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Department of Animal and Aquacultural Sciences
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Yeast (*Cyberlindnera jadinii*): an alternative protein source in pig and poultry feed

Gjær (*Cyberlindnera jadinii*): en alternativ protein kilde i grise-og fjørfefôr

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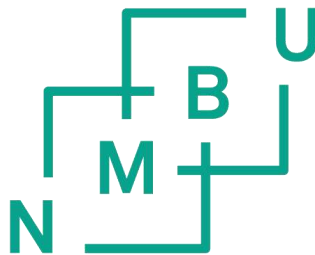Philosophiae Doctor (PhD) Thesis

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Acknowledgments

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Abbreviations

AA	Amino acid(s)
ADG	Average daily gain
AID	Apparent ileal digestibility
Approx.	Approximately
ATTD	Apparent total tract digestibility
BPM	Bacterial protein meal
BW	Bodyweight
CD	Crypt depth
CHO	Carbohydrates
CP	Crude protein
CU10, CJ10	Diet with 10 % CP from yeast
CU20, CJ20	Diet with 20 % CP from yeast
CU30, CJ30	Diet with 30 % CP from yeast
CU40	Diet with 40 % CP from yeast
DM	Dry matter
DN	Digested nitrogen
EC	European Commission
Exp.	Experiment(s)
FCR	Feed conversion ratio (feed:gain)
FM	Fish meal
FN	Fecal nitrogen
HE	Heat energy
IN	Ingested nitrogen
N	Nitrogen
NRC	National research council
RE	Retained energy
RFE	Energy retained as fat
RN	Retained nitrogen
RPE	Energy retained as protein
RSM	Rapeseed meal
RQ	Respiratory quotient
SBM	Soybean meal
SD	Standard deviation
SEM	Standard error of the means
SID	Standard ileal digestibility
UE	Urinary energy
UN	Urinary nitrogen
VH	Villi height

Units

d	day(s)
g	grams
h	hours
kg	kilograms
kJ	kilojoules
MJ	megajoules

Summary

The demand for food protein sources continues to increase rapidly along with the World population. Consequently, the European livestock industry requires a growing supply of feed protein to meet these demands. Furthermore, the European Commission and the United Nations have advised the implementation of sustainable measures in this field. Protein-rich ingredients currently used in European livestock feed, such as soybean meal, fish meal, and rapeseed meal, have all been associated with sustainability issues. Developing alternative and locally-produced protein sources can mitigate the pressure of protein imports to Europe, and especially to Northern Europe, where the capacity to produce protein is limited by the harsh climate. Microbial ingredients such as yeast can be grown on a variety of sugars produced from lignocellulosic biomass, independently of arable land and atmospheric conditions. Recently, *Cyberlindnera jadinii* yeast (previously classified as *Candida utilis*) grown on local lignocellulosic sugars, has shown potential to replace conventional protein sources in diets for pigs and broiler chickens, but little is known of its effects on the growth and digestive performance of these animals.

Three experiments were, therefore, conducted to evaluate the effects of replacing soybean meal, fish meal and rapeseed meal with graded levels of *C. jadinii* in pig (Exp. 1 and 2) and poultry diets (Exp. 3), on growth performance, digestive function, and utilization of protein and energy in young pigs and broiler chickens. *C. jadinii* was grown on second-generation sugars obtained from lignocellulosic biomass of Norway spruce trees (*Picea abies*) by using the Borregaard advanced lignin (BALI) process. The sugars were used in the growth media for the yeast, as described by Øverland and Skrede (2017) and Sharma et al. (2018a). The experiments described here are reported in Papers I, II and III.

In Paper I the effects of dried inactivated *C. jadinii* replacing conventional protein sources in diets for weaned piglets were evaluated in terms of growth performance and digestive function. Forty-eight piglets weaned at 30 days of age (11.06 ± 0.84 kg initial BW) were fed one of four dietary treatments: a conventional control diet with soybean meal, fish meal, rapeseed meal, and potato protein concentrate or one of three experimental diets containing 10, 20 or 40% CP from *C. jadinii* (CU10, CU20, and CU40, respectively). Piglets were equally distributed by litter, gender, and BW and allotted to the dietary treatments, with 12 replicates per treatment. Each pig constituted an experimental unit. Adding *C. jadinii* to diets did not affect piglet growth performance. The CU40 diet had higher apparent total tract digestibility (ATTD) of CP and ash compared with the control diet. The ATTD of neutral detergent fiber was lower in the CU40

diet than in the control. The apparent ileal digestibility (AID) of ash was higher in the CU40 diet compared with the control, while the AID of CP and amino acids were unaffected. Villi-height increased in jejunum and ileum, and villus-height: crypt-depth ratio increased in the jejunum of pigs fed the CU40 diet compared with pigs fed the control diet. Fecal dry matter increased linearly with increasing levels of *C. jadinii* in the diets seven days after weaning and was higher for the pigs fed 40% CP from *C. jadinii* compared with the control group. Trypsin activity and mRNA expression of genes encoding for nutrient transporters in the jejunum did not differ among diets.

In Paper II the protein and energy utilization of the same diets were evaluated in young pigs (16.69 ± 4.45 kg initial BW). Twenty-four intact boars were fed one of the four diets with two pigs per diet per period, during three periods, and a total of six replicates per diet. Each animal constituted an experimental unit. The three periods were divided into a period of adaptation in stables followed by a period of adaptation in balance cages. Each period included an energy and nitrogen balance experiment of four days, including a respiration experiment of 22 h. During the balance period and respiration experiments, the pigs were kept individually, in cages with devices for quantitative collection of feces, urine and feed residues. Individual respiration measurements were performed in open-air-circuit respiration chambers. Growth performance, feed intake, nitrogen, energy metabolism, and apparent total tract nutrient digestibility were similar among dietary treatments.

In Paper III, the effects of dietary *C. jadinii* on growth performance digestive and absorptive capacity of newly-hatched male broiler chickens were evaluated. The dietary treatments consisted of four diets including one control diet based on wheat, oats, maize and SBM, and three diets with 10, 20 and 30% CP from *C. jadinii*. One-thousand broiler chickens (42.0 ± 0.75 g initial BW) were distributed to 20-floor pens with 50 birds each, constituting five replicates per diet. Growth performance and feed intake linearly decreased with increasing dietary inclusion of *C. jadinii*, but they were similar between the control diets and diets with 10% CP from *C. jadinii*. Duodenal crypt depth linearly decreased with increasing amounts of *C. jadinii* in diets, but ileal villus height was similar between the control diet and diet with 30% yeast protein.

Altogether, the results indicate that *C. jadinii* can replace up to 40% CP in conventional young-pig diets and 10% of CP in broiler chicken diets, in terms of growth performance, feed intake, nitrogen, and energy metabolism, digestive function, and intestinal morphometry.

Sammendrag

Behovet for protein i matforsyningen øker i takt med den globale befolkningsveksten. Økt kjøttforbruk på grunn av økt kjøpekraft i befolkningen fører også til økt behov for proteinråvarer i fôrindustrien. Tilgangen på fôrproteinråvarer av høy kvalitet og produsert på en bærekraftig og klimavennlig måte er begrenset. Husdyrproduksjonen i Norge og Europa er i dag avhengig av importerte proteinråvarer. Økt selvforsyningsgrad og behovsdekning for bærekraftige fôrproteinråvarer etter EU-kommisjonen og FN (De forente nasjoner) sine anbefalinger er derfor en av de største utfordring i europeisk husdyrproduksjon. Proteinrike råvarer som i dag brukes i europeisk husdyrfôr, for eksempel soyamel, fiskemel og rapsfrø, er alle forbundet med bærekraftsutfordringer. Å utvikle alternative og lokalproduserte proteinråvarer vil redusere behovet for import til Europa, og spesielt til Nord-Europa, der mulighetene for å produsere vegetabilsk protein er begrenset av klimatiske forhold. Mikrobielle råvarer som gjær kan dyrkes på en rekke sukkerarter, uavhengig av jordbruksarealer og klimatiske forhold. *Cyberlindera jadinii*-gjær (tidligere klassifisert som *Candida utilis*) dyrket på lokale lignocellulosiske sukkerarter har vist potensial til å kunne erstatte konvensjonelle proteinkilder i fôr til gris og fjørfe når det gjelder protein- og aminosyreinnhold. Imidlertid er kunnskapen om fordøyelighet av næringstoffene i *C. jadinii*-gjær og effekter på produksjonsresultater mangelfull.

Totalt tre dyreforsøk ble derfor utført for å undersøke effekten av å erstatte soyamel, fiskemel og rapsfrø med *C. jadinii* i fôr til smågris og slaktekylling, på produksjons resultater, fordøyelighet, absorpsjon og utnyttelse av næringsstoffer og energi. *C. jadinii* ble dyrket på 2. generasjons sukkerarter fra lignocellulosisk biomasse fra Norsk gran (*Picea abies*) ved bruk av prosessen Borregaard advanced lignin (BALI). Sukkerartene ble brukt som vekstmedium for gjæren som beskrevet av Øverland og Skrede (2017) og Sharma et al. (2018a). Dyreforsøkene er beskrevet og rapportert i artiklene I, II og III.

I artikkel I ble effekten av å erstatte konvensjonelle proteinkilder med tørket, inaktivert *C. jadinii* i fôr til avvent smågris undersøkt med hensyn til produksjonsresultater og fordøyelsesfunksjoner. Førti-åtte smågris, avvent ved 30 dagers alder (11,06 ± 0,84 kg startvekt) ble tildelt fire ulike forsøksfôrblandinger: et konvensjonelt kontrollfôr med soyamel, fiskemel, rapsmel og potetprotein, og tre ulike forsøksfôrblandinger med 10, 20 eller 40% av råproteinet (CP) fra *C. jadinii* (CU10, CU20 og CU40). Grisene ble fordelt likt etter kull, kjønn og startvekt, med 12 gjentak per behandling. Økende innhold av *C. jadinii* i forsøksfôret hadde ingen negativ effekt på smågrisens produksjonsresultater, men fordøyelighet og absorpsjon, og gjødselkvaliteten ble forbedret. CU40-forsøksfôret hadde høyere fekal fordøyelighet (ATTD)

av CP og aske sammenliknet med kontrollfôret. ATTD av *neutral detergent fiber* var lavere i CU40-blandingen sammenliknet med kontrollfôret. Den apparent ileale fordøyeligheten (AID) av aske var høyere i CU40-forsøksfôret sammenlignet med kontrollfôret, mens AID for CP og aminosyrer var upåvirket. Villi-høyde økte i jejunum og ileum, og villus-høyde:kryptdybde-forholdet økte i jejunum hos griser som fikk CU40-forsøksfôret sammenliknet med griser som fikk kontrollfôr. Tørrstoff i gjødsel økte lineært med økende nivå av *C. jadinii* i forsøksfôret sju dager etter avvenning og var høyere hos griser som fikk CU40-forsøksfôret sammenlignet med de som fikk kontrollfôr. Det var ikke forskjeller i trypsinaktivitet og mRNA-uttrykk av gener som koder for næringstransportører i jejunum mellom forsøksfôrbehandlingene.

I artikkel II ble utnyttingen av protein og energi for de samme forsøksfôrblandingene beskrevet i artikkel I undersøkt hos ung gris (16,69 ± 4,45 kg startvekt). Tjuefire ukastrerte hanngris ble fordelt på fire forsøksgrupper og ble tildelt en av de fire forsøksfôrblandingene, det vil si totalt seks gjentak per forsøksgruppe. Forsøksperioden omfattet energi- og nitrogenbalansemålinger over fire dager og respirasjonsmålinger over 22 timer. I forsøksperioden var grisene i individuelle bur med kvantitativ oppsamling av avføring, urin og fôrrester. Individuelle respirasjonsmålinger ble utført i respirasjonskammer med åpen lufttilførsel. Det ble ikke avdekt effekter av økende innblanding av *C. jadinii* med hensyn til nitrogen- og energimetabolisme og ATTD av næringsstoffer.

I artikkel III undersøkte vi effektene av økende innblanding av *C. jadinii* i fôr til slaktekylling på produksjonsresultater, fordøyelse og absorbsjonskapasitet. Et kontrollfôr basert på hvete, havre, mais og soyamel ble sammenlignet med tre ulike forsøksfôrblandinger der 10, 20 og 30% av CP kom fra *C. jadinii*. Ett tusen slaktekyllinger (startvekt 42,0 ± 0,75 g) ble fordelt på 20 binger med 50 kyllinger i hver binge, totalt fem gjentak per behandling.Vi fant en lineær reduksjon i fôropptak og produksjonsresultater med økende innhold av gjær i forsøksfôret. Imidlertid var det ingen forskjeller i fôropptak og produksjonsresultater mellom kyllinger som fikk tildelt kontrollfôr sammenlignet med kyllinger som fikk forsøksfôr med 10% av CP fra *C. jadinii*. Kryptdybden målt i duodenum ble redusert lineært med økende mengde *C. jadinii* i forsøkfôret. Imidlertid var villus-høyden målt i ileum den samme hos kyllinger som fikk kontrollfôr sammenlignet med de som fikk forsøksfôr med 30% gjær proteinet.

Samlet sett indikerer resultatene at *C. jadinii* kan erstatte opptil 40% av CP i smågrisfôr og 10% av CP i slaktekyllingfôr uten negative effekter på fôropptak, produksjonsresultater, nitrogen- og energiforbruk, fordøyelse og absorpsjon samt tarmmorfometri.

List of publications

This thesis is based on the papers listed below:

Paper I: Cruz, A., Håkenåsen, I.M., Skugor, A., Mydland, L.T., Åkesson, C.P., Hellestveit, S.S., Sørby, R., Press, C.M., Øverland, M., 2019. *Candida utilis* yeast as a protein source for weaned piglets: Effects on growth performance and digestive function. Livest. Sci. 226: 31-39. https://doi.org/10.1016/j.livsci.2019.06.003

Paper II: Cruz, A., Tauson, A.-H., Matthiesen, C.F., Mydland, L.T., Øverland, M. *Cyberlindnera jadinii* yeast as a protein source for growing pigs: effects on protein and energy metabolism. (Submitted)

Paper III: Cruz, A., Sterten, H., Steinhoff, F.S., Mydland L.T., Øverland, M. *Cyberlindnera jadinii* yeast as a protein source for broiler chickens: effects on growth performance and digestive function. (Manuscript)

1. Introduction

1.1. General introduction

The demand for protein sources for both food and feed continues to increase exponentially along with the increases in the global human population. The consumption of meat in industrial countries *i.e.* Europe, USA and Australia in 2015 was in average 96 kg per person per year, and the estimated global demands for pig and poultry meat were 9.9 and 13.8 kg per person respectively (FAOSTAT). More precisely in Europe, one person consumes on average 24 to 25 kg of poultry meat per year (Table 1). Crude protein (CP) level in Norwegian poultry and pig feed can generally vary from 10 % and up to 24 %, which represents an annual requirement of up to 108 839 tons of protein to meet the demands for pig and poultry feed production (based on *Landbruksdirektoratet*, 2018). While the major part of the poultry and pig meat consumed in Norway was produced nationally, in 2017 446 331 tons of protein ingredients including soy, fish meal, rapeseed, maize were used in the production of the compound feed, of which, only 24 391 tons, representing 5 % were locally produced (Figure 1). Europe continues to be dependent on imported protein-rich ingredients, most markedly soybeans, to feed livestock. In 2012, 69 % of the protein-rich ingredients excluding fish meal used in livestock feed in Europe, were imported (Figure 1), and the self-sufficiency of soybean production was only 3 % (de Visser et al., 2014). Currently, there is a large focus on sustainability and use of locally-produced protein sources in European agriculture, especially in Scandinavia. Developing alternative protein sources from local renewable natural resources through a biorefinery process is considered essential for improving agriculture self-sufficiency and sustainability.

Table 1: Consumption of pork and poultry meat between 2016 and 2019 (kg per person/ year).*

Kg/person/year	2016	2017	2018	2019
Pork				
World	12.28	12.23	12.32	12.31
EU-28	32.16	32.10	32.31	32.06
Norway	20.70	20.24	20.25	20.26
Poultry meat				
World	13.98	13.98	14.20	14.30
EU-28	24.01	24.23	24.47	24.80
Norway	16.79	17.59	18.30	19.02

Source: Organization for Economic Cooperation and Development (OECD) and the Food and Agriculture Organization (FAO), 2018.*data refer to the European Union (EU-28) excluding Iceland.
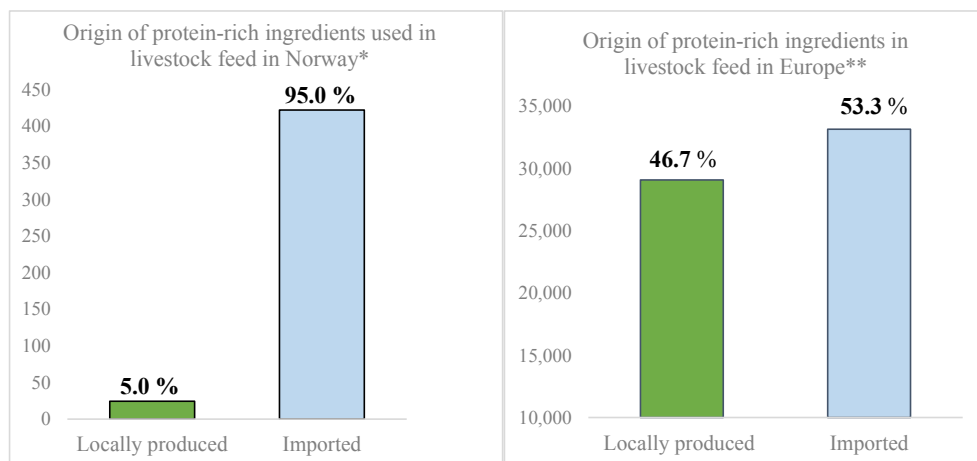
Figure 1: Origin of protein-rich ingredients used in European livestock feed (in thousands of tons). *soybean meal, rapeseed meal, fish meal, and maize gluten. **soybean meal, rapeseed meal, fish meal, and sunflower meal. Sources: Norwegian Agriculture Agency, Norwegian Ministry of Agriculture and Food (*Landbruksdirektoratet*), 2018 (left); data from 2012, Roman et al. 2016, de Visser 2014 (right).

1.2. Traditional protein sources for animal agriculture

Soybean meal (SBM) is one of the most common protein sources in feed used in livestock production and it stands at the top of protein source imports with 201 058 tons imported to Norway in 2017, which is equivalent to 47.7 % of total protein imports and 25.3 % of total imported feed ingredients (Landbruksdirektoratet, 2018). The preference for SBM as a protein source is explained by its high protein content with a favorable amino acid profile and high protein digestibility. However, like other plant-protein sources in animal feed, SBM presents some amino acid (AA) imbalances, which must be compensated by adding crystalline AA or using other high-quality protein sources (Sánchez-Muros et al., 2014). Furthermore, soybeans contain several anti-nutritional factors such as trypsin inhibitors (Liener, 2000) that limit pancreatic-enzyme activity, consequently reducing growth performance in pigs and chicks (Palacios et al., 2004; Yen et al., 1977), the proteins glycinin and β-conglycinin which may act as allergens (Li et al., 1991; Zhao et al., 2010), and the saccharide stachyose, associated with an increased incidence of diarrhea and decreased performance in piglets (Liying et al., 2003).

In compound feed for farm animals produced in Norway, rapeseed meal (RSM) makes up 37.5 % of the protein ingredients, but it is mainly imported, while high-quality locally produced fish meal constitutes < 1 % of the protein ingredients (Landbruksdirektoratet, 2018). Rapeseed meal has a high content in insoluble dietary fiber (Pérez de Nanclares et al., 2017) compared to SBM and FM, and has a moderate content of protein (35 % CP) compared to SBM (46 % CP), FM (68 % CP), and potato protein concentrate (73 % CP). Fish meal is a high-quality protein source with a well-balanced AA profile. The availability of FM is however strictly regulated due to concerns linked to sustainable fishing, as the overuse of this protein source may result in environmental deterioration (Sánchez-Muros et al., 2014). Additionally, the inclusion levels of FM in pig diets are restricted due to the risk of causing off-flavor in frozen pig meat, and the high price (Valaja et al., 1992). Potato protein concentrate is rich in protein and energy but it is

generally used in restricted amounts due to its high price. Field beans and peas may also be used in conventional monogastric nutrition (Figure 2) despite having a CP content of 22 to 38 %, which is lower compared to other protein sources (Griffiths and Lawes, 1978). They are also associated with antinutritional factors (Moseley and Griffiths, 1979). Field beans can, for example, be used to replace 31 % of SBM in broiler chicken diets, without compromising growth performance of the birds (Laudadio et al., 2011).
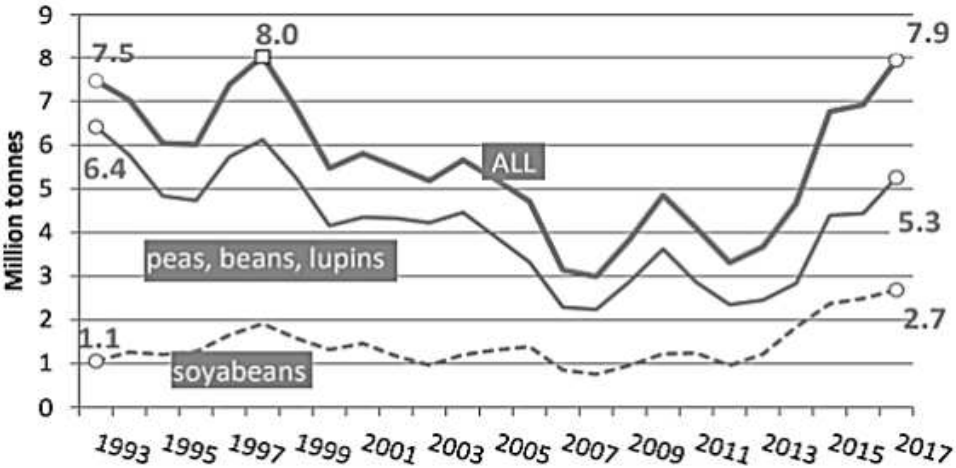
Figure 2: Production of peas, beans, lupins, soybeans, and the total production of protein-rich ingredients in the EU (1993-2017). Source: (European Commission, 2017).

The traditional protein sources used in European livestock production cause sustainability concerns, as the local production of these ingredients cannot meet the demand for protein-rich ingredients in Europe. Additionally, locally produced ingredients are currently not price-competitive, compared with the protein sources offered by other regions of the World, i.e. South America. All things considered, the present need to develop alternative local-based protein sources is clear.

1.3 Alternative protein sources: microbial ingredients

The idea of using yeast as a protein source for humans and animals arose in the early 1900s due to low supplies of food during the first World War. Yeast was a cheap protein source obtained from the byproducts of the fermentation industry (Osborne et al., 1919). The concept of "single-cell protein", currently termed "microbial protein", regained interest in the 1970s. According to Walker (1976), the novelty of microbial protein lies in the development of processes to upscale its production and use in animal diets, an issue which is still faced at present. In recent years, the technology to produce sugars from woody biomass has evolved to allow the development of alternative protein sources based on natural renewable resources, such as lignocellulosic biomass. *Cyberlindnera jadinii*, previously classified as *Candida utilis* (torula yeast), can now be obtained by utilizing second-generation sugars derived from Norway spruce trees (*Picea abies*) as a main growth medium. The process of growing *C. jadinii* on lignocellulosic biomass was described by Sharma et al. (2018a) and Øverland and Skrede (2017).

Dried yeasts are generally available in the market as feed additives, however, they are not traditionally used as a protein source in pig or poultry feed. *Cyberlindnera jadinii* has a similar CP content and essential AA composition compared to conventional protein sources and is thus a potential alternative protein source in feed for monogastric animals. Additionally, *C. jadinii* has shown to have beneficial health effects when fed to Atlantic salmon (Grammes et al., 2013; Sahlmann and Djordjevic et al., 2019, in press). This yeast grown on lignocellulosic biomass does not directly compete with human-food consumption and is a potentially sustainable protein source that can be produced independently of arable land and weather conditions (Couture et al., 2019; Øverland and Skrede, 2017). Although *C. jadinii* has been widely used as a nutritional supplement for farm animals (Nasseri et. al, 2011) little is known about the effects of using *C. jadinii* as one of the main protein sources in diets for pigs and broiler chickens.

