from release 98 was counted using featureCounts v1.4.6-p1 (Liao, Smyth, & Shi, 2014) and differential expression was calculated using DESeq2 v1.22.1 R package (Love, Huber, & Anders, 2014). Differential expression analysis was carried out for ileum and jejunum independently.

To investigate the transcriptomic changes PW and the differences between the treatment groups, significantly differentially expressed genes (DEGs) between timepoints were included in separate heatmaps for each of the dietary treatment groups. Hierarchical clustering was used to group genes with similar expression trends. Genes from each cluster were then extracted and included in search for enriched KEGG pathways using the gprofiler2 version 0.1.5 package in R (Kolberg & Raudvere, 2019) with g:SCS multiple testing correction method applying significance threshold of 0.05. KEGG pathway enrichment analysis was also performed for DEGs between piglets fed the yeast and control diet on day 7 PW.

2.9. Calculations and statistical analyses

Liver index was calculated as: (*Liver weight, kg / Body weight kg*) * 100. AID coefficients were calculated as described by Maynard and Loosli (1969). For digestibility and enzyme activity results, an inter quartile range (IQR) test were run and values outside the range of three times IQR excluded. Two piglets, one from each treatment at day 4 PW, were excluded from the AID dataset due to low yttrium values, which affected all their AID values. Results are presented as mean values with error bars.

Figures were made using the ggplot (Wickham, 2016) and Hmisc package (Harrell Jr, Dupont, & others, 2018) in Rstudio Inc, version 1.1.456. Statistical analysis was performed using the mixed procedure in the SAS® software, V.9.4 (SAS Inst. Inc., Cary, NC). Day zero samples were not included in the statistics. The following model was used for all parameters:

$Y_{ijklmn} = \mu + diet_i + s\_day_j + (diet \times s\_day)_{ij} + breed_k + litter_l + pen_m(diet_i) + e_n$, where Y is one observation on piglet n; μ is the intercept; $diet_i$ is the fixed dietary treatment effect (i = 1,2); s_day_j is the fixed effect of sampling day PW (j = 1:4); $diet_i \times s\_day_j$ is the fixed interaction between dietary treatment and sampling day; $breed_k$ is the fixed effect of the breed of the piglet (k :1 = LLD and k : 2 = LZD); $litter_l$ is the random effect of the l-th litter ~ $N(I \times \sigma^2_{litter})$ here I is an identity matrix of dimensions and σ^2_{litter} is the variance component for litter; $pen_m(diet_i)$ is the random effect of pen within diet each $N(0, I \times \sigma^2_{pen(diet)})$; and e_n is the error of the n piglet $N(0, I \times \sigma^2_{es\_day})$, i.e. assumed heterogeneous per sampling day.

A Pearson correlation matrix between the digestibility coefficients, enzyme activities, morphology, and pH parameters was made using the rcorr function in the Hmsic package v.4.1–1 (Harrell Jr et al., 2018) and was visualized with the corrplot function in the corrplot package v 0.84 (Wei & Simko, 2017).

3. Results

3.1. Growth performance

No health problems related to dietary treatments and no mortality were encountered during the experiment. Average daily gain (ADG) and ADFI did not differ between the treatments. ADG for the whole experimental period was 165 g/day and 163 g/day for control and yeast-fed piglets, respectively. ADFI was 287 g/day and 275 g/day for control and yeast-fed piglets, respectively. Liver index increased from day two to 14 W ($P < 0.001$) but did not differ between treatments ($P = 0.506$).

3.2. Fecal score and ileal DM

Fig. 1 shows the fecal score and ileal DM results. The fecal score increased (from approx. 1.2 to 2.5) for both treatments from day two to four PW. Average fecal scores from day four to seven for both treatments were above 2.5 (2.5 – 3), hence considered piglets being diarrheic. For the remainder of the experimental period, the fecal scores

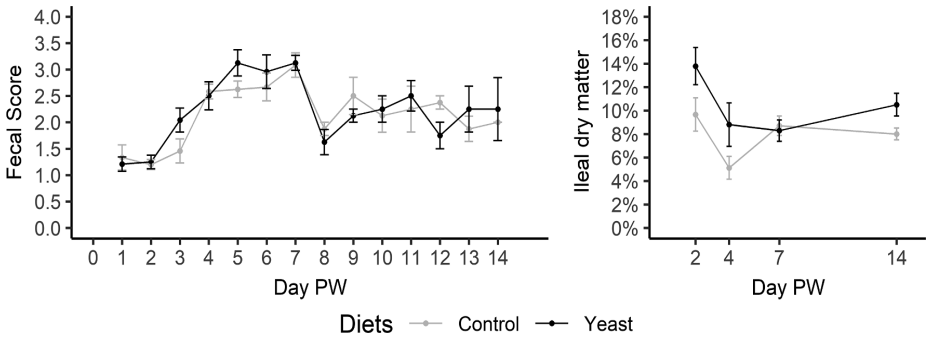

Fig. 1. Fecal score and dry matter content of ileal digesta of PW piglets fed the control diet or the yeast-based diet. Results are presented as mean values with error bars.

were < 2.5. Ileal DM showed changes consistent with fecal scoring. Ileal DM significantly decreased from day two to four PW and increased at day seven and 14 (P = 0.019). Piglets fed the yeast diet had significantly higher DM content in ileum compared with piglets fed the control diet (P = 0.007).

3.3. pH in intestinal segments

There was no significant effect of dietary treatment on pH measured in content from stomach (P = 0.382), duodenum (P = 0.550) or jejunum (P = 0.857). pH differed between sampling days (P < 0.001) in jejunum, where pH increased from 5.5 on day two to 6.2 on day four and 6.4 on day seven, before decreasing to 5.7 on day 14. There was a tendency for a sampling day effect in stomach pH (P = 0.088), but no sampling day effect was found in duodenum (P = 0.468).

3.4. Morphology

In jejunum, CD significantly differed between the PW sampling days (P < 0.001). The CD was lower on day two and four PW compared with day zero but increased from day four to day 14 PW (Fig. 2). No significant difference between sampling days was found for the jejunal VH (P = 0.798). The jejunal VH:CD ratio decreased from day two to 14 PW (P < 0.001). In ileum both VH (P = 0.008) and CD (P < 0.001) increased over time PW. No significant differences between dietary treatments and no significant interaction between diet and sampling day were found for any of these parameters.

3.5. Digestibility

The development of AID PW is shown in Fig. 3. Inclusion of yeast in the PW diet significantly improved AID of CP (P = 0.033) and tended to increase AID of phosphorus (P = 0.089). No significant effect of diet was found for AID of organic matter (P = 0.239), starch (P = 0.652) or fat (P = 0.367). The AID of organic matter tended to be affected by sampling day (P = 0.063). There was no significant interaction between diet and sampling day PW for the AID coefficients.

3.6. Enzyme activities

Development of lumen and brush border enzyme activities in the proximal jejunal mucosa is shown in Fig. 4. A clear increase in trypsin activity PW (P = 0.001) can be observed, whereas lipase activity decreased during the first days PW (P = 0.012). No differences were found for development of amylase activity PW (P = 0.953), and no effect of dietary treatment was found for any of the three enzyme activities measured in lumen of proximal jejunum (trypsin P = 0.757; lipase P = 0.578; amylase P = 0.912). Common for the development of maltase, ALP and LAP activities in the proximal jejunal mucosa, is an increase in activity during the first days PW, with a higher activity present in the yeast fed piglets, followed by a decrease in activity and similar activities between the dietary treatment groups. There was an effect (P < 0.001) of sampling day PW for all three brush border enzymes. Piglets fed the yeast diet had higher (P = 0.014) ALP activity than those fed the control diet, whereas maltase and LAP activity tended (P < 0.1) to be higher for the yeast diet. No interaction between diet and sampling day was found for any of the enzyme activity parameters.

3.7. IgA

IgA concentration showed similar pattern for both diets (P = 0.920) and differed between days PW (P < 0.001), with a decrease from day two to four PW followed by an increase in concentration over time (Fig. 5).

3.8. Correlations between parameters of GIT function

Fig. 6 shows a correlation plot of some of the individual measured parameters, including pH, enzyme activities, digestibility coefficients, ileal DM, VH and CD, and IgA. There were positive correlations between ileal DM and AID of organic matter (r = 0.547, P < 0.001), AID of CP (r = 0.731, P < 0.001), AID of phosphorus (r = 0.534, P < 0.001) and AID of crude fat (r = 0.524, P < 0.001). There were negative correlations between CD in jejunum and ileum and brush border enzymes maltase (jejunal CD: r = -0.439, P < 0.001; ileal CD: r = -0.345, P = 0.003), ALP (jejunal CD: r = -0.404, P < 0.001; ileal CD: r = -0.412, P < 0.001) and LAP (jejunal CD: r = -0.545, P < 0.001; ileal CD: r = -0.565, P < 0.001).

3.9. Gene expression

Using day zero as a baseline, Fig. 7 shows the number of DEGs in proximal jejunum at the different sampling days PW. The number of DEGs increased over time PW. In jejunum, the changes in DEGs were larger in the control than in the yeast piglets, at day four and seven. The number of DEGs at day four were 471 vs. 31 and on day seven 2855 vs. 83, for the control and yeast group, respectively.

Jejunal gene expression patterns over time for control piglets are shown in Fig. 8. The DEGs are divided in two cluster trends; a decreased relative expression PW (cluster 1) and an increased relative expression PW (cluster 2). The DEGs in cluster 1, which were downregulated with time PW, mainly enriched pathways related to environmental information processing and immune system processes. The DEGs in cluster 2 showed an increased expression pattern over time, and enriched pathways were mainly related to endocrine systems, metabolism and others such as genetic information processing (proteasome, protein processing in endoplasmic reticulum and ribosome).

Fewer DEGs were found between timepoints in the yeast group, in total 276 genes. The heatmap in Fig. 9 is also divided in two clusters, where metabolic pathways and protein digestion and absorption are enriched in the cluster with increasing expression trend PW, whereas DEGs in two enriched pathways related to the immune system; hematopoietic cell lineage and B cell receptor signaling pathway, have decreased expression patterns.

Few genes were significantly differentially expressed between dietary treatments on the specified sampling days. In jejunum on day seven, 476 DEGs were found between the control piglets and the piglets fed the yeast diet. Of these DEGs, 469 were upregulated in piglets fed yeast as compared with the control piglets. In total, 38 KEGG pathways were enriched (P < 0.05), i.e., upregulated in piglets fed yeast compared with control piglets on day seven (Fig. 10). Nine of these pathways were related to the immune system, and the most distinctly enriched pathway was intestinal immune network for IgA production, where 29% (12 out of 41) of the genes in this pathway were upregulated in the yeast group. Only seven genes were downregulated on day seven in jejunum of piglets fed yeast compared with control piglets (*COX20*, *DNAJC15*, *GDE1*, *MORC4*, *MRPS21*, *SYDE2* and *ZNF133*).

In ileum, fewer genes were differentially expressed (Table 3). In total, 2090 DEGs were found between any of the timepoints in the control group, with the largest difference being between day two and seven PW with 2038 DEGs. In the yeast fed piglets only 100 DEGs were found between timepoints.

In the ileum of control piglets two opposing cluster trends were identified; cluster 1 included DEGs with an overall increasing expression from day zero to two, followed by a decline below day zero level over time, and cluster 2 included genes with the opposite PW expression trend (Fig. 11). DEGs in cluster 1 enriched pathways involved in genetic information processing, and transport and catabolism (autophagy and mitophagy), along with the NOD-like and RIG-I-like receptor signaling pathways. In cluster 2, the DEGs were mainly enriched signaling pathways in environmental information processing and

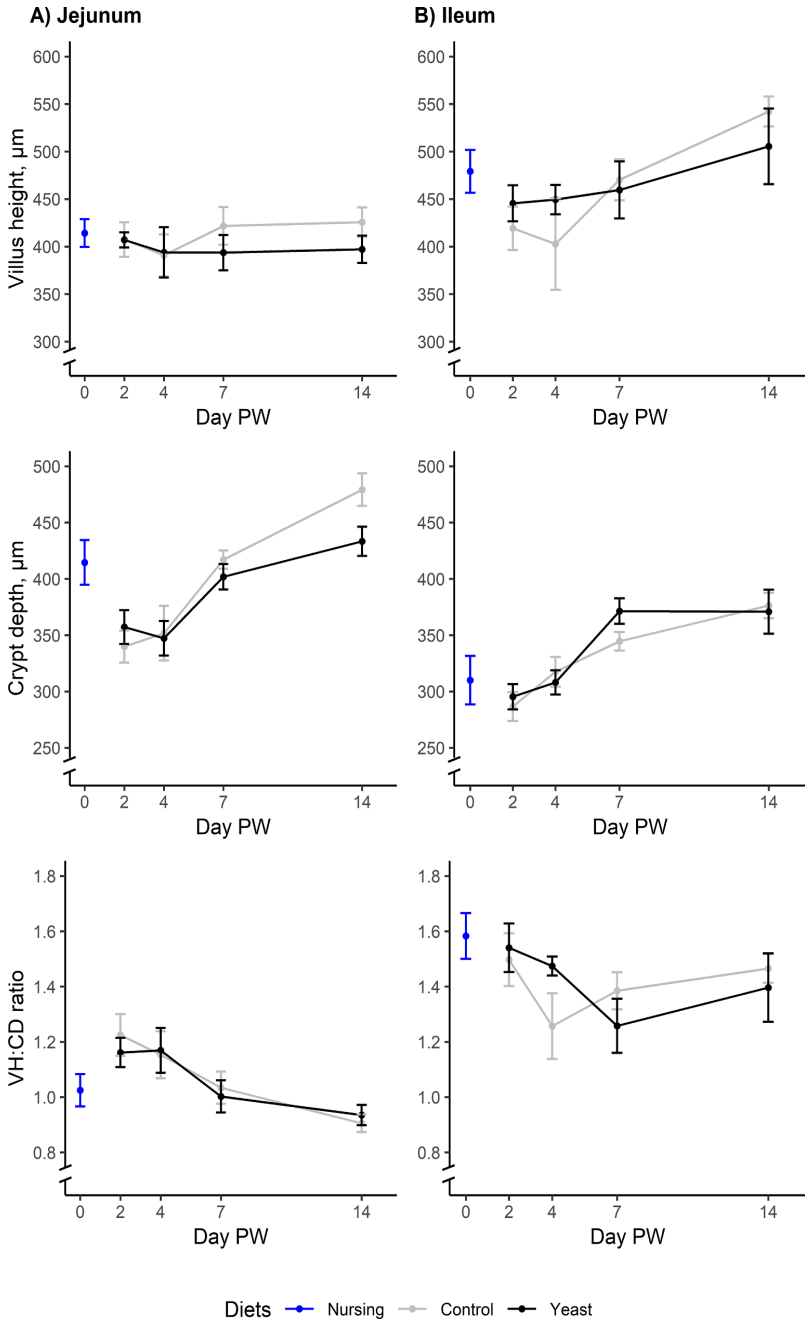

Fig. 2. Villus height (VH) and crypt depth (CD) in jejunum (A) and ileum (B) of PW piglets fed the control diet or the yeast-based diet. VH and CD at day zero (nursing) are indicated in blue. Results are presented as mean values with error bars.

pathways in different organismal systems.

In the ileum of yeast-fed piglets, three cluster trends were found, but in cluster 1 there were too few genes to determine pathway enrichment, and in cluster 3 only one human disease pathway was enriched. Cluster 2 included DEGs with an overall increasing PW relative expression trend. In cluster 2 four pathways were enriched; thyroid hormone synthesis, and three pathways related to protective host response and cytokine signaling networks (Fig. 12).

4. Discussion

In this study, we have investigated the development and maturation of gastrointestinal function and health in PW piglets fed two diets; a control diet or a diet with 14.6% inclusion of *C. jadinii* yeast. The focus

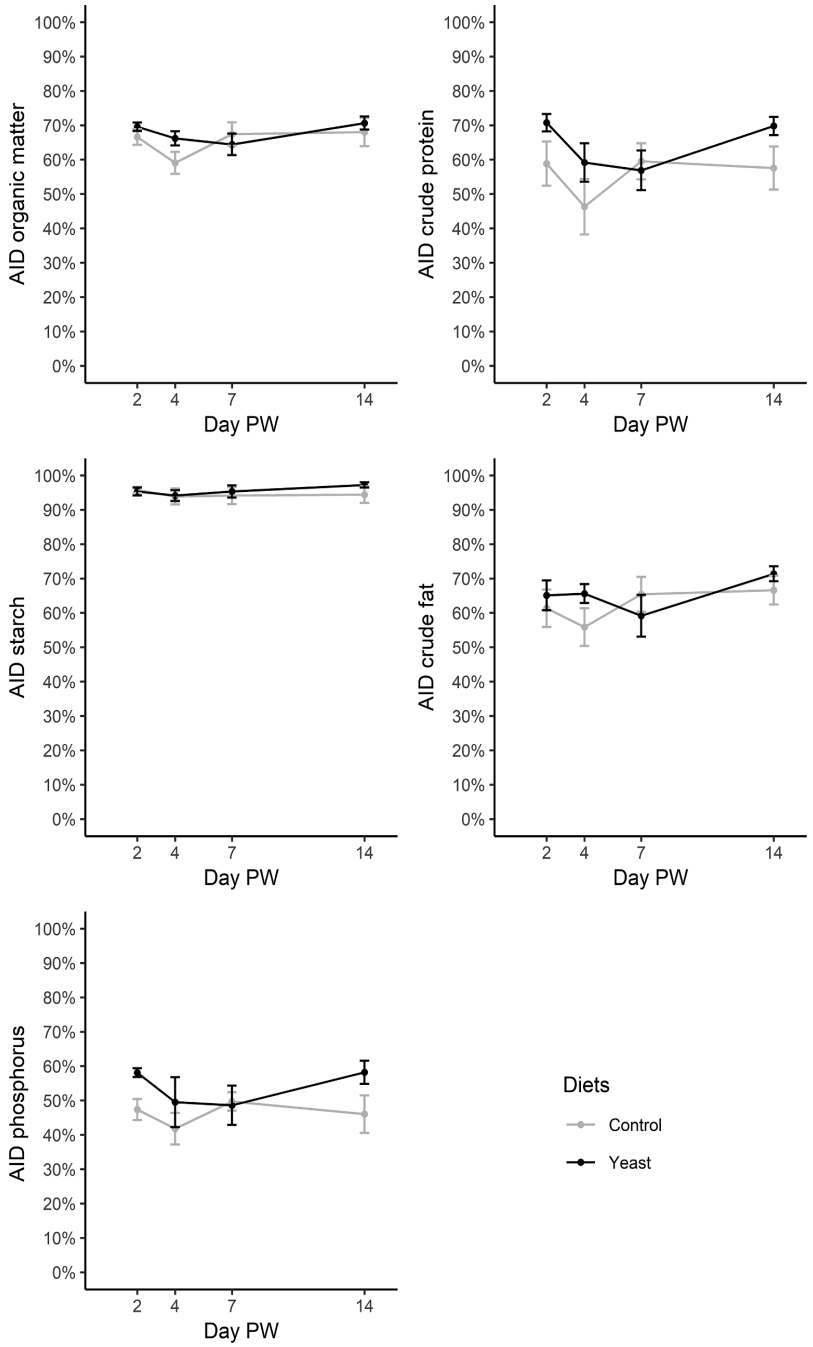

Fig. 3. Apparent ileal digestibility (AID) coefficients of organic matter, crude protein, starch, phosphorus and fat in PW piglets fed the control diet or the yeast-based diet. Results are presented as mean values with error bars.

of the study was on general intestinal function (i.e. pH, AID and enzyme activities), and the health-related parameters fecal score and intestinal morphometry. In addition, we have investigated the jejunal and ileal transcriptome at different time-points during the first week after weaning. In accordance with our previous experiment (Cruz et al., 2019), no differences in ADG or ADFI were observed between dietary treatments in the present experiment, confirming that yeast potentially can replace 40% of CP from the main protein sources traditionally used in piglet diets in Norway.

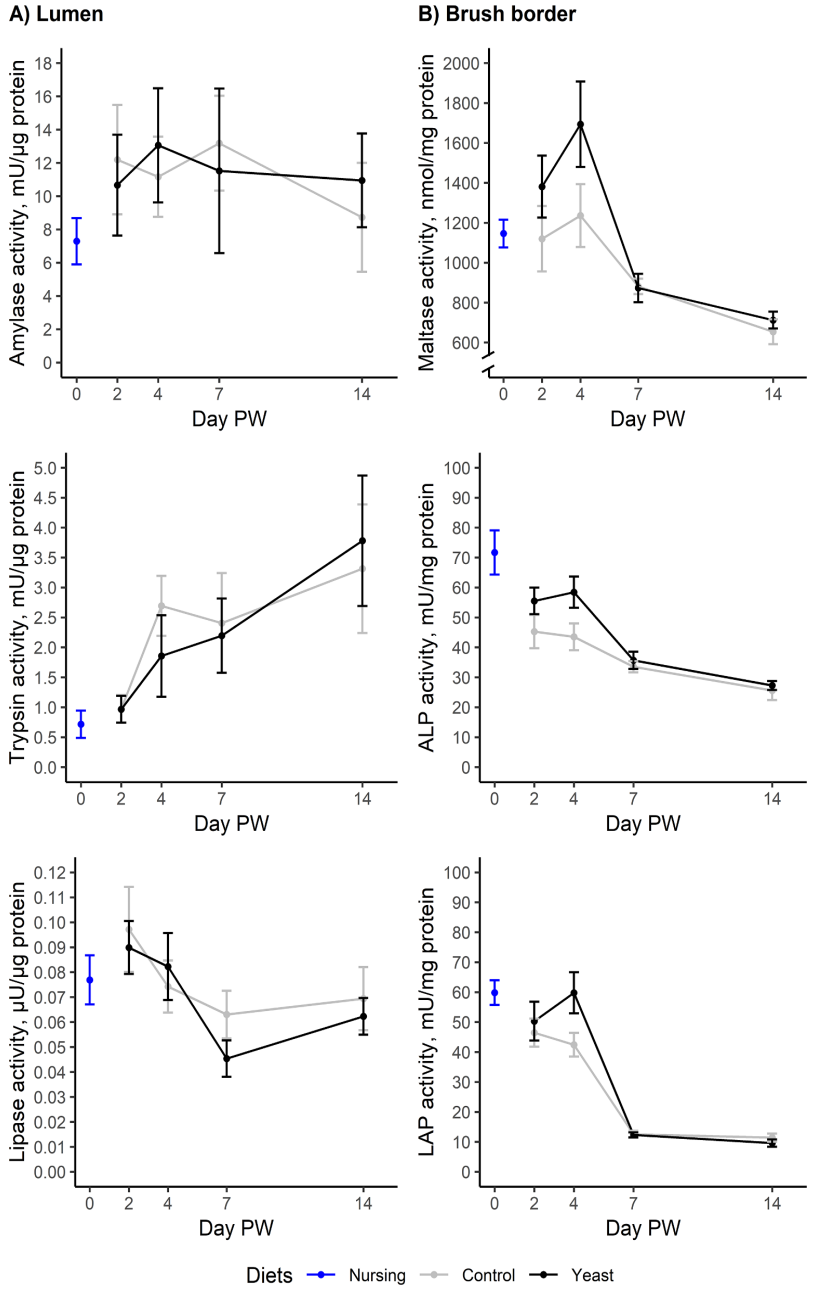

Fig. 4. Lumen (A) and brush border (B) enzyme activities in proximal jejunum of PW piglets fed the control diet or the yeast-based diet. Enzyme activities at day zero (nursing) is indicated in blue. All activities are expressed per µg or mg of protein. Results are presented as mean values with error bars.

Development of general GIT function and health PW

The increased fecal score and diarrhea incidence between day four and seven PW in both treatments are consistent with the cohort study by Madec, Bridoux, Bounaix, and Jestin (1998), that reported PW diarrhea from around day four to nine PW for piglets at approximately the same age. The ileum DM results also corresponded with the observed fecal score pattern. High gastric pH is commonly reported PW and a failure of acidification in the stomach might increase the risk for PW diarrhea (Heo et al., 2013). In all gut segments measured, pH was numerically highest in the first week PW, which correlated positively with increased fecal score and decreased ileal DM. Because pH in the GIT varies with time postprandial, and because the time from feeding to sampling varied among piglets in the present experiment, pH in the different GIT segments showed large variation. Infection of *Escherichia coli* is a major factor causing PW diarrhea, which weakens mucosal and

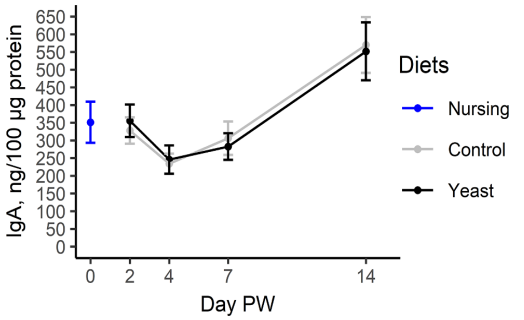

Fig. 5. IgA concentration in proximal jejunal tissue in PW piglets fed the control diet or the yeast-based diet. IgA concentration at day zero (nursing) is indicated in blue. Results are presented as mean values with error bars.

cellular barrier function and increases secretion of water and electrolytes into the small intestinal lumen (Heo et al., 2013). Although, even when not infected, Nabuurs, Hoogendoorn, and Van Zijderveld (1994) reported decreased net absorption of fluids on day four and seven PW compared with suckling piglets, which corresponds to our ileal DM results. No difference in diarrhea was seen between the yeast and the control treatment in our experiment. In our previous experiment (Cruz et al., 2019), we also reported no difference in fecal score between the diets in the second week, but we found improved fecal score with the similar yeast diet in the fourth week PW, and higher DM in feces on day seven and 21 PW.

The piglets digestive capacity increases as it grows older (Manners, 1976). However, abrupt weaning temporarily disturbs GIT maturation. These temporary PW changes were divided into two periods by Montagne et al. (2007). First five days PW constitutes an acute period with deterioration of the GIT structure and function. This initial phase is followed by a more progressive adaptative and maturational phase. The piglets in the study by Montagne et al. (2007) was weaned at 21 days of age, fasted for 48 h and tube fed, controlling the feed intake and simulating a critical weaning situation with no energy intake the first few days. Although our piglets were one week older at weaning and not restricted from feed intake PW, we observed a reduction in some of the functional parameters at the first sampling time-points PW, with an initial recovery on day 14 PW. Even though not significant, AID coefficients for all nutrients showed a numerical decrease in the early PW period at day four and/or day seven. All digestibility coefficients were positively correlated with the DM of ileal digesta, except for starch digestibility. Trypsin activity in jejunal content increased with day PW, which was also reported by Makkink et al. (1994) for piglets weaned at the same age. The reduction in lipase activity until day seven followed by an increased activity has also been reported by several authors (Cera, Mahan, & Reinhart, 1990; Marion et al., 2003) The decrease in lipase activity PW can explain the PW decrease in AID of fat.

Low feed intake during the first days PW is considered the main cause for the functional and structural changes occurring in the acute PW phase (Jayaraman & Nyachoti, 2017; Montagne et al., 2007). In the nursing period, lactose is an important energy source for the intestinal epithelial cells (Spreeuwenberg et al., 2001), and deprivation of luminal substrates for mucosal epithelial cell growth is a consequence of weaning (Montagne et al., 2007). A reduction in villus length equates to a reduction in the mature enterocyte population and affects functional and absorptive capacity. In jejunum, VH remained relatively stable PW, whereas an increase in VH from day two to seven PW was observed in ileum. These results are not consistent with Montagne et al. (2007). These investigators reported that the acute effect of weaning was primarily seen in the proximal part of the small intestine, while changes in ileum occurred five days PW in piglets weaned at 21 days of age and fasted for two days PW. In our study, ileal DM was positively correlated with ileal VH:CD ratio, where piglets with higher villi or lower crypts

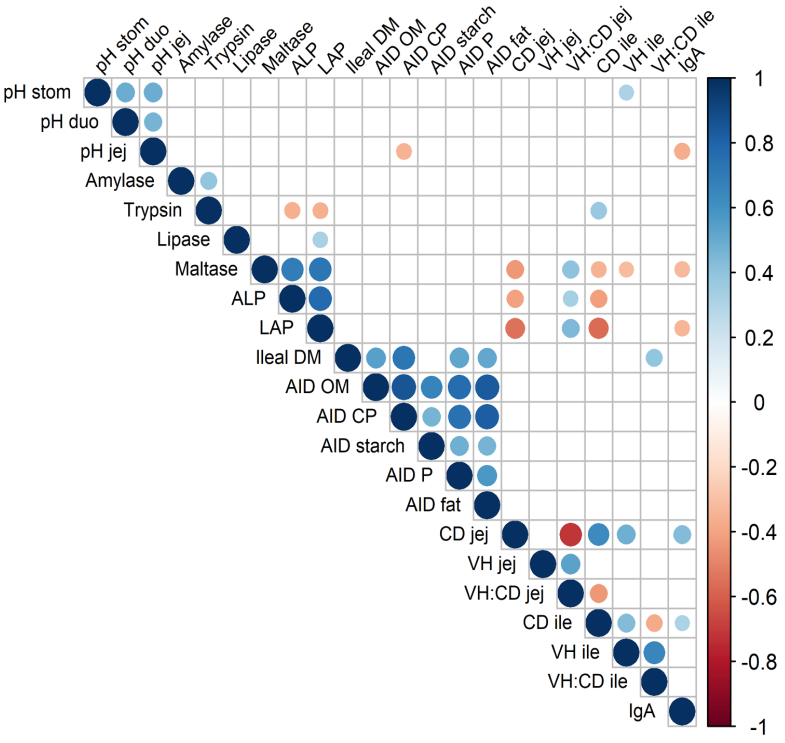

Fig. 6. Correlogram including digestibility coefficients, enzyme activities, pH and morphology parameters. Positive correlations are displayed in blue and negative correlations in red color. Color intensity and the size of the circle are proportional to the correlation coefficients. Only significant correlations ($P < 0.01$) are visualized.

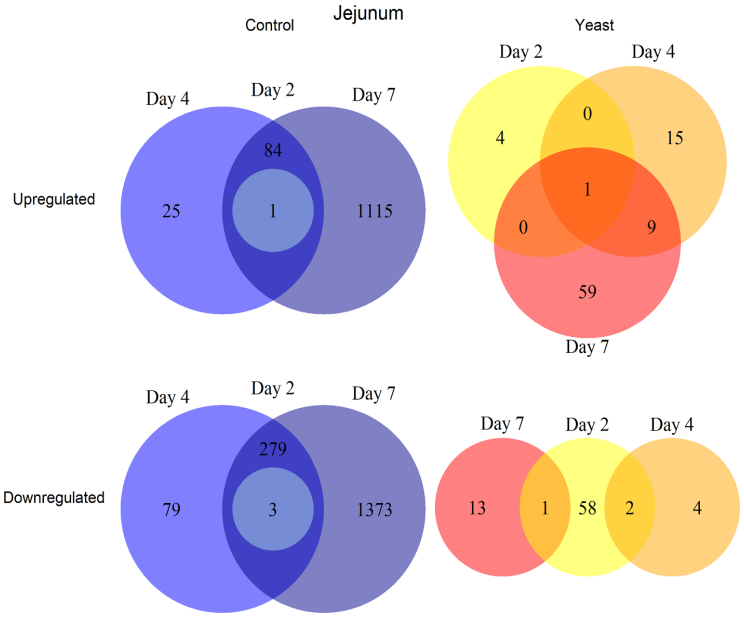

Fig. 7. Venn diagram showing number of DEGs at the different sampling days PW compared with day zero in jejunum.

had less watery content in ileum. These findings support the overall results that functional parameters deteriorated early PW. Shorter villus and deeper crypts have fewer absorptive and more secretory cells, and is associated with PW diarrhea (Nabuurs, Hoogendoorn, Van der Molen, & Van Osta, 1993). CD in both jejunum and ileum increased from day two to seven PW, and correlates negatively with the decreased jejunal activity of the brush border enzymes maltase, ALP and LAP. Increase in CD implies an increased rate of enterocyte production. The continual renewal of the intestinal epithelial cells takes place by differentiation along the crypt-villus axis (CVA) expanding from stem cells located near the base of the crypts (Yang, Xiong, & Yin, 2013). It is reported that enzyme activity, including ALP, aminopeptidase N and sucrase, increases during maturation along the CVA (Fan, Stoll, Jiang, & Burrin, 2001). An increased rate of enterocyte production and migration, indicated by an elongation of the crypts, might lead to more immature cells with lower digestive enzyme activities and nutrient uptake capacity. Yang, Wang, Xiong, and Yin (2016), reported a global change in protein expression during maturation along the CVA. They found an increased mucosal energy metabolism, which is crucial for digestion and absorption processes.

Few differences were found in PW development of GIT function and structure between the control and the yeast fed piglets. However, an overall higher CP digestibility was recorded for the yeast group, but in accordance with our previous study (Cruz et al., 2019), yeast did not affect trypsin activity. Moreover, in our previous study (Cruz et al., 2019), we reported increased ATTD of CP, crude fat and phosphorus in piglets fed a similar yeast-based diet. The increased ALP activity PW in the yeast fed piglets, suggest an improved PW intestinal health. Intestinal ALP is essential for gut homeostasis, and loss of intestinal ALP expression is associated with increased intestinal inflammation and dysbiosis (Fawley & Gourlay, 2016). Enhanced intestinal ALP activity in ileum has been reported by Xiong et al. (2015) when feeding a PW diet containing a yeast product with fermentation products and hydrolyzed yeast cell walls from *Saccharomyces cerevisiae*.

Development of small intestinal gene expression PW

In this study, we have focused on patterns of pathway enrichment PW and on differences between the two feeding groups, rather than on differential expression patterns of singular genes. We investigated the PW gene expression pathway patterns separately for the two feeding groups, followed by a discussion about the differences between diets. It is clear that weaning causes activation of stress responses, alteration in immune functions and metabolism, but the differences vary with time PW (Tao & Xu, 2013). Several studies indicate that weaning before three weeks of age induces more transcriptome alterations than weaning at a later age (Inoue et al., 2015; Li, Rajput, Fernandez, & Moeser, 2018). In our study, piglets were weaned at four weeks of age, making them more developed and robust than many of the piglet studies reported in the literature. However, we found clear changes in gene expression patterns PW.

The DEGs detected in jejunum of the control group PW were mainly classified into three important KEGG categories; nutrient metabolism, environmental information processing and immunoregulation. DEGs in enriched pathways related to metabolism, endocrine systems such as insulin signaling, and some of the cellular processes such as endocytosis and lysosome, showed increased expression in jejunum over time PW. These results indicate an overall increased functionality of the jejunum over time PW, which is contrary to findings reported by Wang et al. (2008) and Bomba et al. (2014). These investigators reported a decline in gene expression related to nutrient metabolism in the first week PW. However, in the study by Wang et al. (2008), piglets were younger, weaned at 21 days of age, and in both studies the piglets were fed a corn- and soybean meal based diet, contrary to our grain based diet. While metabolism related pathways improved in jejunum PW in the present study, a declining expression trend was found for DEGs involved in pathways connected to environmental information processing, immune system and other cellular processes such as cellular senescence, phagosome and regulation of actin cytoskeleton.

Whereas most of the enriched pathways in the immune system were downregulated in jejunum of control piglets over time PW, three enriched pathways (Cytosolic DNA-sensing pathway, NOD-like receptor

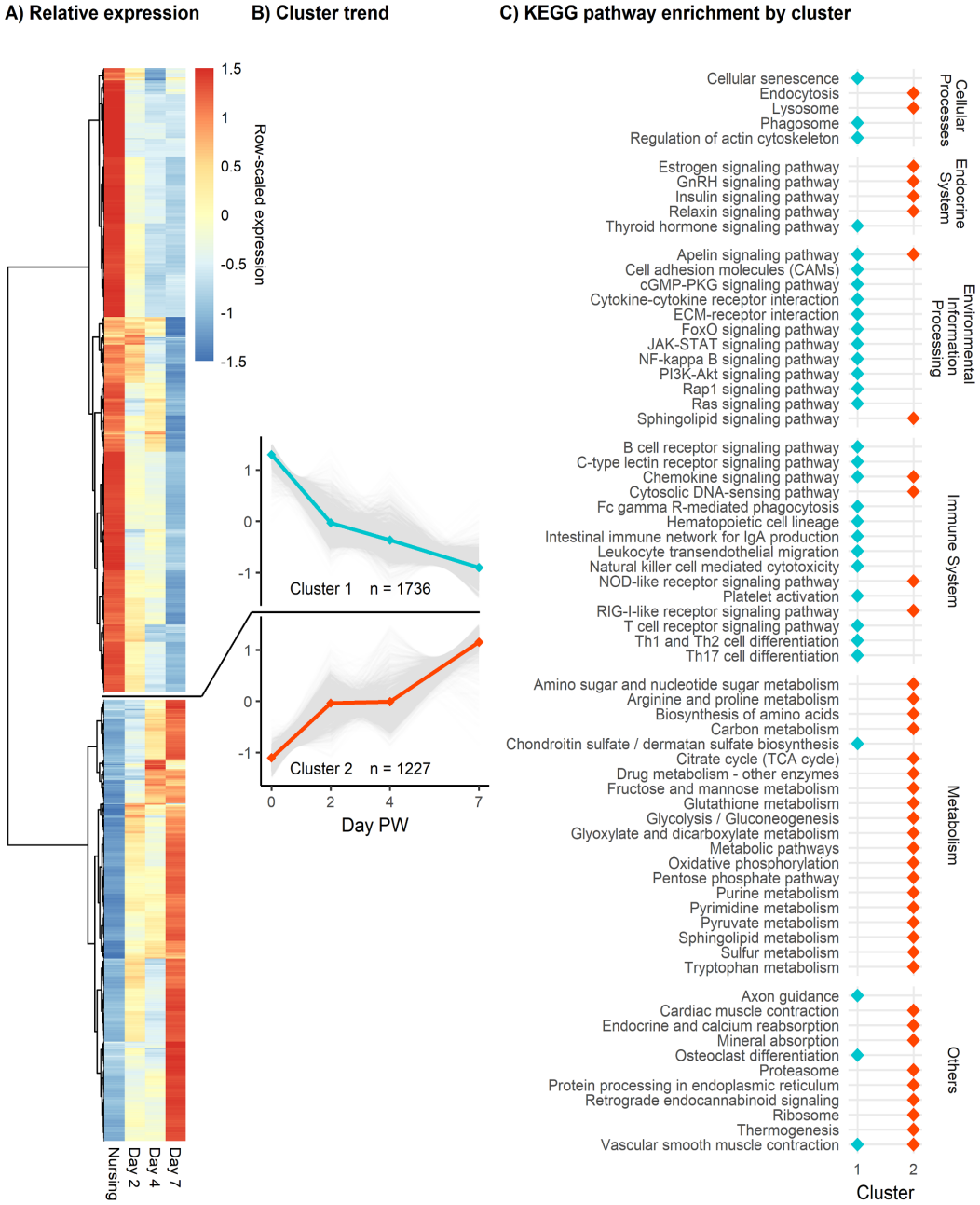

Fig. 8. Jejunal gene expression pattern over time for control piglets. A) Heatmap showing transcriptional changes in jejunum over time for piglets fed control diet. Heatmap includes 2 963 genes differentially expressed (adjusted P < 0.05) between any of the timepoints. Transcript abundance was row-scaled to highlight changes in the expression of individual genes, B) Genes with similar expression patterns across the timepoints were separated into two main clusters using Ward's method. Trend lines are based on mean scaled values in each cluster. C) KEGG pathway enrichment analysis was run on each cluster. Each point represents a significantly enriched pathway (P < 0.05). Pathway parents which includes less than five pathways are gathered and assigned as "Others". Pathways related to human disease were excluded in the figure but can be found in the supplementary data together with an overview of DEGs in pathways (Supplementary data 2).

signaling pathway and RIG-I-like receptor signaling pathway) responsible for pathogen detection and generating innate immune responses were upregulated. The diet change at weaning causes ingestion of new bacteria and feed antigens, which challenges and trains the intestinal tissue. An increased expression of genes in the endocytosis pathway was found in jejunum of the control group PW. By contrast, Wijtten, van der Meulen, and Verstegen (2011), suggest that a PW reduction in endocytosis rate is a strategy to prevent antigen overload excessively activating the immune system. A complex regulation of the GIT immune system is important to prevent excessive activation and

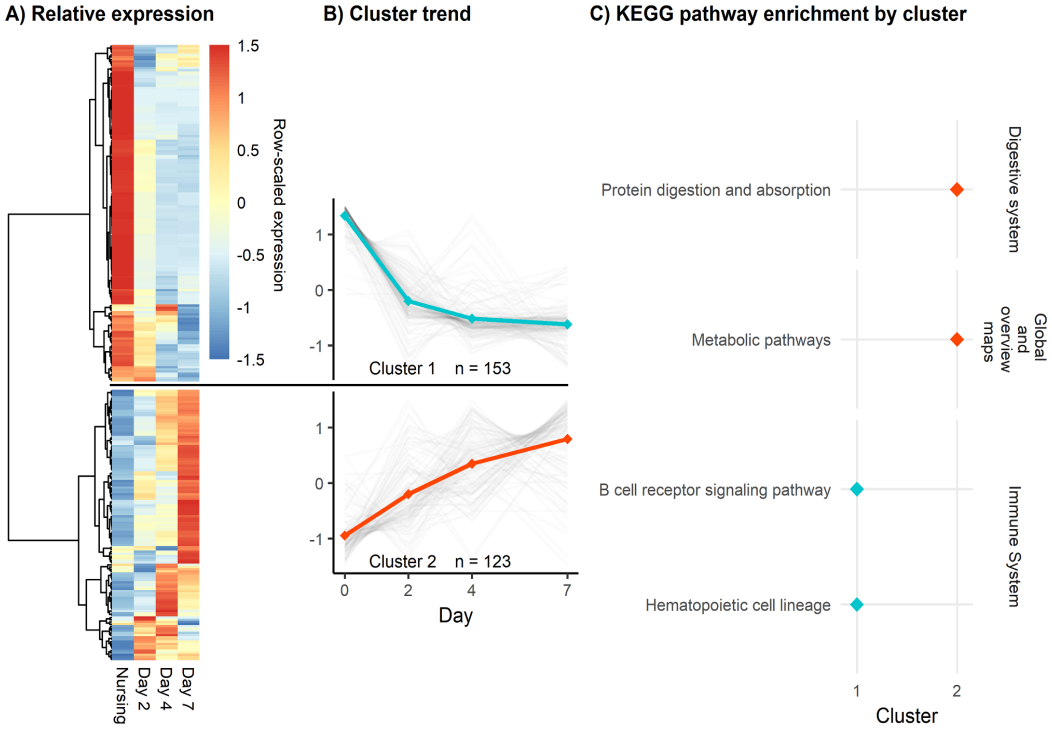

Fig. 9. Jejunal gene expression patterns over time for the yeast-fed piglets. A) Heatmap showing transcriptional changes PW in jejunum for piglets fed yeast diet. Heatmap includes 276 genes differentially expressed (adjusted $P < 0.05$) between any of the timepoints. Transcript abundance was row-scaled to highlight changes in the expression of individual genes, B) Genes with similar expression patterns across the timepoints were separated into two clusters using Ward's method. Three clusters were selected as a minimum number to represent data, and to ensure large enough groups to run pathway enrichment analysis. Trend lines are based on mean scaled values in each cluster. C) KEGG pathway enrichment analysis was run on each cluster. Each point represents a significantly enriched pathway ($P < 0.05$). Pathways related to human disease were excluded in the figure but can be found in the supplementary data together with an overview of DEGs in pathways (Supplementary data 2).

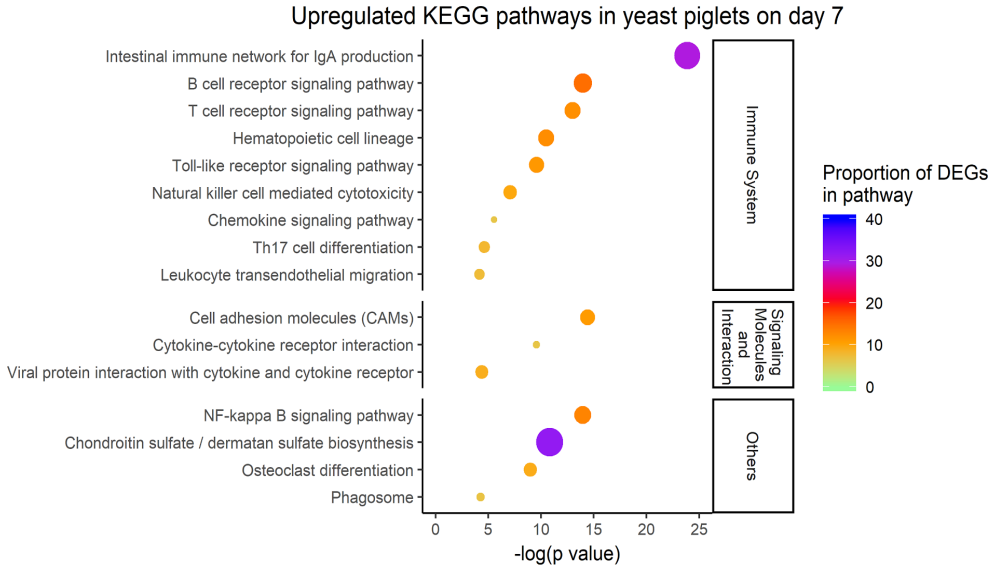

Fig. 10. KEGG pathways upregulated in jejunum on day seven for yeast group compared with control. Pathways are sorted by -log(p value) within pathway parent. High -log(p value) equals low p value. Point color and size represent proportion of DEGs found in pathway. Pathways related to human disease were excluded in the figure but can be found in the supplementary data together with an overview of DEGs in pathways (Supplementary data 3).

Table 3
Number of DEGs in ileum.

	Treatment vs D0		
	Control	Yeast	Yeast vs Control
Upregulated			
Day 2	21	0	0
Day 4	0	1	27
Day 7	28	41	0
Downregulated			
Day 2	1	0	3
Day 4	0	1	0
Day 7	2	2	0

inflammation in response to antigenic substances. Mechanisms to suppress immune activation when challenged with new microorganisms and antigens such as at birth and weaning, are important for an optimal and long-term maturation of the immune system (Moeser et al., 2017). On the other hand, in case of a breach in the epithelial barrier, the GIT immune system must be able to respond rapidly, and this balance of control and reactiveness is critical for optimal GIT health (Moeser et al., 2017).

In ileal tissue from control piglets, DEGs in the NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway, autophagy pathway and mitophagy pathway were upregulated on day two PW compared with pre-weaning levels followed by decreased expression to below pre-weaning level on day seven. Autophagy is activated in response to stress and starvation, which is relevant in the acute PW period, and is the cell's way of removing dysfunctional components. A reduction in VH in ileum was seen between day zero and day two, which corresponds to the upregulation of autophagy in this tissue, while the expression of the pathway is decreased in the following regeneration period. The activation of ubiquitin mediated proteolysis on day 2 with following decline also confirms the increased turnover of damaged tissue in the acute PW phase. An activation of NOD-and RIG-I-like receptor signaling pathway along with apoptosis was also found by Bomba et al. (2014), five days PW in ileum of piglets weaned at day 28. Correspondingly, an opposite trend was seen for ileal genes in the enriched pathways focal adhesion, ECM-receptor interaction and PI3K-Akt signaling, which all play essential roles in cell maintenance, motility and proliferation. Weaning induced enhancement of apoptosis and inhibition of epithelial cell proliferation have been previously reported in jejunal tissue (Zhu et al., 2014).

Comparing PW gene expression between diets

Changes in gene expression PW corresponds with the known functional and structural changes occurring in the intestinal tissue.

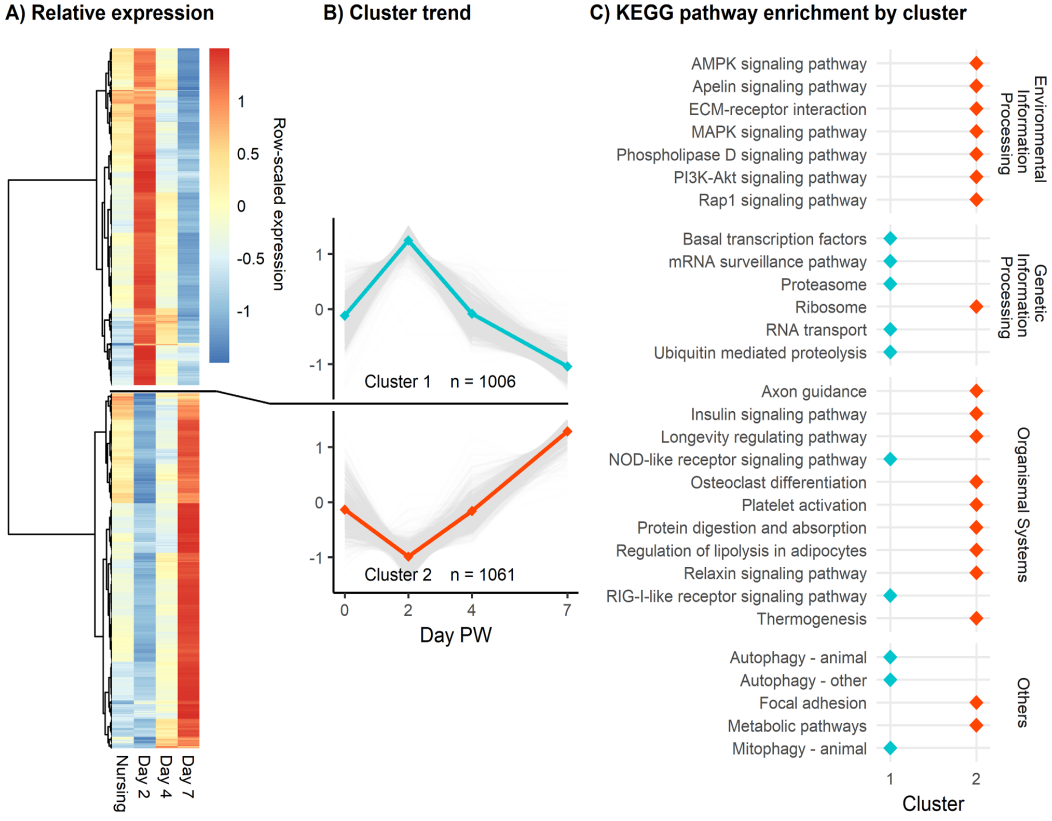

Fig. 11. Ileal gene expression patterns over time in ileum for control piglets. A) Heatmap showing transcriptional changes in ileum over time for piglets fed control diet. Heatmap includes 2067 genes differentially expressed (adjusted $P < 0.05$) between any of the timepoints. Transcript was row-scaled to highlight changes in the expression of individual genes, B) Genes with similar expression patterns across the timepoints were separated into two main clusters using Ward's method. Trend lines are based on mean scaled values in each cluster. C) KEGG pathway enrichment analysis was run on each cluster. Each point represents a significantly enriched pathway ($P < 0.05$). Pathways related to human disease were excluded in the figure but can be found in the supplementary data together with an overview of DEGs in pathways (Supplementary data 2).

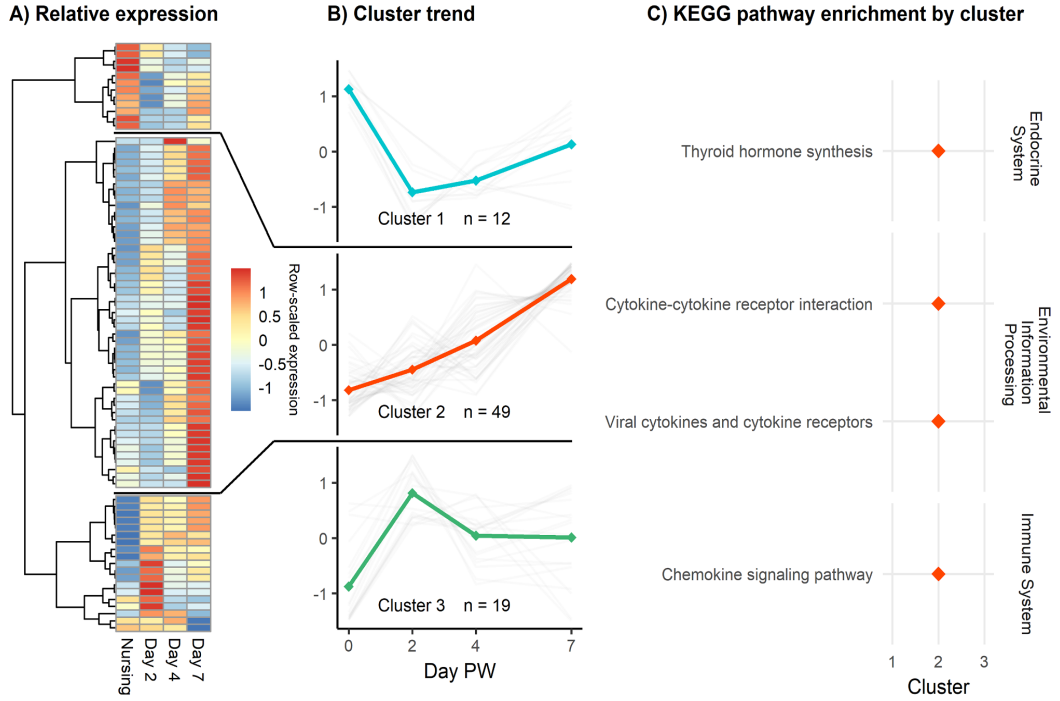

Fig. 12. Ileal gene expression patterns over time in ileum for yeast-fed piglets. A) Heatmap showing transcriptional changes in ileum over time for piglets fed yeast diet. Heatmap includes 80 genes differentially expressed (adjusted P < 0.05) between any of the timepoints. Transcript abundance was row-scaled to highlight changes in the expression of individual genes, B) Genes with similar expression patterns across the timepoints were separated into three main clusters using Ward's method. Cluster 1 contains too few DEGs to determine pathway enrichment. Trend lines are based on mean scaled values in each cluster. C) KEGG pathway enrichment analysis was run on each cluster. Each point represents a significantly enriched pathway (P < 0.05). Pathways related to human disease were excluded in the figure but can be found in the supplementary data together with an overview of DEGs in pathways (Supplementary data 2).

Interestingly, these PW changes in gene expression observed in the control group were not seen to the same extent in the yeast group, suggesting that piglets fed the yeast diet were more tolerant of weaning stress. When plotting the PW expression trend in the yeast-fed piglets for the DEGs in the control group, the overall trend for cluster 1 differs (Figure Supplementary figure 1). For the piglets fed yeast, the overall expression of these immune-related genes declined to a larger extent than for the piglets fed the control from day zero to day two PW, but the immunosuppression seemed to recover faster. This suggests an earlier maturation of the GIT immune system in the yeast fed piglets. On the other hand, there were few DEGs between the two treatments on day two and four PW, whereas significant differences were found on day seven. The genes upregulated in the yeast group on day seven, as compared to the control group, were mostly associated with immune system pathways. Several of the immune system associated enriched pathways in the yeast group on day seven were the same pathways that declined with time PW in the control group. Of the 469 upregulated DEGs in the yeast group compared with control on day seven, 414 were the same genes that were significantly downregulated in the control group compared with day zero. This finding provides evidence that there are different gene expression patterns PW between the two feeding groups. It also suggests that the DEGs on day seven are not caused by a PW upregulation in the yeast group as such, but a reduced expression PW in the control group, as these genes were not found to be significantly enriched with time PW in the yeast group.