The composition of yeast varies depending on strain and fermentation conditions including availability of nitrogen, minerals and other nutrients in growth media (Nasseri et al. 2011). The CP content in yeasts can vary between 30 and 70 % (Nasseri et al., 2011; Øverland and Skrede, 2017). The availability of protein differs among yeasts and can be increased by certain drying methods (Spark et al., 2005). Dried inactivated *C. jadinii* used in our experiments had a similar CP and AA content as SBM (Table 2). The nucleic acid content of microbial ingredients such as yeast and bacterial protein meal is generally higher compared with traditional protein sources. Feed ingredients are not commonly analyzed for nucleotide concentrations, however some studies suggest that SBM, FM, whey and other protein sources contain lower nucleic acid levels (< 0.1 %) than yeast (Mateo et al., 2004; Mateo and Stein, 2004b). Yeast cells are surrounded by a cell wall (Figure 3), which constitutes 15 to 30 % of the dried cell (Nguyen et al., 1998).
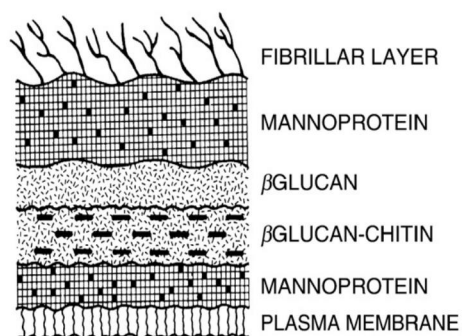
Figure 3: Composition and structure of the yeast cell-wall (Kogan and Kocher, 2007). Reprinted from "Role of yeast cell wall polysaccharides in pig nutrition and health protection. Livestock Science 109 (2007) 161-165" with permission from Elsevier. Copyright © 2007 Elsevier B.V. All rights reserved.

Yeast cell walls are composed of 75 % polysaccharides (dry weight) out of which, 50 to 60 % are *β*-glucans and 40 % mannooligosaccharides linked to proteins (Kogan and Kocher, 2007; Orlean, 1997). In addition, chitin comprises 1 to 2 % of yeast cells (Orlean, 1997).

Cyberlindnera jadinii has a generally favorable AA content when compared to SBM, FM, and RSM (Table 2). The lysine content in *C. jadinii* is considerably high, which is important in pig

and broiler chicken diets because lysine is one of the main growth-limiting AA (NRC, 2012). The tryptophan content in *C. jadinii* is higher than that of fish meal (Table 2). Additionally, threonine and valine contents in *C. jadinii* are higher than in conventional protein sources. However, the content of methionine, and methionine plus cysteine in *C. jadinii* are lower compared to SBM, FM, and RSM, which may cause growth limitations (NRC, 1994) when using *C. jadinii* as one of the main protein sources in diets for broiler chickens. The AA composition of *C. jadinii* and traditional protein sources are compared in Table 2. The content of nucleic acids in *C. jadinii* is approximately 7 % on the dry-matter basis (Maul et al., 1970) and generally, in yeasts, the concentration of nucleotides varies between 3 and 12 % (Bacha et al., 2013; Halász and Lásztity, 1991). Others reported a nucleic acid content of 6 to 8 % (Zee and Simard, 1975), 7.5 to 9.0 % in *C. jadinii* and *C. intermedia* (Castro et al., 1971) and 2.9 to 8.7 % in "food-grade" yeast (Edozien et al., 1970). The nucleic acid composition may vary depending on the temperature that the microbial cells are exposed to (Maul et al., 1970), fermentation conditions, and growth rate of cells.

Table 2 Amino acid (AA) content in traditional protein sources for monogastric animals and AA content of *Cyberlindnera jadinii* grown on lignocellulosic-biomass sugars, used in the experiments.[1]

g/16g N	Traditional protein sources				Alternative protein sources			
	SBM1[a]	FM[b]	RSM[c]	*C. jadinii*[d]	KM[e]	SC[f]	BPM[g]	Insect meal[h]
Arg	7.4	5.9	6.1	5.1	3.9	4.0	6.2	2.8
His	2.7	2.6	2.8	1.8	1.6	1.7	2.3	1.7
Ile	4.6	4.2	3.9	4.5	3.7	3.3	4.5	2.2
Leu	7.7	7.3	7.0	6.6	5.7	5.2	7.5	3.2
Lys	6.2	7.6	5.5	6.4	6.1	6.0	5.8	3.6
Met	1.4	2.8	2.0	1.1	1.4	1.2	2.7	1.0
Phe	5.1	3.9	4.1	3.8	3.5	3.2	4.2	1.9
Thr	3.9	4.2	4.4	5.4	4.2	4.1	4.4	1.9
Val	4.8	4.9	5.1	5.4	3.8	3.9	5.8	2.8
Trp	1.3	1.1	1.3	1.3	0.9	1.1	-	-
Total AA	99.1	92.0	92.4	86.3	-	-	-	-
CP[i]	458	711	388	478	511	460	692	497
Ratios AA/Lys								
Met/Lys	0.23	0.37	0.36	0.17	0.23	0.19	0.47	0.28
Thr/Lys	0.63	0.55	0.80	0.84	0.69	0.68	0.76	0.52
Try/Lys	0.21	0.14	0.24	0.20	0.15	0.18	-	-
Cys/Lys	0.24	0.12	0.45	0.13	-	-	-	-
Arg/Lys	1.19	0.78	1.11	0.80	0.65	0.66	1.07	0.78
Glu/Lys	2.92	1.71	3.07	2.19	-	-	-	-
Met + Cys/Lys	0.47	0.49	0.82	0.30	-	-	-	-
Val/Lys	0.77	0.64	0.93	0.84	0.62	0.65	1.00	0.79
Leu/Lys	1.24	0.96	1.27	1.03	0.94	0.87	1.29	0.88
Ile/Lys	0.74	0.55	0.71	0.70	0.60	0.55	0.78	0.62
His/Lys	0.44	0.34	0.51	0.28	0.26	0.29	0.40	0.47
Ala/Lys	0.71	0.83	0.82	0.92	-	-	-	-

[1]AA, amino acids g/16g N, on crude protein (CP) basis, as is;

[a]SBM soybean meal, CP > 440 g/kg, Centraal Veevoederbureau (CVB), 2005;

[b]FM fish meal (CVB, 2005);

[c]RSM rapeseed meal (CVB, 2005);

[d]*Cyberlindnera jadinii* grown on lignocellulosic sugars from Norway spruce;

[e]KM *Kluyveromyces marxianus,* dry matter (DM) 939 g/kg (Øverland et al., 2013);

[f]SC *Saccharomyces cerevisiae,* DM 968 g/kg (Øverland et al., 2013);

[g]Bacterial protein meal, > 80 % *Methylococcus capsulatus,* (Øverland et al., 2001);

[h]*Tenebrio molitor* (larvae), DM 948 g/kg (De Marco et al., 2015);

[i]Kjeldahl-Nitrogen N × 6.25, g/kg, as is.

The CP content of *C. jadinii* is correlated to the content of RNA in the cell and RNA content can be calculated as described by Brown and Rose (1969; Figure 4). The content of RNA in *C. jadinii* used in our experiments can, therefore, be estimated to approximately 96 g/kg RNA, which is similar to the nucleic acid content in the bacterial meal (95 g/kg) used by Skrede et al. (1998). Approximately 20 % of the nitrogen in yeast is contained in nucleotides (Reed and Nagodawithana, 1991; Shurson, 2018). In the case of the bacterial meal, nucleotides account for approximately 12 % of the nitrogen (Hellwing et al., 2007a).
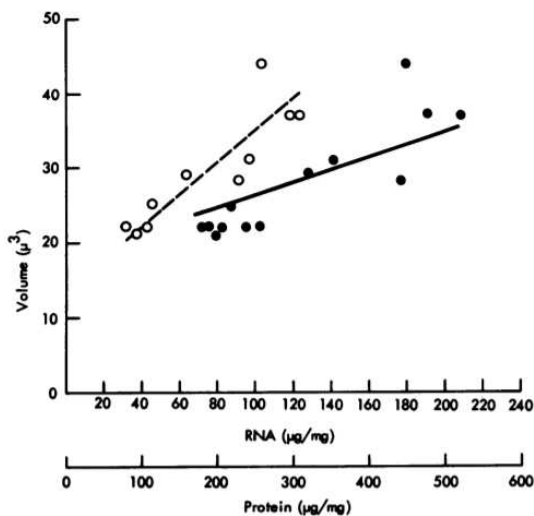
Figure 4 Relationship between RNA content (°), protein content (●) and cell volume of *C. jadinii* grown on media with ammonium sulfate and glucose. Cell volume = 0.218 RNA + 13.2; (r = 0.1); cell volume = 0.033 protein + 18.4; (r = 0.73). Republished from "Effects of Temperature on Composition and Cell Volume of *Candida utilis*. Brown and Rose (1969). Journal of Bacteriology, Jan 1969. p. 261-272" with permission from ASM. *Copyright © 1969, American Society for Microbiology.*

Several other potentially sustainable protein sources were previously proposed as alternatives to the traditional protein sources in pig and broiler chicken diets. Namely, insects and microorganisms, *e.g.* bacteria and yeast, grown on sustainable resources that are not meant for human consumption, such as macroalgae, byproducts of agriculture, aquaculture, and food industry.

Bacterial protein meal (*Methyloccocus capsulatus*, *Alcaligenes acidovorans*, *Bacillus brevis,* and others) grown on natural gas was previously investigated as an alternative protein source in pig and broiler chicken diets (Øverland et al., 2010). Bacteria, in similarity to yeasts, have

rapid growth rates, high protein content (Roth, 1980, and Stringer 1982, in Skrede, 1998) and the ability to grow on substrates that do not directly compete with human food sources, such as natural gas (D' Mello 1972, 1973, in Skrede, 1998). Bacterial protein meal contains 70.2 % CP and 9.5 % nucleic acids, and has a similar AA composition compared to fish meal, although it is lower in lysine and higher in tryptophan (Skrede et al., 1998). Additionally, BPM has a similar amino acid composition when compared with SBM (Øverland et al., 2001). Previous studies in pigs and broiler chickens have shown that the ATTD of nitrogen is 85.4 % for BPM in pigs (AID = 78.1 %) and 80.5 % in broiler chickens (Skrede et al., 1998). Bacterial meal can replace up to 20 and 50 % of the protein in traditional diets - with soybean and fish meal as protein sources - for chickens and pigs respectively (Hellwing et al., 2007a; Hellwing et al., 2007b) without interfering with the efficiency of protein and energy metabolism. Bacterial meal can also replace all the lysine provided by SBM in pig diets without reducing growth performance (Øverland et al., 2001).

Insects, similarly to microbial ingredients, do not require a large area of production, have a high reproduction rate, and are independent of environmental conditions. Their ability to grow on organic material not suitable for human consumption has considerable potential to reduce food waste and decrease costs associated with recycling. They have a higher feed conversion ratio and breeding capacity than other livestock (Nakagaki and Defoliart, 1991) and contribute less to greenhouse-gas emissions (Oonincx et al., 2010). For the same reasons, the black soldier fly (*Hermetia illucens* L.) and the yellow mealworm (*Tenebrio molitor*) have been recently investigated as protein sources in broiler chicken diets. Dietary larval meal of black soldier flies has shown potential to improve BW gain and feed intake of broiler chickens during the starter period and provided efficient nutrient digestion, without interfering with the birds' health (Dabbou et al., 2018; Schiavone et al., 2017b). Their nutrient composition is favorable for livestock animal diets, containing high CP (55.3 %, on DM basis; Renna et al., 2017) and

essential AA (De Marco et al., 2015), and are also a good sources of saturated fatty acids (32 % fat; Schiavone et al., 2017a). Other insects such as *Acridis* (grasshoppers) have higher CP content than SBM and FM (Anand et al., 2008). The average protein content in insects varies from 50 to 82 % in dry weight (Schabel, 2008) being higher than 60 % in larvae (Rumpold and Schlüter, 2013), and the fat content varies from 10 to 30 %, as is (DeFoliart, 1991). Dried housefly larvae (*Musca domestica*) have high CP, in the order of 64 %, high protein digestibility and a favorable AA profile (Hwangbo et al., 2009) compared to conventional protein sources. Insects are also rich in K, Fe, Mg and Se (Finke, 2002), and have higher content in Fe and Ca than beef, pork and poultry meat (Sirimungkararat et al., 2010). The chitin content in insects is approximately 9 % (Sánchez-Muros et al., 2014; Wang et al., 2004), up to nine times higher than in yeast. Chitin may however, act as an anti-nutritional factor in monogastric diets, reducing the digestibility of CP, fat (Kobayashi et al., 2002; Schiavone et al., 2017b) and DM (Khempaka et al., 2006), also reducing nutrient absorption (Razdan and Pettersson, 1994; Schiavone et al., 2017b). Altogether, insects are considered a potentially sustainable alternative ingredient (reviewed by Sánchez-Muros et al., 2014), however, only a few studies are available on their use as animal feedstuffs. Dried housefly pupae grown on poultry manure can completely replace SBM in diets for broiler chickens regarding BW gain and feed conversion ratio (Calvert, 1979). Larvae *Cirina forda* has replaced FM in broiler chicken diets without compromising body weight gain (Oyegoke et al., 2006). The nutrient profile of insects is highly dependent on the composition of the insect-feed (Ramos-Elorduy et al., 2002), similar to how the composition of microbial ingredients is dependent on the nutritional quality of the growth substrate (Martínez-Force and Benitez, 1995; Nasseri et al., 2011; Rodríguez et al., 2011). In addition, the production of insects in Europe, is currently limited by the level of human labor required, the need for upscaling due to low yields, and a lack of standardized measures to ensure product safety and quality (Rumpold and Schlüter, 2013), and consequently, regulatory

constraints, which altogether, still give this novel ingredient little attractiveness compared to traditional protein sources. Some plant protein sources, such as duckweed and leaf protein concentrate have also been proposed as potential protein sources for animal feed (Tallentire et al., 2018).

To cultivate and use *C. jadinii* as a protein source at competitive prices, the utilization of glucose must be replaced by less expensive alternatives such as sustainable local-based sugars obtained from, for example, lignocellulosic biomass and microalgae. Microbial ingredients, like yeast and bacteria, can be produced by utilizing substrates other than lignocellulosic biomass, such as sugars derived from brown macroalgae (Sharma et al., 2018a), hydrolysates from meat and fish industries (Lapeña et al., 2018), and by-products of the agricultural or food industries (Bekatorou et al., 2006).

Brown macroalgae *Saccharina latissima* and *Laminaria* spp. cultivated in Norway have recently been proposed as a sugar source for the production of microbial protein. Although other types of brown macroalgae could serve as potential sugar sources for the production of microbial protein, they are not presently relevant in Europe, and thus not discussed in this thesis. Dried *S. latissima* can contain up to 53 % sugars, up to 24 % total AA and up to 41 % minerals (Sharma et al., 2018b), which are favorable for the growth media of yeast as a source of microbial protein. Their nutritional composition varies seasonally, with light exposure, cultivation depth, and available nutrients (Hånda et al, 2013, Marinho et al., 2015, and Schiener et al., 2015, reviewed by Sharma, 2018). Generally, fresh macroalgae contain high levels of moisture, which considerably increases production energy costs and greenhouse gas emissions, associated with drying processes (Tallentire et al., 2018). Furthermore, macroalgae may contain variable levels of iodine (Sharma et al., 2018b) and heavy metals (Pongratz and Heumann, 1998), which can be potentially toxic for monogastric animals. Dry lignocellulosic biomass, on the other hand, contains 50 to 70 % sugars in the form of cellulose and hemicellulose, 3.8 %

minerals, and a more stable chemical composition compared with macroalgae (Sharma, 2018), but includes a low content of nitrogen, phosphorus, and minerals which are important for the growth media of microbial protein (Sharma, 2018).

In addition to sugars derived from lignocellulose and macroalgae, *C. jadinii* can grow on a variety of low-cost byproducts from food and agricultural industries (Bekatorou et al., 2006), such as salad oil manufacturing wastewater (Zheng et al., 2005), defatted rice polishing (Rajoka et al., 2004), waste of Chinese cabbage (Choi and Park, 2003), apple pomace (Villas-Bôas et al., 2003), pineapple cannery effluent (Nigam, 1998), deproteinized leaf juices of turnip, mustard and cauliflower, molasses, sugar beet pulp and artichoke (Chanda and Chakrabarti, 1996) and from meat industry byproducts such as hydrolysates of beef, pork chicken and salmon (Lapeña et al., 2018).

1.4. Regulatory constraints and limitations

Cyberlindnera jadinii yeast is approved in the European Union for use in compound feed for farm animals (Regulation (EC) No 767/2009; Commission Regulation (EU) No. 575/2011, 12.1.5, yeast and like products) and classified as generally recognized as safe (GRAS) by the United States food and drug administration (FDA). Genetically modified yeasts (brewers and baker's yeast) have become more efficient in substrate conversion (Bekatorou et al., 2006), however, the European regulations concerning the use of genetically modified organisms in animal production are restricting. In the special case of the Norwegian livestock industry, the feed regulations do not allow the use of feed ingredients comprising of more than 0.9 % genetically-modified-organisms. Insect meal is currently not allowed in diets for pigs or broiler chickens under European Union laws (European Commission, 2017) because insects are categorized as "farm animals", and products falling under this category are not presently allowed in diets for these animal species. Additionally, the production of insects and other

alternative protein sources is currently costly and the upscaling must be further improved to compete with existing protein sources (Tallentire et al., 2018). Finally, nitrogen sources used in the growth media of microbial protein must abide by the applicable EU legislation, regarding feed hygiene and quality, and ensure the prevention and control of prionic diseases (Regulation (EC) No. 999/2001).

1.5. Beneficial effects of yeast on animal performance and intestinal function

In the pig and poultry production sectors, several factors may cause changes in growth performance, which in turn may cause economic losses, especially during the starter and grower phases. Yeast can be used in functional feeds to reduce stress factors and help to improve digestive function and growth performance in pigs. As an additive in diets for pigs and broiler chickens, yeast has shown to improve growth rate, feed conversion ratio (Aldabagh and Shareef, 2009; Shen et al., 2009), and gastrointestinal health (Chaucheyras-Durand and Durand, 2010; Line et al., 1998; Shen et al., 2009). Therefore, yeast has become an interesting alternative to the use of growth-promotors and antibiotic additives in the feed (Shurson, 2018). Yeasts can modulate the microbial balance in the gut by binding to pathogenic bacteria, such as *Escherichia coli* (Ewing and Cole, 1994), and reduce enteral colonization by *Salmonella* (Line et al., 1998; Price et al., 2010) and coliform bacteria (White et al., 2002). Yeast-cell-wall components *i. e.* mannan and *β*-glucan can also adsorb mycotoxins in the intestinal tract (Shetty and Jespersen, 2006) reducing their negative effects on enterocyte viability (Alassane-Kpembi et al., 2013). Additionally, cell-wall *β*-glucans from yeast (*Saccharomyces cerevisiae*) produce immune-modulating effects on the gut mucosa by stimulating receptors in monocytes, macrophages, and granulocytes (Kogan and Kocher, 2007), and increase serum IgG (White et al., 2002).

At weaning, piglet growth performance may decline, due to reduced feed intake and challenge to the immune system, resulting in an increased risk of diarrhea during this period. Altogether, these factors contribute to a reduced supply of energy and nucleotides (Sijben et al., 1998), reducing the available energy and protein, necessary for growth. The nucleic acids in microbial ingredients such as yeast can help animals cope with high demands for nucleotides, especially during fast-growing life stages. Young animals *i.e.* weaned piglets, are also especially vulnerable to enteric epithelial damage, requiring fast enterocyte renewal. By including microbial ingredients at the expense of traditional ingredients, especially SBM, in monogastric diets, nitrogen digestibility and utilization may be improved (Spark et al., 2005; Tegbe and Zimmerman, 1977), but few studies are available regarding nucleic acid and nitrogen metabolism in monogastric animal diets with alternative protein sources such as yeast and bacterial meal. Dietary yeast-cell walls at low levels have been associated with improvement in villus height in broiler chicks (Zhang et al., 2005). Increased villus height can be correlated with improved FCR in piglets fed *C. jadinii*-containing diets (Cruz and Håkenåsen et al., 2019), indicating that yeast may have a role in improving nutrient absorption. In addition, live *C. jadinii* contains enzymes such as urease, uricase, and lignocellulolytic enzymes, that could aid the digestion of carbohydrates (Øverland and Skrede, 2017; White, 1956).

1.6. Metabolism of nitrogenous compounds in monogastric animals
1.6.1. Metabolism of protein

The nitrogen digestibility of microbial ingredients depends on the digestibility of the protein, nucleic acids and other non-protein constituents (Skrede et al., 1998). Protein digestion and absorption processes are thought to be similar in pigs and chickens. However, they have different physiological growth rates and different nutrient requirements, and therefore, the digestibility of the protein and AA in the same protein sources may vary among species due to

differences in anatomy, enzyme activity and expression of nutrients transporters. Protein utilization in pigs and broiler chickens is dependent on digestible essential AA reaching the small intestine (Boisen et al., 2000). The levels of CP and essential AA, together with their digestibility, define the protein quality of the ingredients or feed. To evaluate protein quality in feed ingredients and diets for pigs and poultry several digestibility methods exist. Only apparent ileal and total tract digestibility, which is relevant for this thesis, is described in the methodology section. The digestibility of protein in diets for pigs and chickens varies with feedstuffs (Table 3). The diets in the present studies were designed considering that the main limiting AA are digestible lysine for pigs and methionine plus cysteine for broiler chickens. The digestibility of lysine and methionine (AID) in dried brewers' yeast (*S. cerevisiae*) is 87 and 80 % respectively for pigs, and 86 and 79 % (ATTD) respectively for broiler chickens (Centraal Veevoederbureau, 2011, 2005).

Table 3: Crude protein digestibility of selected feed ingredients for monogastric animals.

Ingredient		Pig	Broiler chicken
	CP (g/kg)	AID (%)	ATTD (%)
Wheat	112	80	81
Soybean meal	468	85	87
Rapeseed meal	388	70	76
Fish meal	640	83	88
Yeast (dried *S. cerevisiae*)	468	83	83

Sources: Centraal Veevoederbureau (2016, 2011).

Nitrogen is supplied by the diet in the form of peptides, AA or nucleic acids. The digestion of peptides and nucleic acids begin in the stomach by the action of HCl and pepsin, but the major

steps occur in the small intestine (see also section 1.6.2.). Peptides are cleaved by pancreatic proteases such as chymotrypsin and trypsin, and they are absorbed as AA or peptides in the small intestine through transporters located on the intestinal-villi surface, that are dependent on sodium, hydrogen, and ATP (Krehbiel and Matthews, 2003). The absorbed peptides and free-AA participate in several metabolic pathways, *e.g.* transamination to create new AA, which are essential constituents of muscle tissue and bioactive molecules. Although protein retention is normally prioritized, AA can be metabolized into ATP or glucose *i.e.* gluconeogenesis, which are sources of energy. Ammonia resulting from these processes enters the urea cycle and is excreted as urinary urea in pigs (Michal, 1999). Birds, on the other hand, excrete nitrogenous compounds in the form of uric acid, which is produced through the uric acid cycle (Salway, 2018).

1.6.2. Metabolism of nucleic acids

Among the nucleic acids, purine bases (adenine and guanine) have particular interest regarding alternative feedstuffs such as microbial ingredients, because the content of purine bases is higher in microbial ingredients than in conventional feed ingredients (Mateo and Stein, 2004) and also because purine bases are involved in the metabolic pathways of nitrogen excretion (Michal, 1999). Furthermore, excretion of urinary purine-base derivatives has been shown to increase with increasing levels of microbial protein in pig diets (Hellwing et al., 2007b). The composition of the feed ingredients in terms of nucleic acids was not evaluated in this thesis, but others have reported that the nucleic acid content in yeast and BPM is approximately 3 to 12 % (Bacha et al., 2013; Halász and Lásztity, 1991) and 10 % (Hellwing, 2005), respectively. When used as a protein source, the ribonucleic acid (RNA) in yeast cells can provide nucleotides that influence piglet and broiler chicken growth and intestinal health. Purine and pyrimidine bases are building blocks of nucleotides, RNA, deoxyribonucleic acid (DNA) and adenosine 5'-triphosphate, and intervene in protein synthesis and cell division (Sijben et al.,

1998). The RNA and DNA are originated from dietary, intracellular degradation of purine and pyrimidine bases or from the biosynthesis of AA that are used for re-synthesizing of nucleotides (Sijben et al., 1998).

Nucleic acids, in similarity to protein, are cleaved by pepsin in the stomach but, they are majorly digested in the small intestine by the action of pancreatic endonucleases such as deoxyribonuclease and ribonuclease, and phosphodiesterase and nucleoside phosphorylases, into oligonucleotides, nucleosides and free bases (Liu et al., 2015). Dietary RNA and DNA can be absorbed as nucleosides, free purine or pyrimidine bases to the enterocytes (Wilson and Wilson, 1958, 1962). These nucleotides are absorbed through permease proteins by facilitated diffusion (Michal, 1999). In eukaryotic cells, adenine and guanine are converted to xanthine and hypoxanthine and can follow one of two pathways (Michal, 1999): 1) the salvage pathway by action of the enzyme hypoxanthine ribosyl transferase coupled to PRPP; purine bases are broken down and reconverted into mononucleotides, which can be reabsorbed and used for forming new nucleotides. 2) the excretion pathway by the action of the enzyme xanthine oxidase; this leads to the formation of nitrogenous end-products, *i.e.* uric acid and allantoin (Figure 5). When the salvage pathway is inhibited, *e.g.* the genetic lack of the enzyme hypoxanthine ribosyl transferase, this leads to the accumulation of blood uric acid in humans (gout; Michal, 1999).

The end products of the metabolism of dietary nucleic acids differ in pigs and chickens. Allantoin is produced by the action of the enzyme uriate oxidase in the pig, and uric acid produced by the action of the enzyme xanthine oxidase in the chicken (Figure 5), whereas pyrimidine bases are decomposed to metabolites which enter the citric acid cycle or fat metabolism (Michal, 1999). Urate oxidase is not present in birds, but allantoin may be found in the bloodstream of birds as a result of non-enzymatic oxidation of uric acid, resulting from non-enzymatic oxidations *i.e.* during oxidative stress (Simoyi et al., 2003; Tsahar et al., 2006).

Dietary nucleic acids are important for rapidly dividing tissues such as the intestine, and for the recovery of gut tissues after weaning. In situations of increased demand (after tissue injury or during rapid growth), the capacity for synthesis is limited, thus, dietary nucleotides are considered semi-essential nutrients. Furthermore, the uptake of dietary purines and pyrimidines is energetically more efficient compared with their biosynthesis (Sijben et al., 1998).
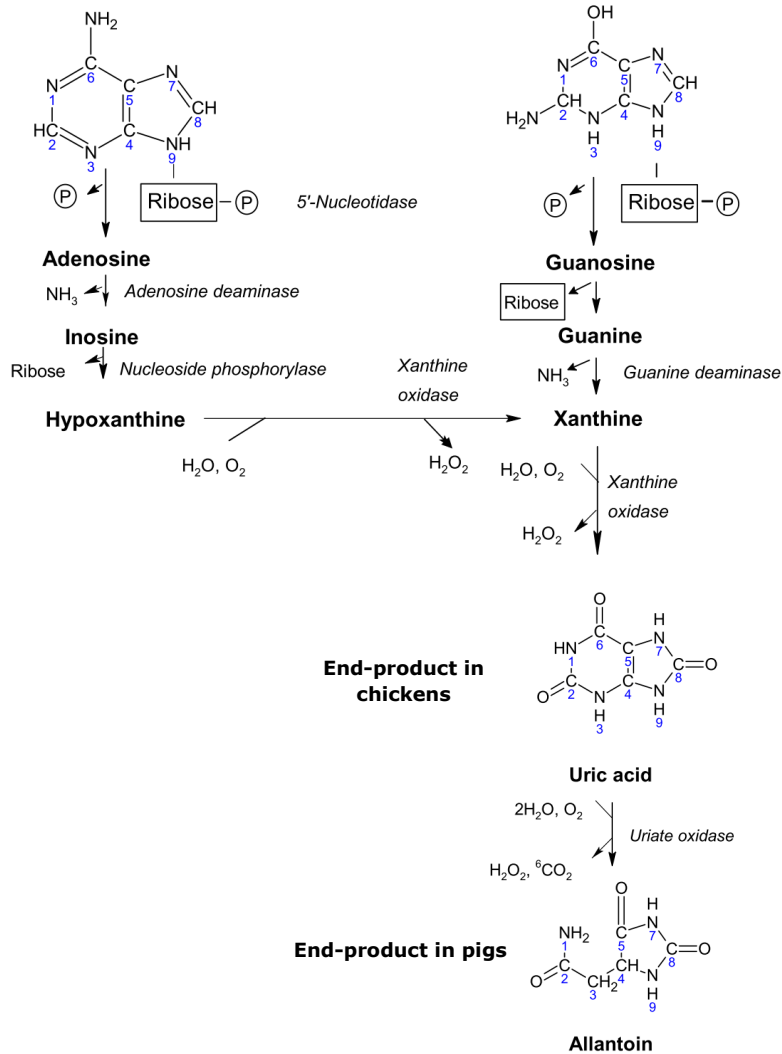
Figure 5: Production of allantoin and uric acid from purine bases. Modified from Greife (1984) and Michal (1999), in Hellwing (2005).

2. Aims of the thesis

2.1. Main objectives

The main aim of this thesis was to investigate dried inactivated *C. jadinii* yeast produced by advanced technology that utilizes sugars from lignocellulosic biomass as growth media, as a renewable and local-based protein source, in diets for young pigs and broiler chickens. The aims derived from an initial concept: to create opportunities to increase national self-sufficiency, and reduce the dependence on imported protein ingredients in animal feed production.

2.2. Sub-objectives

To clarify the main aim, the following sub-objectives were established:

A) Characterize *C. jadinii* yeast grown on lignocellulosic-derived sugars as a feed ingredient in terms of chemical composition and nutritional value;

B) Study the effect of feeding increasing levels of *C. jadinii* on the nitrogen and energy utilization in young pigs;

C) Evaluate the effect of *C. jadinii* on growth performance and digestive function in young pigs and broiler chickens;

2.3. Research questions

To determine the effects of dried inactivated *C. jadinii* yeast as a protein source in monogastric animal diets, three animal experiments were carried out, having as basis the following research questions:

- Can *C. jadinii* serve as a high-quality protein ingredient in pig and broiler chicken diets, and partially replace conventional protein-rich feed ingredients such as soybean meal, fish meal, and rapeseed meal on a protein basis, while obtaining similar growth performance?

- Is the protein and energy utilization affected when *C. jadinii* incrementally replaces conventional protein-sources in pig diets?

- Can dietary *C. jadinii* improve the digestive function and general intestinal health of young pigs and broiler chicks compared to conventional protein-rich ingredients?

3. Methodology
3.1. Growth performance and digestive function in piglets (Paper I)

Paper I aimed to evaluate the effects of *C. jadinii* as a protein source in diets for weaned piglets, on growth performance and digestive function. *C. jadinii* was produced on second generation C5 and C6 sugars obtained from lignocellulosic biomass of Norway spruce trees (*Picea abies*) by using the Borregaard advanced lignin process. The sugars were used in the growth media for fermentation of *C. jadinii*, as described by Øverland and Skrede (2017) and Sharma et al. (2018a). Forty-eight piglets weaned at 30 days of age, with a mean initial BW of 11.06 ± 0.84 kg were provided one of four dietary treatments: a conventional control diet with soybean meal, fish meal, rapeseed meal, and potato protein concentrate or one of three experimental diets containing 10, 20 or 40 % CP from yeast (CU10, CU20, and CU40, respectively). The diets were formulated to be isonitrogenous and isoenergetic based on the chemical content of the ingredients. Yttrium oxide (Y_2O_3; 0.01 %) was used as an inert marker. Piglets were equally distributed according to litter, gender, and BW and randomly allotted to the dietary treatments, with 12 replicates per treatment. Each pig was fed individually, thus, each pig was an experimental unit. Rubber mats were used as bedding material, to minimize interferences with nutrient digestibility. Diets containing Y_2O_3 were provided from d 18. Individual feed leftovers were collected and recorded to calculate feed intake and individual live BW was recorded to calculate ADG and FCR. Fecal score and fecal DM were used to access the occurrence of diarrhea. Apparent total tract (fecal) digestibility of nutrients (ATTD) and apparent ileal digestibility of nutrients (AID) were determined by analyzing the nutritional content of feces and ileal digesta respectively, and the content of marker in diets and biological samples. Gene expression of nutrient transporters and trypsin activity were quantified from ileal samples. Intestinal villi-height (VH) crypt-depth (CD) were measured to assess the effects of *C. jadinii* in the intestinal morphometry and absorptive ability. Piglet health was generally assessed with

histopathological analysis. Diet production, sample collection, sample preparation, and analytical procedures are described in paper I.

3.2. Protein and energy metabolism in young pigs (Paper II)

Paper II aimed to evaluate the metabolism of protein and energy and substrate oxidation in young pigs fed diets with the same type of *C. jadinii* and dietary treatments as in Paper I. Twenty-four intact boars with a mean initial BW of 16.69 ± 4.45 kg were fed one of the four diets (control, CU10, CU20, CU40), with two pigs per diet during three consecutive periods, comprising a total of six replicates per diet. Each pig was fed and weighed individually and thus each pig constituted an experimental unit. Each set of pigs per period comprised of two sets of four litter-mates and each litter-mate was allotted to one of the dietary treatments according to initial BW. The three periods were divided into a seven-day period of adaptation in stables followed by a three-day adaptation period in balance cages. The balance period lasted for four days and it included an energy and nitrogen balance experiment of four consecutive days including a respiration experiment of 22 h. BW was measured individually at four time-points: at the start of the adaptation period (d 1), at the end of the adaptation period (d 7), at the start of the balance period (d 11) and at the end of the balance period (d 14). Pigs were fed *ad libitum* twice daily during 30-min periods, based on estimated feed intake of 2 to 4 % BW, and water was provided *ad libitum*. Uneaten feed was collected and recorded individually to calculate average daily feed intake. The ADG and FCR were calculated from the BW and the average daily feed intake. During the balance period and respiration experiments, the pigs were kept individually, in cages with devices for quantitative collection of feces, urine and feed residues. The ATTD of nutrients was determined using the total collection method during the balance period. Individual respiration measurements were performed in open-air-circuit respiration chambers, as described by Chwalibog et al. (2004). Airflow, O_2, CO_2, and CH_4 concentrations

in the out-going air from each chamber were recorded and gas recovery tests were performed for CO_2 and O_2. Diet production, sample collection, sample processing, and analytical procedures are described in Paper II.

3.3. Growth performance and digestive function in broiler chickens (Paper III)

In Paper III, the effects of dietary *C. jadinii* on the growth performance and digestive function of broiler chicks were evaluated. The same strain of *C. jadinii* used in Papers I and II was used in the experimental diets. The diets were formulated and optimized to meet or exceed the requirements for essential AA and other nutrients (NRC, 1994). Diets were formulated to be isoenergetic, isonitrogenous and free from coccidiostats. The dietary treatments consisted of four starter (S) diets and four grower (G) diets including a control based on wheat, oats, maize, and SBM and three diets with 10, 20 and 30 % CP from *C. jadinii* (CJ10, CJ20 and CJ30). Starter and grower diets were pelleted at 2.5 and 3.5 mm diameter respectively. Each diet was added 0.06 % TiO_2. One thousand broiler chicks (42.0 ± 0.75 g initial BW) were allocated to the dietary treatments and equally distributed to 20 pens with 50 birds each, comprising five replicates per diet. Starter diets were provided from d 1 to 10 and grower diets were provided from d 11 to 30. Birds had *ad libitum* access to feed and water. Litter quality of each pen was assessed weekly. Feed intake and live BW were registered for each pen weekly. Intestinal tissue samples were collected at d 10 for the measurement of VH and CD similarly to Paper I and II. Samples of ileal content were collected at d 28 for determination of AID of nutrients. Diet production, sample collection, sample processing, analytical procedures and calculations were described in Paper III.

4. Main results and discussion

This section combines and discusses the main results of Papers I - III with respect to the effects of dried-inactivated *C. jadinii* as a protein source in pig and broiler chicken diets, on the growth performance, digestive function, energy metabolism, nitrogen metabolism, and intestinal morphometry.

4.1. Inclusion of *C. jadinii* in diets for young pigs and broiler chickens

The diets used in the three experiments were formulated to simulate pig and broiler chicken diets currently used in commercial settings in Northern Europe, so that the results could be extrapolated to practical conditions. The results of the chemical analyses of the diets generally concurred with the calculated nutritional composition, indicating that the nutritional content in diets was correctly predicted (Tables 1, 2; Papers I, II, III), with the exception of a slightly lower lysine and arginine content in the broiler chicken diets with 30 % CP from *C. jadinii*. The chemical composition of the main ingredients and *C. jadinii* is presented in Table 1, Paper I. *Cyberlindnera jadinii* in these experiments had lower CP than previously reported, but CP was higher than *S. cerevisiae* and *K. marxianus* (Øverland et al., 2013), however, the contents of lysine and arginine in *C. jadinii* used in the present studies were higher than reported by Øverland et al., 2013. Among emerging alternative protein-rich ingredients, yeast had higher N content than microalgae and insect meal but lower N content than BPM (Tallentire et al., 2018).

Crude fat and neutral detergent fiber were lower in the *C. jadinii*-containing diets compared with the control diets (Papers I, III), due to the higher content of fiber and fat in traditional protein sources than in *C. jadinii* (Table 1, Paper I). Total phosphorus in *C. jadinii* was 0.943 g/kg, which was lower than in spray-dried *C. jadinii* (3.7 g/kg P; Sharma et al., 2018a) and in dried *S. cerevisiae* (11.6 g/kg P; Sauvant et al., 2004). Pellet durability index increased with increasing levels of *C. jadinii* in the diets and with pellet temperature in the broiler chicken

diets (Paper III). The increase in pellet temperature may be due to the smaller particle size of dried *C. jadinii* than conventional protein-rich ingredients, leading to increased friction in the pellet press. Conditioning temperature is known to increase pellet hardness incrementally (Abdollahi et al., 2010) and could relate to the linear decrease in feed intake (Paper III), however, the effect of pellet hardness on feed intake was not investigated. High temperatures during feed production can lead to changes in protein structure, promote Maillard reactions and heat damage, and alter the digestibility of CP, AA, starch, and energy of the diets (Abdollahi et al., 2010; Pahm et al., 2008). This may explain the linearly decreased digestibility of nutrients in the broiler chicken diets. Furthermore, heat treatment may affect the nutritional quality of lysine, cysteine, and arginine (Ljøkjel et al., 2000), which could explain the reduced growth performance of broiler chicks (Paper III). In the pig diets, however, pellet temperature did not seem to increase with the inclusion of *C. jadinii*.

Replacing conventional protein sources with *C. jadinii* grown on lignocellulosic sugars in monogastric diets has not been reported earlier and is highly relevant to improve the security and sustainability of feed protein ingredients in Northern European countries. This concept was based on previous studies with dried yeast in aquaculture diets (Øverland et al., 2013; Øverland and Skrede, 2017), and the dietary levels were chosen based on previous studies with microbial ingredients as dietary sources of protein for pigs and broiler chickens. *Cyberlindnera jadinii* used in the present experiments, originated from an experimental batch, grown on a media with sugars mainly from hydrolyzed lignocellulosic slurry, during a fed-batch fermentation process. This fermentation process is currently under development, aiming to achieve higher levels of efficiency and yields.

4.2. Effects on growth performance and general health

The findings highlighted the importance of carefully considering different dietary levels of *C. jadinii* for each animal species. In general, the pigs had similar growth performance when fed experimental diets containing *C. jadinii* compared to the control diets and were healthy during the experiments with a few occurrences of loose stools (Papers I, II). Fecal DM in the weanling pigs (Paper I) increased with increasing levels of *C. jadinii* up to 40 % of CP while in Exp. 2, fecal DM was numerically higher in pigs fed the yeast containing-diets than in pigs fed the control diet. The increase in fecal DM in pigs may be explained by the positive effects of *C. jadinii* components such as mannooligosaccharides, *β*-glucans, and nucleic acids, on the intestinal function of piglets (Kogan and Kocher, 2007; Mateo and Stein, 2004), shown more clearly in the younger pigs (Paper I). Post-weaned piglets are especially susceptible to diarrhea and infection which may reflect on growth performance. Low levels of dietary yeast have shown to mitigate these effects by promoting the growth of lactobacillus and reducing the population of coliform bacteria in the hindgut (Kiros et al., 2019; White et al., 2002), the latter being the main cause of diarrhea and consequently reduced welfare in piglets.

Increasing levels of *C. jadinii* in diets was associated with a numerical increase in mortality and decrease in litter quality in broiler chickens, whereas the pigs in Exp. 1 and 2 remained healthy (Paper I, II). In addition, the growth performance of broiler chickens linearly decreased when 20 to 30 % CP of *C. jadinii* replaced SBM on protein basis in the diets (Paper III), which differed from the results obtained in the pig experiments (Papers I, II), where similar or improved growth performance was observed between pigs fed control and *C. jadinii* containing diets. The difference in response to dietary yeast between broiler chickens and pigs could be explained by the ability of the pigs to efficiently utilize nucleic acids in the yeast as components for cell-proliferation and restitution of intestinal tissue (Sijben et al., 1998), whereas in broiler chickens, purine nucleotides are degraded to uric acid and may accumulate in the blood (De Boeck and Stockx, 1978; Simoyi et al., 2003) possibly causing health disturbances and reduced feed intake.

Results supporting the findings in pigs (Papers I, II) were reported in studies with other microbial ingredients. Bacterial protein meal replaced 41 % of the CP n piglet diets without compromising growth performance (Øverland et al., 2010). Interestingly, whey-grown yeast replacing SBM in diets for pigs increased daily weight gain and improved FCR (Spark et al., 2005), and hydrocarbon-grown *S. cerevisiae* replacing 6 to 29 % of CP in FM-based pig diets tended to improve ADG and FCR (Barber et al., 1971).

Results supporting the reduced growth performance in broiler chickens (Paper III) were reported when SBM in broiler chicken diets was replaced with 45 % of CP from molasse-grown yeast (Daghir and Abdul-Baki, 1977), and 59 to 65 % CP from vinasse-grown *C. jadinii* (Rodriguez et al., 2013). On the other hand, broiler chickens maintained their growth performance when dietary SBM was replaced by microbial ingredients at the following levels: 15 to 30 % of CP of from molasse-grown yeast (Daghir and Abdul-Baki, 1977); up to 34 % CP from BPM (Øverland et al., 2010); 39 to 43 % CP from vinasse-grown yeast (Rodriguez et al. 2013). Interestingly, broiler chickens fed diets with 68 % CP from BPM (Schøyen et al., 2007a) and 20 to 22 % CP from distillery-vinasse *C. jadinii* (Rodriguez et al., 2013), had higher BW gain than broiler chickens fed SBM control diets. Seemingly, the effect of dietary yeast on broiler chicken growth performance depends on strain and inclusion levels. The lack of improvement in growth performance and feed intake could be explained by the favorable hygiene and husbandry conditions in Exp. 1 and 2, resulting in generally good health status, *i.e.* the pigs were not exposed to particular stress factors. In the presence of a challenge, for example infection or unfavorable hygiene conditions, effects on growth may be observed, as suggested by Owens and Mccracken (2007).

Broiler chicken diets were provided *ad libitum* (Paper III) whereas pig diets were provided "semi *ad libitum"*, or *ad libitum* during restricted time periods of 30 minutes (Paper I, II). The ratio "feed intake: BW" was approx. 35 % higher in a recent study with the same strain of *C.*

jadinii (Håkenåsen et al., unpublished), where pigs were fed *C. jadinii*-containing diets *ad libitum*. This indicates that these pigs (Paper I, II) might not have expressed their full potential for growth. Feed intake was similar among pigs but linearly decreased with increasing levels of *C. jadinii* in the broiler chickens. Results that support our findings were reported in broiler chickens fed: 1) increasing levels up to 34 % CP of BPM replacing SBM-protein (Øverland et al., 2010); 2) low levels (< 2 %) of vinasse-grown *C. jadinii* at the expense of SBM (Chand and Ullah Khan, 2014). The reduced feed intake in the birds may be explained by a higher nucleic acid content in diets with microbial ingredients (Baker and Molitoris, 1974; Hellwing et al., 2007a; Mateo et al., 2004; Mateo and Stein, 2004) compared with conventional diets. In addition, chitin in yeast-cell walls (Kwiatkowski and Edgar, 2012) may increase gastric viscosity and delay gastric emptying, leading to increased satiety in broiler chickens (Razdan and Pettersson, 1994). In contrast, some studies have reported increased feed intake in broiler chickens fed microbial ingredients as protein sources. Feed intake increased in birds fed diets where SBM was replaced with: 39 to 65 % CP from vinasse-grown *C. jadinii* (Rodriguez et al., 2013); 34 % CP from BPM (Schøyen et al., 2007a); increasing levels of BPM (Schøyen et al., 2007b). The increased feed intake in those cases could be related to increased palatability due to the microbial ingredients. *Cyberlindnera jadinii* could change the flavor of the feed, as it is precepted as sour by humans and used widely used as a flavor enhancer in food. However, birds do not possess such an acute sense of taste and pellets are rapidly swallowed (Owens, 2005; Owens and Mccracken, 2007). Feed intake of the pigs (Paper I, II) did not seem to be affected by the potential flavor of *C. jadinii*. On the other hand, feed intake of pigs fed diets with whey yeast *K. fragilis* replacing 20 to 60 % of SBM, increased compared with pigs fed control diets, which was attributed to a high palatability and tastiness of the diets with yeast (Spark et al., 2005), whereas replacing 80 % of SBM with yeast protein reduced feed intake in piglets (Tegbe and Zimmerman, 1977). Reduced feed intake in the broiler chickens may also have been caused

by a change of taste of the diets with *C. jadinii*, however studies on the palatability of pig and broiler chicken diets containing yeast are scarce.

4.3. Effects on nutrient and energy digestibility

The methodology chosen to evaluate nutrient digestibility is discussed here.

Digestibility values are here presented as "apparent", which can be further corrected to "standard" and "true" digestibility, but this was not in the scope of this thesis. *Post-mortem* collection of ileal content in pigs required well-timed euthanasia, approximately 2.5 h after feeding, to ensure the presence of digesta in the small intestine. This was not an issue in the broiler chickens as they were fed *ad libitum*, however, the amounts of ileal content are limited in a broiler chicken. To overcome this issue, samples from two birds in each pen were pooled. In addition, the Pregl-Dumas method was chosen over the Kjeldahl method, to reduce the amount of sample necessary to determine N content in ileal digesta of birds. Yttrium oxide was chosen as a marker in pig diets because it can be used in smaller quantities compared with chromic oxide (Sales and Janssens, 2003) and is non-toxic for humans. Titanium oxide was chosen as a marker for broiler diets based on previous experience and good recovery rates. Generally, marker methods are a less laborious alternative to the total collection, when the measurement of feed intake or complete collection of feces is not feasible. Values for total tract digestibility by total collection have been reported to be higher than those determined with marker methods (Kavanagh et al., 2001; McCarthy et al., 1974), which can be explained by the fact that the samples used in the total collection method are more representative.

The digestibility of nutrients in pig diets with *C. jadinii* was not affected in pigs aged 63 to 72 days (Table 4, Paper II), but the following effects were observed in weanling piglets aged 53 days (Table 6, Paper I): the ATTD of CP in the CU40 diet was higher than the control, but AID of CP was unaffected; the AID of methionine and alanine tended to be higher in the CU40 diet,

but the AID of other AA was unaffected; the ATTD of neutral detergent fiber was lower for the CU40 diet compared with the control diet; the ATTD of crude fat was higher for the CU40 diet compared with CU10; the ATTD of ash increased in the CU10 and CU40 diets relative to the control diet, and the AID of ash was higher in the CU40 diet; the ATTD of P increased in the CU40 diet compared with the control.

Digestibility of DM, OM, crude fat, carbohydrates plus lignin, and energy was positively correlated with BW of pigs (Paper II), whereas the ATTD of N and ash was unaffected. The ATTD of N and energy in pig diets with *C. jadinii* in Exp. 2 was higher than in Exp. 1, and also higher than in pig diets with 19 to 56 % CP from RSM (Pérez de Nanclares et al., 2017; Pérez de Nanclares et al., 2019) and in diets containing 18 to 53 % of CP from BPM (Hellwing et al., 2007b), but was lower than in diets containing 100 % CP from BPM (Skrede et al. 1998).