The NF-κB signaling pathway, which was enriched in the jejunum of yeast-fed piglets compared with control on day seven, consists of dimers involved in the regulation of immunity, inflammation and cell survival. NF-κB activation has also been shown to regulate intestinal homeostasis through controlling apoptosis (Siggers & Hackam, 2011). Another enriched pathway was the Toll-like receptor signaling pathway, which is responsible for detecting pathogens by responding to the membrane component of Gram-positive or Gram-negative bacteria and for activating innate immune responses. Key genes in this pathway, the transmembrane receptors *TLR1*, *TLR2* and *TLR4* were upregulated in the jejunum of yeast piglets on day seven compared with control. *TLR4* is involved in both defense against pathogens and maintaining tolerance to commensal bacteria (Frosali et al., 2015). In the epithelium, *TLR4* activation is important in recruiting defense against pathogens in case of epithelial injury, and absence might result in severe mucosal damage (Frosali et al., 2015). Similar to *TLR4*, *TLR2* also mediates pro-inflammatory signaling, and activation might suppress mucosal inflammation and enhance barrier integrity (Siggers & Hackam, 2011).

The upregulation of the intestinal immune network for IgA production pathway on day seven in the yeast-fed piglets was not confirmed by quantification of IgA in the jejunal tissue. IgA immunoglobulins are constantly secreted from the tissue and the tissue concentration might therefore not reflect the production of the protein. Improved IgA secretion in jejunum mucosa PW when feeding live or superfine powder of *S. cerevisiae* was reported by Zhy et al. (2017). However, these piglets were weaned at 14 days of age. Moreover, Zhy and coworkers found no effect of heat-killed whole yeast, which is more similar to our yeast product. Although there was no difference in jejunal IgA concentration between the dietary treatments when measured by ELISA, the PW development of IgA concentration matches the PW expression trend in the control group for genes in the intestinal immune network for IgA production pathway. IgA is an important antibody, working as a first-line barrier by limiting antigen transition from the

intestinal lumen to blood and controlling the intestinal microbiota (Pabst, 2012).

Fatty acid-binding protein 6 (*FABP6*), was not involved in any of our significant pathways, but is important in the transportation and metabolism of long-chain fatty acids in the intestine and has been proposed as a potential biomarker for intestinal barrier dysfunction (Celi, Verlhac, Calvo, Schmeisser, & Kluenter, 2019). Intestinal FABP is highly present in mature enterocytes and high concentrations in plasma are correlated with intestinal mucosa damage (Niewold, Meinen, & Van der Meulen, 2004). It is therefore relevant to mention the expression trend of this gene. The jejunal *FABP6* expression decreased for both diets from day zero to day two (Figure Supplementary figure 2B). In the control piglets, the expression remained low, whereas it increased to above day 0 level for the yeast-fed piglets with time PW. The increased expression might be a result of rebuilding after intestinal damage, suggesting that higher expression levels indicate, as previously discussed, an enhanced rebuilding phase in the yeast-fed piglets.

To summarize and conclude, the results showed that yeast is a suitable protein ingredient for PW piglets. For the gut functional parameters, the differences were more significant for sampling day than for dietary treatment. However, inclusion of 14.6% of *C. jadinii* yeast improved PW AID of protein and brush border enzyme activities early PW. Reduction of brush border enzyme activity correlated with an increase in CD indicating a rebuilding process of the intestinal villi, which was also supported by the transcriptomics results where pathways involved in these processes showed the same trends. Although less evident in the yeast-fed group, the PW jejunal gene expression pattern in both dietary treatments showed a decreased PW relative expression of genes in pathways related to immune system processes. There was also an increased PW relative expression of DEGs in metabolic pathways, especially in the control group. The largest differences between the two dietary treatments were seen on day seven PW, where several pathways related to the immune system were upregulated in the yeast-fed piglets compared with control. Thus, there seems to be an PW immunosuppression in jejunum of the control piglets. In ileum, the PW transcriptional changes were also less evident in the yeast-fed piglets compared with control piglets, and the transcriptomic trends match the functional results well, with an early PW critical phase followed by a rebuilding phase. These results indicate that feeding *C. jadinii* yeast can improve PW gut homeostasis and give more robust piglets.

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CRediT authorship contribution statement

Ingrid Marie Håkenåsen: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Visualization, Project administration. **Margareth Øverland:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. **Ragnhild Ånestad:** Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Caroline Piercey Åkesson:** Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Arvind Y.M. Sundaram:** Formal analysis, Writing - original draft, Writing - review & editing. **Charles McLean Press:** Conceptualization, Investigation, Writing - review & editing. **Liv Torunn Mydland:** Conceptualization, Methodology, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104118.

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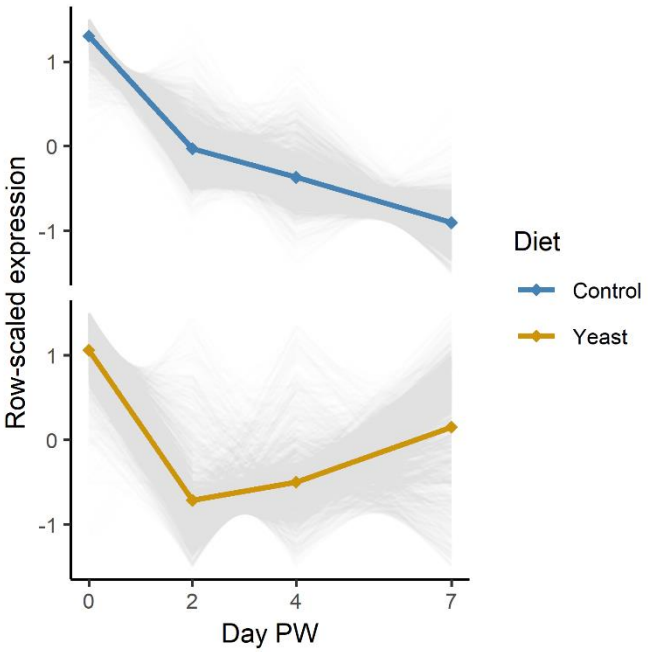
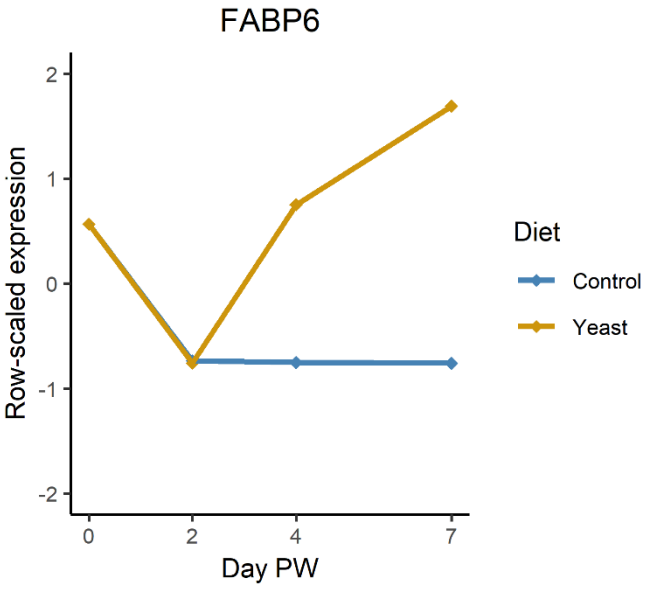


Supplementary figure 1. Expression trends for genes in cluster 1 in Figure 8, for control (blue) and yeast (yellow)



Supplementary figure 2. Post-weaning relative expression trends for the *FABP6* gene.

Supplementary data 1. Raw reads, clean reads and percentage of reads aligning to the genome using hiSat2 for all the samples sequenced using RNA-seq.
Can be found online at: https://doi.org/10.1016/j.jff.2020.104118

Supplementary data 2. Table with ENSEMLE ID's and gene names for differentially expressed genes included in the significantly enriched pathways.
Can be found online at: https://doi.org/10.1016/j.jff.2020.104118

Supplementary data 3. Table with ENSEMLE ID's and gene names for differentially expressed genes included in the significantly enriched pathways in yeast vs. control on day 7 PW.
Can be found online at: https://doi.org/10.1016/j.jff.2020.104118

Paper II

Biochemical, immunological, and metabolic profiling of post-weaning piglets fed *Cyberlindnera jadinii* yeast as a protein source

Ingrid Marie Håkenåsen[1], Mette Skou Hedemann[2], Anna Julie Kjøl Tornes[1], Margareth Øverland[1], and Liv Torunn Mydland[1*]

[1] Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Aas, Norway
[2] Department of Animal Science, Faculty of Science and Technology, Aarhus University, AU-Foulum, Tjele, Denmark

* Corresponding author at: Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway. Tel: +47-67232635. E-mail address: liv.mydland@nmbu.no (L.T. Mydland)

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Abstract

Weaning is a critical time for piglets due to major changes in diet, environment, and exposure to pathogens, as well as stressors such as maternal separation and litter mixing. A feeding trial with piglets in the early post-weaning (PW) phase (day 0 - 14) was conducted to investigate the effects of feeding yeast on the PW development of plasma biochemistry and immunological parameters, as well as on metabolites in plasma and urine. A total of 64 crossbred piglets (10.2 ± 1.6 kg BW; weaned at 27 ± 1 d) were allocated into two dietary groups and received a control diet or a diet containing 14.6 % *Cyberlindnera jadinii* yeast, substituting 40 % of the protein in the control diet. Blood and urine samples were collected on the day of weaning (day zero) and on days 2, 4, 7, and 14 PW for analyses of plasma biochemistry, cytokines, and immunoglobulins. Metabolomic profiling was performed in both plasma and urine. The PW changes in plasma biochemistry, cytokines, and immunoglobulins were time-dependent and not diet-dependent, except for inorganic phosphate and the immunosuppressor IL-1ra. The inorganic phosphate concentrations were higher in yeast fed-piglets PW, whereas the IL-1ra concentrations were higher in control-fed piglets on day two PW, compared with the control piglets on days 4, 7, and 14, and the yeast-fed piglets on all PW sampling timepoints. The plasma cytokine results suggest an early PW acute phase which was less evident in the yeast-fed piglets. Yeast-fed piglets also had lower plasma PW concentrations of IgM compared with control. Betaine was identified as the most discriminating metabolite between dietary treatments in both plasma and urine. These results support our previous findings that feeding *C. jadinii* yeast PW might result in more robust piglets.

Keywords: plasma biochemistry, immunoglobulins, cytokines, metabolites, post-weaning piglets

1 Introduction

Weaning is a challenging occurrence in the piglet's life. They must deal with several stressors, transition in diet, end of the supply of maternal immunoglobulins through milk, and increased exposure to pathogens, while their immune system is not yet fully developed to handle these challenges (Moeser et al. 2017). Yeast is known for their cell wall components to have potential health beneficial effects in post-weaning (PW) piglets (Lee et al. 2021). However, the nutrient availability and functional properties of the yeast are affected by several factors, including the yeast species, fermentation substrates, and downstream processing (Agboola et al. 2021). The yeast species investigated in this study (*Cyberlindnera jadinii*, previously called *Candida utilis*), has previously been investigated in PW piglets, where a similar diet with 40% of crude protein (CP) from *C. jadinii* maintained growth performance and improved the digestive function in a four week period for piglets weaned at 28 days of age (Cruz et al. 2019). Moreover, the inclusion of *C. jadinii* beneficially modulated the microbiome (Iakhno et al. 2020; Lagos et al. 2020), decreased secretion of IgA in the colon (Lagos et al. 2020), and improved the gut homeostasis of PW piglets (Håkenåsen et al. 2020). The *C. jadinii* yeast has also been studied in Atlantic salmon, where it has the potential of reducing soybean meal-induced enteritis (Agboola et al. 2021).

The yeast cell wall mostly consists of complex polysaccharides such as β-d-glucans and α-d-mannans, which by binding to specific immune cell receptors may modulate mucosal immunity (Kogan & Kocher 2007; Shurson 2018). By occupying the receptors of the enteropathogenic bacteria, α-d-mannans may prevent adhesion of the bacteria to the epithelial surface, whereas the β-d-glucan previously has been shown to enhance the functional status of macrophages and neutrophils, modify immunosuppression, and stimulate the release of cytokines such as tumor necrosis factor-alpha (TNFα) (Kogan & Kocher 2007). Several

studies have investigated the effect of live yeast or yeast cell-wall products on the health of PW piglets (Che et al. 2017; Kogan & Kocher 2007; Lee et al. 2021; Long et al. 2021). However, yeast or yeast products are most often included in small amounts in the diet. We wanted to investigate the potential beneficial effect of including inactivated whole yeast as a protein source. Therefore, the objective of this study was to investigate the effect of high dietary inclusion of *C. jadinii* yeast on the PW development of several biochemical indices, pro-and anti-inflammatory cytokines, immunoglobulins, and metabolic profiles in newly weaned piglets.

2 Materials and Methods

2.1 Animals and diets

The experimental design has been explained in details by Håkenåsen et al. (2020). In brief, a total of 64 crossbred ([Norwegian Landrace × Yorkshire z-line] × Duroc [LZD] and Norwegian Landrace × Duroc [LLD]) piglets were selected from eight litters and included in the experiment. Average weaning age was 27 ± 1 days and the average weaning weight was 10.2 ± 1.6 kg. Piglets were allocated to dietary treatment and day of dissection based on litter origin and weaning weight, to make uniform groups. The dietary treatments included 1) a control diet based on wheat, barley, oats, soybean meal (SBM), fishmeal (FM), potato protein concentrate and rapseed oil, and 2) an experimental diet containing 14.6 % *C. jadinii* yeast where the yeast substituted 40 % of the protein in the control diet. Dietary and chemical composition is shown in Table 1. The piglets had *ad-libitum* access to clear drinking water and the experimental diets immediately after weaning and until sampling.

2.2 Sample collection

All animals were euthanized using a captive bolt pistol, followed by exsanguination, and collection of blood and urine samples. To provide a baseline time point to facilitate detection and interpretation of changes due to weaning and potential yeast-induced changes, samples were collected from eight piglets (littermates of piglets included in the experiment) at the day of weaning (day zero). Further, samples were collected from eight piglets from each dietary treatment at day two, four, seven and 14 PW. Blood was collected both in tubes coated with EDTA and heparin during exsanguination and centrifuged at 4°C for 15min (2000 x g). Plasma was aliquoted into cryotubes, immediately stored at -20°C and then at -80°C until analyses. Heparin-plasma was used for analysis of biochemical parameters and metabolomics, whereas EDTA-plasma was used for

analysis of immunoglobulins, cytokines, and chemokine. Urine samples for metabolomics were collected with a needle directly from the urine bladder, postmortem. Samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses.

Table 1 Dietary composition and analyzed chemical composition of the dietary treatments.

	Dietary treatments	
	Control	Yeast
Ingredient[1], g/kg as fed		
Wheat	627.8	593.5
Barley	100.0	100.0
Oats	50.0	50.0
Soybean meal	80.0	19.2
Potato protein conc.	33.8	9.1
Fish meal	20.0	4.8
Rapeseed meal	20.0	4.9
Yeast - *Cyberlindnera jadinii*	-	146.0
Rapeseed oil	19.7	23.4
Vitamins, minerals, and amino acids	48.6	49.0
Yttrium (III) oxide	0.1	0.1
Nutrients, g/kg of DM		
Crude protein	202.0	193.9
Starch	508.0	494.3
Crude fat	45.3	46.2
NDF	110.0	102.3
Ash	52.7	51.2
Gross energy, MJ/kg	18.9	19.0

[1]Detailed information about ingredient origin, and composition of added vitamins, minerals and amino acids can be found in Håkenåsen et al. (2020).

2.3 Analysis of blood biochemistry, immunoglobulins, and cytokines

Biochemical parameters in blood plasma (total protein, albumin, globulin, creatinine, glucose, urea, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), creatine kinase (CK), calcium, inorganic phosphate, and uric acid) were analyzed using the Advia®1800 chemistry system (Siemens Healthcare Diagnostics, Siemens AG, Erlangen, Germany) at the Central Laboratory, Norwegian University of Life Sciences (https://www.sentrallaboratoriet.no/).

The concentrations of immunoglobulin (Ig) A, IgM and IgG in plasma were analyzed using commercial swine-specific enzyme-linked immunosorbent assay (ELISA) kits; Pig IgA ELISA Kit (ab190536) and Pig IgM ELISA Kit (ab190537) from Abcam, Cambridge, UK, and Pig IgG ELISA Kit (CSB-E06804p) from Cusabio, Aachen, Germany. In the IgA and IgM assays, 100 µl of standards and diluted (IgA 1:5000, IgM 1:10000) plasma samples were allocated in duplicates into pre-designated wells on a 96-well plate, whereas in the IgG assay, 50 µl of standards and diluted (1:100) plasma samples were used (duplicates). All ELISA analyses were conducted according to the manufacturer's instructions and the absorbances were read at 450 nm on a SpectraMax M2e Microplate Reader (Molecular Devices).

The bead-based MILLIPLEX assay kit (Milliplex MAP, MerckMillipore [PCYTMG-23K-13PX]) and Luminex technology (Bio-Plex® 200, HTF [171000205], Bio-Rad, USA) was used to analyze the granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN) γ, interleukins (IL)-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and TNFα. The multiplex assay was performed according to the protocol provided by the manufacturer, except for the amount of plasma used in each well. Due to general low values in

the assay for these piglet samples, the sample volume was increased to 50 µl (instead of 25 µl sample and 25 µl assay buffer).

2.4 Gene expression

Full description of the RNA extraction, library construction, RNA sequencing and data analysis can be found in Håkenåsen et al. (2020). In brief, total RNA was extracted from proximal jejunal and ileal tissue following the RNeasy Plus Universal Kit's protocol (Qiagen, Hilden, Germany). High quality samples (RIN ≥ 7) were sequenced at the Norwegian Sequencing Centre (http://www.sequencing.uio.no). Raw reads were cleaned and aligned to the *Sus scrofa* ENSEMBL genome Sscrofa 11.1 release 98 using HISAT v2.1.0 (Kim et al. 2015).

2.5 Metabolomics

2.5.1 Sample Preparation

Urine samples (270 µl) were mixed with 30 µl ice-cold pure acetonitrile containing internal standards (Glycocholic acid: Glycine-1^{13}C, and p-chlorophenylalanine) in a final concentration of 10 µg/mL. Samples were immediately incubated at 4°C for 20 minutes to precipitate protein, followed by centrifugation for 25 minutes at 3.700 rpm at 4°C. The supernatants were collected and again centrifuged for 25 minutes at 3.700 rpm at 4 °C prior to LCMS analysis. Plasma samples (150 µl) were diluted 1:4 with ice-cold acetonitrile containing the internal standards in a final concentration of 10 µg/mL. Samples were mixed and incubated for 10 minutes at 4°C, followed by centrifugation at 3.700 rpm at 4°C for 25 minutes. The supernatants were filtered, transferred, and evaporated to dryness in a vacuum centrifuge (ca. 2.5 h, 805 × g and 30°C). Metabolites were resuspended in 150 µl H_2O:acetonitrile: formic acid (95:5:0.1) and centrifuged for 10 minutes at 3.700 rpm at 4°C prior to LCMS analysis.

2.5.2 Non-targeted UHPLC-MS

UHPLC-MS analysis was performed using a Dionex UltiMate 3000 (Dionex, Sunnyvale, CA, USA) ultra-high pressure liquid chromatography system (UHPLC) coupled with an Impact HD Quadrupole Time-of-Flight (QTOF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) operating in positive electrospray ionization mode (ESI+) and negative electrospray ionization mode (ESI-) using the instrumental parameters previously described by Curtasu et al. (2018). Chromatographic separation of the samples was performed on an HSS T3 C18 UHPLC column, 1.8 µm, 100 x 2.1 mm (Waters Corporation, Milford, MA) equipped with a VanGuard Pre-column, 100Å, 1.8 µm, 2.1 mm × 5 mm (Waters Corporation, Milford, MA). The column was maintained at 30°C and the samples were kept in the autosampler, set to 10°C, for the whole duration of the analysis. The injection volume was set to 3 µl for all matrices. The mobile phases were 0.1 % formic acid in Milli-Q water (A) and 0.1 % formic acid in acetonitrile (B). The flow rate was 0.4 ml/min. Plasma samples were analyzed using the following gradient: 0-12 min, linear gradient from 5 to 100 % B; 12-13 min, 100 % B and return to initial conditions in 0.2 min. The column was re-equilibrated at 5 % B for 2 min in the beginning of each run. For urine samples the gradient was as follows: 0-8 min linear gradient from 5 to 70 % B; 8-8.5 min 70-100 % B; 8.5-9.5 min 100 % B and return to initial conditions in 0.2 min. The column was re-equilibrated at 5 % B for 2 min in the beginning of each run.

The quality of data was assessed by several methods. Blank samples (H_2O + internal standard), blind samples (0.1 % formic acid) and quality control (QC) samples were frequently injected between runs to ensure the quality of data. Carry over effect were assessed by checking chromatograms of blind samples. QC samples were a mixture of equal amount from all urine or plasma samples, respectively, and used to observe retention time stability. Unsupervised multivariate data analysis was used to investigate the relationship between QC

samples (data not shown). The QC samples clustered together with minimal deviation.

1.1 Data processing and statistics

Outliers in the blood parameters were identified using the interquartile range (IQR) method. Outliers were defined as > Q3 + 3* IQR or < Q1 + 3*IQR. Statistical analysis was performed using the mixed procedure in the SAS® software, V.9.4 (SAS Inst. Inc., Cary, NC). Day zero samples were not included in the statistics. The following model was used for all parameters measured in blood:

$Y_{ijklmn} = \mu + diet_i + s\_day_j + (diet \times s\_day)_{ij} + breed_k + litter_l + pen_m(diet_i) + e_n$,

where Y is one observation on piglet n; μ is the intercept; $diet_i$ is the fixed dietary treatment effect (i = 1,2); s_day_j is the fixed effect of sampling day PW (j = 1:4); $diet_i \times s\_day_j$ is the fixed interaction between dietary treatment and sampling day; $breed_k$ is the fixed effect of the breed of the piglet (k :1 = LLD and k : 2 = LZD); $litter_l$ is the random effect of the l-th litter ~ N (I × σ^2_{litter}) here I is an identity matrix of dimensions and σ^2_{litter} is the variance component for litter; $pen_m(diet_i)$ is the random effect of pen within diet each ~ N (0, I × $\sigma^2_{pen(diet)}$); and e_n is the error of the n piglet ~ N (0, I × $\sigma^2_{e\,s\_day}$), i.e. assumed heterogeneous per sampling day. Effects were considered statistically significant when P ≤ 0.050 and tendencies were defined as P-values > 0.050 and ≤ 0.100. Pearson correlations were investigated using the rcorr function in the Hmsic package v.4.6–0 (Harrell Jr et al. 2021). A correlation matrix including biochemical indices, cytokines and immunoglobulins, was visualized with the corrplot function in the corrplot package v 0.90 (Wei et al. 2021). Only significant (P ≤ 0.050) correlations are shown (Supplementary Figure 1).

mzXML files from the untargeted UHPLC-MS analysis were acquired and raw spectral data processed using the XCMS package version 3.4.1 (Benton et al. 2010; Smith et al. 2006; Tautenhahn et al. 2008) in R version 3.6.1 with the following parameters: noise were set to intensity < 1500, with signal to noise ratio cutoff at 6, peak width 10-30 s, m/z tolerance of 0.001, integration method 1 and the meanApex3 function to calculate m/z center. Peaks from different samples were grouped with a bandwidth of 2, width of overlapping m/z slices at 0.008, and common features found in at least 50% of the samples. The median QC sample was used as center for retention time correction with the OBI-Warp method (Prince & Marcotte 2006). Missing peaks were filled in using the peak finder.

Rule based annotation of isotopes and adducts were performed using findIsotpes and findAdducts functions in the CAMERA package version 1.38.0 (Kuhl et al. 2011). When several isotopes were found for a metabolite only $[M+H]^+$ or $[M-H]^-$ were kept in positive and negative mode, respectively. Further data editing involved discarding features with retention time (RT) < 0.4 min, m/z > 800, and features with higher mean values in blinds than QC samples. Urine samples were normalized by creatinine content. Principal component analysis (PCA) was performed in R for separate ionization modes and sample days PW, to look for differences in the metabolic profile between the dietary treatments on each sampling day. For identification of metabolites differing between diets on day 14, Partial least-squares discriminant analysis (PLS-DA) models were built to determine the metabolites responsible for the differences between pigs fed control or yeast diets. Variables for identification were selected using the VIP scores and the scaled regression coefficients. Compounds in both urine and plasma samples were identified based on queries in the METLIN (http://metlin.scripps.edu/), Human Metabolome Database (http://www.hmdb.ca/), and LIPID MAPS

(http://www.lipidmaps.org/) online databases for obtaining possible chemical structures using accurate mass and mass spectrometric fragmentation patterns.

3 Results

3.1 Biochemical parameters

The PW developments of standard blood biochemical parameters are shown in Figure 1. Inorganic phosphate was significantly higher in yeast-fed piglets ($P < 0.001$) compared with control, varying with day PW ($P = 0.037$). Dietary treatment did not affect the total protein ($P = 0.527$), albumin ($P = 0.815$), ALP ($P = 0.811$), AST ($P = 0.632$), creatine kinase ($P = 0.489$), creatinine ($P = 0.705$), globulin ($P = 0.424$), glucose ($P = 0.573$), urea ($P = 0.164$), or calcium ($P = 0.186$) concentrations in plasma. The sampling day PW significantly affected the plasma concentration of total protein ($P = 0.010$), albumin ($P < 0.001$), globulin ($P = 0.001$), creatinine ($P = 0.036$), ALP ($P = 0.003$), AST ($P = 0.024$), and inorganic phosphate ($P = 0.021$). The total protein, albumin and creatinine concentration in plasma decreased from day two until day 14 PW for both dietary treatments, whereas the globulin concentration increased PW. The ALP also had a significant diet*day PW interaction ($P = 0.010$). Whereas the ALP concentration remained relatively stable PW in the yeast-fed piglets, a decreased in concentration from day two to seven PW was detected in the control group. Independent of dietary treatment, the AST increased in concentration from day two to seven PW, before the concentration decreased on day 14 PW. The plasma level of total bilirubin decreased after weaning until day seven, and from day 7 to 14 PW the total-bilirubin level was zero in both dietary groups. Uric acid was not detected in any samples.

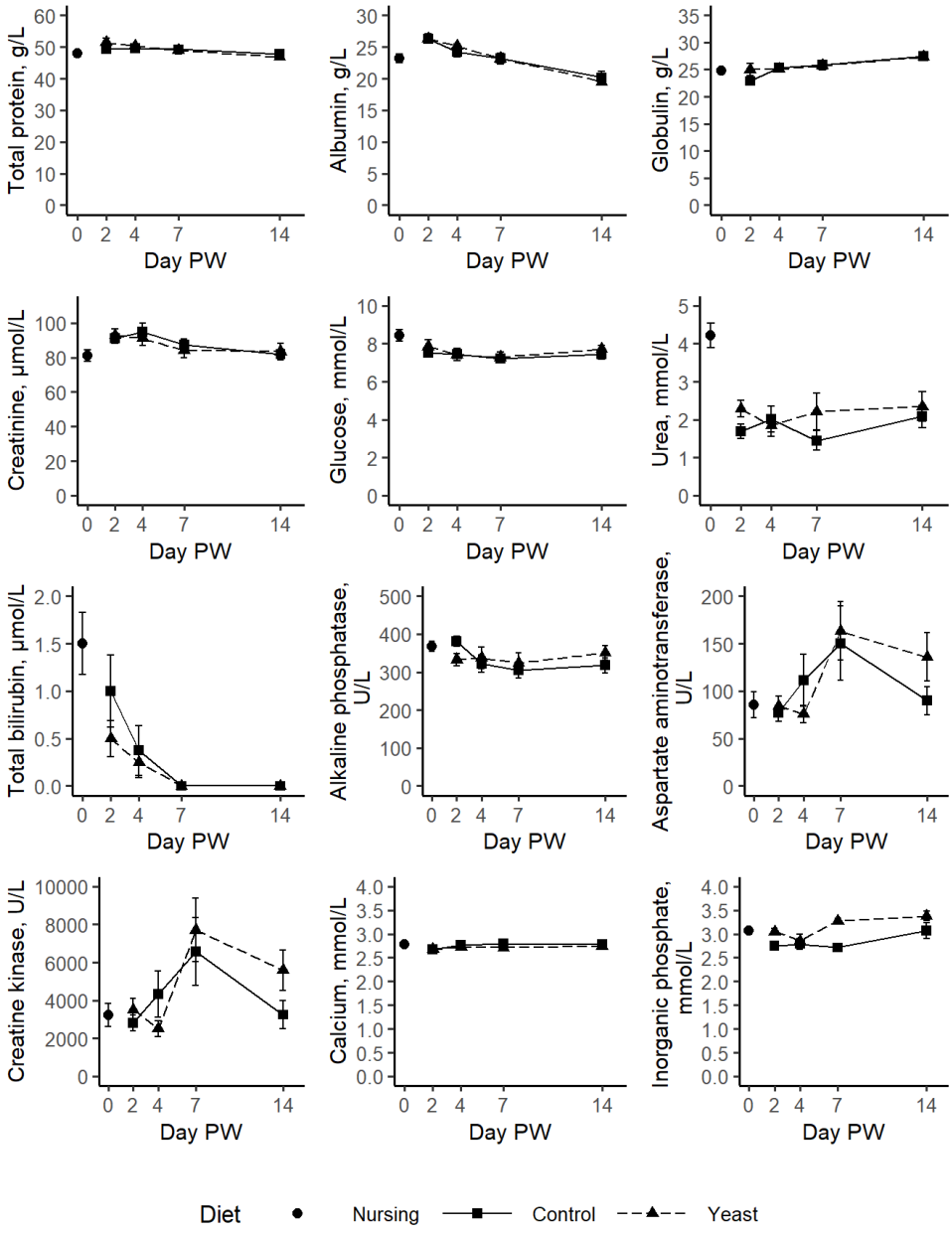

Figure 1 Effect of dietary treatments on the post-weaning (PW) development of plasma biochemical parameters.