The ATTD of energy was slightly lower in salmon diets containing 40 % CP from *C. jadinii* (84 %; Øverland et al., 2013) than in the pig diets containing *C. jadinii* in Exp.1 and 2. The ATTD of OM, fat, and carbohydrates plus lignin was higher in *C. jadinii*-based diets (Exp. 1 and 2) than in RSM-based pig diets (Pérez de Nanclares et al., 2019). Nutrient digestibility in pig diets was also maintained in other studies where microbial ingredients (hydrocarbon-grown *S. cerevisiae,* BPM) replaced SBM and FM (Barber et al., 1971; Hellwing et al., 2007b).

Differences in the ATTD of nutrients between the pig Exp. 1 and 2 can be explained by the positive correlation between the initial BW of the pigs and the ATTD of nutrients (Paper II). Consequently, this can be associated with increased activities of pancreatic enzymes (Jensen et al., 1997) and changes in gut microbiota (Niu et al., 2015) as pigs age. An increase in the ATTD of fat with increasing age was also reported in broiler chickens (Tancharoenrat et al., 2013). Therefore, gut bacteria fermentation in the large intestine may have increased the disappearance of nutrients from feces in older pigs compared with younger pigs. The higher ATTD of nutrients in Exp. 2 compared with Exp. 1, where digestibility was obtained with Y_2O_2, could

alternatively, have been related to differences between the methods. The higher digestibility of yeast-based diets compared with RSM-based diets (Pérez de Nanclares et al., 2019) can be explained by a higher content of fiber in RSM than *C. jadinii*, however, the pig diets in Exp. 1 and 2 had higher levels of wheat and lower levels of barley than the diets used by Pérez de Nanclares et al. (2019). It is noteworthy that the estimate of ATTD of carbohydrates plus lignin, when carbohydrates plus lignin content is calculated from the analytical values for DM, minus the sum of ash, CP and crude fat in the diets and feces may lead to accumulation of analytical errors on the carbohydrate value (Neil, 1978), reducing the accuracy of that value. Live *C. jadinii* cells can provide digestive enzymes, favoring carbohydrate digestion, which could have had a positive effect on protein, fat and mineral digestibility (Øverland and Skrede, 2017; White, 1956), however these were likely inactivated by the temperatures achieved during the processes of yeast-inactivation and drying, or feed production (Villas-Bôas et al., 2002; Roon and Levenberg, 1972; Nishimura et al., 1982). Investigating the viability of endogenous enzymes in *C. jadinii*, prior and post feed production could have been useful.

Higher ash digestibility in pig diets containing *C. jadinii* compared with the control diet (Paper I) could be due to the high bioavailability of the minerals in *C jadinii*, which has a relatively high content of minerals, varying with the growth media (Rodríguez et al., 2011). The higher AID of ash in the pigs concurred with a higher ATTD of P (Paper I). Calculated P in the broiler chicken diets control, CU10, CU20 and CU30 were 6.5, 6.4, 6.4 and 6.2 g/kg respectively. Interestingly, analyzed P in broiler chicken diets with *C. jadinii* seemed dependent on the analytical method. Boiling feed samples with HCl (250°C; method EC No 152/2009; ISO 6491) provided higher values for total P compared with a previous analysis without this step. Content of P in ileal samples was not reevaluated by using this additional step, due to limited amounts of sample, and this may have potentially led to an incorrect estimation of AID.

The AID of CP and starch were not affected by adding 10 to 30 % CP from *C. jadinii* in broiler chicken diets, but the AID of carbohydrates, crude fat, OM, and DM was lower in diets with 30% CP from *C. jadinii* than the control diets (Table 4, Paper III). The lower fat digestibility in diets with increasing levels of yeast might be due to an increase in chitin content in the diets, derived from *C. jadinii*. Chitin can possibly increase the viscosity of the mucous layer and digesta in the small intestine by reacting with gastric acid, and oppose emulsion of fat, thus hindering the digestion and absorption of nutrients (Razdan and Pettersson, 1994). Furthermore, yeast cell walls are poorly digestible (Nguyen et al., 1998; Rumsey et al., 1991b), which may altogether explain the lower digestibility of nutrients in broiler chickens (Paper III). The reduced growth performance in broiler chickens fed the *C. jadinii*-containing diets could be a result of a lower proportion of energy from fat available for growth. The lower AID of carbohydrates in the broiler chicken fed the 30 % CP from *C. jadinii* might explain the lower growth performance. The AID of CP and starch were higher in broiler chickens (Paper III) than in the piglets (Paper I), which could possibly be explained by differences in relative length of the digestive tract in broiler chicken compared with pigs (Stevens and Hume, 1989; Argenzio, 1995; Hellwing, 2005; Denbow, 2015). Interestingly, yeast has previously improved digestive function due to antioxidant activity and mycotoxin binding in pigs (Kogan and Kocher, 2007), which concurs with our results in pigs (Paper I) but opposes our findings in broiler chickens (Paper III). The digestibility of N in *C. jadinii* may be affected by altering the drying method is used during upstream processing. For example, the N-digestibility was higher in pig diets with spray-dried whey yeast than in pig diets with flash-dried (higher temperature) a *S. cerevisiae* and *Kluyveromyces lactis* (Spark et al., 2005). The reduced nutrient digestibility together with reduced feed intake may explain the reduced performance in broiler chickens (Paper IIII). Nevertheless, the slightly lower content of lysine and arginine in the broiler chicken diets, may have caused the reduced performance. There might have been a reduction in available lysine

and arginine in the diet with 30 % *C. jadinii* protein due to 1) a limitation of the analytical method to correctly detect AA in diets with *C. jadinii*; 2) overestimated AA availability in *C. jadinii* for chickens because SID AA for broiler chickens was based on SID CP of *S. cerevisiae* for pigs; 3) Maillard reactions and heat damage, caused by unintentional high pelleting temperatures during pelleting of broiler diets.

4.4. Effects on intestinal morphometry and absorptive capacity

Intestinal villi in duodenum, ileum, and jejunum are responsible for the absorption of digested nutrients and therefore, villus height can be used as an indicator for absorptive ability (Caspary, 1992, cited by Awad et al., 2008; Hernández et al., 2007) in pigs and broiler chickens. The ileal and jejunal VH in young pigs increased in the pigs fed diets with 40 % CP from *C. jadinii* (Paper I) compared with the pigs fed the control diet, while ileal and jejunal VH was maintained for broiler chickens fed 30 % CP from *C. jadinii* compared to the broiler chickens fed the control diets (Paper III). However, ileal VH in the broiler chickens was lower for those fed diets with 20 % CP from *C. jadinii* compared with those fed 30 % CP from *C. jadinii*. Thus, the absorptive capacity expressed by VH in broiler chickens was not affected by adding 30 % of CP from *C. jadinii* at the expense of SBM.

The crypts of Lieberkühn play an important role in cell division and differentiation and are associated with the renovation of the intestinal epithelium (Lallès et al., 2004), secretion of bactericides (*e.g.* lysozyme, defensins) mucus and hormones (Clevers, 2013). Ileal crypts were shorter in pigs fed the diet with 40 % CP from *C. jadinii* compared with the pigs fed the control diet, however, the ileal CD in broiler chickens was not affected by adding increasing levels of *C. jadinii* to the diets. Crypt depth can be associated with the rate of enterocyte synthesis, because enterocytes are formed in the intestinal crypts, migrating towards the apex of the villi as they develop digestive and absorptive capacity (Hampson, 1986). Enterocyte damage for

example by effect of pathogens stimulates the production of intestinal cells in the crypts. Reduced ileal CD could thus indicate that those adverse stimuli are reduced, possibly due to the effects of *C. jadinii*, and therefore the conditions in the intestinal lumen and epithelium are favorable for nutrient absorption. This assumption concurs with the higher VH in the pigs fed the CU40 diet (Paper I). Although high VH in broiler chickens can be associated with good intestinal function and growth performance (Hernandez et al 2006), according to Svihus (2014), the increase in intestinal VH might be observed in birds as part of a compensation system that attempts to acquire more nutrients when nutrient absorption is deficient. Increased VH and VH:CD partly explain the increase in ATTD of CP in *C. jadinii*-containing diets in Exp. 1, due to an increased absorption capacity in the piglets fed the CU40 diet compared with the piglets fed the control diets. The higher ATTD of P in the CU40 pig diets compared with the control diets in Exp. 1 may thus have been due to increased VH (Heidarieh et al., 2013). Higher VH:CD can be associated with higher expression of genes involved in nutrient sensing and transportation of glucose, fatty acids and peptides in the jejunum (Heim et al. 2015) implying improved absorption ability, although this was not the case in the pigs in Exp. 1.

4.5. Effects on nitrogen and energy utilization

Replacing traditional protein sources with *C. jadinii* in pig diets did not affect energy and protein utilization in young pigs, which concurs with the maintenance of growth performance and digestive function results (Papers I, II).

Digested nitrogen was similar among pig diets (Table 5, Paper II), which explains the similarities in nutrient digestibility in the pigs (Table 4, Paper II). The RN values in pigs (Paper II) agreed with a previous study where BPM was used as a protein source (Hellwing et al., 2007b). Supporting our results, RN was similar among pigs fed diets replacing conventional protein sources with 6 to 29 % CP from hydrocarbon-grown *S. cerevisiae* replacing FM (Barber

et al., 1971), and up to 52.5 % of CP from BPM replacing SBM (Hellwing et al., 2007b). Lower RN was reported when replacing SBM with 18.7 to 56.0 % of the CP from RSM (Pérez de Nanclares et al., 2019). Interestingly, increasing levels of *K. fragilis* (6 to 17 %) replacing 20 to 60 % of dietary SBM, increased RN in pigs (Spark et al., 2005). Nevertheless, RN may decrease in pigs fed diets with microbial ingredients compared with conventional diets, because non-protein N in yeast cells is not directly available for protein synthesis (Skrede et al., 1998), partly explaining the reduced performance in the broiler chicken experiment (Paper III).

The utilization of Digested N for retention expressed as RN : DN was higher in pigs fed *C. jadinii*-containing diets (Paper II) than in pigs fed diets with 17.5 to 52.5 % CP from BPM (Hellwing et al. 2007b). Additionally ratios RN : IN and RN : DN in the pigs fed *C. jadinii*-containing diets in Exp. 2 (Paper II) were higher than in pigs fed RSM-based diets (Pérez de Nanclares et al., 2019). The higher RN : DN might be explained by a higher capacity for N retention in intact pigs than castrated pigs as shown by Tauson et al. (1998). Additionally, the perforated rubber mats used in the metabolism cages in Exp. 2 may have contributed to losses of nitrogenous material and the higher RN values as compared with previous studies. The higher RN (Paper II) compared with the previous studies, concurs with the lower UN, and FN.

 Nitrogen excretion in urine and feces in Exp. 2 was not affected by adding *C. jadinii* to pig diets but was lower (Paper II) than in RSM-fed pigs (Peréz de Nanclares et al., 2019). Additionally, N excretion was previously found to decrease in pigs fed diets with increasing levels of whey yeast *K. fragilis* (Spark et al., 2005). Allantoin is a nitrogenous end-product of the metabolism of purine bases, excreted through urine in pigs (Michal, 1999). The concentration of allantoin in urine was not measured in the present studies, however, previous research indicates that N losses as allantoin in urine of pigs fed diets with yeast have relatively low importance, because of the high biological value of yeast, which was found to have more than 80 % protein digestibility and 60 % protein retention (Spark et al., 2005). Blood urea and

uric acid levels in pigs fed whey yeast were investigated by Spark et al. (2005), who found values within reference limits, however, there were differences among pigs fed diets with different yeast strains. In other studies, the inclusion of BPM and yeast in pig diets led to increased allantoin levels in pigs, as discussed by Hellwing et al., (2007c). More recently, diets containing yeast protein concentrate led to higher N excretion in broiler chickens than diets based on SBM, BPM, insect meal, algae, and duckweed (Tallentire et al., 2018).

Energy metabolism measured by indirect calorimetry was similar among dietary treatments (Table 6, Paper II). Experimental period tended to affect DE and ME intakes, which can be explained by an effect of initial BW among the periods. The DE, ME, HE and RE and energy retained as protein were similar among pigs fed control and diets with yeast, agreeing with the findings of Hellwing et al. (2007b), in a study investigating another microbial ingredient in pig diets. The DE and ME were higher (Paper II) than for pigs fed diets with 18.7 to 56.0 % of CP from RSM (Pérez de Nanclares et al., 2019), explained by the higher content of fiber in RSM, than in SBM and *C. jadinii*. Pigs fed RSM-based diets produced more CH_4 and CO_2 (Pérez de Nanclares et al., 2019) than pigs fed yeast-containing diets (Table 6, Paper II). This may be explained by an increase in the passage of undigested nutrients to the hindgut and a higher fermentation rate by intestinal microflora in RSM-based diets. Mean oxidized protein and mean oxidized carbohydrates were similar among dietary treatments. Net oxidation of fat was zero because all pigs had RQ values > 1, indicating *de novo* lipogenesis from dietary carbohydrates (Chwalibog and Thorbek, 2000). Carbohydrate oxidation was higher and protein oxidation was lower (Paper II) than the values reported by Chwalibog et al. (1998). Differences in substrate oxidation may be caused by dissimilar collection procedures, where the mats in metabolism cages (Exp. 2) may have caused losses on nitrogenous material.

4.6. Challenges and limitations of the experiments

A few challenges were encountered when investigating the effects of *C. jadinii* as a protein source in monogastric animal diets, which may be relevant for future studies:

1) Feed ingredients with fine-particle size such as dried yeast in large quantities, when associated with low-fat content in the mixture may increase friction and resistance in the pellet press. As a result, energy expenditure and pellet temperature increased while producing diets with the highest levels of *C. jadinii*, compared with the control diets.

2) There is a general challenge in extrapolating growth performance results from experimental conditions to production conditions. Under practical conditions, animals are kept and fed in larger groups and hygiene conditions may be unfavorable compared to the present experiments; finally, in Exp. 3, obtaining individual feed intake was not feasible in practice.

3) *C. jadinii* grown on lignocellulosic biomass was available in limited amounts, as the production process was still under development. The fermentation yields and the nutritive quality of *C. jadinii* depended on the quality of the nitrogen source, growth media, and batch (discussed by Sharma, 2018, and Lapeña et al., 2019, submitted), which could be further optimized in future studies.

4.7. Implications and impact on sustainability

The work described in this thesis envisioned securing competitive and locally-produced protein sources, enabling the production of sustainable livestock feed. These studies contributed to increasing the knowledge about alternative protein-rich ingredients currently under development, more specifically *C. jadinii* grown on local lignocellulosic biomass.

Cyberlindnera jadinii rapidly grows on inexpensive substrates, producing high cell density (Bekatorou et al., 2006). This yeast has advantages over SBM, RSM, and FM: low arable land use, independent of climate and fishing restrictions (Tallentire et al., 2018). *C. jadinii* has shown

potential to replace conventional protein-rich ingredients, additionally providing intestinal health benefits for newly-weaned piglets and potential to partially replace soybean in diets for broiler chickens. *C. jadinii* replaced 79 % of the main protein sources on a weight basis, in young pig diets, without compromising growth performance. In addition to intestinal health benefits, *C. jadinii* has the potential to become a highly-valuable-functional-protein source for pig diets. Given a successful upscaling of *C. jadinii* grown on lignocellulosic sugars, this microbial ingredient could potentially change the European feed market situation, which is now highly dependent on imported protein-rich feedstuffs, such as SBM.

Sustainable monogastric animal diets will likely rely on several alternative protein sources (*e.g.* yeast, insects, BPM) to promote the variety of feed ingredients. For example, duckweed ponds can be used as a manure management option in pig (Tallentire et al., 2018; Xu and Shen, 2011) or poultry production, where N excretion might be increased by diets with yeast or BPM. Thus creating integrated environment-friendly solutions in livestock systems. Recently, the environmental burdens of broiler chicken diets with alternative protein sources have been investigated, and diets containing yeast protein concentrate had lower agricultural land use than soybeans (Tallentire et al., 2018). Yeast produced from first-generation sugars such as wheat require a large agricultural land area (Tallentire et al. 2018), while *C. jadinii* in the present studies was grown on a coproduct from the forestry industry, which is assumed to have a lower environmental impact. Another recent study has shown that livestock diets containing yeast protein, instead of soybean protein, have a markedly lower environmental impact, measured in CO_2 equivalent, and also lower water consumption associated with their production (Couture et al., 2019).

C. jadinii grown on lignocellulosic sugars, used in these studies, is an alternative protein rich-feedstuff in an early phase of development. The full potential of this yeast as a feed protein source has not yet been uncovered. Recent investigations in the Centre for Research and

Innovation, Foods of Norway indicate that by modifying growth conditions (Lapeña et al. 2019, submitted) and by optimizing downstream processing methods (Hansen et al., 2018) the nutritional value of *C. jadinii* and other yeast strains may be further increased, thus increasing its competitivity as a feed ingredient for livestock diets.

5. Concluding remarks and future studies

5.1. Concluding remarks

The results of the experiments indicate that *C. jadinii* can replace 10 % and 40 % of the total crude protein in traditional broiler chicken and pig diets respectively, while maintaining growth performance and digestive function. Regarding pig diets, this represents 79 % of the protein-rich ingredients on a weight basis. Further assessing the costs of producing *C. jadinii* from local lignocellulosic biomass is encouraged, to evaluate the economic feasibility to use yeast as a protein source for these animals. To fully assess the feasibility of using lignocellulosic-sugar grown *C. jadinii* as a protein source will require close cooperation among feed companies, biorefineries, and research sites.

5.2. Future studies

Recommendations for further studies:

- The maintained performance with improved general health of the piglets in Exp. 1 suggests that *C. jadinii* can be included at levels higher than 40 % of the CP in the diets, thus further investigating the optimal inclusion level of *C. jadinii* in pig diets would be interesting;

- An assessment of the economic viability of *C. jadinii* grown on lignocellulosic biomass as a feed ingredient in the context of the Norwegian livestock production should be performed;

- The nutritional composition and nutritional value of downstream-processed yeast using different methods such as sonication, pulse electric field, heat treatment, and enzymatic hydrolysis should be evaluated in experiments with pigs and chickens. In addition, *β*-glucanase levels in the digesta of pigs and broiler chickens should be measured to provide recent knowledge about yeast-*β*-glucan digestion in monogastric animals;

- The effects of other yeast strains and fermentation media, such as yeast grown on agricultural co-products should be explored in experiments with pigs and broiler chickens;

- The effects of *C. jadinii* or other yeast strains on intestinal morphometry should be evaluated in older broiler chickens and pigs;

- The utilization of purine bases from yeast should be investigated in experiments with pigs and broiler chickens;

- The chemical composition *i.e.* P, MOS, *β*-glucans, and enzymes of *C. jadinii* grown on different media should be further evaluated;

- The effects of feed production on the physio-chemical properties of diets containing *C. jadinii* should be explored.

6. Reference list

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Paper I

Paper I

Candida utilis yeast as a protein source for weaned piglets: Effects on growth performance and digestive function

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A B S T R A C T

Yeast such as inactivated *Candida utilis* produced from lignocellulosic biomass from underutilized wood co-products as a second-generation sugar source is a potentially sustainable protein feed ingredient in diets for piglets. This study aimed to evaluate the effects of *C. utilis* added to diets for weaned piglets on growth performance and digestive function when replacing main protein sources. Forty-eight piglets weaned at 30 days of age, with a mean starting weight of 11.06 ± 0.84 kg were fed one of four dietary treatments for 28 days: a conventional control diet with soybean meal, fishmeal, rapeseed meal, and potato protein or one of three experimental diets containing 10, 20 or 40% crude protein (CP) from yeast (CU10, CU20, and CU40, respectively). Adding yeast to diets did not affect growth performance compared with the control. The diet with 40% CP from *C. utilis* had higher apparent total tract digestibility (ATTD) of CP compared with the control ($P = 0.034$) and higher ATTD of ash ($P < 0.001$) compared with the control. The ATTD of neutral detergent fiber decreased in the CU40 diet compared with the control ($P = 0.006$). The apparent ileal digestibility (AID) of ash increased ($P = 0.001$) in the CU40 diet compared with the control, while the AID of CP and amino acids was unaffected. Villi-height increased in jejunum ($P = 0.007$) and ileum ($P = 0.047$), and villus-height: crypt-depth ratio increased ($P = 0.001$) in jejunum of piglets fed the CU40 diet compared with the control. Fecal dry matter increased linearly with increasing levels of *C. utilis* in the diets at day 7 after weaning ($P = 0.001$) and was higher for the CU40 group compared with the control group at day 21 after weaning ($P = 0.027$). Trypsin activity and messenger RNA expression of nine genes encoding for nutrient transporters in the jejunum did not differ among diets. Collectively, the results indicated that *C. utilis* can replace 40% of CP from the main protein sources traditionally used in diets for weaned piglets while maintaining growth and improving digestive function.

1. Introduction

The livestock industry in Norway is challenged by a high dependence on imported feed ingredients such as soybean meal because of a limited supply of locally produced protein resources (de Visser et al., 2014; Øverland and Skrede, 2017). To improve national self-sufficiency of food, it is necessary to develop alternative methods to acquire protein resources. Recent advances in biorefining technology using lignocellulosic biomass as a source of second-generation sugars enable the production of locally-produced protein sources such as yeast (Øverland and Skrede, 2017). Yeast cells and their derivatives are known for their β-glucan, mannooligosaccharide and nucleic acid contents, to induce

immunostimulant effects in piglets (Hahn et al., 2006; White et al., 2002) and reduce post-weaning diarrhea. Dietary yeast for pigs has shown beneficial effects on health when used in small amounts (White et al., 2002), but limited information exists on the nutritional value of yeast in larger amounts as a protein source in piglet diets. However, Spark et al. (2005) demonstrated that growth performance in piglets improved when 20 to 60% of soybean meal was replaced by 6 to 17% dietary yeast, due to a reduction in the content of anti-nutritional factors in the diet. *Candida utilis* yeast (more recently classified as *Cyberlindnera jadinii*) grown on lignocellulosic biomass has not been previously tested in diets for pigs. The aim of this study was therefore to determine the effects of this locally-produced *C. utilis* as a protein

source on the growth performance and digestive function of weaned piglets.

2. Materials and methods

2.1. Animals and facilities

All the animals were handled in accordance with the applicable laws and regulations controlling experiments with live animals in Norway (the Animal Welfare Act of 28 of December 2009 and the local legislation derived from the directive 2010/63 EU of the European Parliament and Council of 22 September 2010 on the protection of animals used for scientific purposes). The experiment was approved by the Norwegian Food Safety Authority (identification number: 11314). The experiment was performed at the Center for Livestock Production, Norwegian University of Life Sciences, Aas, Norway, from February to March of 2017 and lasted for twenty-eight days. Twelve sows (Norwegian Landrace × Yorkshire) inseminated with boar semen (Duroc) provided the piglets for this experiment. At approximately thirty days of age (29.6 ± 1.05 standard deviations [SD]), and an average initial body weight of 11.06 kg ± 0.84 SD, twenty-five surgically castrated-male piglets, and twenty-three intact-female piglets, were equally distributed by litter, gender, and weight and randomly allotted to four dietary treatments, with twelve replicates per treatment. Pigs within the same pen received the same diet. At the stipulated feeding times, each pig was separated from the others in an individual feeding stall for 30 min to measure individual feed intake. Thus, each pig constituted an experimental unit. All piglets were healthy at the start of the experiment. Each group of four piglets was kept in a concrete-floored, partially slatted pen of 3.35 × 2.25 m with individual feeding areas of 0.37 × 1.35 m each. A rubber mat of approximately 90 × 100 cm was used as a replacement for other bedding materials, to minimize interference with the measurements of digestibility and gastrointestinal health effects of the diets. Heating lamps were installed over the rubber mats to provide comfortable resting areas and the pens were equipped with activity enrichment toys. The room temperature was kept on average at 19.05 °C ± 1.74 SD, with 8 h of light and 16-h darkness cycles. During the hours of darkness, a night light was used.

2.2. Yeast single-cell protein

Candida utilis biomass (LYCC 7549; Lallemand Yeast Culture Collection) was produced by Lallemand Inc, Salutaguse, Estonia. Second generation sugars were obtained from lignocellulosic biomass of Norway spruce trees (*Picea abies*) by using the Borregaard Advanced Lignin process at Borregaard AS, Sarpsborg Norway (Patent "Lignocellulosic biomass conversion by sulfite pretreatment"; EP2376642B1 EP Grant). The C5 and C6 sugars were used in the growth media for the yeast, as described by Øverland and Skrede (2017) and Sharma et al. (2018).

2.3. Diets and feeding

The diets were formulated in collaboration between Felleskjøpet Fôrutvikling A.S. and the Norwegian university of life sciences and produced at the Center for Feed Technology (Fôrtek), Norwegian university of life sciences, Aas, Norway. Feed ingredients were ground through a 3 mm die using a hammer mill (Roskamp, California, USA). Fine materials were transported into an automated dosing and batching system (Abel Company, Wisconsin, USA). All ingredients were mixed with a twin shaft paddle mixer (Dinnissen, Netherlands). The mash was conditioned at 74 to 76 °C and pelleted (Twin Pass, Muench, Germany). The finished pellets were cylindrical 3 × 10 mm and the pellet temperature varied from 82.4 to 93.4 °C. The dietary treatments consisted of one control diet and three experimental diets. The experimental diets consisted of a gradual replacement of the main sources of CP, soybean

Table 1

Dietary composition of the experimental diets.

Item	Diet[a] Control	CU10	CU20	CU40
Formulation, g/kg, as is				
Wheat	624	616	608	593
Barley	100	100	100	100
Oats	50	50	50	50
Yeast meal[b]	0	36	73	146
Soybean meal[c]	80	65	50	19
Fish meal[d]	20	16	13	5
Potato protein concentrate[e]	38	30	23	9
Rapeseed meal[f]	20	16	12	5
Rapeseed oil	22	22	23	25
Sodium chloride	6	6	6	5
Monocalcium phosphate	13	14	14	16
Limestone	9	9	9	9
Iron (Fe)	0.4	0.4	0.4	0.4
Vitamin + trace-mineral premix[g]	4.8	4.9	4.9	5.0
L-Lysine	6.3	6.3	6.1	5.8
L-Methionine	2.1	2.3	2.5	3.0
L-Threonine	2.8	2.8	2.6	2.4
L-Valine	1.0	1.0	1.0	1.0
L-Tryptophan	0.9	0.9	0.9	1.0
Calculated content				
Net energy[h] (MJ/kg)	9.94	9.94	9.94	9.94
Crude protein	170	170	170	170
Crude protein from *Candida utilis* (%)	0.0	10.0	20.1	40.3

[a] Control diet (Control); diet with 10% crude protein (CP) from *Candida utilis* (CU10); diet with 20% CP from *C. utilis* (CU20); diet with 40% CP from *C. utilis*. (CU40).

[b] Dried inactivated *C. utilis*: dry matter (DM) 970 g/kg, CP (N × 6.25) 470 g/kg, crude fat 16 g/kg, ash 78 g/kg, gross energy 19.9 MJ/kg; essential amino acid content in g/16 g N: 24.4 Arg, 8.5 His, 21.6 Ile, 31.6 Leu, 30.6 Lys, 5.2 Met, 18.4 Phe, 25.6 Thr, 25.9 Val, 6.2 Trp.

[c] Non-GMO soybean meal, Denofa AS, Fredrikstad, Norway: DM 881 g/kg, CP 458 g/kg, crude fat 10 g/kg, ash 56 g/kg, neutral detergent fiber (NDF) 89 g/kg, gross energy 17.5 MJ/kg.

[d] Norsildmel AS, Egersund, Norway: DM 917 g/kg, CP 684 g/kg, crude fat 73 g/kg, ash 145 g/kg, NDF 5 g/kg, gross energy 19.4 MJ/kg.

[e] Cargill, Denmark: DM 914 g/kg, CP 725 g/kg, crude fat 30 g/kg, ash 20 g/kg, gross energy 21.8 MJ/kg.

[f] Expeller pressed rapeseed meal, Mestilla, UAB, Klaipeda, Lithuania: DM 889 g/kg, CP 350 g/kg, crude fat 88 g/kg, ash 59 g/kg, NDF 161 g/kg, gross energy 19.1 MJ/kg.

[g] Provided per kg of diet: 120 mg Fe (FeSO$_4$); 60 mg Mn (MnO); 120 mg Zn (ZnO); 26 mg Cu (CuSO$_4$); 0.60 mg I (Ca (IO$_3$)); <1.0 g Se; 8000 IU vitamin A; 45 mg dl-α-tocopheryl acetate; 105 mg ascorbic acid; 1500 IU cholecalciferol; 4.64 mg menadione; 3 mg thiamin; 5.63 mg riboflavin; 45 mg niacin; 15 mg pantothenic acid; 20 µg cyanocobalamin.

[h] Calculated based on Central Veevoederbureau (2005).

meal, potato protein concentrate, fishmeal and rapeseed meal with drum dried and inactivated *C. utilis* corresponding to 10, 20 or 40% of the total CP content. Thus, the diets were coded in order as control, CU10 (10% CP from yeast), CU20 (20% CP from yeast) and CU40 (40% CP from yeast). The chemical composition of soybean meal, potato protein concentrate, fishmeal, rapeseed meal and *C. utilis* is shown under Table 1. A replica of each diet was produced in separate batches and added the inert marker Yttrium (III) oxide (Y$_2$O$_3$) was added at 0.01% to these replicas. The diets were formulated to be isonitrogenous and isoenergetic based on the analyzed chemical content of the ingredients (Table 1). The analyzed chemical composition of the diets is shown in Table 2. Piglets were fed three times per day during the first 14 days and two times per day during the remaining period. Feed was provided *ad libitum* during restrictive time periods and the amounts of feed were adjusted weekly, based on estimated feed intake of 3 to 5% of the live body weight. Water was accessible *ad libitum* via automatic drinkers. Diets containing Y$_2$O$_3$ were provided from day 18 of the experiment for the determination of apparent total tract digestibility

Table 2
Analyzed chemical composition of experimental diets.

| Item, g/kg | Diet[a] | | | |
	Control	CU10	CU20	CU40
Dry matter	882	878	885	890
Crude protein	177	169	170	174
Crude fat	36	40	45	43
Starch	443	448	455	458
Ash	54	48	50	52
Neutral detergent fiber	97	96	96	85
Gross energy (MJ/kg)	16	17	17	17
Essential AA[b] (g /16 g N)				
Arg	9.3	9.1	8.8	8.7
His	3.7	3.6	3.5	3.4
Ile	7.1	6.8	6.8	6.6
Leu	12.5	12.1	11.7	11.2
Lys	13.1	13.0	12.8	12.3
Met	4.4	4.5	4.5	4.7
Phe	7.9	7.6	7.4	7.0
Thr	9.5	9.6	9.3	9.5
Val	9.5	9.3	9.2	9.1
Trp	2.8	2.9	2.9	2.8
Non-essential AA (g/16 g N)				
Ala	7.2	7.3	7.3	7.8
Asp	14.4	13.8	13.2	12.6
Gly	7.6	7.4	7.2	7.0
Glu	35.0	34.8	34.7	34.3
Cys	2.6	2.5	2.4	2.2
Tyr	3.1	3.3	3.1	3.1
Pro	11.9	11.7	11.6	10.9
Ser	8.5	8.6	8.3	8.4

[a] Control diet (Control); diet with 10% crude protein from *Candida utilis* (CU10); diet with 20% crude protein from *C. utilis* (CU20); diet with 40% crude protein from *C. utilis* (CU40).

[b] Amino acids.

(ATTD) and apparent ileal digestibility (AID). Individual feed leftovers were collected after each meal and recorded weekly for calculating average daily feed intake (ADFI). Individual live body weight was recorded weekly for calculating average daily gain (ADG) and feed conversion ratio (FCR). A cumulative feed sample from each diet was collected for analysis of dry matter, ash, starch, CP, crude fat, neutral detergent fiber, gross energy and amino acids (AA).

2.4. Fecal score and dry matter

Fecal score was registered daily for 28 days and for each pen ($n = 12$) on a scale from 1 to 4, according to consistency (1 = dry and hard; 2 = normal; 3 = pasty, with loss of normal shape; 4 = watery) to assess the presence of diarrhea (fecal score ≥ 3) (Pedersen and Toft, 2011). In addition, fecal samples were collected weekly by pen for determination of dry matter.

2.5. Sample collection

On the last day of the experiment, the piglets were fed 2.5 h before euthanasia, to ensure the presence of enough intestinal content for sample collection. All animals were euthanized with a captive bolt pistol and exsanguination. Intestinal content and tissue samples were collected from the aboral portion of jejunum and ileum. Jejunal content was collected for analysis of trypsin activity. Intestinal segments, heart, lung, liver, kidney, and other organs with gross lesions were collected for morphological studies. Samples of jejunum were collected for quantification of nutrient transporter expression. Total liver weight was recorded, and liver index was calculated as: liver index = liver weight (kg)/live body weight (kg).

2.6. Digestibility

For determination of the ATTD, individual fecal samples were collected from the floor after defecation, consecutively from experiment days 21 to 25. The fecal samples were pooled, freeze-dried, ground at 0.5 and 1 mm and homogenized before analyses. Immediately after slaughter, intestinal contents were collected from the last two meters of the ileum and jejunum from each animal, for determination of AID. The intestinal content and fecal samples were analyzed for Y_2O_3 concentrations and nutritional content based on the methods described by Austreng et al. (2000). Apparent digestibility of nutrients was calculated as described by Maynard and Loosli (1969).

2.7. Morphology and intestinal morphometry

To evaluate the general health status of the pigs, all abdominal and thoracic organs and the remaining carcass, were evaluated for gross lesions while sampling. Gross lesions were recorded, and additional samples were taken for histology and/or microbiology when indicated. In addition, histomorphology was performed on tissues from the heart, lung, liver, and kidney from all pigs. Heart, lung, liver, kidney and intestinal tissue samples for histology were collected within 20 min of euthanasia and fixed in 10% formalin. The gut tissues from the 48 individuals were sectioned along the mesenteric attachment and the serosal surface was placed on a piece of cardboard prior to formalin fixation. After 48 h of fixation, the tissues were routinely processed, embedded in paraffin and cut in 4 μm sections. Sections were deparaffinized in xylene and rehydrated in graded alcohol before routine staining with hematoxylin and eosin. Formalin-fixed, paraffin-embedded tissue sections were also stained with high iron diamine and alcian blue (HID-AB). Digital images of the intestinal sections were captured using an Axiocam 105 color digital sight camera configured with a Zeiss Lab.A1 microscope. Morphometric measurements were performed using the software ImageJ 1.51k (National Institutes of Health, USA). For villus height (VH) and crypt depth (CD) measurements and VH:CD calculations, villi, and crypts were chosen from the stem of mucosal folds not containing Peyer's patches. The longest villi in proximity to well-oriented crypts were selected and micrographs were captured at $10 \times$ magnification, while the longest crypts in the same micrographs were selected for measurements of the CD. VH was measured by drawing a segmented line through the villus center extending from the tip to the villus-crypt-junction. CD was measured from the villus-crypt junction to the basement membrane of the deepest portion of the crypt, adjacent to the *tunica muscularis mucosae*. Between three and six villi and crypts were measured in each intestinal segment from each piglet. VH:CD for each intestinal segment was calculated using the mean VH and mean CD of the villus-crypt complexes.

2.8. Enzyme activity

Approximately 100 mg of contents from the jejunum were collected and snap-frozen at $-80\,°C$. The samples were thawed, homogenized and centrifuged at $21,100 \times g$ for 5 min at $4\,°C$. The supernatant was analyzed for trypsin activity and total protein concentration using commercial kits according to manufacturer's instructions (Trypsin Activity Assay kit, Abcam, Cambridge, UK and Bio-Rad Protein Assay, Bio-Rad, California, USA).

2.9. Gene expression of intestinal nutrient transporters

2.9.1. RNA extraction

Total RNA from jejunum was extracted from 7 pigs fed the control diet and 8 pigs fed the CU40 diet, using TRIzol TM protocol (Invitrogen) followed by RNeasy Plus Mini protocol (Qiagen). After the first washing step, on-column DNAse treatment was performed using the PureLink DNase kit (Invitrogen). RNA purity and quality were measured using

Table 3
Primers used for real-time quantitative PCR.

Primer name	Abbreviation	Sequence (5´−3´)[a]	Product size (bp)	Accession number
Fatty acid binding protein 1	FABP1	F-CTTCTCCGGCAAATACCAAG R-CCCGGTAGTGATGGTCAACT	160	NM_001004046.2
Fatty acid binding protein 2	FABP2	F- TAACTACAGCCTCGCAGACG R- GACCATTTCATCCCCGATAA	139	NM_001031780.1
Fatty acid binding protein 6	FABP6	F- GTGCGACATAGAGACCATCG R- TAGTTGGGGCTGTTCACCA	87	NM_214215.2
Peptide transporter 1	PEPT1	F- AATTGTGTCGTTGTCCAT R-AAGTCTGTGACTCATTG	78	NM_214347.1
Glucose transporter type 2	GLUT2	F-GTTCATGGTGGCCGAGTT R-ATTGCGGGTCCAGTTGC	82	NM_001097417.1
Glucose transporter type 4	GLUT4	F- TAAGACAAGATGCCGTCGGG R-GAGAAGACGGCGAGGACAAG	98	NM_001128433.1
Sodium-glucose cotransporter 1	SGLT1	F-TGTCTTCCTCATGGTGCCAA R-AGGAGGGTCTCAGGCCAAA	149	NM_001164021.1
Monocarboxylate transporter 1	MCT1	F-GGTGGAGGTCCTATCAGCAG R-AAGCAGCCGCCAAAATCAT	74	NM_001128445.1
Alkaline phosphatase, intestinal	ALPI	F-AGGAACCCAGAGGACCATTC R-CACAGTGGCTGAGGGACTTAGG	83	XM_003133729.4
β-actin	ACTB	F-CCAGGTCATCACCATCGG R-CCGTGTTGGCGTAGAGGT	158	XM_021086047.1
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	F- ACACTCACTCTTCTACCTTTG R- CAAATTCATTGTCGTACCAG	90	NM_001206359.1

[a] F, forward, R, reverse.

NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Only high quality (RNA integrity number ≥7) samples were used for quantitative real-time PCR (polymerase chain reaction) analysis.

2.9.2. cDNA synthesis and quantitative real-time PCR

The gene expression for the following nutrient transporters was measured: *glucose transporter 2, glucose transporter 4, sodium-glucose cotransporter 1, monocarboxylate transporter 1, fatty acid binding protein 1, fatty acid binding protein 2, fatty acid binding protein 6, peptide transporter 1* and *intestinal alkaline phosphatase.* The primers used for quantitative real-time PCR are shown in Table 3. Complementary DNA (cDNA) synthesis was performed using the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies). The quantitative real-time PCR was performed in a total volume of 20 μL using 10 μL LightCycler 480 SYBR Green I Master, 2 μL primers, 3 μL Milli-Q water and 5 μL cDNA diluted 1:50. The specificity of PCR amplification was confirmed with melting curve analysis. The PCR conditions were: 95 °C for 10 min, 95 °C for 10 s, 60–64 °C for 10 s depending on the primers, 72 °C for 10 s, in a total of 40 cycles. Samples were analyzed using the Light-Cycler 480 System (Roche Diagnostics, Mannheim, Germany). *Glyceraldehyde-3-phosphate dehydrogenase* and *β-actin* were tested as reference genes, but only *β-actin* showed stable expression across samples and treatments and was used in the analysis. All reactions were performed in duplicate and the transcriptional levels of selected genes were quantified relative to the expression of *β-actin* using a mean $-\Delta\Delta Ct$ value.

2.10. Chemical analysis

The chemical analyses of ingredients, feed, ileal and fecal samples were performed by the LabTek group, Norwegian university of life sciences, Norway. Ingredients and diets were ground at 1 mm and 0.5 mm for chemical analysis of main nutrient content. The diets were analyzed in triplicate for dry matter, ash, starch, CP, crude fat, neutral detergent fiber, energy content and AA including tryptophan. Fecal samples and ileal content were freeze-dried, homogenized and analyzed in duplicate for dry matter, ash, starch, and CP. Fecal samples were additionally analyzed for crude fat, neutral detergent fiber, and gross energy content. Ileal samples were also analyzed for AA and

tryptophan. Dry matter, ash, CP (Kjeldahl-nitrogen × 6.25) and AA were determined according to the methods described in the European Commission Regulation (EC) No 152/2009. AA were analyzed using the Biochrom 30 Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK). Tryptophan was analyzed on a Dionex UltiMate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) with a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan). Neutral detergent fiber was analyzed as described by Mertens (2002) using the Ankom200 Fiber Analyzer (ANKOM Technology, Macedon, New York, USA). Gross energy content was determined by a PARR 1281 Adiabatic Bomb Calorimeter (Parr Instruments, Moline, Illinois, USA) (method International Organization for Standardization, 1998). Crude fat was determined using Accelerated Solvent Extraction (ASE350, Dionex Corporation, Sunnyvale, California, USA). Feed samples were extracted with 70% petroleum-ether and 30% acetone at 125 °C. Ileal and fecal samples were extracted with 80% petroleum and 20% acetone at 125 °C. Starch was hydrolyzed with α-amylase and amyl glucosidase-enzymes to glucose, and glucose concentration was determined using a spectrophotometer (MaxMat PL II Multianalyzer, France) as described by McCleary et al. (1994). Yttrium (Y-89) concentrations in samples were determined 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, at the Department of Environmental Sciences, Norwegian university of life sciences. The samples were 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.

2.11. Statistical analysis

For statistical analyses of performance, digestibility and fecal score, the general linear model procedure with the least square means method in SAS software 9.4 (SAS Inst. Inc., Cary, North Carolina, USA) was used with the STDERR PDIFF options and adjusted for TUKEY to investigate differences ($P < 0.05$) between the dietary treatment groups. P-values between 0.05 and 0.1 were considered tendencies. The CONTRAST statement was used to investigate differences between the control and the yeast diets. Linear correlations between amounts of CP from yeast in the experimental diets and the growth performance parameters were investigated using the linear regression procedure. For the statistical

analyses of VH, CD and VH:CD, Graph pad prism 7.0 (2016 GraphPad Software, Inc., California, USA) was used to investigate associations between each diet, VH, CD, VH:CD, ADG and FCR by performing unpaired *t*-tests and determining Pearson's' correlation coefficients. When a parameter was measured for each animal such as ADFI, ADG, FCR, digestibility, liver index, and enzyme activity, the piglet was considered the statistical unit and data were analyzed according to the model $Y_{ijkmn} = \mu + \alpha_i + \beta_j + \tau_k + \eta_{m \cdot}(\beta_j) + \varepsilon_{ijkmn}$, where Y_{ijkmn} is the dependent variable (animal), μ represents the overall sample mean, α_i the dietary treatment effect (i = 1, 2, 3, 4), β_j the litter effect (1,2,..12), τ_k the effect of sex (k = female, male), $\eta_{m \cdot}(\beta_j)$ the random effect of pen (m = 1, 2,.. 12) when given same dietary treatment and ε_{ijkmn} the residual error. The model was reviewed for each group of parameters; when no effect on nutrient digestibility was shown, the variable sex was excluded from the model. Litter and pen showed no significant effect on the liver index and were therefore removed from the statistical model for this analysis. In statistical analyses of fecal dry matter and fecal score, pen constituted the experimental unit. Diets were included as explanatory effects according to the following model $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$. For the gene expression analysis of nutrient transporters in jejunum, a two-sample *t*-test was performed to investigate differences between the control and yeast groups. Based on chemical composition, it was suspected that there was an error in the batch of the diet CU20 that included the digestibility marker, thus the results from this diet were removed from the statistical analysis. This included ADG, FCR, fecal dry matter between days 14 and 28, AID, ATTD, trypsin activity, and intestinal morphometry.

3. Results

3.1. General health and growth performance

There was no mortality during the experimental period. During the first three days of the experiment, fecal score was 1 for all pens (n = 12). There were some occurrences of diarrhea (fecal score ≥3) during the experimental period, especially during the second week. The average fecal score for the overall period were 2.3, 2.1, 2.1 and 2.1 for the control, CU10, CU20, and CU40 diets respectively (P = 0.563). During the second week of the experiment, the average fecal score were 2.7, 2.8, 2.6 and 2.4 for the control, CU10, CU20, and CU40 diets respectively (P = 0.550). During the fourth week of the experiment, the average fecal score for the pigs fed the control diet was higher compared to the pigs fed the yeast-containing diets (2.4 for the control group vs. 2.1 and 1.8 for the CU10 and CU40 groups respectively; P = 0.044). Fecal dry matter (%) increased linearly with increasing levels of dietary yeast (P = 0.001) at day 7 of the experimental period (18.3 for the control group vs. 22.5, 24.1, 27.7 for CU10, CU20 and CU40, groups respectively) and was higher for pigs fed the CU40 diet compared with the pigs fed the control diet (30.4 vs. 27.1; P = 0.027) at day 21. The liver index increased in piglets fed the CU40 diet compared with piglets fed the control diet (3.20 vs. 2.89; P = 0.022). The yeast diet CU40 tended to improve FCR during the last two weeks of the experiment (P = 0.099). In general, no statistical differences among dietary treatments were observed in ADG, ADFI, and FCR (Table 4).

3.2. Digestibility and enzymatic activity

Results for the apparent digestibility of AA and other nutrients are shown in Tables 5 and 6, respectively. There were no differences in AID of dry matter, CP or most AA among diets, however, AID of methionine and alanine tended to be highest in the CU40 diet (P = 0.064, P = 0.084). AID of ash was higher in the group fed the CU40 diet (P = 0.001). AID of starch tended (P = 0.096) to be lower in the CU10 diet. ATTD of CP of the CU40 diet was higher than the control (P = 0.034). ATTD of neutral detergent fiber was lower for the CU40 diet compared with the control (P = 0.006). ATTD of crude fat was

Table 4

Effects of dietary *Candida utilis* on the growth performance of weaned piglets[a]

Item	n	Diet[b]				SEM[c]	*P*-value
		Control	CU10	CU20	CU40		
Initial BW[d], kg	48	11.08	11.06	11.13	11.00	0.12	0.986
Final BW[d], kg	48	21.07	20.46	19.62	20.64	0.25	0.203
Average daily gain, g							
Day 0–14	47	181	175	208	195	13.09	0.297
Day 14–28	36	486	482	–	516	17.89	0.338
Overall period	36	334	328	–	352	12.15	0.335
Average daily feed intake, g							
Day 0–14	47	275	263	294	278	10.89	0.233
Day 14–28	36	639	651	–	651	16.44	0.834
Overall period	36	457	457	–	467	11.67	0.790
FCR[e], g/g							
Day 0–14	47	1.59	1.53	1.49	1.43	0.074	0.460
Day 14–28	36	1.32	1.38	–	1.27	0.033	0.099
Overall period	36	1.38	1.41	–	1.33	0.030	0.228

[a] Results are given as least square means. Values with a distance from the grand mean larger than 3 times the interquartile range were excluded from the analysis.
[b] Control diet (Control); diet with 10% crude protein from *Candida utilis* (CU10); diet with 20% crude protein from *C. utilis* (CU20); diet with 40% crude protein from *C. utilis* (CU40).
[c] SEM, pooled standard error of the means.
[d] BW, live body weight.
[e] FCR, feed conversion ratio, calculated as feed: gain.

Table 5

Effects of feeding diets with up to 40% crude protein from *Candida utilis* on the apparent ileal digestibility of amino acids in weaned piglets.[a]

Item	n	Diet[b]			SEM[c]	*P*-value
		Control	CU10	CU40		
Apparent ileal digestibility,%						
Arg	35	84.0	84.2	85.9	0.87	0.234
His	36	81.9	81.1	83.3	0.98	0.307
Ile	35	80.7	80.0	80.2	1.07	0.868
Leu	35	83.1	82.8	83.5	0.92	0.863
Lys	36	87.3	87.1	88.8	0.75	0.255
Met	36	90.9	90.6	92.3	0.52	0.064
Phe	36	82.9	81.7	82.9	0.95	0.567
Thr	36	81.8	80.4	78.3	1.17	0.131
Trp	36	83.7	84.0	84.3	0.72	0.830
Val	36	81.4	80.1	80.9	1.09	0.719
Ala	36	74.9	74.4	78.5	1.31	0.084
Asp	36	75.9	75.0	78.5	1.21	0.127
Cys	36	72.8	72.3	72.7	1.51	0.974
Glu	36	86.0	86.5	87.6	0.93	0.463
Gly	36	60.3	56.0	61.8	4.50	0.647
Pro	35	74.5	77.1	74.3	2.42	0.679
Ser	36	79.1	77.8	77.5	1.24	0.621
Tyr	36	71.7	70.8	71.9	1.35	0.829

[a] Results are shown as least square means. Values with a distance from the grand mean larger than 3 interquartile range were excluded from the analysis.
[b] Control diet (Control); diet with 10% crude protein from *Candida utilis* (CU10); diet with 40% crude protein from *C. utilis* (CU40).
[c] SEM, standard error of the mean.

higher for the CU40 diet compared with CU10 (P = 0.035). ATTD of ash increased in the CU10 and CU40 diets relative to the control diet (P < 0.001). ATTD of phosphorus increased in the CU40 diet compared with the control (P < 0.001). No differences in trypsin activity among dietary treatments were observed (P = 0.812). Numerical means for trypsin activity for the control, CU10 and CU40 diets were 2.20, 2.45 and 2.43 U/mg protein respectively.