3.2 Cytokines

The PW developments of cytokine concentrations in plasma are shown in Figure 2. The concentration of IL-1ra differed by dietary treatment ($P = 0.031$) and day PW ($P < 0.001$). The dietary treatment effect on IL-1ra plasma concentrations also differed with time PW ($P = 0.002$), where the concentration in plasma from control piglets on day two PW was significantly higher compared with the control piglets on day four, seven and fourteen, and yeast piglets on all sampling timepoints PW. Dietary treatment did not affect the PW plasma concentrations of TNFα ($P = 0.508$), IL-1β ($P = 0.136$), IL-2 ($P = 0.892$) or IL-8 ($P = 0.729$), whereas a tendency for lower plasma concentrations of IL-10 was found in the yeast-fed piglets compared with control ($P = 0.052$). However, the concentrations of these cytokines differed by day PW. TNFα plasma concentration was significantly higher on day two and four compared with day fourteen ($P < 0.001$). Independent of dietary treatment, IL-1β and IL-10 plasma concentrations were significantly higher on day two PW compared with day seven PW (IL-1β: $P = 0.035$; IL-10: $P = 0.001$). A tendency for effect of day PW was also found for IL-2 ($P = 0.092$) and IL-8 ($P = 0.092$) plasma concentrations. In addition, IL-2 was the only cytokine which significantly differed between breeds ($P = 0.023$), where the LLD piglets had higher IL-2 plasma levels compared with the LZD piglets. Dietary treatment or sampling day PW did not affect the plasma concentrations of IL-1α (Diet: $P = 0.914$; Day: $P = 0.423$), IL-12 (Diet: $P = 0.342$; Day: $P = 0.729$), or IL-18 (Diet: $P = 0.257$; Day: $P = 0.235$). GM-CSF was not detected in any samples, whereas IFNγ was only detected in 23 of the 72 samples. The other samples had negative fluorescence intensity when subtracting the background intensity, and was therefore registered as NA. However, it is worth mentioning that the treatment groups where IFNγ were detected in most samples was the day zero, and pigs fed the yeast diet on day two and four PW. Negative fluorescence intensity was also the reason for low detection rate for IL-4 and IL-6, with no clear pattern for the treatment groups.

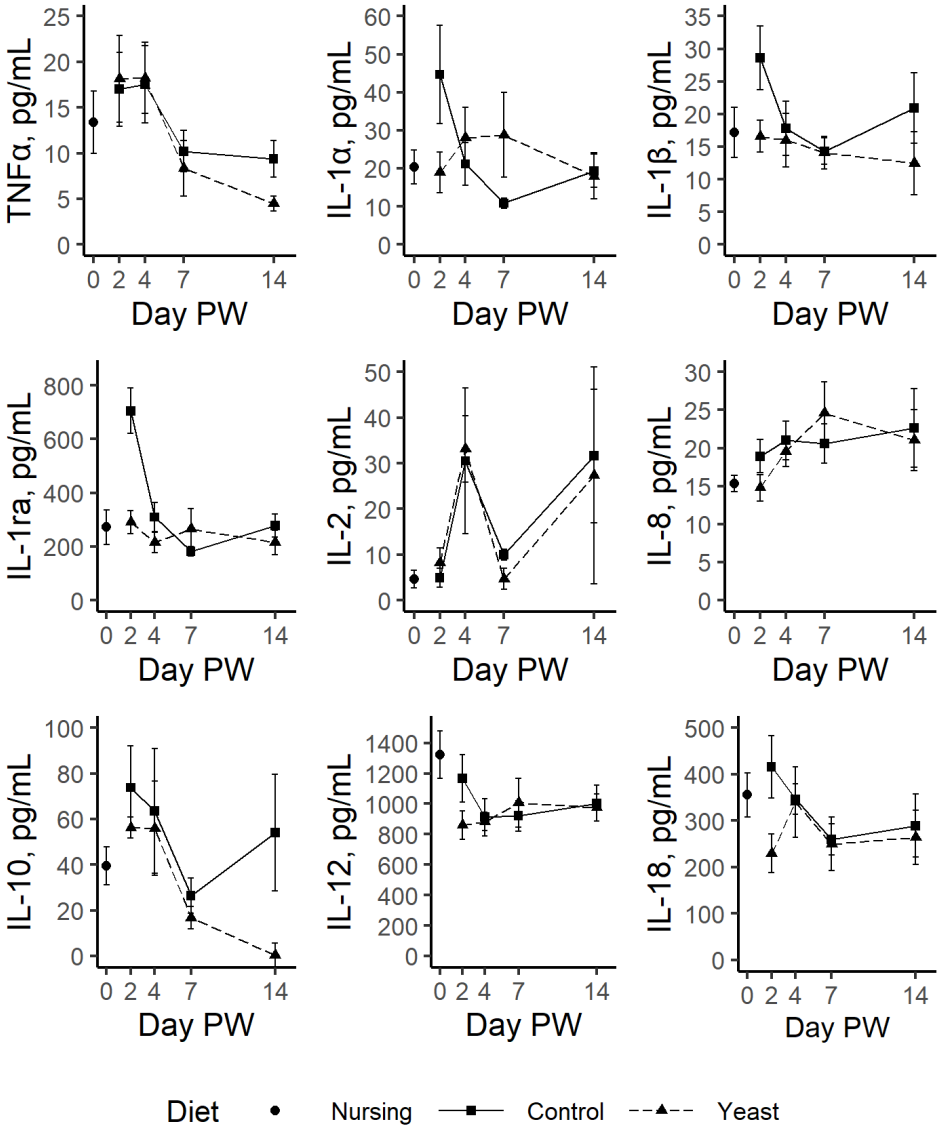

Figure 2 Effect of dietary treatment on the post-weaning (PW) development of cytokine concentrations in plasma.

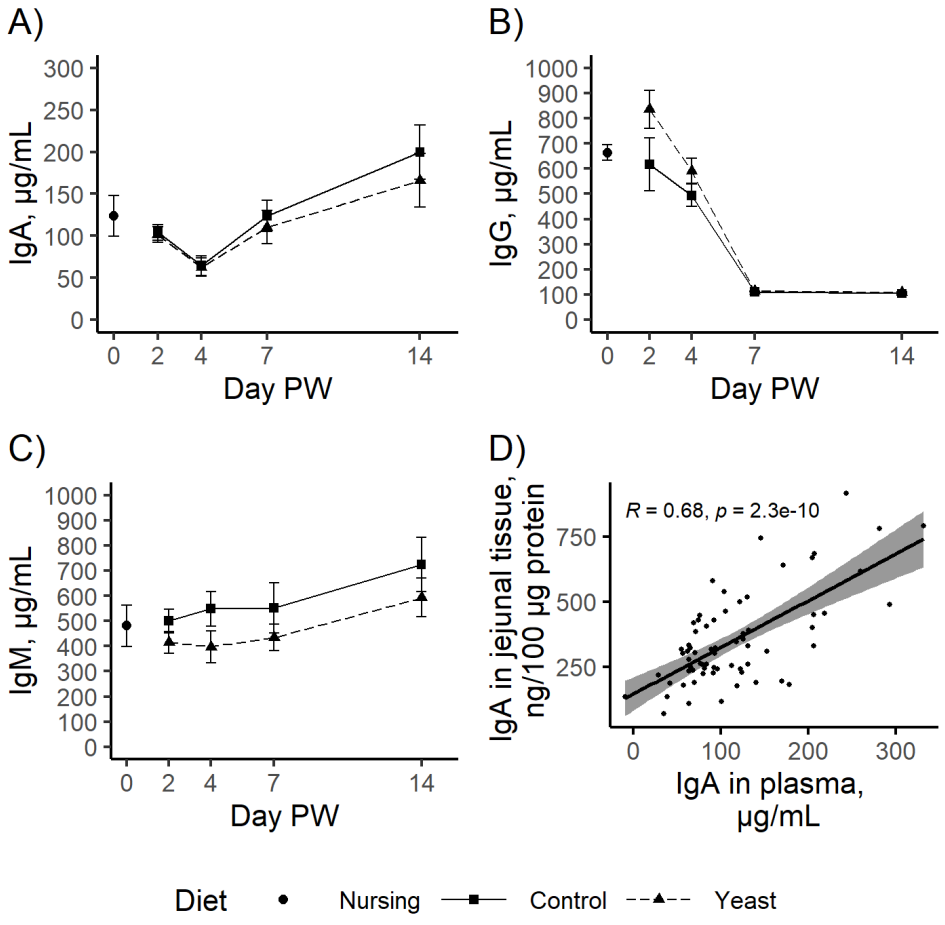

Figure 3 A), B) and C) Effect of dietary treatment on the development of immunoglobulins (µg/ml) in plasma of post-weaning (PW) piglets. D) Correlation plot between IgA levels in plasma and IgA concentration in jejunal tissue from Håkenåsen et al. (2020).

3.3 Immunoglobulins

The developments of IgA, IgG, and IgM in plasma PW are shown in Figure 3 A, B and C. IgM in plasma was significantly reduced PW in the yeast-fed piglets compared with the control piglets ($P = 0.014$). For both dietary treatments, the concentration of IgM increased PW ($P = 0.027$). There were no significant differences in IgA ($P = 0.428$) or IgG ($P = 0.181$) concentrations between the dietary treatments, but the concentrations significantly differed with day PW. The concentration of IgA in plasma decreased PW until day four PW, before it increased to above day zero level at day 14 ($P < 0.001$). The IgG concentration in plasma decreased until day 7 PW and remained at a stable low level until day 14 ($P < 0.001$). A summary of mean values, standard error of mean (SEM) and P-values for all plasma biochemistry, cytokines, and immunoglobulins can be found in Supplementary Table 1.

A significant positive correlation (Figure 3D; $r = 0.68$, $P < 0.001$) was observed between the IgA concentration in plasma, and the IgA concentration previously detected in jejunal tissue (Håkenåsen et al. 2020). The relationship between the currently detected ALP in plasma and the previously detected ALP in jejunal tissue (Håkenåsen et al. 2020) was also investigated, but the parameters did not correlate ($r = 0.12$, $P = 0.318$). There was also no correlation between creatinine in plasma and urine ($r = 0.18$, $P = 0.134$). A correlation plot, comparing the plasma biochemical indices, cytokines, and immunoglobulins is presented in Supplementary Figure 1. There was a strong positive correlation ($r = 0.96$, $P < 0.001$) between the AST and CK levels in plasma. Positive correlations were also found between total protein and albumin ($r = 0.71$, $P < 0.001$), albumin and IgG ($r = 0.52$, $P < 0.001$), IL-1α and IL-2 ($r = 0.55$, $P < 0.001$), IL-1α and IL-10 ($r = 0.50$, $P < 0.001$), IL-1β and IL-1ra ($r = 0.61$, $P < 0.001$), IL-1β and IL-2 ($r = 0.55$, $P < 0.001$), IL-1β and IL-10 ($r = 0.65$, $P < 0.001$), IL-2 and IL-10 ($r = 0.86$, $P < 0.001$), IL-10 and IL-18 ($r = 0.50$, $P < 0.001$), and IgA and IgM ($r = 0.51$, $P <$

0.001). Whereas there was a negative correlation between albumin and IgA (r = -0.50, P < 0.001).

Transcriptomic analyses of local responses in jejunal and ileal tissue from the same experiment have earlier only been presented as KEGG pathway analyses in Håkenåsen et al. (2020). Jejunal and ileal gene expression levels for a few selected cytokines (TNFα, IL-1α, IL-1β, IL-10, IL-18, and IFNγ) from day zero to seven can be seen in Supplementary Figure 2 and 3 in the current article. However, there were no relationships between the PW levels in blood and the local gene expressions of the same cytokines in jejunal or ileal tissue (Supplementary Table 2).

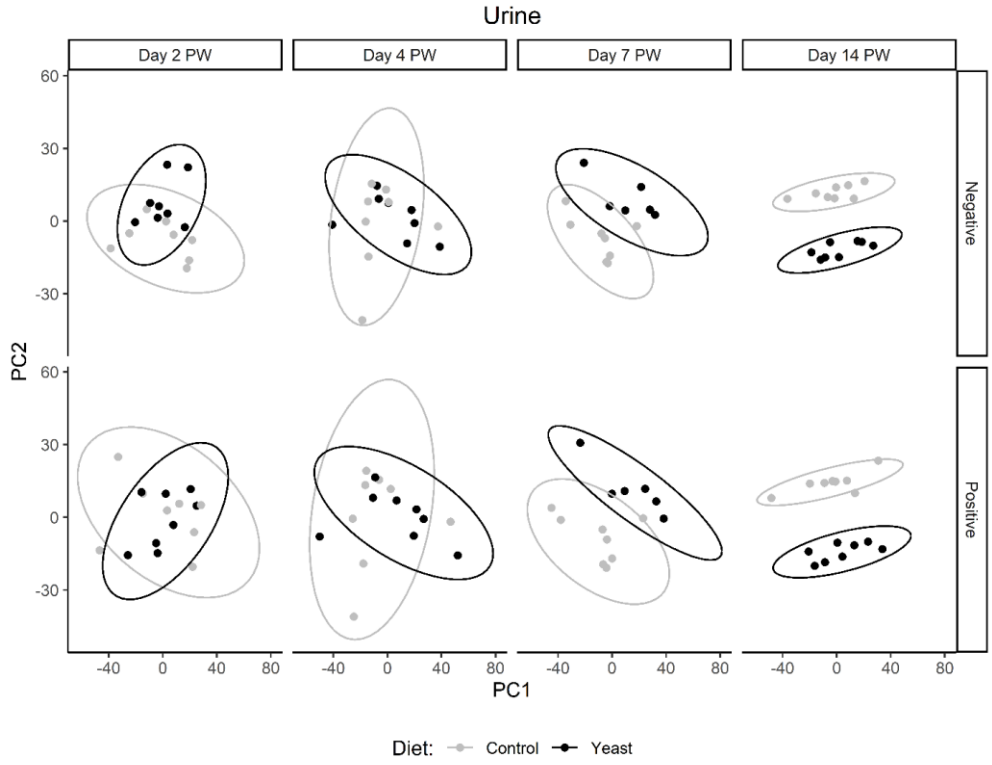

Figure 4 PCA plot for UHPLC-MS of urine samples. PCA is run individually for each day in each mode.

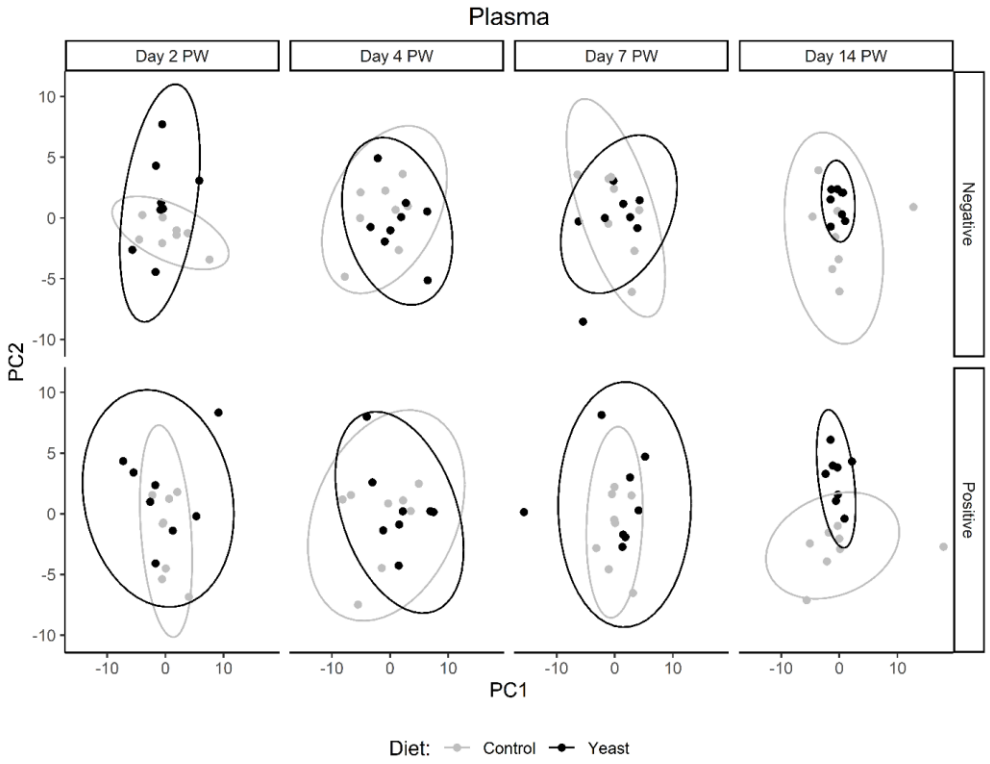

Figure 5 PCA plot for UHPLC-MS of plasma samples. PCA is run individually for each day in each mode.

3.4 Metabolic profiles

The metabolic profile in urine and plasma was investigated using UHPLC-MS. PCA models for each day PW was plotted to examine the separation in the metabolic profiles between the dietary treatments. For urine, the PCA models showed an increased separation of the dietary treatments according to day post weaning (Figure 4). On day 14 PW, the two dietary treatment was clearly separated in terms of metabolic profiles. The separation in metabolic profile between dietary treatments was not so clear in the plasma samples (Figure 5), but on day 14 PW yeast-fed piglets seemed to be grouped closer together. Metabolites were identified based on their characteristic fragmentation pattern. In urine, the metabolites causing separation according to diet, were highly dominated by glucuronidated compounds (Table 2). Compounds related to intake of soybean, e.g., genistein, daidzein and equol, were identified in piglets fed the control diet whereas a major part of the glucuronide conjugates excreted by yeast-fed piglets remained unidentified. Furthermore, betaine was a highly discriminating metabolite excreted by yeast-fed piglets. Betaine was identified as the most discriminating metabolite in plasma as well (high level in yeast fed piglets). Phosphatidylcholines were also dominating in plasma from piglets fed the yeast diet, along with several amino acids, purine, and hypoxanthine (Table 3).

Table 2. List of urine metabolites identified in the metabolomics analysis that significantly contribute to group separation in the PLS-DA analysis. The dietary treatment discriminating metabolites are listed according to VIP score in positive and negative mode, respectively.

Metabolites	Diet[1]	Ion	Mode	RT	Mz	VIP score
Betaine	Yeast	[M+H]+	Pos	0.73	118.0863	133.33
Creatinine	Control	[M+H]+	Pos	0.75	114.0662	17.42
N1-Methyl-2-pyridone-5-carboxamide	Yeast	[M+H]+	Pos	1.46	153.0660	14.96
Unknown	Control	[M+H]+	Pos	2.59	486.1978	12.31
Unknown	Yeast	[M+H]+	Pos	5.51	526.2655	11.82
Unknown	Yeast	[M+H]+	Pos	6.47	510.2705	11.65
Genistein 5-O-glucuronide	Control	[M+H]+	Pos	4.46	447.0928	9.86
Unknown	Yeast	[M+H]+	Pos	0.72	193.1550	9.74
N-Oleoyl phenylalanine	Control	[M+H]+	Pos	5.39	430.3324	8.85
Daidzein (isoflavone)	Control	[2M+H]+	Pos	4.22	431.0978	8.43
Daidzein (isoflavone)	Control	[2M+H]+	Pos	3.86	431.0979	8.29
Unknown	Control	[M+H]+	Pos	5.32	444.3116	7.51
Unknown	Control	[M+H]+	Pos	4.75	436.1609	7.24
Feruloylcholine	Yeast	[M+H]+	Pos	3.72	280.1546	7.22
Unknown	Control	[M+H]+	Pos	5.80	412.3218	6.74
N-Methylethanolaminium phosphate	Yeast	[M+H]+	Pos	0.71	156.0422	6.68
Retinoyl b-glucuronide	Yeast	[M+H]+	Pos	6.80	477.2492	6.32
Unknown	Yeast	[M+H]+	Pos	6.45	475.2335	5.73
Unknown	Yeast	[M+H]+	Pos	1.49	218.1390	4.93
Pantothenic acid	Yeast	[M+H]+	Pos	2.36	220.1182	4.73
Glycitein 7-O-glucuronide	Control	[M+H]+	Pos	3.91	461.1085	4.36
Unknown	Control	[M+H]+	Pos	5.16	416.3166	4.36
Unknown	Yeast	[M+H]+	Pos	6.23	530.2968	4.21
Unknown	Yeast	[M+H]+	Pos	4.10	302.1238	4.12
Glucuronide conjugate	Control	[M+H]+	Pos	2.12	303.0714	3.95
4-Hydroxyretinoic acid glucuronide	Yeast	[M-H]-	Neg	6.45	491.2284	20.35
2-Phenylethanol glucuronide	Control	[M-H]-	Neg	4.81	297.0978	17.30
Glucuronide conjugate	Yeast	[M-H]-	Neg	5.51	507.2232	13.15
Glucuronide conjugate	Yeast	[M-H]-	Neg	6.81	493.2441	12.73
p-Cresol glucuronide	Yeast	[M-H]-	Neg	4.09	283.0818	12.53

Glucuronide conjugate	Yeast	[M-H]-	Neg	3.87	299.0770	9.62
Equol 7-O-glucuronide	Control	[M-H]-	Neg	4.76	417.1188	9.52
Genistein 5-O-glucuronide	Control	[M-H]-	Neg	4.47	445.0772	9.16
Glucuronide conjugate	Control	[M-H]-	Neg	5.51	387.1659	9.10
Glucuronide conjugate	Yeast	[M-H]-	Neg	6.23	511.2545	8.53
Glucuronide conjugate	Control	[M-H]-	Neg	3.63	299.0768	8.18
C6H12O6	Yeast	[M-H]-	Neg	0.74	179.0561	7.74
Glucuronide conjugate	Control	[M-H]-	Neg	5.06	431.1919	7.74
Glucuronide conjugate	Yeast	[M-H]-	Neg	2.52	303.0720	7.63
Unknown	Control	[M-H]-	Neg	2.58	259.0757	7.10
Daidzein 4'-O-glucuronide	Control	[M-H]-	Neg	3.85	429.0824	6.38
6-Hydroxy-5-methoxyindole glucuronide	Control	[M-H]-	Neg	3.18	338.0878	6.32
Daidzein 4'-O-glucuronide	Control	[M-H]-	Neg	4.21	429.0823	5.79
p-Cresol glucuronide	Yeast	[2M-H]-	Neg	4.09	567.1711	5.77
Pantothenic acid	Yeast	[M-H]-	Neg	2.34	218.1033	5.42
Glucuronide conjugate	Yeast	[M-H]-	Neg	5.88	509.2390	5.31
Phenylacetylglutamine	Yeast	[M-H]-	Neg	4.56	265.1192	5.30
Glucuronide conjugate	Yeast	[M-H]-	Neg	5.94	511.2541	4.65
Unknown	Yeast	[M-H]-	Neg	0.68	78.9591	4.51
Unknown	Yeast	[M-H]-	Neg	2.09	177.0227	3.98
Unknown	Control	[M-H]-	Neg	2.13	301.0564	3.95
Glutaric acid	Yeast	[M-H]-	Neg	1.06	133.0506	3.79
Glucuronide conjugate	Control	[M-H]-	Neg	3.15	399.0930	3.73
Hippuric acid	Control	[M-H]-	Neg	3.59	178.0507	3.18

[1] Metabolite highest in control or yeast diet.

Table 3. List of plasma metabolites identified in the metabolomics analysis that significantly contribute to group separation in the PLS-DA analysis. The dietary treatment discriminating metabolites are listed according to VIP score in positive and negative mode, respectively.

Metabolite	Diet[1]	Ion	Mode	RT	Mz	VIP score
PC (18:2/0:0)	Yeast	[M+FA]-	Neg	9.31	564.3305	7.83
PC (20:4/0:0)	Yeast	[M+FA]-	Neg	9.35	588.3305	6.55
PC (18:1/0:0)	Yeast	[M+FA]-	Neg	10.09	566.3462	4.27
5'-Oxoinosine	Yeast	[M-H]-	Neg	1.36	267.0734	4.12
Glucuronide conjugate	Yeast	[M-H]-	Neg	4.14	283.0824	3.06
Ketoleucine/ketoisoleucine	Yeast	[M-H]-	Neg	3.35	129.0558	2.90
4-Hydroxyretinoic acid glucuronide	Yeast	[M-H]-	Neg	6.57	491.2286	2.72
Tryptophan	Yeast	[M-H]-	Neg	2.92	203.0827	2.30
Purine	Yeast	[M-H]-	Neg	0.85	119.0350	2.23
Sulfate	Control	[M-H]-	Neg	0.92	96.9602	2.14
Ketoleucine/ketoisoleucine	Control	[M-H]-	Neg	3.68	129.0558	1.43
Unknown	Yeast	[M-H]-	Neg	2.10	115.0401	1.38
Sulfate	Control	[M-H]-	Neg	0.80	96.9602	1.32
Unknown	Control	[M-H]-	Neg	4.80	225.0769	1.21
Glutamic acid	Control	[M-H]-	Neg	0.73	146.0459	1.11
PC (17:0/0:0)	Yeast	[M+FA]-	Neg	10.39	554.3463	1.04
6-Hydroxy-5-methoxyindole glucuronide	Control	[M-H]-	Neg	3.21	338.0880	1.03
PC (18:0/0:0)	Yeast	[M+FA]-	Neg	11.07	568.3619	0.92
Betaine	Yeast	[M+H]+	Pos	0.73	118.0864	25.64
Methionine	Yeast	[M+H]+	Pos	0.99	150.0585	4.01
PC (18:2/0:0)	Yeast	[M+H]+	Pos	9.32	520.3407	3.31
Fragment of methionine	Yeast	[Fragment]	Pos	0.99	133.0320	2.89
PC (18:1/0:0)	Yeast	[M+H]+	Pos	10.09	522.3564	2.24
(±)-2-Methylthiazolidine	Yeast	[M+H]+	Pos	0.99	104.0530	2.20
Fragment of valine	Yeast	[Fragment]	Pos	0.84	72.0809	1.68
PC (18:0/0:0)	Yeast	[M+H]+	Pos	11.08	524.3721	1.01
Creatine	Yeast	[M+H]+	Pos	0.75	132.0768	0.86
PC (16:0/0:0)	Yeast	[M+H]+	Pos	9.75	496.3408	0.83
Fragment of tryptophan	Yeast	[Fragment]	Pos	2.92	188.0708	0.79
N-Methyl-2-pyridone-5-carboxamide	Yeast	[M+H]+	Pos	1.46	153.0661	0.74
PC (18:2/0:0)	Yeast	[M+Na]+	Pos	9.32	542.3226	0.69
PC (20:4/0:0)	Yeast	[M+Na]+	Pos	9.35	566.3226	0.64
4-Trimethylammoniobutanoic acid	Control	[M+H]+	Pos	2.64	100.1124	0.53
PC (18:1/0:0)	Yeast	[M+Na]+	Pos	10.09	544.3383	0.53
Unknown	Control	[M+H]+	Pos	5.73	440.3167	0.50
PC (17:0/0:0)	Yeast	[M+H]+	Pos	10.4	510.3567	0.50
Hypoxanthine	Yeast	[M+H]+	Pos	0.98	137.0460	0.47

Unknown	Control	[M+H]+	Pos	5.26	416.3168	0.47
Hypoxanthine	Yeast	[M+H]+	Pos	1.36	137.0460	0.44
Unknown	Yeast	[M+H]+	Pos	0.98	102.0551	0.39
Unknown	Control	[M+H]+	Pos	8.53	398.2422	0.37
Fragment of phenylalanine	Control	[Fragment]	Pos	2.18	120.0810	0.35
Piperidine	Yeast	[M+H]+	Pos	1.26	86.0966	0.34
PC (18:0/0:0)	Yeast	[M+Na]+	Pos	11.08	546.3540	0.31
Tryptophan	Yeast	[M+H]+	Pos	2.92	205.0975	0.27
PE (18:2/0:0)	Yeast	[M+H]+	Pos	9.27	478.2935	0.25
Phenylalanine	Control	[M+H]+	Pos	2.18	166.0865	0.25
Proline	Control	[M+H]+	Pos	0.75	116.0708	0.22
Proline betaine	Control	[M+H]+	Pos	0.76	144.1021	0.21
PC (16:0/0:0)	Yeast	[M+Na]+	Pos	9.75	518.3227	0.20
Creatinine	Control	[M+H]+	Pos	0.74	114.0664	0.19

[1] Metabolite highest in control or yeast diet.