3.3. Messenger RNA (mRNA) expression of nutrient transporters in jejunum

Results for expression of nutrient transporters are shown in Table 7.

Table 6

Effects of dietary *Candida utilis* on the apparent ileal digestibility and apparent total tract digestibility of nutrients in weaned piglets.[*]

Item	Diet[**]					P-value
	n	Control	CU10	CU40	SEM[***]	
Apparent ileal digestibility,%						
Dry matter	36	73.1	74.1	73.6	1.33	0.865
Crude protein ($N \times 6.25$)	36	76.7	76.1	78.8	1.40	0.389
Starch	34	98.2	98.0	98.6	0.20	0.096
Ash	36	39.9[a]	45.3[a]	54.9[b]	2.29	0.001
Apparent total tract digestibility,%						
Dry matter	35	83.2	83.5	83.9	0.28	0.264
Starch	35	99.7	99.7	99.7	0.02	0.545
Crude protein ($N \times 6.25$)	35	78.3[a]	79.8[ab]	80.0[b]	0.44	0.034
Crude fat	35	71.0[ab]	69.4[a]	74.4[b]	1.25	0.035
Neutral detergent fiber	35	36.1[a]	33.2[ab]	25.5[b]	2.10	0.006
Ash	35	55.0[a]	59.1[b]	59.6[b]	0.66	< 0.001
Ca	34	61.0	63.0	63.6	1.19	0.219
P	35	51.0[a]	54.1[a]	58.0[b]	0.92	< 0.001
Gross energy	35	82.4	83.0	83.2	0.28	0.164

a-b Means within a row with different superscripts differ ($P < 0.05$).

[*] Results are given as least square means. Values with a distance from the grand mean larger than 3 times the interquartile range were excluded from the analysis.

[**] Control diet (Control); diet with 10% crude protein from *Candida utilis* (CU10); diet with 40% crude protein from *C. utilis* (CU40).

[***] SEM, pooled standard error of the means.

The expression of the selected genes was not affected by dietary treatments. Although statistically not different between treatments, the gene with the highest expression in CU40 fed pigs compared with the control was the *intestinal alkaline phosphatase* encoding a digestive brush-border enzyme, while the *fatty acid binding protein 6*, regulating uptake, transport, and metabolism of fatty acids, had the lowest expression in pigs fed the CU40 diet compared with the pigs fed the control diets.

3.4. Morphology

Macroscopic evaluation of the pigs revealed a mild to moderate hyperkeratosis of the cutaneous mucosa of the ventricle in all animals and was independent of the dietary treatments. Sixteen of 48 pigs (33.3%) had peritonitis, 13 of these pigs (27.1%) had chronic peritonitis comprising mild fibrous thickening of peritoneum over cecum and colon, and three pigs (6.3%) presented signs of active inflammation with hyperemia and sparse amounts of small fibrin flakes. A navel abscess was observed in one animal and a small abscess was observed on the thigh of another animal. Two pigs (4.2%) presented mild chronic thickening of the mitral valve. Renal cysts were observed in two animals (4.2%). *Staphylococcus aureus* and *Streptococcus dysgalactiae* were isolated from a bacterial culture of the navel abscess. Histomorphological evaluation of the lungs demonstrated very mild to mild, multifocal to diffuse, subacute interstitial pneumonia in forty-five of the piglets (93.8%). Seven of twelve animals in the control group and

Table 8

Effects of dietary *Candida utilis* on the intestinal morphometry of weaned piglets.[a]

Item	Diets[b]				P-value
	Control	SEM[c]	CU40	SEM[c]	
Jejunum					
VH (µm)	430.8	20.86	520.6	21.60	0.007
CD (µm)	356.2	12.15	342.4	13.55	0.455
VH:CD	1.22	0.06	1.53	0.06	0.001
Ileum					
VH (µm)	409.9	20.43	414.9	10.86	0.047
CD (µm)	314.3	9.29	286.5	5.69	0.018
VH:CD	1.31	0.07	1.45	0.04	0.089

[a] VH, villus height; CD, crypt depth. Results are given as means of three to six observations per gut segment per piglet, $n = 24$.

[b] Control diet (Control); diet with 40% crude protein from *Candida utilis* (CU40).

[c] SEM, standard error of the mean.

one of twelve animals in the CU40 group had very mild multifocal hepatitis, with infiltrations of few aggregates of neutrophils, lymphocytes, and macrophages multifocally in the liver parenchyma. No specific findings were observed in the myocardium and kidney samples.

3.5. Intestinal morphometry

Results for intestinal morphometry are presented in Table 8. VH, CD, and VH:CD were compared between the control and CU40 diets. Pigs fed the CU40 diet had longer VH in the jejunum ($P = 0.007$) and ileum ($P = 0.047$) compared with pigs fed the control diet. VH:CD in jejunum increased in pigs fed the CU40 diet compared with the control diet ($P = 0.001$). The ileal CD measurements differed between these two feeding groups. Ileal crypts were deeper in the control group than in the group receiving the CU40 diet ($P = 0.018$). Ileal VH was negatively correlated with FCR (Fig. 1) in pigs fed the CU40 diet ($r = -0.61$, $P = 0.035$). No correlation between ileal VH and FCR was found in the control group.

4. Discussion

The reliance on imported protein-rich ingredients in Norway has attracted increased interest in the research and development of competitive locally-produced protein sources. The present study demonstrated the potential of *C. utilis* yeast as an alternative protein source to soybean meal, fishmeal, rapeseed meal, and potato protein concentrate in pelleted diets for weaned piglets. Weaning is a critical life-stage for piglets because they are exposed to several stress factors (social, nutritional and immunological) that frequently result in diarrhea and reduced growth performance. This study showed that it is possible to replace 40% of CP by using *C. utilis* in commercial-like diets for weaned piglets while maintaining ADG and FCR. Similar results in growth performance have been reported in other studies using yeast as a protein source for pigs or Atlantic salmon (*Salmo salar*), while others have

Table 7

Jejunal expression of genes involved in the regulation of nutrient uptake in piglets fed diets with 40% crude protein from *Candida utilis* compared with the control diet as measured by quantitative real-time PCR.[a]

Item				Gene[b]					
	FABP1	FABP2	FABP6	ALPI	SGLT1	PEPT1	MCT1	GLUT2	GLUT4
Mean, $-\Delta\Delta Ct$	0.23	−0.23	−1.00	0.37	−0.08	−0.40	0.30	−0.01	0.24
P-value	0.43	0.52	0.62	0.23	0.81	0.21	0.15	0.97	0.57

[a] Results are presented as mean $-\Delta\Delta Ct$ ($n = 8$) relative to the control group ($n = 7$). Transcriptional levels of selected genes were normalized to *β-actin*.

[b] FABP1, Fatty acid binding protein 1; FABP2, Fatty acid binding protein 2; FABP6, Fatty acid binding protein 6; ALPI, Alkaline phosphatase, intestinal; SGLT1, Sodium-glucose cotransporter 1; PEPT1, Peptide transporter 1; MCT1, Monocarboxylate transporter 1; GLUT2, Glucose transporter type 2; GLUT4, Glucose transporter type 4.
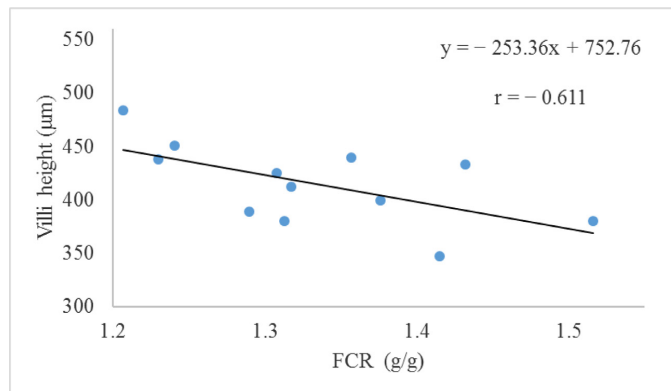
Fig. 1. Relationship between average ileal villi height and overall feed conversion ratio (FCR), in piglets fed diets with 40% crude protein from *Candida utilis*. FCR, Feed conversion ratio, g feed intake per g weight gain; ($n = 12$). 95% confidence interval -0.8776 to -0.0584; $P < 0.05$.

reported improved growth performance. Improvement in growth performance may occur because of increased ADFI (Lezcano et al., 2013) due to enhanced palatability of the feed (Ati et al., 2009). Hu et al. (2014) observed improved FCR of piglets fed diets containing 8% CP derived from baker's yeast (AB Yestex), but they did not observe differences in ADFI among piglets fed the control compared with those fed the yeast-based diets. Øverland et al. (2013) reported no difference in ADFI, growth rate or FCR between Atlantic salmon fed a fishmeal-based control diet and a test diet containing 28.3% *C. utilis*, replacing 40% of the CP. However, at high levels, yeast may reduce the palatability of the diet, as shown in a study with diets containing up to 75% dried brewers' yeast fed to rainbow trout (*Oncorhynchus mykiss*) and consequently lower feed intake (Rumsey et al., 1991a). In a study with broiler chickens, the partial replacement of soybean meal with 10% vinasse yeast resulted in a higher weight gain compared with the control diet, while at the higher inclusion levels of 20 or 30%, the addition of yeast to diets led to reduced weight gain and increased FCR (Lezcano et al., 2013). Unlike the findings in the present study, Lezcano et al. (2013) observed that feed intake increased by including 10, 20 and 30% of *C. utilis* yeast in diets compared with the control.

Yeast cell-walls are rich in mannooligosaccharides, which can bind glycoprotein receptors in pathogenic bacteria and limit their attachment to the intestinal mucosa (Refstie et al., 2010). This mechanism promotes the passage of pathogenic bacteria through the intestine without causing infection and reduces the consumption of dietary protein by pathogenic bacteria, which could otherwise be available for digestion and absorption (Ewing and Cole, 1994; Kogan and Kocher, 2007). In the present study, providing 40% of yeast CP resulted in higher ATTD of CP of pigs fed the CU40 diets compared with the control. The improved ATTD of CP could be a result of the observed increased VH and VH:CD, which indicates an increased intestinal absorption area in the piglets fed the CU40 diet compared with the piglets fed the control diets. In our study, the dietary treatments did not induce differences in the expression of the nine selected genes involved in nutrient sensing and transportation, however, an increase in VH:CD can be associated with higher expression of genes coding for nutrient transporters. Heim et al. (2015) found a connection between increased VH:CD in ileum and higher expression of nutrient transporter *sodium-glucose cotransporter 1* in piglets, implying improved absorption ability. In this study, no differences in the expression of *sodium-glucose cotransporter 1* were observed in the jejunum.

The activity of digestive enzymes may be affected by yeast, either directly or indirectly, although differences for trypsin activity were not observed in our study. Live yeast can provide digestive enzymes, favoring efficient digestion of complex carbohydrates which could

potentially also exert a positive effect on protein, fat and mineral digestibility (Øverland and Skrede, 2017). However, the enzymes provided by *C. utilis* in this experiment were likely inactivated during the downstream processing of this ingredient. The increased ash digestibility in the diets containing *C. utilis* compared with the control diet could be due to a high bioavailability of the minerals in the yeast. In accordance with our results, Kim et al. (2000) reported higher phosphorus digestibility in boars fed diets containing brewers' yeast. Improved mineral digestibility may have been related to increased VH (Heidarieh et al., 2013). The lower digestibility of the neutral detergent fiber in diets with yeast, especially in the CU40 diet compared with the control, could be due to the cell wall of the yeast, which constitutes on average 29% of dried yeast cells (Nguyen et al., 1998), and possesses low digestibility (Rumsey et al.,1991b).

Growth performance relies on healthy intestinal tissue capable of absorbing nutrients in the amounts necessary to meet the nutritional requirements for maintenance and growth. VH, CD, and VH:CD measurements in jejunum and ileum can be used as indicators of general intestinal function and health. Our results showed longer VH in yeast-fed piglets compared with the control group and inversely the CD was shorter in the pigs fed the yeast-based diet CU40 compared to the control group. Heidarieh et al. (2013) discussed the relationship between improved FCR and increased VH in pigs and Shen et al. (2009) concluded that dietary yeast culture supplementation at 0.5% had a positive effect on growth performance of nursery pigs by improving jejunal VH and VH:CD. The longer VH in piglets fed yeast-based diets in our study could be suggestive of a reduced contact between pathogenic bacteria and the intestinal wall in the yeast-fed group, and thus less damage to the villi, compared with the pigs fed the control diet. Deeper intestinal crypts in the control pigs fed the control diet could be the result of increased cell proliferation to repair damaged villi tissue, caused by the adherence of intestinal pathogens to the intestinal mucosa in these pigs. Due to the adsorbing properties of *C. utilis*, this might have been prevented in the yeast fed pigs (Ewing and Cole, 1994; Kogan and Kocher, 2007). Alternatively, increased VH and consequently VH:CD could be related to the modulating effect of yeast in gut immune responses (Shen et al., 2009). Shen and co-workers demonstrated a comparable effect of 0.5% of yeast culture supplementation and antibiotic growth promoters on the growth performance of nursery pigs, which provides evidence for the yeast's ability to counteract pathology and promote health. We thus speculate that the mechanisms involved in the intestinal health effects of dietary *C. utilis* are mainly immune-and-microbial modulation as previously described in other studies with pigs (Hahn et al., 2006; Shen et al., 2009) and fish (Siwicki et al., 1994).

Pathogens and inflammatory processes in the intestine may interfere

with the efficiency of nutrient absorption. Repartitioning of energy from growth to inflammation and immune-stimulation processes may, in turn, lead to reduced animal performance (Fox et al., 2005; Grammes et al., 2013). Mannooligosaccharides, β-glucans and nucleic acids in *C. utilis* may contribute to improve intestinal health (Refstie et al., 2010), and reduce inflammation (Grammes et al., 2013), which could explain the increased intestinal absorption surface indicated by our study. This explanation is further supported by the correlation between FCR and the ileal mucosal-surface area, found in our study where the FCR decreased with increasing ileal VH in the CU40 group.

Post-weaning diarrhea is often associated with a decrease in productive performance in piglets and can be assessed in herds by subjective fecal score or determination of fecal dry matter. These methods provide indications though they are not standard methods to determine diarrhea (Pedersen et al., 2011). The linear increase in fecal dry matter at day 7 with increasing levels of yeast in the diets suggest an improvement of the intestinal health status.

The CP content in *C. utilis* attracted our interest as a potential and competitive protein source when compared to soybean meal. *C. utilis* used in the present study contained 48% CP (on dry matter basis) and had a high content of threonine, but a low content of methionine, cysteine, and arginine when compared with other commonly used protein sources in Norwegian pig diets. However, the low content of the mentioned essential AA was considered when formulating the diets by adjusting to a similar AA level by addition of crystalline AA. The protein level in *C. utilis* was on average similar to those reported by Martin et al. (1993) (52.0%) and Olvera-Novoa et al. (2002) (46.1%), while Øverland et al. (2013) reported higher CP level (59.8%). *C. utilis* is also rich in nucleic acids, which are known to have positive effects on intestinal development and regeneration (Mateo et al., 2004). Endogenous nucleotides depleted during stressful periods, such as weaning, may be restocked by nucleic acid-rich compounds such as yeast, which in turn may have a role in preventing losses in growth performance, common for pigs during this life-stage (Mateo et al., 2004). In our experiment, *C. utilis* may have to some extent contributed to maintain the nucleic acid balance in the intestine and promote better intestinal health, also expressed by the increased VH. These results agree with the documented positive effects of *C. utilis* (Grammes et al., 2013) and *S. cerevisiae* (Refstie et al., 2010) on intestinal health.

5. Conclusions

Replacing up to 40% of CP from the traditional protein sources with CP from the yeast *C. utilis* in piglet diets had no effect on feed intake and growth rate of the piglets while the ATTD of CP was improved. Adding *C. utilis* to diets also resulted in longer intestinal villi, increased VH:CD and improved fecal consistency in the piglets. These findings suggest that *C. utilis* can replace 40% of CP from the main protein sources traditionally used in Norway while maintaining growth performance and improving digestive function.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Paper II

Paper II

1   *Cyberlindnera jadinii* **yeast as a protein source for growing pigs: effects on protein and**

2   **energy metabolism**

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12

13 **Abstract**

14 Inactivated *Cyberlindnera jadinii* yeast (previously classified as *Candida utilis*) produced

15 from local lignocellulosic biomass-based sugars is an interesting alternative protein source in

16 diets for young pigs. The objective of this study was to evaluate the effects of diets containing

17 *C. jadinii* on the nitrogen and energy metabolism and apparent total tract digestibility (ATTD)

18 of major nutrients and energy in young pigs. Twenty-four intact boars with a mean initial

19 body weight of 16.69 ± 4.45 kg were fed one of four diets: a conventional control diet for

20 young pigs with soybean meal, fish meal, rapeseed meal and potato protein concentrate as

21 major protein sources or one of three experimental diets containing 10, 20, and 40 *%* of CP

22 from *C. jadinii* (CU10, CU20 and CU40, respectively). The pigs were equally distributed to

23 the dietary treatments according to initial body weight (BW) and litter, comprising a total of

24 six replicates per diet. The experiment was carried out during three periods of 14 days, each

25 divided into a period of adaptation in stables (day 1 to 7) followed by an adaptation period in

26 metabolism cages (day 8 to 10), preceding the balance and respiration experiments (days 11 to

27 15). Each period included an energy and nitrogen balance experiment of four consecutive

28 days including a 22 h respiration experiment by means of indirect calorimetry. Adding *C.*

29 *jadinii* to diets did not affect the growth performance or the ATTD of nutrients and energy in

30 the diets. The energy and nitrogen metabolism was not affected by partially replacing the

31 main protein sources with *C. jadinii*. Collectively, the results indicate that CP from *C. jadinii*

32 can replace up to 40% of dietary CP from conventional protein sources while maintaining the

33 efficiency of nitrogen and energy metabolism in young pigs.

34

35 **Keywords:** nitrogen, energy, metabolism, total tract digestibility, yeast, growing pig

Introduction

Protein production in Europe is currently insufficient to supply the demands of the livestock sector (de Visser et al., 2014; Roman et al., 2016). In addition, the prices of locally produced protein-rich ingredients offer little competition to those available through imports, leading to a heavy reliance on imported protein sources, such as soybean meal (SBM) (Tallentire et al., 2018; van Krimpen et al., 2013). Identifying alternative ingredients is, therefore, crucial to maintaining sustainability in agriculture. The use of new technology allows the production of yeast from local non-food biomass that does not compete directly with human food, offering potential to improve self-sufficiency and sustainability (Schader et al., 2015; Øverland and Skrede, 2017). *Cyberlindnera jadinii* (previously classified as *Candida utilis*) grown on sugars from lignocellulosic biomass, has high crude protein (CP) with a favorable amino acid composition, resembling high-quality protein sources such as SBM. Yeasts are additionally rich in proteins, mannans, *β*-glucans (Kogan and Kocher, 2007; Kollár et al., 1997) and nucleotides (Bacha et al., 2013; Halász and Lásztity, 1991), which can improve growth rate and gut function in young pigs (Cruz and Håkenåsen et al., 2019; Mateo et al., 2004; Mateo and Stein, 2004; Shurson, 2018). Yeast is commonly used as a feed additive (< 1 % of the formulation), but less information is available on yeast as a potential protein source in pig diets. In comparison, fish meal (FM) is a high-quality protein source commonly used in Europe, however, the availability of this resource is limited (Hardy, 1996; Tallentire et al., 2018). Furthermore, rapeseed meal (RSM) is partially available in northern Europe but has a lower nutritional value in terms of CP and essential amino acids (Van Zanten et al., 2015) and a higher content of fiber and glucosinolates compared with SBM (Mejicanos et al., 2016; Pérez de Nanclares et al., 2017). Field beans and peas may also be used in conventional diets but they have lower CP (22 to 38 %) compared to conventional protein sources (Griffiths and Lawes, 1978) and are associated with antinutritional factors (Moseley and Griffiths, 1979).

61 Presently, limited information exists about the utilization of energy and nitrogen (N) of pigs

62 fed diets with moderate inclusion levels of *C. jadinii, i.e.* 10 to 40 % of the dietary CP. Energy

63 balance measurements are interesting to evaluate for newly introduced feed ingredients

64 (Noblet, 2007) and N balance measurements provide valuable information about the

65 utilization of dietary protein for retention in body tissues. It was questioned if protein and

66 energy from *C. jadinii* can be equally utilized by pigs as those provided by conventional high-

67 quality protein sources. Therefore, we hypothesized that *C. jadinii* is a viable alternative

68 protein source that can replace up to 40 % of the CP from conventional protein sources in

69 diets for young pigs while N and energy utilization, growth and digestive performance are

70 maintained. The objectives of this study were, thus, to evaluate the effects of diets containing

71 up to 40 % of CP from *C. jadinii,* which replaced the main protein sources in pig diets by

72 measuring 1) the N and energy metabolism and 2) the apparent total tract digestibility

73 (ATTD) of major nutrients and energy among pigs.

74

75 **1. Materials and Methods**

76 All animals were handled and cared for in accordance with the Animal Welfare Act of 28

77 December 2009, and the research protocol complied with the guidelines of The Animal

78 Experiments Inspectorate, Ministry of Environment and Food, Copenhagen, Denmark,

79 regarding animal experimentation and care (license no. 2015-15-0201-00685).

80

81 *1.1.   Animals and facilities*

82 Twenty-four crossbred-intact boars (Danish Landrace × Yorkshire) were obtained from a

83 commercial herd and housed at the experimental facilities at Rørrendegård experimental farm,

84 Taastrup, Denmark. The experiment was carried out with four experimental diets during three

85 consecutive periods, with two pigs per diet per period and a total of six replicates per diet.

86 Each animal was fed and weighed individually and thus each pig constituted an experimental

87 unit. Each set of piglets per period comprised two sets of four litter-mates. Each litter-mate

88 was allotted to one of the four dietary treatments. The piglets were equally distributed to the

89 dietary treatments according to initial body weight (BW). The three periods were divided into

90 a seven-day period of adaptation in stables (where the animals adapted to the experimental

91 facilities and diets) followed by a three-day adaptation period in metabolism cages, which

92 preceded the balance and respiration experiments. Each balance period lasted for four days

93 and it comprised an energy and N balance experiment of four consecutive days including a

94 22-h respiration experiment by means of indirect calorimetry. The BW was measured

95 individually at four time-points: at the start of the adaptation period (day 1), at the end of the

96 adaptation period (day 7), at the start of the balance period (day 11) and at the end of the

97 balance period (day 15). Mean BW at the beginning of the adaptation period was (mean ± SD)

98 16.69 ± 4.45 kg and the pigs were on average 54 ± 4.3 days old. Straw bedding was provided

99 to ensure the comfort of the animals during the adaptation period in pens, while during the

100 balance period, rubber mats were placed inside the metabolism cages. Environment enriching

101 toys were available for each pig throughout the experiment. Fecal score was registered daily

102 and for each pen (n = 4) during the adaptation period and for each pig after the pigs were

103 transferred to the metabolism cages, on a scale from 1 to 4, according to consistency and

104 shape (1 = dry and hard; 2 = normal "sausage-shaped"; 3 = loose stools, pasty consistency

105 with some loss of normal shape; 4 = watery diarrhea with complete loss of normal shape) to

106 monitor the occurrence of diarrhea and loose stools (fecal score ≥ 3) (Pedersen and Toft,

107 2011). Room temperatures (°C) in the adaptation stables and the metabolism room were 17.6

108 ± 0.97 and 20.7 ± 1.42, respectively.

109

110

1.2. Diets and feeding

Cyberlindnera jadinii (LYCC 7549; Lallemand Yeast Culture Collection) was obtained from Lallemand Inc, Salutaguse, Estonia. Sugars used in the growth media for *C. jadinii* were obtained from lignocellulosic biomass of Norway spruce trees (*Picea abies*) by using the BALI (Borregaard Advanced Lignin) process at Borregaard AS, Sarpsborg Norway (Patent "Lignocellulosic biomass conversion by sulfite pretreatment"; EP2376642B1 EP Grant) as described by Øverland and Skrede, (2017) and Sharma et al. (2018). *C jadinii* was inactivated and drum-dried prior to being included in the diets.

The dietary treatments consisted of one control diet and three diets containing 3.6 %, 7.3 % and 14.6 % of *C. jadinii*, which partially replaced conventional protein sources: fishmeal, rapeseed meal, soybean meal, and potato protein concentrate. Crude protein (CP) in the diets was replaced at the rates of 0, 10, 20 and 40 % respectively, based on the standardized ileal digestibility (SID) values of CP and of essential AA for pigs in the conventional feedstuffs. The SID of CP and AA for *C. jadinii* was estimated based on the SID of CP for brewer's yeast (*Saccharomyces cerevisiae*) in pigs (Centraal Veevoederbureau, 2005). All diets were thus formulated to be isonitrogenous and isoenergetic and to meet or exceed pig nutritional requirements for energy, amino acids, and all other nutrients. The diets (Table 1) were formulated by Felleskjøpet Fôrutvikling, A.S. (Trondheim, Norway) in cooperation with NMBU and produced at Fôrtek (Aas, Norway). The feed mixture was pelleted through a 3.5 mm die and representative samples of each diet were collected and analyzed for chemical composition (Table 2). Feed production was described by (Cruz and Håkenåsen et al., 2019). Pellet durability index (%) was 96.6, 97.0, 97.7, and 98.1 for the control, CU10, CU20, and CU40 diets, respectively.

During the adaptation period, animals were offered a commercial weaner diet (Nutrimin A/S, Ans, Denmark), which was gradually replaced by 50, 75 and 100 % of the respective

136 experimental diets during the first three days. The animals were fed individually, twice daily

137 at 0800h and 1500h. Feed amounts were adjusted daily to *ad libitum* based on the initially

138 estimated feed intake of 2 to 4 % of BW. Water was provided *ad libitum* via automatic

139 drinkers. Individual feed leftovers were collected after each meal and recorded daily for

140 calculating average daily feed intake. Average daily gain (ADG) and feed conversion ratio

141 (FCR) were calculated from the individual BW and feed intake. A cumulative feed sample

142 from each diet was collected for analysis of DM, ash, starch, N, crude fat, neutral detergent

143 fiber, gross energy and amino acids (AA).

144

145 ### *1.3. Experimental procedures*

146 During the balance period and respiration experiments, the pigs were kept individually, in

147 stainless steel metabolism cages (1.65 × 0.75 m) with devices for quantitative collection of

148 feces, urine and feed residues which were collected quantitatively daily (between 0800h and

149 1200h) during the balance periods. Urine was collected in flasks containing 30 ml of 5 %

150 sulfuric acid solution. Citric acid (1 % solution) was used to rinse the metabolic cages and

151 was collected separately at the end of each collection. All samples of feces, urine, feed

152 residues, and citric acid solutions were frozen at − 20 °C after the collections and thawed at

153 the end of each period. Feces were individually homogenized, and urine, feed residues, and

154 citric acid solutions were individually mixed. All samples were subsampled: approximately

155 20 % of feces and 10 % of urine were frozen at − 20 °C; feed residues and citric acid rinse

156 solution were sampled representatively and frozen at − 20 °C. Feed samples of each diet were

157 collected daily, pooled per period and frozen at − 20 °C.

158 The individual respiration measurements were performed in two open-air-circuit respiration

159 chambers with a volume of 3500 L. Construction and function of the respiration chambers

160 have been described by Chwalibog et al. (2004). The airflow through the chambers and the

161   concentrations of O_2, CO_2 and CH_4 in the out-going air from each chamber were recorded

162   automatically every third minute. Gas recovery tests were performed for CO_2 and O_2 in both

163   chambers with the following results: Chamber A CO_2 = 1.0968 and O_2 = 1.0757; Chamber B

164   O_2 = 1.0016 and O_2 = 1.0333. The temperature was set at 20 °C, the relative humidity varied

165   between 60 and 65 % and 12 h light-dark cycles were operated. All pigs were continuously

166   monitored by video surveillance during the respiration experiments.

167   At the end of each experimental period, the animals were euthanized by captive bolt pistol

168   and exsanguination.

169

170   *1.4.   Analytical methods*

171   Fecal samples were freeze-dried. Feces and diets were ground to pass a 1 mm sieve before

172   chemical analysis. Feed residues were analyzed for dry matter content (DM). It was assumed

173   that the chemical composition of the DM was equal to that in the DM of the feed. Wet fecal

174   samples were analyzed for N and DM. Diets and freeze-dried fecal samples were analyzed for

175   DM, ash, crude fat, and gross energy. Diets were additionally analyzed in triplicate for amino

176   acids (AA) including tryptophan. Urine and citric acid rinse samples were analyzed for N

177   content. The DM was measured by drying to constant weight at 105 °C. Ash was determined

178   by incineration at 525 °C. The N content was determined by the Kjeldahl-N method using the

179   Tecator-Kjeltec system 1030 (Tecator AB, Höganäs, Sweden). Fat content was determined by

180   petroleum ether extraction in a Soxtec system 2043 (Foss, Hillerød, Denmark) after HCl

181   hydrolysis. The gross energy in feed and feces was determined using an IKA Calorimeter

182   system (IKA GmbH and Co. KG, Staufen, Germany). Besides the above analyses, the diets

183   were analyzed for neutral detergent fiber as described by Mertens (2002), using the

184   Ankom200 Fiber Analyzer (ANKOM Technology, Macedon, New York, USA). Amino acids

185   were determined on a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd, Cambridge, UK).

186   Tryptophan was analyzed (method EC 152/2009) on a Dionex UltiMate 3000 HPLC system

187   (Dionex Softron GmbH, Germering, Germany) with a Shimadzu RF-535 fluorescence

188   detector (Shimadzu Corporation, Kyoto, Japan). Starch was hydrolyzed with α-amylase and

189   amyl glucosidase-enzymes to glucose, and glucose concentration was determined using a

190   spectrophotometer (MaxMat PL II Multianalyzer, France) as described by McCleary et al.

191   (1994).

192

193   *1.5.   Calculations*

194   The CP content was calculated as $N \times 6.25$. Total carbohydrate and lignin (CHO + L) content

195   in the diets and feces was calculated as $CHO + L = DM - ash - CP - crude\ fat$. The apparent

196   total tract digestibility (ATTD) of nutrients and energy was calculated as ATTD $(\%) = [($

197   nutrient intake $-$ nutrient in feces) / nutrient intake $] \times 100$ (Maynard and Loosli, 1969).

198   Nutrient intake was calculated based on feed intake (feed intake = amount of feed provided $-$

199   feed residues) and the analyzed nutrient content of the feed. Nutrients in feces were calculated

200   based on the total weight of the feces collected and the analyzed nutrient content in fecal

201   samples.

202   Retained nitrogen (RN) was calculated as RN (g) = IN $-$ UN $-$ FN $-$ NCA, where, IN is the

203   ingested nitrogen, UN the urinary nitrogen, FN the fecal nitrogen, and NCA the concentration

204   of nitrogen in the citric acid rinse solution. Digestible nitrogen (DN, g) was calculated as DN

205   = IN $-$ FN. Energy in urine (UE, kJ) was calculated as UE = 53.5 kJ/g $\times$ UN (g) (Chwalibog

206   et al., 2004). Energy in methane (ECH_4, kJ) was calculated as ECH_4 = 39.6 kJ/l $\times$ CH_4 (l)

207   (Brouwer, 1965). Metabolizable energy intake (MEi, kJ) was calculated as MEi = GEi $-$ FE $-$

208   UE $-$ ECH_4, where GEi is the gross energy intake. Heat production (HE) was calculated

209   based on the 22-h measurements of gas exchange and the mean UN according to Brouwer

210   (1965) as HE (kJ) = 16.18 (kJ/l) $\times$ O_2 (l) + 5.02 (kJ/l) $\times$ CO_2 (l) $-$ 2.17 (kJ/l) $\times$ CH_4 (l) $-$ 5.99

kJ/g × UN (g), where 22-h volumes of O_2, CO_2, and CH_4 were extended to 24 h by multiplication with 24/22. Retained energy (RE, kJ) was calculated as RE = ME − HE. Energy retained as protein (RPE, kJ) was calculated as RPE = RN (g) × 6.25 × 23.86 (kJ/g) and energy retained as fat (RFE, kJ) as RFE = RE – RPE. The respiratory quotient (RQ) was determined as RQ = CO_2 production / O_2 consumption. The oxidation of protein, carbohydrate and fat were calculated according to Chwalibog et al. (1992) and validated for RQ values below and above one (Chwalibog and Thorbek, 1995).

1.6.   Statistical Analysis

Statistical analysis of growth performance, ATTD, metabolism of N and energy were performed using the general linear model multivariate procedure of SPSS statistics software v25 (IBM Corporation, Armonk, New York, 2017). Fixed effects of diet (n = 4) and period (n = 3), were included in the statistical model for the growth performance, ATTD, and N and energy metabolism analyses. Interactions between diet and period were investigated and removed from the model when non-significant. Dietary treatment means were separated using the estimated marginal means (an equivalent of the least square means) option of the general linear model. Pearson's' correlation was used to investigate relationships between initial BW and the ATTD of nutrients. Effects were considered significant if $P < 0.05$.

2. Results and discussion

2.1.   General health and growth performance

Pigs generally remained healthy throughout the experiment, however, there were some occurrences of loose stools (fecal score > 2), but no watery diarrhea was observed (fecal score = 4). Means for DM of feces (%) during the balance period were 26.2, 27.8, 31.4 and 28.4 for pigs fed the control, CU10, CU20, and CU40 diets (P = 0.112). Fecal scores of the pigs were

236   not affected by dietary treatment ($P > 0.1$). On the contrary, feeding piglets increasing levels

237   of dietary *C. jadinii,* replacing up to 40 % of the CP, induced a linear increase in fecal DM

238   (Cruz and Håkenåsen et al., 2019), possibly explained by the positive effects of the

239   mannooligosaccharides, *β*-glucans and nucleic acids present in the yeast-cells on the intestinal

240   function of piglets (Kogan and Kocher, 2007; Mateo et al., 2004; Mateo and Stein, 2004).

241   In general, ADG, feed intake, and FCR of the pigs were not affected by dietary treatment ($P >$

242   0.1, Table 3). Nevertheless, there was an effect of period on those values ($P < 0.01$) which

243   reflected differences in initial BW among periods ($P < 0.001$; Table 3), with the exception of

244   FCR, that was not affected by period during the balance experiment ($P > 0.1$). The pigs in

245   period 1 were heavier than the pigs in period 2 and 3 ($P < 0.01$), and pigs in period 2 were

246   heavier than in period 3 ($P < 0.05$) but initial BW did not differ among dietary treatments ($P =$

247   0.994). Results that support our findings have previously been reported by Øverland et al.

248   (2010) who concluded that bacterial protein meal (BPM) could replace 41 % of the dietary CP

249   for piglets without compromising growth performance, and more recently, Cruz and

250   Håkenåsen et al. (2019) who found no overall changes in growth performance of piglets fed

251   up to 40 % CP from the same *C. jadinii*. However, hydrocarbon-grown *S. cerevisiae* has

252   previously replaced 6 to 29 % of CP in FM-based diets for pigs, resulting in a tendency for

253   better ADG and FCR in the pigs fed yeast-containing diets compared with the pigs fed FM

254   control diets (Barber et al., 1971).

255

256   ***2.2.   Apparent total tract digestibility (ATTD)***

257   The ATTD of DM, organic matter (OM), N, ash, crude fat, carbohydrates and energy in

258   young pigs fed the control diet and diets with different levels of *C. jadinii* (Table 4), was not

259   affected by dietary treatment ($P > 0.1$). The ATTD of DM, OM, crude fat, carbohydrates and

260   energy increased with initial BW (Pearsons' r = 0.57, 0.60, 0.58, 0.58, 0.60; P < 0.005), while

261   the ATTD of N and ash was unaffected by initial BW (P > 0.1).

262   The ATTD of N and energy in this study was generally higher for all diets compared with the

263   ATTD of N in pigs fed diets with up to 40 % CP from *C. jadinii* (Cruz and Håkenåsen et al.,

264   2019), which might be related to lower initial BW and younger age of the pigs in the previous

265   study. The ATTD of N was generally lower in our study (81.7 vs. 85.0 %) compared with

266   Skrede et al. (1998), where the sole source of protein for pigs was BPM (88 % *Methylococcus*

267   *capsulatus*). The ATTD of N in pigs fed 6 to 29 % of total CP from hydrocarbon-grown *S.*

268   *cerevisiae* was not affected (Barber et al., 1971), which agreed with our results. The ATTD of

269   N in high-RSM diets was lower (Pérez de Nanclares et al., 2017) than that in the yeast-

270   containing diets in our experiment, which is likely due to the high content of fiber in the diets

271   based on RSM. However, as protein digestibility varies with protein intake (Noblet et al.,

272   2004), comparisons of ATTD between studies may be difficult (Eggum, 1973; Fan et al.,

273   1994). The ATTD of N increases with N intake when the intake of CP is low, as a result of a

274   relatively lower loss of endogenous N through feces. The ATTD of energy in the *C. jadinii*-

275   based diets in our study was higher than in pig diets with 18.7 to 56.0 % of CP from RSM

276   (Pérez de Nanclares et al., 2017), despite the slightly lower CP and gross energy levels in our

277   diets. The ATTD of energy was also higher in pig diets in our study compared with pig diets

278   containing 17.5 to 52.5 % of CP from BPM (Hellwing et al., 2007), despite the similar energy

279   level but higher CP content compared with the diets in the present study.

280   The positive correlation between the initial BW of the pigs and the ATTD of DM, OM,

281   energy, crude fat, and carbohydrates in this study, supports evidence that the ATTD of

282   nutrients increases with BW in young pigs, which, can be associated with age differences in

283   these pigs. This is further supported by an increasing trend in the activities of pancreatic

284   trypsin, chymotrypsin, and amylase with increasing age of the pigs from d 35 to 56 observed

by Jensen et al. (1997). The apparent digestibility of crude fiber and N in pigs increases with age (from 60 to 150 days), due to changes in the microbiota populations of the gut over time (Niu et al., 2015). Differences in the ATTD of nutrients between this study when pigs were 63 to 72 days old and the previous when pigs were on average 53 days old (Cruz and Håkenåsen et al., 2019) are likely to be explained by the lower age of the pigs at the time-point for sample collections. Alternatively, the generally high ATTD of nutrients compared with the study by Cruz and Håkenåsen et al. (2019), where digestibility was obtained by the inert marker method, using yttrium oxide, could be related to differences between the marker and total collection methods. However, ATTD measured with yttrium oxide was similar compared with total collection measured in minks (Vhile et al., 2007) and it was previously demonstrated that ATTD is highly correlated between pigs and minks fed BPM (Skrede et al., 1998). Thus, it is suggested that the differences in ATTD observed between the present study and that by Cruz and Håkenåsen et al. (2019) are more likely due to differences in age rather than the method. In similarity to our study, the ATTD of nutrients in growing-pig diets with 17.5 to 52.5 % of CP from BPM gradually replacing SBM, was not affected by the dietary treatments (Hellwing et al., 2007). The ATTD of OM, fat, carbohydrates + lignin, and energy was lower in pig diets with 18.7 to 56.0 % of CP from RSM (Pérez de Nanclares et al., 2019), most likely due to the high content in fiber in the RSM-based diets. In broiler chickens, the ATTD of fat was found to generally increase with age (Tancharoenrat et al., 2013), which agrees with our findings in pigs. The estimate of ATTD of carbohydrates + lignin was based on the calculated values for carbohydrate content, calculated from the analytical values for dry matter, ash, CP and crude fat in the diets and feces. This leads to accumulation of all analytical errors on the carbohydrate value (Neil, 1978), reducing the accuracy of that value, compared to those of the other nutrients here evaluated.

2.3. Nitrogen metabolism

Nitrogen metabolism in young pigs fed the experimental diets (Table 5) did not differ among dietary treatments or among periods ($P > 0.1$). The RN of the pigs in our experiment was correlated with initial BW (Pearson's $r = 0.81$; $P < 0.01$), which agrees with the study conducted by Barber et al. (1971). This relationship can be explained by an intensive growth in piglets after weaning, often accompanied by increasing N retention until the maximum capacity for N retention is reached (Tauson et al., 1998). It would be relevant to investigate the maximum N retention capacity for older cross-bred pigs, in further studies.

Retained N was similar in growing pigs fed diets with 6 to 29 % of CP from hydrocarbon-grown *S. cerevisiae* yeast compared to pigs fed FM-based control diets (Barber et al., 1971), and was similar among pigs fed diets with up to 52.5 % of CP from BPM, (Hellwing et al., 2007) and pigs fed control diets, which supports our results. However, RN was generally higher in the pigs in our study compared with the pigs fed BPM (Hellwing et al., 2007), which concurs with a comparably lower UN, and FN in the pigs in our study.

Yeast cells contain more non-protein N in the form of nucleic acids than most conventional protein sources, which can influence N digestibility and thus N metabolism (Skrede et al., 1998). This may reflect the slightly lower RN in the pigs fed the yeast-containing diets in our study and in the pigs fed BPM (Hellwing et al. 2007) compared with pigs fed control diets, indicating an increase in nucleic acids not directly available for protein synthesis in the diets, as a consequence of adding incremental levels of these microbial ingredients. The nucleic acid content of the yeast was not measured in our study but it has been previously reported that ribonucleic and deoxyribonucleic acids comprise approximately 7 to 9 % of the composition of *C. jadinii* (Castro et al., 1971; Maul et al., 1970) equivalent to approximately 10 % of the N in *C. jadinii*, while they comprise < 0.1 % of the composition of other major protein sources

334 (Mateo et al., 2004; Mateo and Stein, 2004) and 1 % of the total N in FM (Barber et al.,

335 1971).

336 Pigs fed diets with 18.7 to 56.0 % of the CP from RSM (Pérez de Nanclares et al., 2019),

337 ingested and digested less N, and excreted more N than the pigs in our experiment, which

338 explains the higher RN in our experiment compared with the previous. The ratio RN : DN is a

339 measure of the efficiency of utilizing DN for retention, and this was higher (80 to 83 vs. 64%)

340 for the pigs in our study than those fed diets with 17.5 to 52.5 % CP from BPM (Hellwing et

341 al. 2007). Ratios RN : IN and RN : DN were also generally higher in pigs in our experiment

342 compared with the pigs fed RSM-based diets (Pérez de Nanclares et al., 2019) resulting from

343 higher RN in the present experiment. The higher RN : DN in the present experiment, as

344 compared to the previous studies (Hellwing et al., 2007; Pérez de Nanclares et al., 2019)

345 might be explained by the use of intact pigs, which have higher capacity for N retention than

346 castrated pigs (e.g. Tauson et al., 1998). Additionally, rubber mats with openings were used in

347 the metabolism cages in this experiment, to improve the comfort of the pigs, and this may

348 have contributed to some loss of nitrogenous material compared with previous studies without

349 mats.

350

351 ### 2.4.   *Energy metabolism and substrate oxidation*

352 Energy metabolism, measured by the consumption of O_2, and the production of CO_2 and of

353 CH_4 (Table 6) did not differ among dietary treatments ($P > 0.1$). Period tended to affect DE

354 ($P = 0.065$) and ME ($P = 0.064$) intakes, which can be explained by an effect of initial BW as

355 discussed above. The DE, ME, HE and RE and energy retained as protein did not differ

356 between pigs fed the control and pigs fed yeast-containing diets, agreeing with the findings of

357 Hellwing et al. (2007), despite the slightly higher energy retained as protein in the present

358 study. The DE and ME were, however, lower for pigs fed diets with 18.7 to 56.0 % of CP

359   from RSM (Pérez de Nanclares et al., 2019) compared with the pigs in our study, which is

360   likely associated with the higher content of fiber in barley and RSM, compared with wheat,

361   and *C. jadinii*. Additionally, those pigs produced higher amounts of methane and CO_2 (Pérez

362   de Nanclares et al., 2019) compared to the pigs fed yeast-based diets in our study. The lower

363   methane production in the present experiment compared with the previous experiment by

364   Pérez de Nanclares et al. (2019) might be explained by an increase in the passage of

365   undigested nutrients to the hindgut and a higher fermentation rate by intestinal microflora, due

366   to the higher fiber content in the barley and RSM-based diets.

367   Mean oxidized protein (7 to 8 % of HE) and mean oxidized carbohydrates (92 to 93 % of HE)

368   were similar among dietary treatments ($P > 0.1$). Net oxidation of fat was zero because all

369   pigs had RQ values > 1, indicating *de novo* lipogenesis from dietary carbohydrates

370   (Chwalibog and Thorbek, 2000). Thus, in previous studies with zero net oxidation of fat, HE

371   was mainly made up by carbohydrate oxidation (85 %) while protein oxidation made up the

372   remaining (15 %) (Chwalibog et al., 1998). In our experiment carbohydrate oxidation was

373   higher and protein oxidation was lower than the values from Chwalibog et al. (1998). The

374   differences in substrate oxidation between experiments may also be caused by differences in

375   collection procedures where perforated rubber mats in the metabolism cages may have caused

376   losses on nitrogenous material in this study. Few studies have been performed to evaluate the

377   metabolism of protein and energy in pigs fed diets containing microbial protein thus, further

378   assessments with *C. jadinii* as a protein source for pigs are encouraged.

379

380   **3. Conclusions**

381   Replacing up to 40 % of CP from SBM, FM, RSM, and potato protein concentrate with

382   inactivated *Cyberlindnera jadinii* yeast CP in diets for young pigs had no adverse effects on N

383   and energy metabolism or on feed intake, growth rate, general animal health and performance,

384   compared with the control diet. The ATTD of dietary nutrients and energy was not affected

385   by dietary treatment but was affected by the initial BW of the piglets. Altogether, the results

386   suggest that partially replacing conventional protein sources with inactivated *C. jadinii* yeast

387   in diets for young pigs is possible without compromising energy and protein metabolism.