4 Discussion

In this study, we have investigated the effect of dietary inclusion of *C. jadinii* yeast as a protein source on the PW development of plasma biochemical indices, cytokines, immunoglobulins, and metabolic profile of plasma and urine in piglets. Most significant differences in plasma levels were according to the day PW, whereas few parameters significantly differed between the dietary treatments.

4.1 Biochemical parameters

Reference intervals for biochemical indices in blood are usually set for adult individuals, but the biochemical and metabolic changes that the piglets undergo at weaning might be not be comparable to the adults. Yu et al. (2019), have established serum biochemical reference intervals for some of the relevant parameters for piglets at day zero, seven and 14 PW for piglets weaned at 21 days of age, which will be used for comparisons.

The total protein and albumin level in plasma decreased with time from day two PW. The plasma total protein were inside the reference interval by Yu et al. (2019), except on day seven PW where our values was higher. However, the reference intervals were made for serum, and total protein is known to be higher in plasma compared to serum (Miles et al. 2004). Corresponding to the decrease in albumin, the plasma concentration of globulin increased. Numerically, the total protein concentration was higher on day two PW compared with day zero and decreases back to day zero level on day 14 PW. Dehydration could be a cause of increased total protein and albumin levels (Sutherland et al. 2014). Water intake was not monitored and the water intake at weaning is known to be excessively (McLeese et al. 1992; Torrey et al. 2008), especially at earlier weaning ages (Worobec et al. 1999). Bomba et al. (2014), also observed increase in total protein and albumin levels early PW and explained it by PW diarrhea causing

dehydration. Watery feces were observed for the piglets from day four to seven PW (Håkenåsen et al. 2020).

Creatinine levels were within the PW reference interval by Yu et al. (2019). ALP is in general high in young growing animals. The ALP concentration in plasma showed different pattern PW for the dietary treatments, where the yeast-fed piglets remained relatively stable PW, whereas control-fed piglets showed a reduction from day two to seven. Tao et al. (2016), reported reduced ALP serum concentration in piglets until day seven PW, which corresponds to the result for our control piglets, and suggested this was a result of adverse intestinal health in the first week PW. The ALP plasma levels did not correlate to the previously measured intestinal ALP activity for the same piglets (Håkenåsen et al. 2020). AST and CK are indicators of liver or muscular inflammation and damage. These two parameters were strongly correlated and plasma levels increased PW, with the highest levels on day seven PW. Contrary, Yu et al. (2019), reported lower reference interval for AST on day seven compared with day zero and 14 PW. However, these piglets were weaned one week earlier, and when comparing to age, AST was within the reference intervals. The increase in AST and CK on day seven PW corresponds to the previously reported PW diarrhea period from day four to seven PW (Håkenåsen et al. 2020). Higher AST levels was also reported by Feng et al. (2020) in the treatment group with the highest diarrheal index, and increased AST and CK has been reported in relation with transportation stress (Sutherland et al. 2010). The observed development of AST and CK levels in plasma might reflect stress and suboptimal intestinal health in the piglets, leading to suboptimal muscle and liver conditions.

Total plasma bilirubin levels were within normal range at all sampling points (Klem et al. 2010; Perri et al. 2017). Low post-weaning plasma levels of total bilirubin have also been observed by Sauerwein et al. (2005) and Petrovič et al.

(2009). Hyperbilirubinemia combined with e.g., decreased blood enzyme activities may indicate difficulty to adapt to solid diets during weaning (Vasilevskaya et al. 2021). However, the low values in the present study give no indication of problems with hemoglobin turnover, hemolysis, or other metabolic processes. Another sign of efficient metabolism could also be observed for the nucleic acids from the yeast, as uric acid could not be detected in any plasma samples. Yeast-fed piglets had overall higher levels of inorganic phosphate in the plasma, but levels were within the reference intervals (Cooper et al. 2014). Improved apparent total tract digestibility of phosphorus has previously been reported, when feeding the same yeast species as in the present study (Cruz et al. 2019; Lagos & Stein 2020), indicating high phosphate availability in the yeast.

4.2 Cytokines

Cytokines are involved in response to disease or infection. They could be divided in pro- and anti-inflammatory cytokines, but one should do so with care as the functionality might depend on the biological process (Dinarello 2000). Therefore, it is difficult to interpret the results with certainty. There were some differences in the PW development of plasma cytokine levels, but only IL-1ra was affected by the dietary treatment and then only on day two PW (2.4-fold higher in control piglets). The results corresponds to Lagos et al. (2020), which found no differences in plasma levels of IL-1α, IL-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, or TNFα on day 7 and 28 PW, when feeding the same amount and strain of yeast.

The IL-1 receptor antagonist (IL-1ra) is an anti-inflammatory cytokine which prevents uncontrolled activation of the IL-1 receptor by competing with IL-1β for binding (Schett et al. 2016). IL-1ra also has been reported to be a key immunosuppressor during the course of porcine reproductive and respiratory syndrome virus infection (Nedumpun et al. 2019). The balance between the levels

of IL-1β and IL-1ra are influencing the severity of an infection (Hurme & Santtila 1998). In general, IL-1ra are found in higher levels in plasma compared to IL-1β (Schett et al. 2016), which corresponds to our results. IL-1α and IL-1β are proinflammatory cytokines and members of the IL-1 family, which have a central role in regulation of immune and inflammatory responses to infections. IL-1α is produced and stored in cells, including epithelial cells. After epithelial damage and cell necrosis, the IL-1α is released and acts as an alarmin molecule, communicating tissue damage and triggering the early phase of inflammation (Schett et al. 2016). No significant difference in IL-1α plasma level was found in this study, but there were some differences in the IL-1β plasma levels with time PW. Control piglets also had numerically higher levels of both IL-1α and IL-1β on day two PW. IL-1β is important in the defense against infections. At the systemic level, the main action of IL-1β is induction of fever response by the hypothalamus and signaling inflammatory pain (Schett et al. 2016). The IL-1β and IL-1ra levels in plasma were correlated and followed the same patterns PW for the two dietary treatments. Whereas the yeast group showed stable PW levels, both IL-1ra and IL-1β were clearly increased at day two PW, indicating an early PW inflammatory response for the control group. An increase in plasma IL-1β levels have been reported in growing barrows after injections with *Escherichia coli* lipopolysaccharide (LPS) (Rakhshandeh & De Lange 2012). Moreover, McCracken and coworkers (1995), observed a diet-independent increase in IL-1 on day two PW in piglets weaned at 19 days of age and fed a cereal or milk-based diet.

The IL-10 is an anti-inflammatory cytokine important for controlling the duration and magnitude of the inflammatory response by inhibiting the production of proinflammatory cytokines (Celi et al. 2019; Sun et al. 2015). The level of IL-10 in plasma differed with day PW. There was no significant effect of dietary treatment, but a large numerical difference was observed on day 14 PW, where

the IL-10 level in plasma was 1.9-fold higher in the control piglets. Contrary, Li et al. (2005) reported higher IL-10 plasma level when feeding β-glucans extracted from the *Saccharomyces cerevisiae* yeast. They also reported increased IL-10 plasma levels when pigs were challenged with LPS. Whereas, in a study by Zhong et al. (2012), they found no differences in IL-10 levels in serum, but increased IL-10 in the jejunum of piglets weaned at 21 days of age and given glutamine supplementation compared with control. A lack of correlation between interleukin levels in blood and local gene expression of interleukins in the gut, was also observed in the present experiment.

The level of TNFα in plasma decreased PW, and was below reference values for this age pigs (Yu et al. 2019). By contrast, Yu et al. (2019) observed an increase in TNFα PW. Whereas, Hu et al. (2013) reported a mRNA expression pattern of TNFα PW in jejunal mucosa similar to our study in piglets weaned at 21 days of age. However, they found that the PW TNFα gene expression differed between the different intestinal segments. In the distal small intestine and colon, the increase in TNFα mRNA expression PW occurred later and stayed higher compared with weaning levels. TNFα is important for cell signaling in inflammatory responses. The PW decline in TNFα levels reported in the present study could indicate that the piglets quickly adapted to the new feed antigens and were living in a low infectious pressure environment. Pié et al. (2004), also reported increased TNFα mRNA levels in the mid small intestine on day one PW, before it declined and returned to PW levels already on day two PW. They also found this pattern for other cytokines and suggested that the cytokine response could be divided into two periods: an early acute response occurring from the time of weaning to day two PW, and a later long-lasting response from day two to eight days PW. Even though measured systemically and not in the gut, and with different analysis methods, our results somewhat corresponds to this theory of Pié et al. (2004). The plasma concentration of several cytokines numerically

increased early PW before the concentration decreased. Activation of the immune system steels energy and might reduce growth performance (Huntley et al. 2018). The general PW cytokine patterns of the present study suggest the acute phase to be less evident in yeast-fed piglets.

4.3 Immunoglobulins

Immunoglobulins are important in identifying and neutralizing pathogenic bacteria and viruses. In this study, IgA, IgG, and IgM differed with time PW. All three immunoglobulins showed different PW patterns. Contrary, Tao et al. (2016), reported no significant differences in either of these immunoglobulins in the first week PW. The IgA plasma concentration correlated with the previously reported jejunal IgA concentration (Håkenåsen et al. 2020). The IgA concentration decreased PW until day four, before it increased to above day zero level on day 14 PW. When challenging piglets with *E.coli*, Sugiharto et al. (2014), also found an increased plasma IgA concentration from day zero to eleven PW, but by contrast, they did not observe a drop in concentration on day four. IgA is the major immunoglobulin in sows milk, with a half time of 2.7 days (Porter 1976). The abruption of milk by weaning could explain the drop in IgA levels PW. Lagos et al. (2020), reported a tendency for lover levels of IgA in plasma when feeding the same level and species of yeast as in the present study compared with control. By contrast, in the present study, there were no effect of feeding yeast on the IgA levels in plasma, but a numerically lower concentration was seen in the yeast group on day 14.

The level of IgM increased with time PW and was higher in the control piglets compared with the yeast-fed piglets. Increased IgM levels PW (from day seven to 21 PW) was also reported by Moore et al. (2011), when weaned at 21 days of age. However, they found no difference between pigs fed yeast protein concentrate or nucleotides compared with the control. It should also be mentioned

that their diet changed from first (< 7 days PW) to second (< 21 days PW) stage weaner diet. In the present study, the concentration of IgG in plasma was greatly reduced until day seven PW. Contrary, Sugiharto et al. (2014), reported no change in IgG concentration PW when challenging with *E.coli*. Whereas, Rooke et al. (2003), reported increased IgG plasma concentrations PW weaning when weaned at 28 days of age. The piglets in our experiment were in general good health and there was a low infection pressure in the facilities, which can explain contradictory results when compared with other studies with higher infection pressure.

4.4 Metabolomic profiles

Metabolomics is the study of metabolites and their alterations under certain conditions. Weaning is clearly an occurrence affecting the piglet's digestive system and metabolism. We conducted an LC-MS-based untargeted metabolomic analysis of plasma and urine from the piglets, to study the metabolic changes during a two-week period PW. As shown in the PCA scores plot for urine, there was an increased separation of metabolites with time PW between piglets fed the two dietary treatments. On day 14 PW, the treatment groups were clearly separated, indicating a dietary treatment effect on the metabolic profile of urine. The separation was not as evident in the PCA scores plot for plasma, but there was a clearer grouping of the dietary treatments on day 14 PW in plasma also. Plasma homeostasis is important for maintaining normal physiological activities. Therefore, it is expected that the plasma would be less affected by dietary changes than urine. Although, Sugiharto et al. (2014) previously reported separation in the plasma metabolic profile of piglets between day zero, four, and eleven PW, when challenged with *E.coli*. These piglets were fed a milk replacer until day four PW.

In the present study, betaine was found to be the most discriminating metabolite between dietary treatments, with considerable higher PW levels in both urine and

plasma in yeast-fed piglets. Betaine is a commonly used feed additive that can also be found naturally in plants, animals, and microorganisms, and the yeast diet showed a much higher level of betaine than the control diet (data not shown). Betaine serves as a methyl donor for important metabolic pathways, and have been reported to positively affect e.g., daily gain, body composition and β-oxidation of fatty acids in pigs, as well as to increase serum growth hormone (GH) and insulin-like growth factor-1 (IGF-1) levels (Fu et al. 2021). Further, the high level of betaine in plasma of yeast-fed piglets, can probably be linked to the higher level of several phosphatidylcholines, as well as creatine (Bertolo & McBreairty 2013). In the study of Sugiharto et al. (2014), the plasma betaine levels were decreasing after weaning and exposure to *E. coli*. It has also been reported that betaine acts as an osmoprotectant, which increases the water-retention capacity of the gut tissue (reviewed in Fu et al. 2021). Thus, the high level of betaine may partly explain the increased ileal dry matter in yeast-fed piglets during the first days after weaning in the present experiment (Håkenåsen et al. 2020). The European Food Safety Authority (EFSA) have concluded that betaine is safe for piglets at the maximum supplementation rate of 2000 mg/kg complete feed (EFSA 2013). Both diets contain SBM, however, a large proportion of the SBM were replaced by yeast in the yeast diet. This could be observed in the urine of control-fed piglets as higher levels of a whole range of isoflavone glucuronides and sulfate conjugates. However, there is little knowledge concerning the biological activity of these compounds (Shelnutt et al. 2000). Piglets from both diet groups also excreted a high number of glucuronide conjugated metabolites in urine. Glucuronidation is a metabolic process where xenobiotics are capped by conjugation to increase their polarity and thereby ease their elimination from the body (Gibson & Skett 2013). Identification of these glucuronidated metabolites is challenging but the diverse glucuronides are most probably derived from dietary and microbial metabolism and hence represents features of the dietary treatments.

5 Conclusion

In conclusion, the PW changes in blood biochemistry, cytokine, and immunoglobulin concentrations were in general time-dependent and not diet-dependent. The plasma cytokine results suggest an early PW acute phase which was less evident in the yeast-fed piglets. The metabolic profiles of plasma and urine for control and yeast-fed piglets was separated by time PW. Betaine was identified as the most discriminating metabolite between dietary treatments in both plasma and urine. These results support our previous findings that dietary inclusion of *C. jadinii* yeast result in more robust piglets PW.

6 Supplementary

Supplementary Figure 1 Correlogram visualizing correlations between plasma biochemical indices, cytokines, and immunoglobulins. Only significant correlations ($P < 0.05$) are visualized. Positive correlations are displayed in blue and negative correlations in red color.

Supplementary Figure 2 Effect of dietary treatment on the jejunal post-weaning (PW) gene expression development of some selected cytokines.

Supplementary Figure 3 Effect of dietary treatment on the ileal post-weaning (PW) gene expression development of some selected cytokines.

Supplementary Table 1

Excel sheet with mean values, standard error of mean (SEM) and P-values for plasma biochemistry, cytokines, and immunoglobulins.

Supplementary Table 2 Pearson correlations between gene expression in jejunal and ileal tissue and the plasma levels of some selected cytokines.

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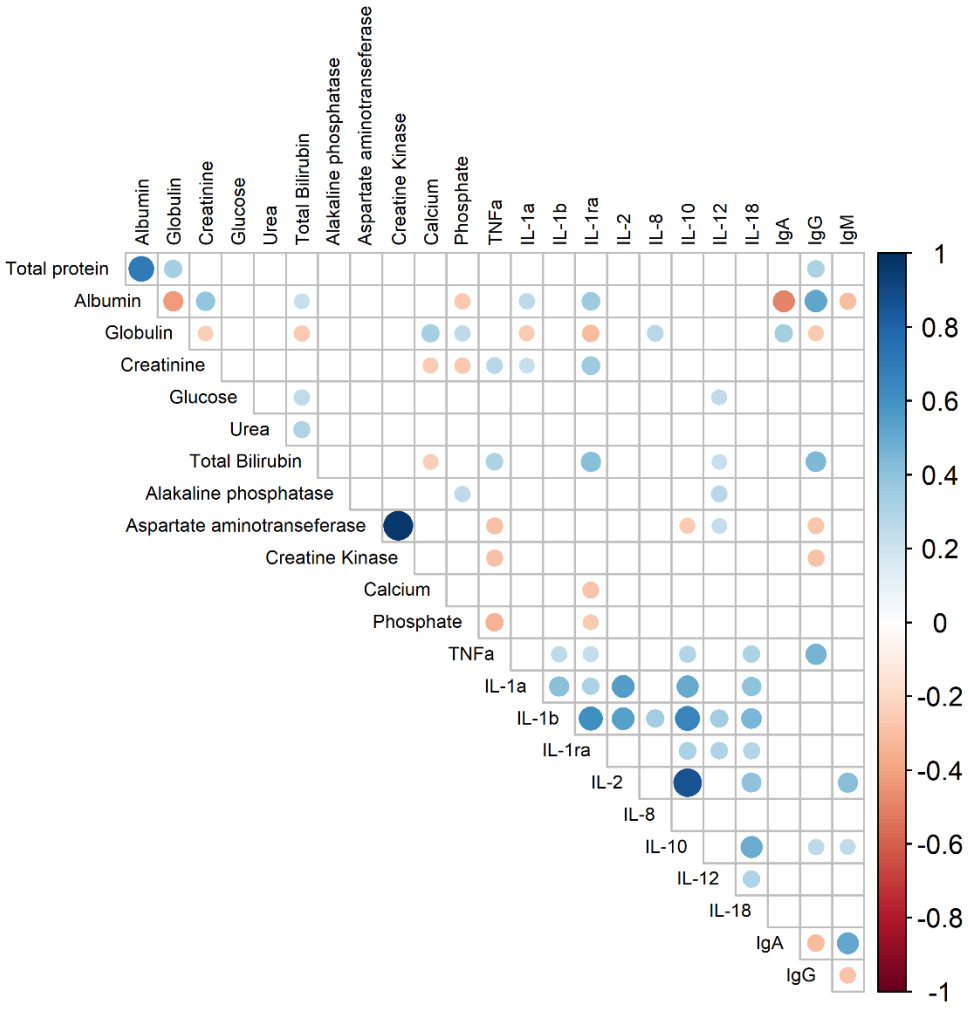
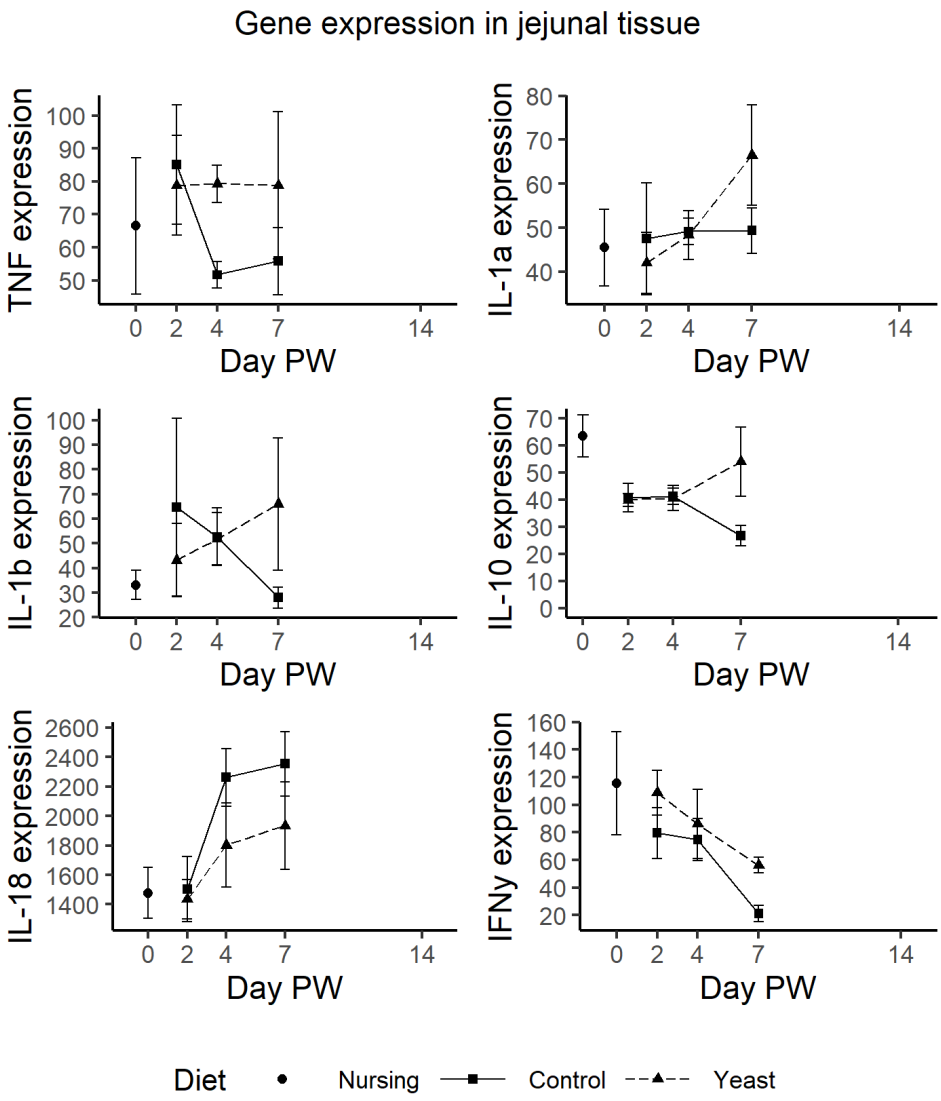
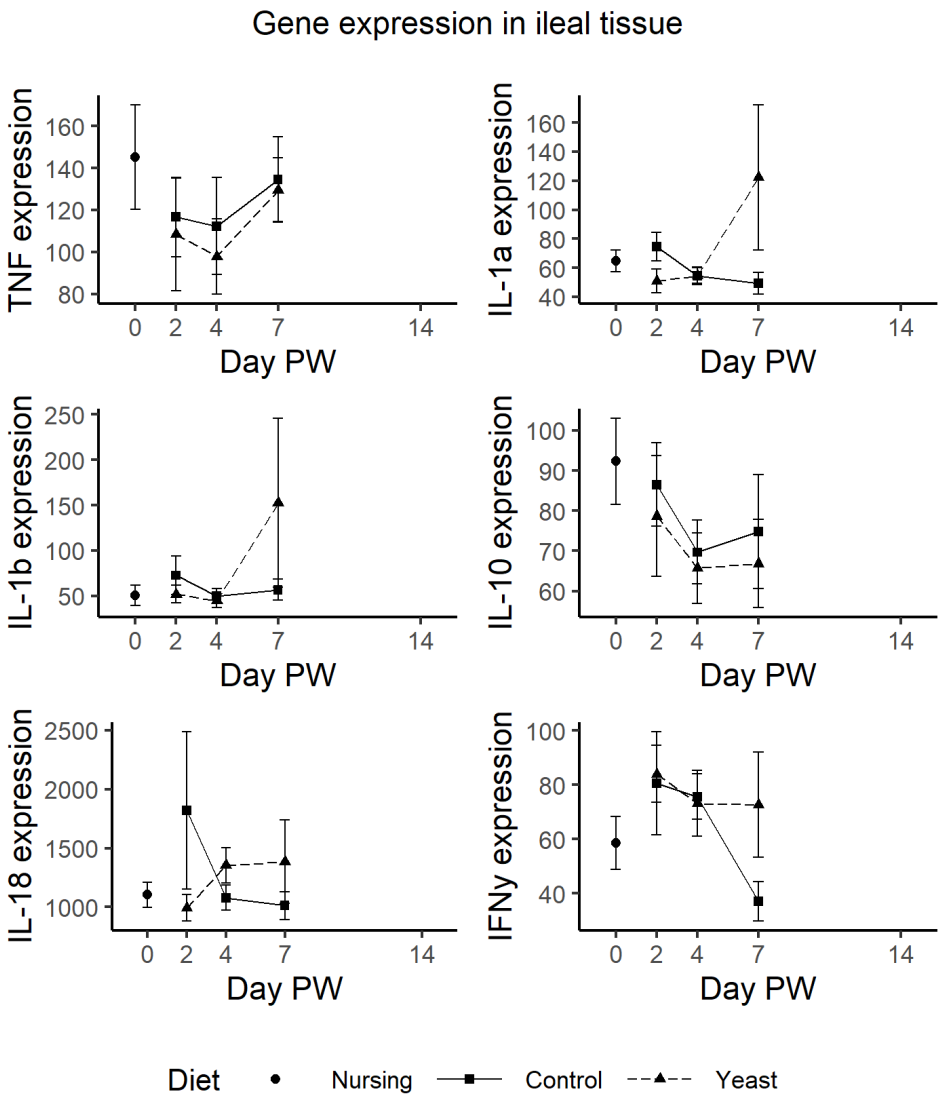


Supplementary Figure 1 Correlogram visualizing correlations between plasma biochemical indices, cytokines, and immunoglobulins. Only significant correlations ($P < 0.05$) are visualized. Positive correlations are displayed in blue and negative correlations in red color.



Supplementary Figure 2 Effect of dietary treatment on the jejunal post-weaning (PW) gene expression development of some selected cytokines.



Supplementary Figure 3 Effect of dietary treatment on the ileal post-weaning (PW) gene expression development of some selected cytokines.

Supplementary Table 1 Mean values, standard error of mean (SEM) and P-values for plasma biochemistry, cytokines, and immunoglobulins.

Item	n	Mean values									SEM	P-value diet	P-value day	P-value diet*day	P-value breed
		Nursing	Control, day 2 PW	Yeast, day 2 PW	Control, day 4 PW	Yeast, day 4 PW	Control, day 7 PW	Yeast, day 7 PW	Control, day 14 PW	Yeast, day 14 PW					
Albumin, g/L	72	23.2	26.3	26.3	24.2	25.2	23.1	23.2	20.2	19.5	0.4	0.815	<0.001	0.497	0.392
Alkaline phosphatase, U/L	72	367.1	380.5	333	322.4	337.1	305	323.6	317.5	350.5	7.1	0.811	0.003	0.01	0.216
Aspartate aminotransferase, U/L	71	85.4	76.8	84.5	111.4	75.9	150.1	163.2	89.8	135.9	7.8	0.632	0.024	0.294	0.978
Creatine kinase, U/L	71	3218.4	2795.4	3513.8	4332.5	2512.9	6580.7	7704.9	3247.5	5581.1	384.9	0.489	0.062	0.288	0.907
Inorganic phosphate, mmol/L	71	3.1	2.8	3	2.8	2.9	2.7	3.3	3.1	3.4	0.04	<0.001	0.021	0.037	0.198
Globulin, g/L	72	24.8	22.9	25	25.4	25.1	25.9	25.8	27.5	27.4	0.3	0.424	0.001	0.426	0.362
Glucose, mmol/L	70	8.4	7.5	7.9	7.4	7.4	7.2	7.3	7.4	7.7	0.1	0.573	0.255	0.726	0.448
Calcium, mmol/L	70	2.8	2.7	2.7	2.8	2.7	2.8	2.7	2.8	2.7	0.01	0.186	0.173	0.75	0.985
Creatinine, µmol/L	72	81.1	91.1	93	95	91.1	87.4	84.1	82	83.5	1.3	0.705	0.036	0.752	0.122
Total bilirubine, µmol/L	72	1.5	1	0.5	0.4	0.2	0	0	0	0	0.1	Did not converge	Did not converge	Did not converge	Did not converge
Total protein, g/L	72	47.9	49.2	51.4	49.6	50.2	49.2	49	47.8	46.9	0.3	0.527	0.01	0.452	0.132
Urea, mmol/L	72	4.2	1.7	2.3	2	1.9	1.4	2.2	2.1	2.4	0.1	0.164	0.662	0.467	0.979
IgA, µg/mL	71	123.8	104	101.3	64.4	62.6	123.3	110.1	199.5	194.1	8	0.428	<0.001	0.949	0.413
IgG, µg/mL	72	663.9	616.2	835	493.7	591	110.7	112.8	103.9	108.1	36.4	0.181	<0.001	0.422	0.835
IgM, µg/mL	72	480.5	499.6	414.4	548.5	396.9	551.5	433.9	723	592.7	25.9	0.014	0.027	0.903	0.24
TNFa, pg/mL	69	13.4	17	18.1	17.6	18.2	10.2	8.4	9.4	4.5	1.2	0.508	<0.001	0.314	0.553
IFNy, pg/mL	23	321.6	713.8	425.8	481.2	388.1	348.2	315	215.3	NA	47.8	Too few data	Too few data	Too few data	Too few data
IL1a, pg/mL	70	20.4	54.7	18.9	21.2	28.1	27.4	28.8	19.3	18	2.5	0.914	0.423	0.13	0.985
Il1b, pg/mL	69	17.2	39.5	16.6	17.8	16	50.7	26.8	20.9	12.5	1.4	0.136	0.035	0.145	0.581
IL1ra, pg/mL	71	271.8	705.4	290.5	309.5	215	298	265.4	278.7	215.5	24.8	0.031	<0.001	0.002	0.939
IL2, pg/mL	45	4.6	137.6	8.2	30.5	33.1	176.8	57.2	31.6	27.3	4.1	0.892	0.092	0.838	0.023
IL4, pg/mL	28	55.5	412.5	12.7	62	137.3	451.5	470.2	54.9	92.7	23.9	0.311	0.4	0.305	0.81
IL8, pg/mL	67	15.3	18.9	14.8	21	33.6	20.6	49.8	22.7	83.1	1	0.729	0.092	0.493	0.653
IL10, pg/mL	66	39.4	198.1	56.3	121.8	55.9	290.9	94.9	54	28.7	5.7	0.052	0.001	0.444	0.118
IL12, pg/mL	72	1324	1166.4	858.9	911.1	882.9	919.9	1008.2	1003.7	975.5	41.9	0.342	0.729	0.433	0.284
IL18, pg/mL	70	355.4	415.7	228.9	346.6	340.1	405.4	342.9	289.2	264.1	19	0.257	0.235	0.209	0.489

Supplementary Table 2 Pearson correlations between gene expression in jejunal and ileal tissue and the plasma levels of some selected cytokines.

Pearson correlation between the level in plasma and gene expression in jejunum	**R**	**P-value**
IFNy*	-0.27	0.281
TNFa	0.18	0.257
IL-1a	0.04	0.823
IL-1b	0.02	0.885
IL-2	-0.04	0.835
IL-10	0.02	0.907
IL-18	0.21	0.173
Pearson correlation between the level in plasma and gene expression in ileum		
IFNy*	-0.34	0.165
TNFa	-0.03	0.838
IL-1a	-0.06	0.69
IL-1b	-0.06	0.713
IL-2	-0.26	0.173
IL-10	0.09	0.577
IL-18	0.06	0.729

*Few data

Paper III

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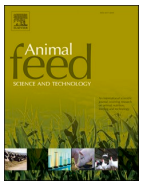

Full-fat insect meal in pelleted diets for weaned piglets: Effects on growth performance, nutrient digestibility, gastrointestinal function, and microbiota

Ingrid Marie Håkenåsen, Guro Holseth Grepperud, Jon Øvrum Hansen, Margareth Øverland, Ragnhild Martinsen Ånestad, Liv Torunn Mydland *

Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Aas, Norway

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ABSTRACT

Insects, such as the black soldier fly larvae (BSFL), are suggested as a sustainable novel protein source for pigs. The BSFL contains chitin, medium-chain fatty acids, and antimicrobial peptides, which could improve the gastrointestinal function and health of the post-weaning pig. The objective of this study was to investigate the effect of increased inclusion of full-fat BSFL in diets for post-weaning pigs on growth performance parameters, digestibility of nutrients, gut morphology, and the microbial community in the colon. Eighty crossbred pigs were weaned at approximately 32 days of age, with an average weaning weight of 10.6 ± 0.8 kg. For four weeks, pigs were fed: a control diet or one of three diets containing increasing amount of full-fat BSFL meal at 4.76%, 9.52%, and 19.06%. The average daily gain (ADG) for the overall experimental period showed a negative cubic effect of dietary treatment, where the ADG was highest for pigs fed the control diet and lowest for pigs fed the diet with 4.76% BSFL ($P = 0.031$). Increased level of full-fat BSFL in the diet did not affect feed efficiency or fecal consistency. A linear reduction in the coefficient of apparent total tract digestibility (CATTD) of crude protein ($P = 0.011$) was found for increasing inclusion of BSFL, whereas for crude fat both the coefficient of apparent ileal digestibility ($P = 0.043$) and the CATTD ($P < 0.001$) increased linearly. Jejunal, ileal, or colonic morphometry was not affected by the BSFL inclusion. No differences in the short-chain fatty acid concentrations were detected among the dietary treatments, but a few minor changes in the colon microbiota were observed. At the phylum level, the colon microbiota was dominated by Bacteroidota and Firmicutes, but there was no clear pattern relationship with the BSFL inclusion level. At the genus level, the inclusion of BSFL in the diet reduced the relative abundance of *Lactobacillus* ($P = 0.015$) compared to the control. Collectively, the results indicate that up to

Abbreviations: ADF, acid detergent fiber; ADFI, average daily feed intake; ADG, average daily gain; aNDF, amylase-treated neutral detergent fiber; ASV, amplicon sequence variant; BSFL, black soldier fly larvae; CD, crypt depth; CF, crude fat; CAID, coefficient of apparent ileal digestibility; CATTD, coefficient of apparent total tract digestibility; CP, crude protein; CSID, coefficient of standardized ileal digestibility; DM, dry matter; FTIR, Fourier Transform Infrared Imaging; G:F, gain:feed ratio; GIT, gastrointestinal tract; MCFA, medium-chain fatty acid; PCoA, principal coordinate analyses; PW, post-weaning; SCFA, short-chain fatty acid; SEM, standard error of the mean; VH, villus height.

* Correspondence to: Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway.

E-mail address: liv.mydland@nmbu.no (L.T. Mydland).

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19.06% of full-fat BSFL meal could be included in a balanced diet for post-weaning pigs with only minor effects on growth performance, general gut function, and gut health.

1. Introduction

The world is facing a growing food demand by an ever-increasing number of people. To meet this challenge, new resources must be considered by using innovative solutions. Insects have been proposed as a high quality, efficient, and sustainable alternative protein source (Veldkamp et al., 2012). The black soldier fly larvae (BSFL; *Hermetia illucens*) is an easily reared species, capable of efficient conversion of a wide range of organic materials (Wang and Shelomi, 2017), and do not accumulate pesticides or mycotoxins (Purschke et al., 2017; Leni et al., 2019). The BSFL has an overall favorable amino acid profile but is limiting in sulfur-containing amino acids and contains a high amount of minerals, especially calcium (Finke, 2013).

A well-functioning gastrointestinal tract (GIT) is of importance to the overall growth performance and health of pigs in all stages, especially for the newly weaned pigs. If not defatted, BSFL is high in fat, especially in lauric acid (12:0; Finke, 2013) which is categorized as a medium-chain fatty acid (MCFA) with antimicrobial effects, especially against gram-positive bacteria (Zentek et al., 2011). Insects and insect larvae also contain chitin, a dietary polysaccharide that can function as a prebiotic and an immunostimulant (Song et al., 2014). Finally, antimicrobial peptides, which are part of the insect immune system, are effective antimicrobial agents with low risk of development of bacteria resistance (Lewies et al., 2019). The antimicrobial peptides have good potential as health promoters in livestock, even though there is limited information about the *in vivo* effects (Wang et al., 2016).

Insects are, therefore, interesting to investigate both as a feed ingredient and as a functional ingredient with potential health beneficial effects. Recently, there has been a high focus devoted to the potential of insect meals in diets for monogastric farm animals (Józefiak et al., 2018; Spranghers et al., 2018; Biasato et al., 2019; Nogales-Mérida et al., 2019), however, there is a scarcity of literature on the use of full-fat BSFL in diets for pigs. The objective of this study was to investigate the effect of increasing inclusion of

Table 1
Dietary composition of experimental diets and calculated ratio of crude protein CP and crude fat (CF) from black soldier fly larvae (BSFL) meal.

	Dietary treatments			
Ingredients, g/kg as fed[a]	Control	BSFL5	BSFL10	BSFL20
Wheat	507.7	502.0	496.6	485.1
Barley	200.0	200.0	200.0	200.0
Oats	50.0	50.0	50.0	50.0
BSFL meal[b]	–	47.6	95.2	190.6
Soybean meal[c]	70.8	59.7	48.2	25.5
Soy protein concentrate[d]	36.1	27.1	18.1	–
Fish meal[e]	34.1	25.5	17.1	–
Rapeseed oil	32.8	25.1	17.4	1.3
Saturated vegetable fat[f]	11.0	8.1	5.1	–
Rapeseed meal[g]	10.0	10.0	10.0	10.0
Monocalcium phosphate	12.9	12.0	11.2	9.5
Limestone	8.2	6.7	5.1	2.1
Sodium chloride	5.3	5.4	5.5	5.8
Selenium premix	0.9	0.9	0.8	0.8
Iron(II) fumarate	0.4	0.4	0.4	0.4
Micromineral premix[h]	2.0	2.0	2.0	2.0
Vitamins[i]	3.0	3.0	3.0	3.0
L-Lysine·HCl	6.8	6.8	6.9	6.9
L-Methionine	2.4	2.5	2.6	2.8
L-Threonine	2.8	2.9	2.9	3.0
L-Valine	1.1	0.8	0.5	0.0
L-Tryptophan	0.9	0.8	0.7	0.6
Betaine	0.7	0.7	0.7	0.7
Yttrium(III) oxide	0.1	0.1	0.1	0.1
Ratio CP from BSFL (% of total CP)	0	10	20	39
Ratio CF from BSFL (% of total CF)	0	19	39	69

[a] Chemical composition of main ingredients are provided in Supplementary Table 1.
[b] HiProMine S.A., Poznanska Str, Poland.
[c] Non-GMO soybean meal, Denofa AS, Fredrikstad, Norway.
[d] AX3 Gastric, TripleA a/s, Hornsyld, Denmark.
[e] Nordsildmel AS, Egersund, Norway.
[f] AkoFeed Gigant 60, AAK AB, Malmö, Sweden.
[g] Expeller-pressed rapeseed cake, Mestilla, UAB, Klaipeda Lithuania.
[h] "Mikro-Svin"; provided per kilogram of diet: Zn, 120 mg as ZnO; Fe, 120 mg as $FeSO_4$; Cu, 26 mg as $CuSO_4$; S 13.2 mg as $FeSO_4$ and $CuSO_4$; Mn 60 mg as MnO; Mg, 3.4 mg as $CaMg(CO_3)_2$; I, 0.6 mg as $Ca(IO_3)_2$.
[i] Provided per kilogram of diet: vitamin A, 0.7 g; vitamin E v5, 1.2 g; vitamin ADKB mix 0.8 g; vitamin C (Stay C 35%), 0.3 g.

full-fat BSFL in diets for post-weaning (PW) pigs, focusing on growth performance parameters, nutrient digestibility, gut morphology, and microbial community in the colon.

2. Materials and methods

A 27-day experiment was performed in February 2019 at the Center for Livestock Production (SHF, Norwegian University of Life Sciences, Ås, Norway), which is an animal experimental unit approved by the National Animal Research Authority (permit no. 174). All pigs were handled under the applicable laws and regulations controlling experiments with live animals in Norway regulated by the "Animal Welfare Act" and "The Norwegian Regulation on Animal Experimentation" derived from the "Directive 2010/63/EU on the protection of animals used for scientific purposes."

2.1. Animals and housing

Eighty crossbred [(Norwegian Landrace x Yorkshire z-line) x Duroc] weaned pigs were included in the experiment. All piglets had access to the sows feed during the nursing period. The experiment was conducted as a randomized complete block design. Piglets were selected from eleven litters (four or eight pigs from each litter depending on the litter size), based on their weaning weight to create a uniform group, and then equally distributed to the four dietary treatments based on litter, sex and weight. The average weaning age was 32.8 ± 1.6 days and the average weaning weight was 10.6 ± 0.8 kg. There were five pens per treatment with four pigs per pen, containing two gilts and two barrows. No pigs in the same pen were siblings. Three of the pens per treatment were installed with rubber mats. The remaining pens had wood shavings as bedding material. The pen size was 1.6 m^2. The room temperature was logged every morning, and the average temperature for the experimental period was 21.6 ± 1.4 °C.

2.2. Dietary treatments

Meal of BSFL was produced at HiProMine S.A., Poznan, Poland. The BSFL feed had a DM level of 22% and consisted of 17% wheat middlings and 83% fresh vegetable mix, consisting of apples (15%), carrots (50%), potatoes (15%), and cabbage (20%). Fresh vegetable pre-consumer waste was ground (2000 rpm, 55kW, HPM milling system, Robakowo, Poland) to pass a 2 mm screen and offered *ad libitum* to the BSFL. Substrates were not contaminated by any animal products in accordance with EC regulation (No 1069/09). At the prepupal stage (10th day of rearing), larvae were harvested, sieved through a 3 mm screen, and washed with water on drum separator at 90 °C for 10 min (HPM cleaning system, Poland).

The dietary treatments included a control diet and three experimental diets with increasing inclusion of BSFL at 4.76% (BSFL5), 9.52% (BSFL10), and 19.06% (BSFL20; Table 1). Diets were formulated in collaboration with Felleskjøpet Fôrutvikling AS (Trondheim, Norway) using their optimization least-cost program based on the Dutch energy evaluation system (Blok et al., 2015). Diets were formulated based on net energy and coefficients of standardized ileal digestibility (CSID) to be isoenergetic, balanced for digestible amino acids, and to meet or exceed the nutrient requirements of pigs (NRC, 2012). Literature values from Finke (2013) for the amino acid content in the BSFL meal were used in the diet formulation. The CSID for the amino acids in the BSFL meal were set to 0.83. Yttrium oxide (Y_2O_3) was included as an inert marker in the diets (0.01%) for digestibility calculations. Pelleted diets were produced by the Center for Feed Technology (FôrTek, Norwegian University of Life Sciences, Ås, Norway). The feed mash was ground in a Münch hammer mill (HM 21.115, Wuppertal, Germany) fitted with a 3 mm screen before pelleting. The mash was steam conditioned at 82 °C in a double-pass pellet-press conditioner (Münch-Edelstahl, Germany) before pelleting (Münch-Edelstahl, Germany, 2 × 17 kW) through a 3.5 mm die with a production rate of 700 kg/h. Pigs had *ad-libitum* access to the experimental diets immediately after weaning through automatic feeders (FRH-2 Domino A/S, Tørring, Denmark) with 43 cm feeding space. The automatic feeders were checked daily and refilled when needed. Feed residues were registered weekly and average daily feed intake (ADFI) per pen was calculated. Clean drinking water was always available from a drinking nipple next to the feeder.

The farm had an ongoing issue with edema disease and symptoms resulted in antibiotic treatment of five pigs on days 8–10 PW. After one pig died without any registered symptoms on day 11 PW, all pigs were treated with intramuscular antibiotic injections (Borgal vet., Ceva Santé Animale, Libourne, France) for three consecutive days (11–13 PW). After treatment, all pigs appeared healthy throughout the experiment.

2.3. Sample collection

Fecal consistency was assessed daily and registered for all pens based on the four category scale developed by Pedersen and Toft (2011). A higher score indicated more watery feces. Scores one and two were considered normal while scoring three and four were considered as diarrhea. The daily fecal score was registered as a pen average with 0.25 intervals on the scale. All pigs were weighed weekly and average daily gain (ADG) and gain:feed ratio (G:F) was calculated per pen. Fecal samples were also collected every week for the determination of the fecal DM. An approximately equal amount of feces from each pig was pooled for the pen before oven drying at 103 °C for 24 h. Fresh individual fecal samples were collected on days 21, 22, 25, 26, and 27 for determination of the coefficient of apparent total tract digestibility (CATTD) of nutrients. Individual samples from all days were pooled, freeze-dried, and ground using a Retsch ZM 100 centrifugal mill (Retsch, Haan, Germany) fitted with either a 0.5 mm or a 1 mm screen before chemical analysis. Apparent digestibility of nutrients was calculated as described by Maynard and Loosli (1969).

2.4. Terminal sample collection

Only pigs from the pens with rubber mats (n = 3 pens per treatment, a total of 47 pigs) were included in the terminal sampling at day 28 and 29 PW. Pigs were fasted from the evening before but had access to feed three hours before euthanasia. Euthanasia was done using a captive bolt pistol followed by exsanguination. Immediately after exsanguination, the abdominal cavity was opened, and the GIT was removed. pH was measured in stomach and jejunal content. Intestinal content was collected from jejunum (first 30 cm post the hepatopancreatic duct) and from the spiral colon. Tissue from oral jejunum (15 cm post the hepatopancreatic duct), aboral ileum (15 cm from the ileocecal valve), and the spiral colon were collected for histological assessment. Intestinal content from the last two meters of the small intestine was collected and stored at −20 °C for determination of the coefficient of apparent ileal digestibility (CAID) of nutrients. For seven of the pigs (three control pigs, two pigs fed BSFL10, and two pigs fed BSFL20), samples were incorrectly collected from jejunum instead of ileum, and therefore excluded from analyzes. The ileal contents were freeze-dried and homogenized using a batch mill (A11 basic Analytical mill, IKA, England). Liver weight was recorded to calculate liver index: [liver weight (kg)/live body weight (kg) × 100].

2.5. Chemical analyses

Pooled feed samples for each diet, collected from the feed storage during the experiment when preparing feeding, were ground using a Fritsch Pulverisette 19 cutting mill (Fritsch GmbH, Idar-Oberstein, Germany) fitted with either a 0.5 mm screen or a 1.0 mm screen before the chemical composition of nutrients were analyzed in triplicates (Table 2–4). The chemical analyses were performed by the LabTek group at the Department of Animal and Aquacultural Science, Norwegian University of Life Sciences, Ås, Norway. The DM content was determined by drying to constant weight at 103 ± 2 °C (ISO 6496, 2001), and ash was determined by complete combustion at 550 °C for at least 4 h (ISO 5984, 2002). Gross energy (GE) content was determined using a PARR 6400 Automatic Isoperibol Calorimeter (Parr Instruments, Moline, IL, USA) according to ISO 9831 (1998). Crude protein (CP) was analyzed with the Kjeldahl method according to Commission Regulation (EC) No 152/2009 (European Commission, 2009), using a Digestor 2520 (FOSS Analytical, Hillerød, Denmark) and the Kjeltec 8400 analyzer (FOSS Analytical, Hillerød, Denmark). Crude fat (CF) was analyzed using Accelerated Solvent Extraction (ASE 350, Thermo Fisher Scientific, Waltham, MA, USA). Extraction was conducted with 80% petroleum ether and 20% acetone at 125 °C. Starch was determined using an enzymatic-colorimetric method according to McCleary et al. (1994), with some modifications. In brief, starch was degraded with heat-stable α-amylase and amyloglucosidase-enzymes to glucose. Glucose concentration was then determined using a spectrophotometer (RX Daytona +, Randox Laboratories Ltd., Crumlin, UK). Acid detergent fiber (ADF) and amylase-treated neutral detergent fiber (aNDF) were analyzed using the Ankom200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). For aNDF analysis, the sample was treated with α-amylase and analyzed according to Mertens (2002). To determine ADF content, samples was digested with 1 *N* sulfuric acid containing 20 g cetyl trimethylammonium bromide according to the manufacture's instruction. Amino acids in diets and ileal samples were analyzed according to Commission Regulation (EC) No 152/2009 (European Commission, 2009). Amino acids were analyzed on a Biochrom 30+ Amino Acid Analyzer with an autosampler (Biochrom Ltd., Cambridge, UK). Tryptophan was analyzed in diets on a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) with a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan). The fatty acid composition of the diets was analyzed according to O'fallon et al. (2007) by synthesizing the fatty acids to fatty acid methyl esters

Table 2
Analyzed and calculated chemical composition of dietary treatments.

	Dietary treatments[a]			
Items, g/kg DM	Control	BSFL5	BSFL10	BSFL20
Dry matter, g/kg	890.3	888.9	887.7	893.0
Ash	54.3	52.3	52.5	52.9
Crude protein	196.8	196.1	199.8	206.8
Starch	512.5	523.8	504.1	488.0
Crude fat	69.3	79.7	78.6	89.1
aNDF[b]	124.1	122.9	118.3	131.0
ADF[c]	52.9	52.0	50.2	53.8
Gross energy (MJ/kg DM)	19.5	19.6	19.8	19.9
Phosphorus	7.4	7.3	7.6	7.0
Calculated net energy (MJ/kg)	10.47	10.47	10.47	10.47
Amino acids, calculated SID[d] (g/kg)				
Lysine	12.5	12.5	12.5	12.5
Methionine + Cysteine	7.4	7.4	7.4	7.4
Threonine	7.9	7.9	7.9	7.9
Tryptophan	2.8	2.8	2.8	2.8
Valine	8.4	8.4	8.4	8.5

[a] BSFL: black soldier fly larvae (dietary inclusion level 5%, 10%, and 20%).
[b] aNDF: amylase-treated neutral detergent fiber.
[c] ADF: acid detergent fiber.
[d] SID: Standardized ileal digestibility.

Table 3
Analyzed amino acid (AA) composition of dietary treatments.

		Dietary treatments[a]			
Items[b]	BSFL meal	Control	BSFL5	BSFL10	BSFL20
Indispensable AA, g/kg					
Arginine	16.7	8.6	8.5	8.1	7.8
Histidine	9.0	3.7	3.8	3.9	4.3
Isoleucine	16.4	5.7	5.8	5.7	6.2
Leucine	30.4	10.6	10.5	10.4	10.8
Lysine	21.9	11.8	12.5	11.7	12.7
Methionine	6.5	4.0	4.3	4.1	4.6
Phenylalanine	15.0	6.7	6.5	6.4	7.0
Threonine	15.1	7.1	7.6	7.2	7.9
Tryptophan		2.5	2.1	2.5	2.5
Valine	21.5	7.0	7.1	7.0	7.5
Dispensable AA, g/kg					
Alanine	27.8	5.7	6.2	6.5	7.6
Aspartic acid	33.2	11.8	12.0	11.6	11.8
Cysteine	2.7	2.3	2.3	2.2	2.1
Glutamic acid	43.8	34.9	33.2	33.2	33.6
Glycine	18.2	5.7	5.8	5.8	6.3
Proline	20.6	10.4	10.3	10.7	11.7
Serine	14.2	6.6	6.5	6.4	6.5
Tyrosine	20.3	3.0	3.2	3.9	5.7
Total amino acids	333.4	145.6	146.1	144.7	154.1

[a] BSFL: black soldier fly larvae (dietary inclusion level 5%, 10%, and 20%).
[b] Determined using water-corrected molecular weights.

Table 4
Analyzed fatty acid composition of dietary treatments, and calculated sum of saturated-, monounsaturated-, and polyunsaturated fatty acids.

		Dietary treatments[a]			
Items, g/kg DM	BSFL meal	Control	BSFL5	BSFL10	BSFL20
C12:0	95.8	0.40	6.11	11.7	23.1
C14:0	19.3	0.42	1.35	2.33	4.25
C15:0	0.44	0.04	0.06	0.07	0.09
C16:0	31.8	13.3	12.4	11.4	9.96
C17:0	0.60	0.07	0.08	0.10	0.13
C18:0	7.05	4.31	3.49	2.76	1.54
C20:0	0.29	0.24	0.20	0.15	0.09
C21:0	1.73	0.01	0.09	0.28	0.32
C22:0	0.09	0.14	0.11	0.08	0.04
C23:0	–	0.02	0.01	0.01	–
C24:0	–	0.05	0.04	0.03	0.02
Sum saturated fatty acids	157.1	19.02	23.95	28.89	39.57
C14:1	0.36	–	0.02	0.04	0.08
C16:1	5.18	0.40	0.58	0.76	1.13
C18:1n9t	0.10	0.46	0.37	0.21	0.04
C18:1n9c	33.5	25.9	22.8	19.0	12.2
C20:1	0.22	0.89	0.73	0.55	0.22
C21:1n9	–	0.05	0.05	0.04	0.02
C24:1	–	0.11	0.08	0.04	0.03
Sum monounsaturated fatty acids	39.35	27.81	24.63	20.64	13.72
C18:2n6c	40.8	21.1	22.0	21.7	21.1
C18:3n3	5.31	4.56	4.15	3.56	2.39
C18:3n6	0.05	0.13	0.11	0.08	0.02
C20:2	0.12	0.13	0.11	0.09	0.05
C20:4n6	–	0.02	0.02	0.01	–
C20:5n3	–	0.30	0.23	0.16	–
C22:2	–	0.04	0.02	0.02	0.02
C22:5n3	–	0.03	0.02	0.01	–
C22:6n3	–	0.36	0.30	0.20	–
Sum polyunsaturated fatty acids	43.30	26.67	26.96	25.83	23.58

[a] BSFL: black soldier fly larvae (dietary inclusion level 5%, 10%, and 20%).

(FAME), in which concentrations were determined using a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA). Total phosphorus was analyzed after combustion and acid digestion (European Commission, 2009) using a commercial spectrophotometric kit (PH8328, Randox laboratories, County Antrim, UK). Yttrium concentration was determined after acid decomposition in a microwave digestion system (Start D, Milestone S.r.l., Sorisole, Italy), using a microwave plasma atomic emission spectrometer (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA). The chitin content of the BSFL meal was determined according to Finke (2007).

Trypsin and lipase activities were analyzed in jejunal content. Ice-cold Milli-Q water (1.5 mL; 600 µl for lipase analysis) was added to approximately 100 mg of the jejunal content, homogenized using a bead mill (TissueLyser, Qiagen Retsch, Haan, Germany) and sonicated in an ice-cold bath for three minutes (T 460/H, Elma Schmidbauer GmbH, Ransbach-Baumbach, Germany). After centrifugation at 21,100 × *g* for 10 min at 4 °C, the supernatant was collected, aliquoted, stored at −80 °C, and used for the analysis of lipase, trypsin, and total protein. Total protein concentration was determined in microtiter assay according to the Quick Start Bradford Protein Assay protocol (Bio-Rad Laboratories, Oslo, Norway). Absorbance was measured using a SpectraMax M2e Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA). Lipase and trypsin activity were analyzed using commercial kits (Lipase Activity Assay Kit III, MAK048–1KT, fluorometric, Sigma-Aldrich, Merck KGaA; Trypsin Activity Assay Kit, ab102531, colorimetric, Abcam), according to the manufacturer's protocols.

Short-chain fatty acids (SCFA) were analyzed in colon content. Samples were thawed on ice and 500 mg of the colon content were mixed with 500 µl ice-cold internal standard solution (2-methyl valeric acid in 5% formic acid), before sonication for 5 min in cold water. After centrifugation for 15 min at 4 °C with 15,000 × *g*, the supernatant was transferred to a spin column (45 kDa; VWR International, Radnor, PA, USA) and centrifuged again with the same parameters. SCFA concentration was determined by capillary gas chromatography on a stabilwax-DA, 30 m × 0.25 mm × 0.25 µm capillary column (Restek Corporation, Bellefonte, PA, USA) installed on a Trace 1300 gas chromatograph equipped with an AS 1310 autosampler, split injection, a flame ionization detector and Chromeleon software (Thermo Fisher Scientific, Waltham MA, USA). The initial oven temperature was 90 °C, held for 2 min, followed by a temperature increase of 10 °C/min to 150 °C and 50 °C/min to 250 °C and then held for 1 min. Helium was used as the carrier gas at a flow rate of 3 mL/min. The injector temperature was set at 260 °C and the detector temperature was set at 275 °C.

2.6. Morphology and morphometry

Jejunal, ileal, and colon morphology were blindly assessed and villus heights (VH) and crypt depths (CD) were measured by Aquamedic AS, Oslo, Norway. Gross pathological observations were recorded before tissue sampling. Samples were fixated in 10% formalin up to 48 h before processing following standard histological methods for gut tissue. Sections were stained with hematoxylin and eosin and evaluated by light microscopy, where the evaluation was done on morphological characteristics such as epithelial cell and barrier morphology and integrity, crypt changes such as hyperplasia, dilation or abscessation, degenerative and inflammatory mucosal changes including increased numbers of intraepithelial lymphocytes and infiltration by leukocytes. Methodologies of the evaluation protocol are described by Day et al. (2008) and Pérez de Nanclares et al. (2017). The morphological characteristics evaluated were graded using a semi-quantitative scoring system where score 0 is normal, score 1 represent mild changes, and score 2, 3, and 4 represent moderate, marked, and severe changes, respectively. Measurements of VH and CD were made on scanned whole-section images of the respective tissues captured using the PreciPoint M8 Microscope and Scanner (PreciPoint, Freising, Germany), and obtained using the ViewPoint software (PreciPoint, Freising, Germany). A minimum of three well-oriented crypts and villi were measured from each of the sections.

2.7. Extraction of DNA and 16S rRNA sequencing

Total DNA was extracted from bacteria in approximately 190 mg of colon content using QIAamp Fast DNA Stolen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. The DNA concentration was determined using NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples were stored at −20 °C until further analysis. Before preparing for 16S rRNA sequencing, samples were normalized to 10 ng/µl. The V3-V4 regions of the bacterial 16S rRNA gene were amplified using the primers Pro341f (5'-CCTACGGGNBGCASCAG-3') and Pro805r (5'-GACTACNVGGGTATCTAATCC-3'). The library preparation was conducted using the Miseq Reagent Kit V3 (Illumina, San Diego, CA, USA) according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). For indexing, Nextera XT index kit V2 was used (Illumina, San Diego, CA, USA). After the indexing reaction, all samples were measured by Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) using Qubit 1X dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). An equal amount of each sample was pooled together, and spiked with 5% PhiX Control (Illumina, San Diego, Waltham, MA, USA). For sequencing, 10 pm of the pooled sample was loaded to a flow cell. The sequencing analysis was performed on the Miseq System (Illumina, San Diego, CA, USA). The clustering density was 1256 k/mm^2 and 88.3% of clusters were passing filter.

Raw sequence data were analyzed using DADA2 v. 1.12.1 (Callahan et al., 2016) in R v. 3.5.0 (R Core Team, 2019). Default parameters were used if other is not specified. In brief, primers were removed, and the forward reads were truncated at 280 bp and the reverse reads at 250 bp. Max expected errors were set to 7. This allowed 73% of the reads to pass the quality control. Error rates were estimated, and the core sample inference algorithm applied. The denoised read pairs were merged, and chimeras removed. The Silva v. 138 database (Quast et al., 2013; Yilmaz et al., 2014) was used as a reference database for the taxonomy assignment. A phyloseq object was built with the phyloseq v.1.26.1 for further analyses (McMurdie and Holmes, 2013). Figures were made with ggplot2 v.3.2.1 (Wickham, 2016).

2.8. Statistical analysis

Outliers in the data were identified using the interquartile range method. Values outside the range of three times the interquartile range outside the lower and higher quartile were defined as outliers. Results are presented as mean values and standard error of the mean (SEM). Statistical analysis was performed using R v.4.0.3 (R Core Team, 2019) in Rstudio v.1.3.1093 (Rstudio, Boston, MA, USA). A linear mixed-effects procedure was run, using the lme4 1.1-23 (Bates et al., 2015) and lmerTest 3.1-2 (Kuznetsova et al., 2017) packages. Initially, the following model was used for variables from individual samples (pH, digestibility, enzyme activity, intestinal morphology and morphometry, liver index and SCFA):

$$Y_{ijklm} = \mu + diet_i + sex_j + bedding_k + litter_l + \varepsilon_{ijklm}$$

where Y is one observation in pig n; μ is the intercept; $diet_i$ is the fixed dietary treatment effect (i = 1:4); sex_j is the fixed effect of the sex of the pig (j = 1,2); $bedding_k$ is the fixed effect of bedding material, rubber mat or wood shavings (k = 0,1); $litter_l$ is the random effect of the lth litter (l = 1:11) $\sim N(0, \sigma^2_{litter})$ and ε_{ijklm} is a random residual $\sim N(0, \sigma^2_{\varepsilon})$. Pen (1:20) $\sim N(0, \sigma^2_{pen})$ was considered as a random effect in the model, but the effect of pen was tested with a likelihood-ratio test and found insignificant ($P > 0.05$) for all the variables. Hence, the pen was removed from the final model. An orthogonal polynomial contrast matrix, adjusted for BSFL inclusion levels, was specified using the contr.poly function in the stats base package (R Core Team, 2019). The bedding was not included in the model when analyzing the terminal sampling parameter, as it was constant. Effects are considered statistically significant when $P < 0.05$ and tendencies are defined as *P*-values between 0.05 and 0.10. For the effects of sex and bedding material, only significant results are presented.

For all pen-level parameters (growth performance, fecal DM and fecal score), the following model was used with the lm function in the stats base package:

$$Y_{ikn} = \mu + diet_i + bedding_k + \varepsilon_{ikn}$$

where Y now is one observation in pen n.

The Fisher's exact test was used to analyze data from the macroscopic evaluation of the intestinal segments. Histopathological findings was analyzed using the Kruskal-Wallis Rank Sum Test in the stats package (R Core Team, 2019).

To assess diversity in the microbial communities within pigs, Shannon alpha-diversity indices were calculated at the amplicon sequence variant (ASV) level. A comparison of the Shannon indices among dietary treatments was done using the Kruskal-Wallis Rank Sum Test. The beta-diversity (among pig variation in microbial communities) was assessed by principal coordinate analyses (PCoA) with the Bray-Curtis, unweighted, and weighted UniFrac distance matrices. Beta dispersion (variances) was calculated within each dietary group and a permutation-based test of multivariate homogeneity applied (vegan package v.2.6–6; Oksanen et al., 2019). Fulfilling the assumption of homogeneity in group variance, a PERMANOVA test was performed on the distance matrices with the dietary group as the independent variable. Pairwise PERMANOVA tests were performed to compare beta diversity among dietary treatments. *P*-values were adjusted for multiple testing with the method by Benjamini and Hochberg (1995).

The relative abundance was calculated, and the statistical difference in relative abundance among dietary treatments was analyzed with the Kruskal-Wallis test with dietary treatment as the explanatory variable.

To test for a statistical difference in relative abundance among dietary treatments, the Kruskal-Wallis test with dietary treatment as the explanatory variable was applied. Two-sample Wilcoxon tests, also known as the Mann-Whitney test, was used for pairwise comparison between the diets if the Kruskal Wallis test gave a significant effect of dietary treatment. *P*-values were corrected for multiple testing with the Benjamini and Hochberg (1995) method.

To test for covariation between the microbiota and SCFA profile, dissimilarity indices with the Bray-Curtis indices were calculated separately for the microbiota and SCFA data with the vegdist function, and the covariation tested with the mantel function in the vegan package (Oksanen et al., 2019).

Table 5
Growth performance parameters for post-weaning (PW) pigs (n = 5) fed increasing levels (5%, 10%, and 20%) of black soldier fly larvae meal (BSFL).

		Dietary treatments					*P*-value		
Day PW	Items[a]	Control	BSFL5	BSFL10	BSFL20	SEM[b]	Linear	Quadratic	Cubic
	Initial BW	10.7	10.6	10.6	10.6	0.1	0.842	0.893	0.992
	Final BW	25.7	24.0	25.2	24.9	0.3	0.662	0.317	0.056
0–14	ADFI	485	448	459	464	9	0.633	0.324	0.430
	ADG	432	392	425	427	7	0.682	0.267	0.060
	G:F	0.87	0.88	0.93	0.92	0.01	0.052	0.303	0.209
14–27	ADFI	943	867	918	917	15	0.939	0.348	0.152
	ADG	683	608	661	636	15	0.502	0.470	0.085
	G:F	0.72	0.70	0.72	0.69	0.01	0.411	0.880	0.332
0–27	ADFI	712	650	680	682	11	0.679	0.233	0.162
	ADG	552	496	538	528	9	0.720	0.279	0.031
	G:F	0.79	0.76	0.79	0.77	0.01	0.949	0.776	0.187

[a] BW: body weight, kg; ADG: average daily gain, g; ADFI: average daily feed intake, g; G:F: gain to feed ratio.
[b] SEM: standard error of mean.

3. Results

3.1. Growth performance

The ADG for the overall experimental period showed a negative cubic effect of dietary treatment, where the ADG was highest for pigs fed the control diet and lowest for pigs fed the BSFL5 (Table 5). The same tendencies were found for the ADG during 0–14 days and 14–27 days, and for the final BW. There was also a tendency for increased G:F ratio for day 0–14 PW with increasing inclusion of BSFL. The ADFI was not affected by dietary inclusion of BSFL. Pigs in pens with wood shavings had higher ADG ($P = 0.040$) for day 14–27 PW.

3.2. General intestinal function

There were no differences in pH in stomach or pH in jejunum among the dietary treatments (Supplementary Table S2). There were no differences in fecal DM or fecal score (Table 6) among the dietary treatments. The graph of the overall development in the fecal score during the experimental period (Supplementary Figure S1) shows an increased score (>2) in the first week PW, which seems to stabilize below score two after day 12, but again increasing to score >2 on day 23–25. Pens with wood shavings had lower fecal scores in week 3 ($P = 0.049$), and numerical lower average fecal score all experimental weeks.

3.3. Digestibility and enzymatic activity

There were no differences in jejunal trypsin or lipase activity among the dietary treatments (Supplementary Table S2). The CAID of crude fat increased linearly with increasing inclusion of BSFL in the diet, whereas the BSFL inclusion level had a positive quadratic effect on the CAID of ash. No differences among dietary treatments were found for the CAID of the other main nutrients (Table 7). Gilts had higher CAID of CP ($P = 0.030$), CF ($P = 0.025$) and ash ($P = 0.028$), compared to barrows.

The CAID of lysine was linearly reduced with increased inclusion of BSFL. A positive quadratic relationship was found between the BSFL inclusion level and CAID of histidine, isoleucine, leucine, and phenylalanine. For the CAID of tyrosine, there was both a linear decrease and a positive quadratic effect of the BSFL inclusion level. For the CAID of the other amino acids and total amino acids, no differences were found among the dietary treatments (Table 8). An effect of sex was found for alanine ($P = 0.013$), arginine ($P = 0.004$), aspartic acid ($P = 0.007$), histidine ($P = 0.008$), isoleucine ($P = 0.007$), leucine ($P = 0.009$), lysine ($P < 0.001$), methionine ($P = 0.009$), phenylalanine ($P = 0.012$), serine ($P = 0.004$), threonine ($P = 0.007$), valine ($P = 0.013$), and total amino acids ($P = 0.022$). All higher for gilts compared to barrows.

The CATTD of CP was linearly decreased with increasing inclusion of BSFL, whereas by contrast, the CATTD of CF and phosphorus increased (Table 9). A linear and positive quadratic relationship was found between BSFL inclusion level and CATTD of ash, where the CATTD of ash was reduced compared to control, when adding 5% BSFL, but increased with BSFL inclusion level. Gilts had higher CATTD of CP ($P = 0.035$), starch ($P = 0.015$) and phosphorus ($P = 0.005$). Using wood shavings as bedding material in the pens gave higher CATTD of CP ($P = 0.035$), CF ($P = 0.001$), ADF ($P = 0.002$) and ash ($P = 0.010$).

3.4. Intestinal morphology, morphometry, and liver index

Macroscopic evaluation of the intestinal tissue during sampling revealed some changes, mostly in jejunum and colon, with reddening of the mucosa, and low muscle tone and no mucosal folding in the jejunum (Table 10 and Fig. 1). The macroscopic appearance was distributed equally with no clear differences among pigs fed the different dietary treatments.

Table 6
Fecal dry matter (DM) and fecal score in post-weaning pigs (n = 5) fed increasing levels (5%, 10%, and 20%) of black soldier fly larvae meal (BSFL). A higher fecal score indicates more watery feces.

	Dietary treatments					*P*-value		
Items	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Fecal DM, %								
Day 7	26.6	25.7	25.3	24.3	0.7	0.271	0.917	0.898
Day 14	27.8	26.1	26.9	26.3	0.3	0.190	0.385	0.144
Day 21	26.3	26.2	26.1	24.7	0.4	0.128	0.520	0.937
Day 27	26.7	26.3	27.3	26.7	0.4	0.879	0.805	0.489
Total average	26.8	26.1	26.4	25.5	0.3	0.182	0.987	0.482
Fecal Score								
Week 1	1.82	1.87	1.87	1.84	0.06	0.954	0.777	0.932
Week 2	1.83	1.99	1.88	1.91	0.06	0.861	0.656	0.395
Week 3	1.73	1.78	1.56	1.72	0.06	0.801	0.445	0.269
Week 4	1.86	2.00	1.86	1.95	0.05	0.785	0.947	0.312
Overall period	1.82	1.92	1.80	1.86	0.05	0.960	0.990	0.344

[a] SEM: standard error of mean.

Table 7
Coefficients of apparent ileal digestibility (CAID) in post-weaning pigs (n = 12; average body weight 25.5 kg) fed increasing levels (5%, 10%, and 20%) of black soldier fly meal (BSFL).

	Dietary treatments					*P*-value		
Items	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Dry matter	0.711	0.674	0.680	0.680	0.010	0.375	0.294	0.470
Crude protein	0.697	0.659	0.666	0.676	0.014	0.624	0.303	0.543
Starch	0.974	0.949	0.953	0.958	0.005	0.448	0.131	0.361
Crude fat	0.817	0.817	0.848	0.868	0.010	0.043	0.894	0.445
Ash	0.309	0.281	0.266	0.407	0.020	0.069	0.031	0.793

[a] SEM: standard error of mean.

Table 8
Coefficients of apparent ileal digestibility (CAID) for amino acids (AA) in post-weaning pigs (n = 12; average body weight 25.5 kg) fed increasing levels (5%, 10%, and 20%) of black soldier fly larvae meal (BSFL).

	Dietary treatments					*P*-value		
Items[a]	Control	BSFL5	BSFL10	BSFL20	SEM[b]	Linear	Quadratic	Cubic
Indispensable AA								
Arginine	0.811	0.802	0.784	0.782	0.008	0.076	0.560	0.701
Histidine	0.783	0.763	0.741	0.757	0.006	0.073	0.030	0.581
Isoleucine	0.778	0.757	0.738	0.775	0.008	0.835	0.038	0.720
Leucine	0.790	0.768	0.752	0.781	0.008	0.671	0.047	0.843
Lysine	0.865	0.864	0.841	0.842	0.005	0.020	0.297	0.231
Methionine	0.873	0.876	0.855	0.884	0.005	0.494	0.053	0.080
Phenylalanine	0.795	0.760	0.736	0.778	0.008	0.445	0.005	0.740
Threonine	0.758	0.752	0.738	0.764	0.009	0.922	0.241	0.705
Valine	0.792	0.763	0.746	0.772	0.009	0.409	0.055	0.941
Dispensable AA								
Alanine	0.692	0.673	0.683	0.740	0.012	0.105	0.119	0.745
Aspartic acid	0.675	0.655	0.667	0.699	0.013	0.466	0.278	0.646
Cysteine	0.637	0.564	0.604	0.600	0.019	0.635	0.351	0.229
Glutamic acid	0.811	0.770	0.797	0.813	0.011	0.719	0.243	0.237
Glycine	0.425	0.397	0.432	0.531	0.032	0.220	0.402	0.747
Proline	0.635	0.667	0.732	0.742	0.022	0.069	0.478	0.595
Serine	0.735	0.709	0.706	0.715	0.010	0.486	0.241	0.753
Tyrosine	0.576	0.503	0.411	0.481	0.017	0.036	0.005	0.321
Total amino acids	0.750	0.733	0.734	0.756	0.010	0.795	0.279	0.817

[a] Determined using water-corrected molecular weights.
[b] SEM: standard error of mean.

Table 9
Coefficients of apparent total tract digestibility (CATTD) in post-weaning pigs (n = 20; average body weight 25 kg) fed increasing levels (5%, 10%, and 20%) of black soldier fly larvae meal (BSFL).

	Dietary treatments					*P*-value		
Items	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Dry matter	0.834	0.828	0.830	0.827	0.002	0.292	0.682	0.490
Crude protein	0.796	0.784	0.775	0.773	0.004	0.011	0.184	0.886
Starch[b]	0.997	0.997	0.998	0.998	<0.001	0.051	0.339	0.065
Crude fat	0.757	0.780	0.794	0.800	0.004	<0.001	0.058	0.985
ADF[c]	0.303	0.279	0.284	0.292	0.011	0.800	0.395	0.686
aNDF[d]	0.399	0.369	0.375	0.394	0.007	0.954	0.109	0.507
Phosphorus	0.513	0.515	0.553	0.564	0.006	<0.001	0.574	0.092
Ash	0.587	0.574	0.576	0.612	0.005	0.014	0.015	0.911
Energy	0.826	0.820	0.823	0.818	0.002	0.255	0.794	0.416

[a] SEM: standard error of mean.
[b] 26 feces samples had starch levels below lower detection limit.
[c] ADF: acid detergent fiber.
[d] aNDF: amylase-treated neutral detergent fiber.

Several pigs had morphological changes in jejunum. Occurrence of enterocyte vacuolization in jejunum differed among dietary treatments ($P < 0.001$), where all control pigs scored “normal,” whereas pigs fed the highest inclusion level all scored “mild.” Six pigs fed the BSFL5 and five pigs fed BSFL10 scored “mild.” In addition, three pigs fed the BSFL10 scored “moderate.” The remaining pigs

Table 10
Summary of the main intestinal macroscopic findings in post-weaning pigs (n = 12) fed increasing levels (5%, 10%, and 20%) of black soldier fly larvae meal (BSFL).

	Dietary treatments				
Items[a]	Control	BSFL5	BSFL10	BSFL20	*P*-value
Reddening of the jejunal mucosa	3	3	1	2	0.702
Jejunum with low muscle tone and no mucosal folding	2	4	2	1	0.523
Reddening of the ileal mucosa		1	2	1	0.893
Reddening of the colonic mucosa	5	6	4	6	0.864

[a] Numbers represent total samples observed with specific changes (N = 47).

scored normal. For the number of intraepithelial lymphocytes, all pigs scored normal except two pigs in the control group ($P = 0.082$). Dietary treatment did not affect lamina propria lymphocyte infiltration ($P = 0.899$), lamina propria edema ($P = 0.311$), or submucosal edema ($P = 0.581$) in jejunum. In the ileum, all tissue sections were observed to have a normal and healthy mucosal appearance.

In the colon, mild to moderate inflammatory morphological changes were observed. The inflammation was largely characterized by an increased content of lymphocytes and plasma cells in the inter-crypt compartment (Fig. 2). Other notable changes observed were mild crypt dilatation as well as a few samples showing crypt abscessation characterized by an accumulation of sloughed-off epithelial cells and neutrophils. However, there was no effect of dietary treatment on the inter-crypt area lymphocyte infiltration ($P = 0.240$) or crypt dilatation ($P = 0.908$). Dietary treatment did not influence VH, CD, or VH:CD ratio in jejunum, ileum, or colon, as shown in Table 11.

There were no differences in liver index among the dietary treatments (Supplementary Table S2). Barrows had higher liver index compared to gilts ($P = 0.008$).

3.5. SCFA and microbial community in the colon

There was no difference in the SCFA profile in the colon among the dietary treatments, except a negative cubic tendency in the level of isovaleric acid (Table 12). The SCFA profile differed between sexes. Barrows had higher levels of acetic acid ($P = 0.035$), propionic acid ($P = 0.003$), and of total SCFA ($P = 0.040$), but lower levels of isobutyric acid ($P = 0.045$) and isovaleric acid ($P = 0.047$) compared to gilts.

There was no difference in alpha diversity indices in the colon of piglets fed the different dietary treatments (Fig. 3a; N = 45, $P = 0.355$) as measured by the Shannon diversity index. Beta-diversity was assessed by several distance methods (Bray-Curtis, unweighted and weighted UniFrac). There were no differences in colon microbiota variance (beta dispersion) among the dietary treatments with neither of the distance methods. The PERMANOVA test showed effect of dietary treatment for the Bray-Curtis ($R^2 = 0.094$; $P = 0.029$) and unweighted UniFrac ($R^2 = 0.082$; $P = 0.041$) distances, but not for the weighted UniFrac distances ($R^2 = 0.084$; $P = 0.203$). However, when adjusted for multiple testing, the pairwise PERMANOVA tests gave no differences among any of the dietary treatments. The PCoA plot using Bray-Curtis distances (Panel b in Fig. 3) shows no clear grouping of dietary treatments.

At the phylum level, the colon microbiota was dominated by Bacteroidota and Firmicutes (Fig. 3, Panel c). The relative abundance of Bacteroidota differed among the dietary treatments ($P = 0.026$), whereas a tendency was found for the Firmicutes ($P = 0.055$). The Bacteroidota and Firmicutes were contributing 46.3% and 44.2% to the control group, 51.0% and 39.4% to the BSFL5 group, 50.0% and 42.1% to the BSFL10 group, and 48.1% and 38.9% to the BSFL20 group, respectively. There was also an effect of dietary treatment on Campilobacterota ($P = 0.042$) and Thermoplasmatota ($P = 0.002$), but the relative abundance of these two phyla were low in all groups (Campilobacterota: 1.0% in Control, 1.5% in BSFL5, 0.6% in BSFL10 and 0.5% in BSFL20; Thermoplasmatota: <0.01% in all groups).

Fig. 4 shows the relative abundance of the top 10 most abundant genera in the colon. *Prevotella* was the most abundant genus, contributing 15.2%, 18.2%, 14.0% and 13.6% to the colon microbiome in pigs fed control, BSFL5, BSFL10, and BSFL20, respectively. An effect of dietary treatment was found for the relative abundance of *Lactobacillus*, where control pigs had higher relative abundance of *Lactobacillus* compared to pigs fed the BSFL20 diet. An effect of dietary treatment was also found for the *Rikenellaceae RC9* gut group, where higher relative abundance was found in the colon of pigs fed the BSFL10 diet compared to pigs fed the BSFL5 diet. No covariation was found between the colonic SCFA concentrations and the microbiota composition (*i.e.*, the overall ASV data; $P = 0.366$).

4. Discussion

The interest of using insects in animal feed is increasing (DiGiacomo and Leury, 2019; Varelas, 2019), and inclusion of BSFL have been investigated in diets for pigs (Spranghers et al., 2018), chicken (Józefiak et al., 2018), and fish (Nogales-Mérida et al., 2019; Weththasinghe et al., 2021), but to this date, there is limited information about the effect of high dietary inclusion levels. The present study demonstrated that up to 19% full-fat BSFL meal can be included as an alternative protein and energy source in pelleted diets for weaned piglets without affecting growth performance.

For a four-week period PW, pigs fed the control diet had the highest ADG, with a cubic effect of the BSFL inclusion level. Dietary inclusion of BSFL did not alter the ADFI or G:F. There was, however, a tendency for increased G:F with increased inclusion of BSFL the

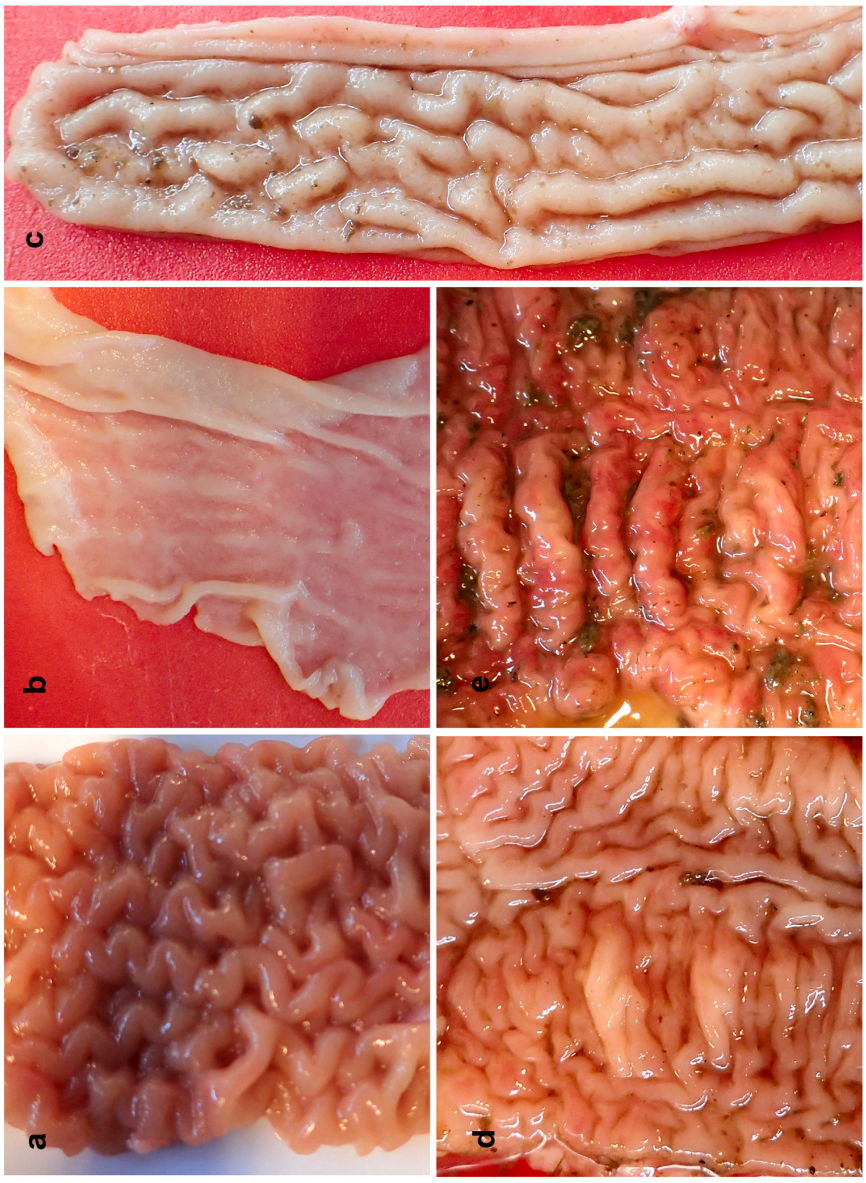

Fig. 1. Main findings from the visual inspection of intestinal tissue prior to the collection of tissue for histology. Panel a: Normal jejunum mucosa. Panel b: Jejunal mucosal with low muscle tone and complete loss of mucosal folds. Panel c: Normal appearance of ileum mucosa as observed for most of the samples. Panel d: Normal and healthy appearance of the colon. Panel e: Colon mucosa with mild mucosal reddening.

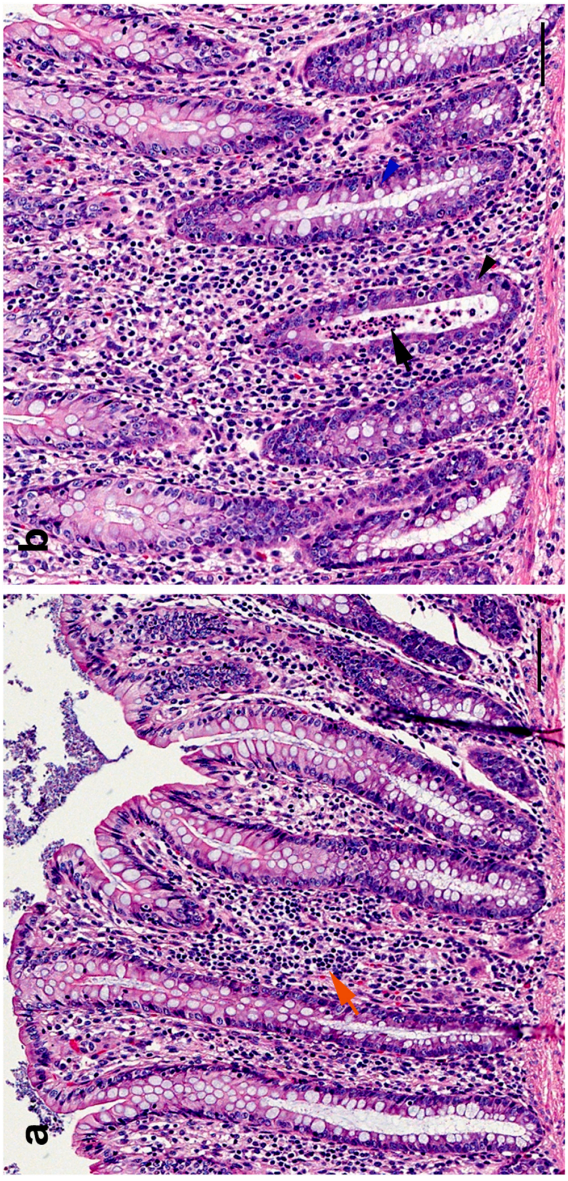

Fig. 2. Representative images of the morphological appearance of the colon tissue showing the histopathological changes of a: lymphocytic and plasma cell infiltration of the inter-crypt area (orange arrow), and b: crypt abscessation (black arrow) characterized by an accumulation of neutrophils in the crypt lumen and surrounded by crypt epithelial cells that have reduced numbers (black arrowhead) of goblet cells compared to healthy crypt epithelium (blue arrowhead). Scale bars in both images represent a distance of 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 11
Intestinal morphometry in post-weaning pigs (n = 12) fed increasing levels (5%, 10%, and 20%) of black soldier fly larvae meal (BSFL).

	Dietary treatments					*P*-value		
Items[a]	Control	BSFL5	BSFL10	BSFL20	SEM[b]	Linear	Quadratic	Cubic
Jejunum								
VH, μm	454	457	472	465	13	0.747	0.747	0.757
CD, μm	413	389	368	398	12	0.724	0.238	0.861
VH:CD ratio	1.23	1.25	1.37	1.30	0.06	0.628	0.620	0.681
Ileum								
VH, μm	232	233	250	247	7	0.316	0.680	0.544
CD, μm	134	148	150	137	4	0.909	0.136	0.775
VH:CD ratio	1.92	1.64	1.75	1.96	0.6	0.493	0.118	0.381
Colon								
CD, μm	486	479	480	482	9	0.907	0.754	0.966

[a] VH: villus heigh, CD: crypt depth.
[b] SEM: standard error of mean.

Table 12
Short-chain fatty acid (SCFA) and ammonia concentration in colon content in post-weaning pigs (n = 12) fed increasing levels (5%, 10%, and 20%) of black soldier fly larvae meal (BSFL).

	Dietary treatments					*P*-value		
Items, μmol/g	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Acetic acid	56.5	51.9	52.7	52.1	1.63	0.428	0.492	0.565
Butyric acid	13.5	10.4	12.4	12.6	0.62	0.926	0.165	0.110
Isobutyric acid	0.87	0.69	0.87	0.78	0.06	0.774	0.807	0.218
Isovaleric acid	1.05	0.79	1.11	0.92	0.08	0.793	0.979	0.093
Propionic acid	25.6	21.8	24.2	24.5	0.84	0.941	0.367	0.168
Valeric acid	1.96	1.75	1.70	1.77	0.11	0.533	0.405	0.936
Total SCFA	99.8	87.4	93.0	92.3	2.68	0.562	0.310	0.232
Ammonia, mM	3.00	2.72	2.87	2.70	0.08	0.309	0.724	0.344

[a] SEM: standard error of mean.

first 14 days PW. These results are partly consistent with Yu et al. (2020), which reported a linear improvement in both ADG and feed to gain ratio when feeding increasing BSFL inclusion from 0% to 4% in the two first weeks PW, whereas no differences were found for a four-week feeding period. Contrary, a study by Spranghers et al. (2018) showed no differences in performance when feeding up to 8% full-fat BSFL for 15 days, after weaning at 21 days of age.

Insects contain varying levels of chitin, depending on both types of insects and life stage (Finke, 2013). This polysaccharide is the major component of the insect's cuticle and creates a strong skeleton together with minerals and proteins. The exoskeleton of insects is different from crustaceans by having more amino acids strongly bound to the chitin (Finke, 2007; Andersen, 2010) and the degree of chitin deacetylation is dependent on the insect life stage (Smets et al., 2020). Inclusion of chito-oligosaccharide (derivates from chitosan) in a corn-soybean meal diet has been shown to reduce the incidences and score of diarrhea in piglets weaned at 16 days of age (Liu et al., 2008), and in piglets weaned at 21 days of age and challenged with *Escherichia coli* (Xiao et al., 2014). No differences in fecal score or fecal DM were found among the dietary treatments in this study, which may be due to the complexity of the chitin in the insect cuticle and the low degree of acetalization and solubility compared to the chito-oligosaccharides (Guan et al., 2019). However, this could also be related to the antibiotic treatment on days 11–13 PW as discussed below. The increased fecal score in the first week PW for all treatments, shown in the supplementary figure, is in accordance with the critical period for PW diarrhea (Madec et al., 1998). The increase in fecal score in the last experimental week could be related to increased stress from sampling, as individual fecal samples for digestibility were collected daily in this period.

There is scarce information regarding the nutrient digestibility of insects and only a few studies including BSFL. Newton et al. (1977) found similar CATTD of CP in five weeks old barrows fed 33% BSFL meal compared to the control diet. In the present study, there was reduced CATTD of CP with increased inclusion of BSFL, but no difference in trypsin activity was found. A decline in the CATTD of CP was also reported by Yu et al. (2020) when including low amounts of BSFL (<4%). Reduced CP digestibility with increased dietary inclusion of BSFL has also been reported for broiler chickens (Cullere et al., 2016), Atlantic salmon (Belghit et al., 2019; Weththasinghe et al., 2021), and rainbow trout (Renna et al., 2017). The CP digestibility is found to negatively correlate with chitin content in BSFL, *in vitro* (Marono et al., 2015). Because of the nitrogen-rich chitin, the Kjeldahl-N method overestimates the protein content of insects (Jonas-Levi and Martinez, 2017). This is supported by the fact that the CAID of total amino acids were not affected by dietary treatment. Janssen et al. (2017) suggested using a conversion factor of 5.6 g CP/g N to avoid the overestimation of protein content in BSFL.

Limited information is available about the amino acid digestibility of BSFL in pig diets. However, the amino acid digestibility of full-fat black soldier fly prepupae was recently investigated by Tan et al. (2020) and Crosbie et al. (2020). Tan et al. (2020) found the CAID of amino acids in black soldier fly prepupae to be between 0.641 and 0.821, and the CSID to be between 0.767 and 1.177.

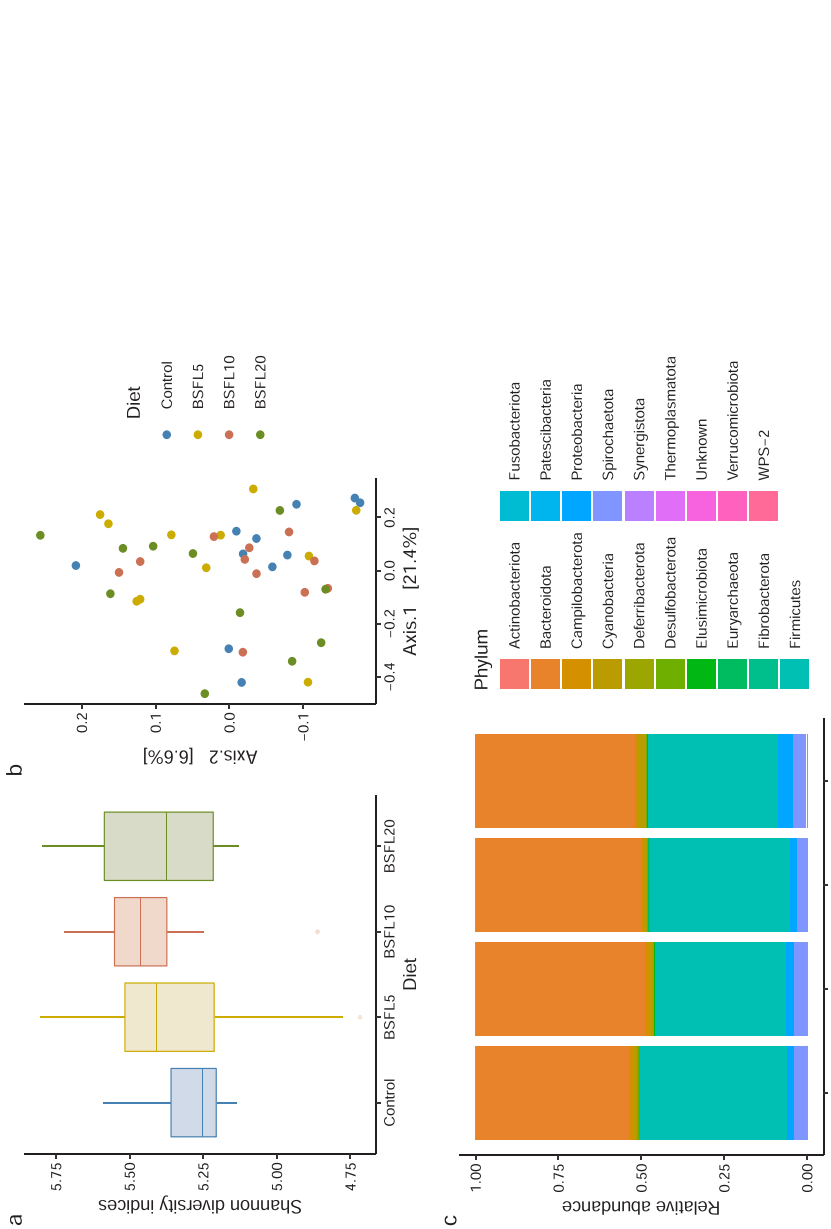

Fig. 3. Colon microbiota diversity in pigs fed an increased amount of BSFL. Panel a: Boxplot of Shannon diversity indices. The median value is represented as the lines inside the boxes. Panel b: Principal coordinate analysis plot with Bray-Curtis distances of colon microbiota colored by diet. Panel c: Average relative abundance of the microbial populations at the phylum level for each of the dietary treatments.

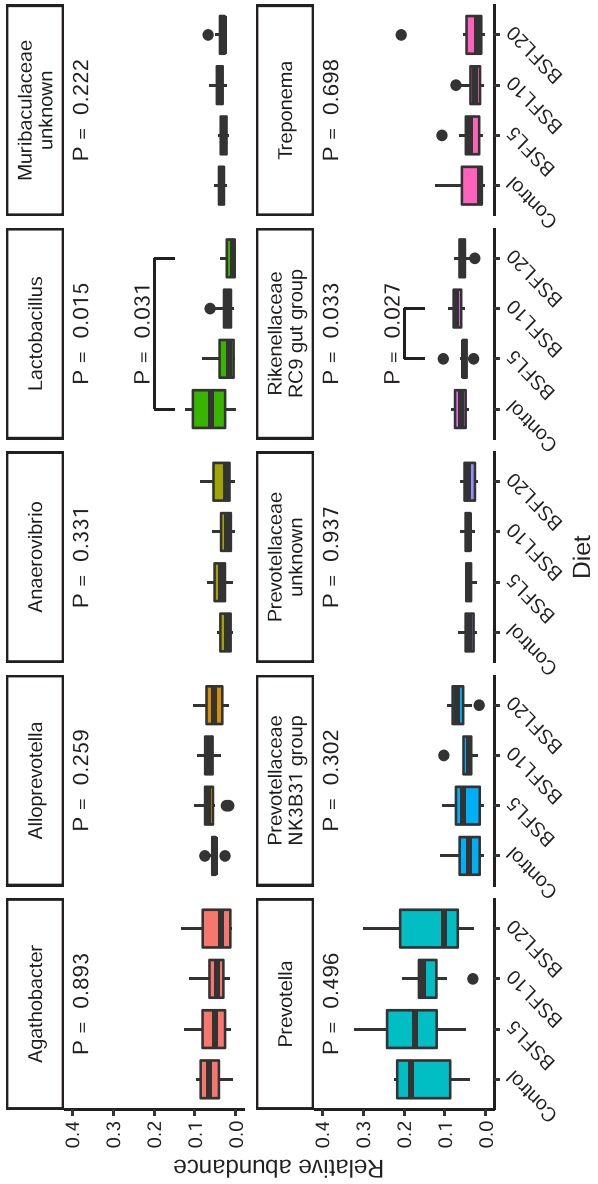

Fig. 4. Relative abundance of top 10 abundant genera. Brackets denote significant differences among dietary treatments.

Whereas, Crosbie et al. (2020) reported CAID of amino acids to be between 0.616 and 0.874, and CSID values to be between 0.814 and 1.001 in full-fat BSFL meal. During the diet optimization in the present study, the CSID for all amino acids in the BSFL meal were set to 0.83 because there was no reliable published CSID data at that time. All dietary treatments were formulated to have the same level of standardized ileal digestible lysine, but the CAID of lysine decreased with increased inclusion of BSFL. Tan et al. (2020) reported a CSID of 0.776 for lysine in BSFL. The reduced CAID of lysine in the present experiment, might therefore be explained by an overestimation of the CSID of lysine in the BSFL meal when formulating the experimental diets. A reduced apparent digestibility coefficient of lysine with increased inclusion of the same BSFL meal was also found in Atlantic salmon (Weththasinghe et al., 2021). However, Crosbie et al. (2020) reported the CSID of lysine to be 0.868, indicating more research is needed to define a CSID of lysine in BSFL. Maillard reactions are also known to reduce lysine availability in feed (Fastinger and Mahan, 2006; Moughan and Rutherfurd, 2008), but there were no differences in conditioner or pellet temperature during feed production (data not shown). On the other hand, the CAID of proline tended to increase with increased inclusion of BSFL. Proline is high in endogenous losses, attributed to low reabsorption of mucin (Stein et al., 1999), but no information about BSFL affecting endogenous losses was found in the literature. In general, the CAID of amino acids in the control diets was slightly lower compared with the control diet in a previous experiment with this age pigs (Cruz et al., 2019). Especially the CAID of tyrosine was in general low and differed among the dietary treatments. The reported CSID for tyrosine in BSFL (0.824; Tan et al., 2020) is only slightly lower than the coefficient used in the diet formulation (0.83). In the method used for amino acid analysis, glucosamine, resulting from hydrolyzed insect chitin, give a ninhydrin-positive peak close to the tyrosine peak in the chromatogram, which may cause a higher uncertainty for accurate tyrosine analyses.

There was a linear increase in the CAID and the CATTD of CF with increased inclusion of BSFL, whereas lipase activity measured in the oral part of jejunum showed no difference. The increased digestibility of CF might be caused by a dilution of endogenous fat loss as explained by Jørgensen et al. (1993). However, the authors believe that the total increase in the CATTD of CF from 0.76 to 0.80 is only partly explained by this dilution factor due to the high level of fat in all diets. The fat from BSFL amounted to be 19–69% of the total dietary fat and was affecting the fatty acid profile of the diets. Saturated fatty acids increased with increased BSFL inclusion, whereas the total amount of mono- and polyunsaturated fatty acids decreased. The increase in saturated fatty acids was dominated by lauric acid (C12:0). The lauric acid is a major constituent of the BSFLs fat. It is synthesized by the larvae, and therefore present at a high level independent of diet (Spranghers et al., 2017; Ewald et al., 2020). Finke (2013) reported that lauric acid constituted 42% of the total fatty acids in the BSFL. The MCFAs are rapidly absorbed in the mucosa and, unlike long-chain fatty acids, directly into the portal vein for transportation to the liver (Odle, 1997; Zentek et al., 2011). The differences in fatty acid composition among the diets are therefore believed to be the cause of the differences in the CATTD of CF. Higher digestibility of fat was also reported with 8% (Spranghers et al., 2018) and 33% inclusion of BSFL (Newton et al., 1977). On the contrary, Yu et al. (2020) found a lower CATTD of CF when including 4% full-fat BSFL. Their reported CATTD of CF was overall low (<70%). Different feed ingredients causing a different fatty acid profile of the diets, and low inclusion level of BSFL limiting the differences in fatty acid profiles among diets could explain the contradictory result by Yu et al. (2020).

Gilts showed higher CAID and CATTD of CP, CF, and ash, as well as for the CAID of several amino acids, compared to barrows. Higher CATTD of CP in gilts is previously reported by (Sheikh et al., 2017), including a nonsignificant increase in the CATTD of ether extract. The use of wood shavings as bedding material also increased the numerical CATTD of all nutrients except for aNDF. This is probably a result of pigs eating the wood shavings. Dietary fiber is known to decrease the CATTD of nutrients (Wilfart et al., 2007), but when not included in the dietary composition it can disturb digestibility calculations.

The MCFA can directly supply the enterocytes with energy and thereby improve morphological changes in periods with nutrient deficiency such as weaning (Zentek et al., 2011). It is well known that weaning causes villus atrophy. However, both in this study and the study by Spranghers et al. (2018), no effect of BSFL inclusion was found on intestinal morphometry. The effect of MCFA on morphology could be more evident the first days PW as the VH in the small intestine seems to recover within two weeks PW (Håkenåsen et al., 2020). Biasato et al. (2019) also reported no difference in intestinal morphometric indices after feeding pigs diets with up to 10% defatted BSFL. In accordance with our results, they also observed greater morphometric indices in the jejunum than the ileum.

The gut health was mildly suboptimal due to the observation of inflammation and edema in the jejunum and colon of some piglets. However, except for the jejunal enterocyte vacuolization, the general histopathological findings did not appear to be associated with the dietary treatments. The results are consistent with Biasato et al. (2019) which replaced soybean meal with defatted BSFL, concluding that up to 10% BSFL inclusion does not negatively affect gut morphology or histological features. The mild morphological changes (submucosal and lamina propria edema) in some of the pigs could be related to the edema disease outbreak. The mild to moderate enterocyte vacuolization at the folder tips of the jejunum could be related to an accumulation of lipids (or other nutrients) in the enterocytes. Randazzo et al. (2021) observed increased enterocyte vacuolization in the medium intestine of fish fed diets with both *Hermetia illucens* prepupae meal and vegetable-protein mixture and demonstrated by Fourier Transform Infrared Imaging (FTIR) analysis that this was due to improved lipid absorption. No special staining or FTIR analysis for further characterization of the vacuoles were conducted in the present study, thus further investigation is needed in this regard.

The microbiota of the gut is involved in the nutritional, physiological, and immunological functions of the pig (Fouhse et al., 2016). Variation in the diet composition is one of the most important factors affecting the GIT microbial ecosystem of the pig (Rist et al., 2013). The microbial community in the colon was dominated by Bacteroidota and Firmicutes, also reported by Yu et al. (2019). Yu and coworkers found no differences in phyla abundances when including 4 or 8% full-fat BSFL in diets for finishing pigs. By contrast, the relative abundance of Bacteroidota, Firmicutes, Campilobacterota, and Thermoplasmatota differed among the dietary treatments in the present study, but there was no clear dose-effect of the dietary BSFL inclusion. However, the highest relative abundance of Firmicutes and lowest relative abundance of Bacteroidota was found in pigs fed the control diet. In accordance with Yu et al. (2019), no

significant differences in Shannon indices were observed.

Prevotella was the most abundant genus in the colon of the pigs regardless of dietary treatment. This is in correspondence with several authors reporting *Prevotella* to be the most abundant genus in the fecal microbiome in the PW period (Isaacson and Kim, 2012; Guevarra et al., 2018; Wang et al., 2019). *Lactobacillus* was more abundant in the colon of the control piglets. This result is contrary to what recently was reported by Yu et al. (2019), where inclusion of 4% BSFL increased the abundance of *Lactobacillus*. In the study by Yu et al. (2019), finishing pigs were fed corn-based diets contrary to our PW pigs, which were fed wheat and barley-based diets. Also, when they increased the inclusion to 8% BSFL, Yu et al. (2019) did not find the same beneficial effects on the colonic microbiota as when feeding the 4% inclusion of BSFL. *Lactobacillus* is a genus of gram-positive lactic acid-producing bacteria in general considered favorable in the gut microbiome. It is known that lauric acid, abundantly found in BSFL fat, has antimicrobial effects, especially against gram-positive bacteria (Zentek et al., 2011). Spranghers et al. (2018), observed inhibition of the growth of lactobacilli in an *in vitro* study with BSFL fat but did not observe differences *in vivo* in the small intestine. They also observed that a high amount of the lauric acid was absorbed already in the stomach and therefore had little opportunity to modulate the colon microbiome. In rainbow trout, it is shown that the BSFL life stage and lipid content are important factors influencing the gut microbiome (Huyben et al., 2019).

In the present experiment, the content of several protein and fat ingredients, including soybean meal, soy protein concentrate, fish meal, rapeseed oil, and saturated vegetable fat, was reduced at the expense of increased BSFL inclusion in the diets. It is therefore not possible to differentiate if the observed chances are due to the BSFL inclusion or the reduction of other ingredients. It is also important to mention the treatment with antibiotics of all pigs in the second week of the experiment. The effect of the antibiotic on the gut microbiota is antibiotic specific (Fouhse et al., 2016), and it is therefore difficult to know what impact the antibiotic treatment of the pig in this experiment had on their gut microbiota. Indeed, the antibiotic treatment could have affected microbiota results and be the reason for the small differences in the colon microbiome among the dietary treatments. Because of the antibiotic treatment in the second experimental week, it might be that the feeding period was too short to cause significant changes in the gut microbiome. Also, therapeutic doses of antibiotics might suppress the innate immune defenses and contribute to increased disease susceptibility (Fouhse et al., 2016). Almost half of the pigs, independent of dietary treatment, had reddening of the colonic mucosa, indicating some inflammation in the colonic intestinal wall. However, the overall Shannon diversity indices reported were similar to what was reported by Yu et al. (2019) in finishing pigs.

5. Conclusion

To conclude, up to 19.06% of full-fat BSFL meal could be included in a balanced diet for PW pigs with minor effects on performance results. Increased inclusion of BSFL decreased the CATTD of CP and increased CATTD of CF but did not affect the general gut function, as assessed by enzyme activity and morphometry. There was a mild to moderate enterocyte vacuolization at the jejunal folder tips in piglets fed insect meal. Some changes in the colon microbiota composition were observed, such as decreased relative abundance of *Lactobacillus* with dietary inclusion of BSFL. However, there are limited information and contradictive results on the effect of BSFL on the gut microbiome in pigs, thus further investigations are needed.

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CRediT authorship contribution statement

IMH, **JØH**, **MØ**, and **LTM** contributed to the conceptualization and study design. **IMH**, **JØH**, **LTM**, **GHG**, and **RMÅ** contributed to sample collections. **RMÅ** conducted enzyme activity analyses, 16S rRNA extraction, and sequencing. **IMH** and **GHG** performed statistical analysis on growth performance, liver index, fecal consistency, and digestibility data. **IMH** performed statistical analysis on pH, enzyme activities, short-chain fatty acids, and raw sequence data. **IMH** wrote the original draft. All authors critically reviewed the original draft and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2021.115086.

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Supplementary Table S1 Nutrient composition (g/kg) of feed ingredients.

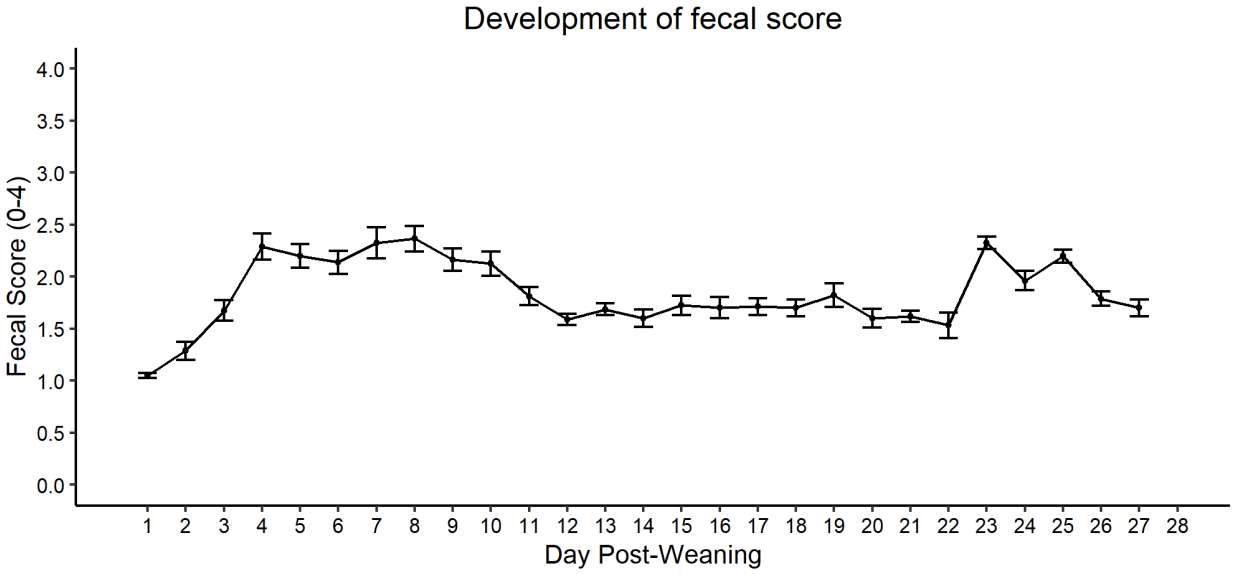
Ingredient	Dry matter	Crude protein	Starch	Crude fat	ADF	aNDF	Ash	Chitin	Energy (MJ/kg)
Wheat	867	124	488	14	45	116	14		16
Barley	858	92	564	11	82	206	17		16
Oats	858	88	408	44	150	270	23		17
BSFL meal[a]	905	380	-	289	-	-	84	7.2	-
Soybean meal	876	426	-	11	128	156	53		17
Fish meal	920	679	-	101	-	-	147		19
Rapeseed meal	928	342	-	100	179	223	53		20

[a] Ca: 17.4 g/kg, Mg: 2.4 g/kg, total P: 7.8 g/kg.

Supplementary Table S2 pH, enzyme activities and liver index in post-weaning pigs (average body weight 25 kg) fed increasing levels (5, 10, and 20%) of black soldier fly larvae meal (BSFL).

		Dietary treatments					P-value		
	N	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
pH stomach	47	3.8	3.9	3.7	3.6	0.1	0.579	0.751	0.598
pH jejunum	47	5.4	5.3	5.3	5.2	0.1	0.479	0.971	0.649
Trypsin jejunum, mU/µg protein	42	1.04	0.90	1.06	0.68	0.14	0.457	0.584	0.767
Lipase jejunum, mU/mg protein	45	0.07	0.07	0.08	0.06	0.01	0.839	0.810	0.839
Liver index	44	2.65	2.58	2.64	2.74	0.04	0.127	0.456	0.624

[a] SEM: standard error of mean



Supplementary Figure S1 Development of the overall fecal score during the experimental period.

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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