388

389   *Conflict of interest statement*

390   The authors declare that they have no conflict of interest.

391

397

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545

546

547 **Table 1**

548 Dietary composition and calculated content of nutrients and energy of experimental diets for
549 young pigs with increasing levels of inactivated *Cyberlindnera jadinii* yeast.
550

Ingredients, g/kg	Diets[1]			
	Control	CU10	CU20	CU40
Wheat	624	616	608	593
Barley	100	100	100	100
Oats	50	50	50	50
Cyberlindnera jadinii[2]	0	36	73	146
Soybean meal[3]	80	65	50	19
Fish meal[4]	20	16	13	5
Potato protein concentrate[5]	38	30	23	9
Rapeseed meal[6]	20	16	12	5
Rapeseed oil	22	22	23	25
Sodium chloride	6	6	6	5
Monocalcium phosphate	13	14	14	16
Limestone	9	9	9	9
Iron (Fe)	0.4	0.4	0.4	0.4
Vitamin + trace-mineral premix[7]	4.2	4.2	4.2	4.2
L-Lysine	6.3	6.3	6.1	5.8
L-Methionine	2.1	2.3	2.5	3.0
L-Threonine	2.8	2.8	2.6	2.4
L-Valine	1.0	1.0	1.0	1.0
L-Tryptophan	0.9	0.9	0.9	1.0
Calculated content				
Net energy[8] (MJ/kg)	9.94	9.94	9.94	9.94
Crude protein g/kg	170	170	170	170
Digestible protein (SID) g/kg	140	140	140	140
Digestible Lys (SID) g/kg	12.0	12.0	12.0	12.0
SID Met g/kg	4.6	4.7	4.8	4.9
SID Thr g/kg	7.6	7.6	7.6	7.6
SID Val g/kg	8.0	8.0	8.0	8.0
SID Try g/kg	2.6	2.6	2.6	2.6
Crude protein from *C. jadinii* (%)	0.0	10.0	20.1	40.3

551 [1]Control diet; diet with 10 % crude protein (CP) from *Cyberlindnera jadinii* (CU10); diet with 20 % CP from *C. jadinii*
552 (CU20); diet with 40 % CP from *C. jadinii*. (CU40).
553 [2]Dried, inactivated: dry matter (DM) 970 g/kg, CP (N × 6.25) 470 g/kg, crude fat 16 g/kg, ash 78 g/kg, gross energy 19.9
554 MJ/kg; essential amino acid content in g/16g N: 24.4 Arg, 8.5 His, 21.6 Ile, 31.6 Leu, 30.6 Lys, 5.2 Met, 18.4 Phe, 25.6 Thr,
555 25.9 Val, 6.2 Trp.
556 [3]Non-GMO soybean meal, Denofa AS, Fredrikstad, Norway: DM 881 g/kg, CP 458 g/kg, crude fat 10 g/kg, ash 56 g/kg,
557 neutral detergent fiber (NDF) 89 g/kg, gross energy 17.5 MJ/kg.
558 [4]Norsildmel AS, Egersund, Norway: DM 917 g/kg, CP 684 g/kg, crude fat 73 g/kg, ash 145 g/kg, NDF 5 g/kg, gross energy
559 19.4 MJ/kg.
560 [5]Cargill, Denmark: DM 914 g/kg, CP 725 g/kg, crude fat 30 g/kg, ash 20 g/kg, gross energy 21.8 MJ/kg.
561 [6]Expeller pressed rapeseed meal, Mestilla, UAB, Klaipeda, Lithuania: DM 889 g/kg, CP 350 g/kg, crude fat 88 g/kg, ash 59
562 g/kg, NDF 161 g/kg, gross energy 19.1 MJ/kg.
563 [7]Provided per kg of diet: 120 mg Fe ($FeSO_4$); 60 mg Mn (MnO); 120 mg Zn (ZnO); 26 mg Cu ($CuSO_4$); 0.60 mg I (Ca
564 (IO_3)); < 0.3 mg Se; 8000 IU vitamin A; 45 mg dl-α-tocopheryl acetate; 105 mg ascorbic acid; 1500 IU cholecalciferol; 4.64
565 mg menadione; 3 mg thiamin; 5.63 mg riboflavin; 45 mg niacin; 15 mg pantothenic acid; 20 μg cyanocobalamin.
566 [8]Calculated based on Centraal Veevoederbureau (2005).

567

568 **Table 2**

569 Analyzed chemical composition of experimental pig diets with increasing levels of inactivated
570 *Cyberlindnera jadinii* yeast.

Item, g/kg	Diets[1]			
	Control	CU10	CU20	CU40
Dry matter	877	914	881	887
Crude protein	183	180	174	176
Crude fat	39	48	48	49
Starch	443	448	455	458
Ash	48	51	49	50
Carbohydrates + lignin[2]	608	635	610	612
Neutral detergent fiber	97	96	96	85
Gross energy (MJ/kg)	16.5	16.8	17.3	16.7
Essential AA[3] (g /16g N)				
Arg	9.3	9.1	8.8	8.7
His	3.7	3.6	3.5	3.4
Ile	7.1	6.8	6.8	6.6
Leu	12.5	12.1	11.7	11.2
Lys	13.1	13.0	12.8	12.3
Met	4.4	4.5	4.5	4.7
Phe	7.9	7.6	7.4	7.0
Thr	9.5	9.6	9.3	9.5
Val	9.5	9.3	9.2	9.1
Trp	2.8	2.9	2.9	2.8
Non-essential AA (g/16g N)				
Ala	7.2	7.3	7.3	7.8
Asp	14.4	13.8	13.2	12.6
Gly	7.6	7.4	7.2	7.0
Glu	35.0	34.8	34.7	34.3
Cys	2.6	2.5	2.4	2.2
Tyr	3.1	3.3	3.1	3.1
Pro	11.9	11.7	11.6	10.9
Ser	8.5	8.6	8.3	8.4

571 [1]Control diet; diet with 10 % crude protein (CP) from *Cyberlindnera jadinii* (CU10); diet with 20 %
572 CP from *C. jadinii* (CU20); diet with 40 % CP from *C. jadinii* (CU40).
573 [2]Calculated as carbohydrates + lignin = dry matter - ash - CP - crude fat
574 [3]Amino acids.

575

Table 3

577 Growth performance of young pigs fed diets with increasing levels of inactivated
578 *Cyberlindnera jadinii* yeast.[1]

Performance	Diets[2]				SEM[3]	*P*-value	
	Control	CU10	CU20	CU40		diet	period
Initial BW	16.97	16.64	16.58	16.58	1.13	0.994	< 0.001
Final BW	25.27	24.56	23.75	23.93	1.43	0.873	< 0.001
Weight gain (g/day)							
Overall	593	566	511	525	34.1	0.338	0.002
Week1	428	472	351	352	43.1	0.163	< 0.001
Week 2	758	660	672	698	63.7	0.708	0.001
Feed intake (g/day)							
Overall	858	831	792	802	49.1	0.778	< 0.001
Week 1	533	542	472	481	43.0	0.578	0.007
Week 2	1169	1107	1195	1104	68.5	0.865	< 0.001
FCR[4]							
Overall	1.46	1.46	1.55	1.52	0.05	0.494	< 0.001
Week 1	1.64	1.29	1.58	1.42	0.25	0.754	< 0.001
Week 2	1.66	1.70	1.65	1.60	0.11	0.940	0.125

579 [1]Values presented as least square means (n = 24); BW, body weight (kg).
580 [2]Control diet; diet with 10 % crude protein (CP) from *Cyberlindnera jadinii* (CU10); diet with
581 20 % CP from *C. jadinii* (CU20); diet with 40 % CP from *C. jadinii* (CU40).
582 [3]SEM, pooled standard error of the means.
583 [4]FCR, feed conversion ratio, calculated as feed intake (g) : BW gain (g).
584

585 **Table 4**

586 Effects of increasing dietary levels of inactivated *Cyberlindnera jadinii* yeast on the apparent

587 total tract digestibility (ATTD) of nutrients and energy in young pigs.[1]

ATTD, %	Diets[2]				SEM[3]	P-value	
	Control	CU10	CU20	CU40		Diet	Period
Dry matter	87.2	86.9	87.7	87.6	0.5	0.611	0.053
Organic matter	88.5	88.3	89.1	89.0	0.4	0.538	0.020
Nitrogen	81.7	80.4	82.5	81.7	1.0	0.525	0.590
Ash	63.0	62.3	65.1	64.2	2.1	0.789	0.812
Crude fat	79.1	81.7	81.0	82.5	1.0	0.160	0.013
Carbohydrates + lignin	91.2	91.1	91.6	91.6	0.3	0.560	0.003
Energy	86.3	86.2	87.2	87.0	0.5	0.447	0.036

588 [1]Values are presented as least square means ($n = 24$).
589 [2]Control diet; diet with 10 % crude protein (CP) from *Cyberlindnera jadinii* (CU10); diet with
590 20 % CP from *C. jadinii* (CU20); diet with 40 % CP from *C. jadinii* (CU40).
591 [3]SEM, pooled standard error of the means.
592
593
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596

597 **Table 5**

598 Effects of increasing dietary levels of inactivated yeast *Cyberlindnera jadinii* on the nitrogen
599 metabolism in young pigs.[1]

Item, g/kg$^{0.6}$	Diets[2]				SEM[3]	*P*-value	
	Control	CU10	CU20	CU40		Diet	Period
Ingested nitrogen	5.46	5.08	5.09	5.37	0.23	0.540	0.093
Digested nitrogen	4.46	4.09	4.20	4.39	0.21	0.572	0.184
Excreted nitrogen	1.78	1.82	1.76	1.80	0.11	0.979	0.083
Fecal nitrogen	0.997	0.987	0.893	0.985	0.06	0.550	0.076
Urinary nitrogen	0.758	0.790	0.837	0.795	0.09	0.934	0.659
UN:FN[4]	0.777	0.808	0.950	0.818	0.10	0.619	0.849
Retained nitrogen	3.705	3.307	3.362	3.592	0.19	0.405	0.198
RN:IN[5]	0.677	0.652	0.660	0.668	0.02	0.716	0.509
RN:DN[6]	0.830	0.808	0.800	0.818	0.02	0.674	0.683

600 [1]Values are presented as least square means, ($n = 24$).
601 [2]Control diet; diet with 10 % crude protein (CP) from *Cyberlindnera jadinii* (CU10); diet with
602 20 % CP from *C. jadinii* (CU20); diet with 40 % CP from *C. jadinii* (CU40).
603 [3]SEM, pooled standard error of the means.
604 [4]UN, urinary nitrogen; FN, fecal nitrogen.
605 [5]RN, retained nitrogen; IN, ingested nitrogen.
606 [6]DN, digested nitrogen.

607

608 **Table 6**

609 Effects of increasing levels of inactivated *Cyberlindnera jadinii* yeast on the energy
610 metabolism and substrate oxidation in young pigs.[1]

Item, kJ/kg$^{0.6}$	Diets[2]				SEM[3]	*P*-value	
	Control	CU10	CU20	CU40		Diet	Period
Digestible energy	2646	2623	2651	2798	122.1	0.736	0.065
Metabolizable energy	2602	2576	2604	2749	120.2	0.737	0.064
Heat production	1317	1399	1297	1272	73.0	0.645	0.749
Retained energy	1285	1177	1306	1477	121.7	0.398	0.146
RE:ME[4], *%*	49	46	50	53	3.0	0.482	0.467
Energy retained in protein	550	487	497	532	27.4	0.352	0.224
Energy retained in fat	736	691	809	945	102.4	0.345	0.169
O_2 consumed (l/kg$^{0.60}$)	60.17	63.63	59.52	58.03	3.46	0.706	0.801
CO_2 produced ((l/kg$^{0.60}$)	69.35	74.53	67.72	67.28	3.74	0.515	0.399
CH_4 produced (l/kg$^{0.60}$)	0.09	0.09	0.05	0.09	0.03	0.689	0.562
Respiratory quotient	1.16	1.17	1.14	1.16	0.03	0.927	0.114

611 [1]Values are presented as least square means ($n = 24$).
612 [2]Control diet; diet with 10 *%* crude protein (CP) from *Cyberlindnera jadinii* (CU10); diet with
613 20 *%* CP from *C. jadinii* (CU20); diet with 40 *%* CP from *C. jadinii* (CU40).
614 [3]SEM, pooled standard error of the means.
615 [4]RE, retained energy; ME, metabolizable energy.
616
617

Paper III

Paper III

1 **YEAST AS PROTEIN SOURCE FOR BROILER CHICKENS**

2

3 *Cyberlindnera jadinii* **yeast as a protein source for broiler chickens:**

4 **effects on growth performance and digestive function**

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12

13 **Metabolism and Nutrition**

14

ABSTRACT

This study evaluated the effects of inactivated yeast *Cyberlindnera jadinii* (previously classified as *Candida utilis*) grown on local lignocellulosic sugars on the growth performance and digestive function of Ross 308 broiler chickens. A thousand male chicks were allocated to 20 pens (experimental units). Four diets were randomly allocated to the pens in a total of five replicates with 200 birds per replicate. The diets consisted of one conventional wheat-oat-soybean meal-based control diet, and three diets with increasing levels of *C. jadinii* replacing 10, 20 and 30 % crude protein (CP) from soybean meal. In general, the results have shown that feed intake and weight gain of the birds decreased linearly, and feed conversion ratio increased linearly ($P < 0.01$) with increasing levels of *C. jadinii* in the diets. However, growth performance and feed intake were similar between birds fed control diets and diets with 10 % *C. jadinii* CP. Mortality rates and litter quality were not affected by dietary treatments. The apparent ileal digestibility (AID) of dry matter, crude fat, organic matter, and carbohydrates was higher in control diets than diets with 30 % *C. jadinii* CP ($P < 0.05$), and linearly decreased ($P < 0.01$) with incremental levels of dietary *C. jadinii*. The AID of CP, starch, ash, and phosphorus was unaffected by dietary treatments. Ileal villus height at day 10 was maintained in birds fed diets with 30 % *C. jadinii* CP compared with the birds fed control diets but was lower for birds fed diets with 10 and 20 % *C. jadinii* protein ($P < 0.05$). To conclude, up to 10 % *C. jadinii* CP can replace soybean meal CP in broiler chicken diets, maintaining growth performance and digestive function of the birds. Higher levels of *C. jadinii* may decrease bird performance. Altogether, this study suggests the potential of *C. jadinii* as a local-based protein source, to partially replace soybean meal in broiler chicken diets, contributing to a more sustainable feed.

39

40 **Keywords:** *Cyberlindnera jadinii*, yeast, broiler chicken, growth performance, digestive

41 function, alternative protein source

42

43

INTRODUCTION

Global poultry production has rapidly increased over the last decades and the demand for poultry meat is expected to increase in the next years. On average, a European person currently consumes around 25 kg of poultry meat annually (OECD and FAO, 2018). Europe is heavily dependent on imported feed protein sources such as soybean meal (SBM), but to increase self-sufficiency, it is important to develop local sustainable high-quality protein sources. Recently, there is an increased interest in alternative protein feedstuffs, such as the inactivated yeast *Cyberlindnera jadinii* (previously classified as *Candida utilis*) produced on sugars from lignocellulosic biomass such as Norwegian spruce tree (*Picea abies*; Øverland and Skrede, 2017). *C. jadinii* is a potentially sustainable alternative protein source with a low carbon footprint compared with soy protein concentrate (Couture et al., 2019). Yeasts have mostly been used in animal feed in small amounts as a feed additive for their functional properties, but limited information exists on the use of yeast as a protein source. Different yeast strains such as *C. jadinii, Saccharomyces cerevisiae,* and *Kluyveromyces marxianus* have recently been evaluated as replacement of high-quality fish meal in diets for Atlantic salmon (*Salmo salar*), with best results for *C. jadinii*-containing diets, due to a high crude protein (CP) content of 56 % and a high CP digestibility (Øverland et al., 2013). Extensive knowledge exists about the use of *C. jadinii* as a feed additive (Shurson, 2018). Moderate levels of *C. jadinii* in diets for monogastric animals (20 to 40 % of the CP), have shown to increase feed intake and growth rate in broiler chicks (Rodriguez et al. 2013), improve digestive function, and maintain growth performance in weaning piglets (Cruz and Håkenåsen et al., 2019). In addition, 40 % of CP from *C. jadinii* in diets have shown to maintain growth performance in Atlantic salmon (*Salmo salar*) (Øverland et al., 2013) compared with a fish meal-based control. *C. jadinii* also contains a wide range of bioactive components such as *β*-glucans, mannooligosaccharides and nucleic acids (Maul et al., 1970; Nguyen et al., 1998; Kogan and Kocher, 2007) shown to improve digestive function and health of farm animals,

70　especially during critical life stages (Gopalakannan and Arul, 2009; Grammes et al., 2013;

71　Cruz and Håkenåsen et al., 2019; Øvrum Hansen et al., 2019). Altogether, this suggests that

72　*C. jadinii* can serve as a potential local-based and sustainable protein ingredient. A study was,

73　therefore, performed to evaluate the effects of *C. jadinii* grown on lignocellulosic sugars on

74　production parameters and intestinal morphometry of broiler chickens, when gradually

75　replacing SBM in broiler chicken diets, providing indications about the future use of yeast as

76　a potential protein source.

77

78　**MATERIALS AND METHODS**

79　All birds were handled and cared for according to local welfare laws and regulations (the

80　Animal Welfare Act of 28 of December 2009 and the local legislation derived from the

81　directive 2010/63 EU of the European Parliament and Council of 22 September 2010 on the

82　protection of animals used for scientific purposes).

83

84　*Diets and experimental design*

85　The diets were formulated to meet or exceed the requirements for protein, essential amino

86　acids and all other nutrients (NRC, 1994). All diets were isoenergetic, isonitrogenous, equal in

87　digestible lysine, methionine + cysteine, threonine, tryptophan, arginine per energy unit, and

88　equal in the concentration of Na, Ca, P. Additionally all diets were free from coccidiostats

89　(Tables 1 and 2). The dietary treatments consisted of four diets for the starter period (d 0 to 9)

90　and four diets for the grower period (d 10 to 30), for both periods including a control diet

91　based on wheat, oats and soybean meal (**Control**) and three diets where 10, 20 and 30 *%* of

92　the CP originating from SBM was replaced by CP from *C. jadinii* (**CJ10, CJ20,** and **CJ30**).

93　The experimental diets were produced at the Center for Feed Technology (FôrTek), Aas,

94　Norway. The starter and grower diets were pelleted with a diameter of 2.5 and 3.5 mm,

95 respectively at the minimum pelleting temperature of 82 °C for microbial control. Each diet

96 was added 0.06 % titanium dioxide (TiO_2) as a digestibility marker. Representative samples

97 of main raw materials and finished feed were collected in duplicate from each diet. Pellet

98 hardness was evaluated by pellet durability index (Holmen NHP200, UK) in three replicates

99 per diet, at the Feed quality Lab, Norwegian University of Life Sciences, Aas, Norway.

100 The 1,000 male Ross 308 broiler chickens were distributed in 20 pens of 5.3 m^2, each

101 containing 50 birds, constituting a total of five replicates per diet. The mean initial BW was

102 42.0 g ± 0.75 SD in all treatments. All birds had *ad libitum* access to feed and water. Bedding

103 composed of wood shavings was renewed weekly. The starter feed was provided from day 0

104 until day 9 and the grower feed from day 10 to slaughter (day 30). Accumulated feed intake

105 was recorded for each pen weekly and live BW were registered for each pen weekly and at

106 slaughter. Room temperature was kept on average at 32, 28, 25 and 23 °C from days 1 to 7, 8

107 to 14, 15 to 21, and 22 to 28 respectively. During the first 24 h the birds were kept with 23 h

108 light: 1 h darkness and during the remaining period (d 2 to 30) with 16 h light: 8 h darkness

109 cycles. The relative humidity (%) was in average 26.3, 35.9, 39.0 and 41.6 for days 1 to 7, 8

110 to 14, 15 to 21, and 22 to 28, respectively. Litter quality was scored weekly for each pen on

111 days 4, 11, 18, 25 and 28 on a 0 to 5 scale according to the visual and tactile estimation of

112 water content, where 1 was considered completely dry and 5 completely wet. The number of

113 dead birds was recorded daily for all pens and water intake was measured daily for 10 of the

114 pens. At slaughter (d 30) all birds were inspected for general health (foot lesions, systemic

115 diseases, ascites) and the carcass weights (eviscerated) were registered individually.

116

117 ***Yeast microbial ingredient***

118 *Cyberlindnera jadinii* (LYCC 7549; Lallemand Yeast Culture Collection) was produced by

119 Lallemand Inc, Salutaguse Estonia. Second generation sugars were obtained from

120 lignocellulosic biomass of Norway Spruce trees (*Picea abies*) by using the Borregaard

121 advanced lignin process at Borregaard AS, Sarpsborg Norway (Patent "Lignocellulosic

122 biomass conversion by sulfite pretreatment"; EP2376642B1 EP Grant). The C5 and C6 sugars

123 were used in the growth media for the yeast, as described by Øverland and Skrede (2017), and

124 Sharma et al. (2018). The chemical composition of *C. jadinii* is presented as a footnote in

125 Table 1.

126

127 *Sample collection*

128 Ten birds per diet were euthanized by cervical dislocation on d 10 and d 28 for sample

129 collection. The abdominal cavity was sectioned caudal to the sternum and the ileum, ceca, and

130 Meckel's diverticulum was located and exposed. On d 10, tissue from duodenum, jejunum,

131 and ileum was collected from 10 birds per dietary treatment, for intestinal morphometry

132 measurements. Four-to-five-cm pieces of distal duodenum, jejunum, and ileum were

133 collected, rinsed, and placed in buffered formalin solution (10 %). On d 28 individual ileal

134 content samples were collected from Meckel's diverticulum to the ileocecal junction, for

135 measurement of apparent ileal digestibility (AID) of nutrients of 10 birds per diet. The digesta

136 samples were placed into locked plastic containers, preserved on ice for transportation and

137 stored at − 20 °C until freeze-drying.

138

139 *Chemical analysis*

140 The chemical composition of the experimental diets and the raw materials are presented in

141 Table 2. Ileal digesta samples from two birds per pen were pooled before analysis (n = 5 pens

142 per diet) due to the small quantity of each individual sample, to ensure enough material for

143 chemical analysis. The pooled samples were ground through a 1 mm and 0.5 mm screen,

144 homogenized and analyzed in triplicate for dry matter, ash, starch, nitrogen, crude fat, and

145   TiO_2. Diets were freeze-dried, ground at 1 mm and 0.5 mm (0.5 mm for nitrogen analysis),

146   homogenized and analyzed in duplicate for dry matter, ash, crude protein, starch, crude fat

147   and in triplicate for phosphorus and TiO_2. Dry matter, ash, and AA were determined

148   according to the methods described in the European Commission Regulation (EC) No

149   152/2009. Nitrogen was analyzed with the Pregl-Dumas method: freeze-dried ileal content

150   and diets submitted to combustion at 1,150°C (Elemental Analysesysteme GmbH, Hanau,

151   Germany) and N_2 molecules are separated and measured by thermal-conductivity detection.

152   Crude fat was determined using Accelerated Solvent Extraction (ASE350, Dionex

153   Corporation, Sunnyvale, CA, USA): feed samples were extracted with 70 % petroleum-ether

154   and 30 % acetone at 125 °C; ileal samples were extracted with 80 % petroleum and 20 %

155   acetone at 125 °C. Starch was hydrolyzed with α-amylase and amyl glucosidase-enzymes to

156   glucose, and glucose concentration was determined using a spectrophotometer (MaxMat PL II

157   Multianalyzer, France) as described by McCleary et al. (1994). Phosphorus was analyzed with

158   the spectrophotometric method, according to the Commission Regulation (EC) No 152/2009

159   and ISO 6491. Crude fiber, calcium, sodium, and potassium were analyzed at Eurofins Food

160   & Feed Testing AS, Moss, Norway. Crude fiber was analyzed according to the method

161   (Fibertec) ISO 5498. Calcium, sodium, and potassium were analyzed according to the method

162   ISO 17294-2. TiO_2 concentrations in ileal samples and diets were determined using the

163   method described by Short et al. (1996).

164

165   ***Gut morphometric indices***

166   Formalin-fixed samples were dehydrated in graded levels of alcohol, paraffin-embedded,

167   sectioned, and stained with hematoxylin-eosin. Four histological cuts per animal were used

168   for measurements. The ileal villi heights (VH) and crypt depths (CD) were measured using

169   the software ViewPoint v0.8.2.7 (Precipoint GmbH, Freising, Germany) on images captured

170   from an Olympus BX43 light microscope (Olympus Norge AS, Asker, Norway) mounted

171   with IDS UI3260CP-C-HQ 2.3 MP camera (IDS Imaging Development Systems GmbH,

172   Obersulm, Germany) using the whole-slide scanning software, MicroVisioneer

173   (MicroVisioneer Freising, Germany). The five longest and well-oriented villi in proximity to

174   well-oriented crypts were selected from each individual and micrographs were captured at 20

175   × objective magnification, and the five adjacent crypts in the same micrographs were selected

176   for measurements of CD. The VH were measured by drawing a segmented line through the

177   villus center extending from the tip of the mucosal epithelium to the villus-crypt junction. The

178   CD were measured from the villus-crypt junction to the tunica *muscularis mucosae*. Ratios

179   VH:CD were calculated using the mean VH and mean CD of five observations per animal

180   where possible, with a minimum of three observations per animal.

181

182   ***Calculations and statistical analysis***

183   Feed conversion ratio (FCR) was calculated as FCR = total feed intake (g) / total live weight

184   gain (g). Live weights at d 10 and 30 were estimated based on a growth curve $y = ax^3 + bx^2$

185    $+ cx + d$, established from average live weights for each pen at days 0, 7, 14, 21 and 28. The

186   AID of dry matter, starch, CP and crude fat was calculated as AID $(\%)$ = $(1 - ((T_{abs}F) / (T_{abs}I)$

187    $\times (N_{il} / N_f))) \times 100$, where: $T_{abs}F$ is the TiO_2 absorbance at 410 nm in the feed sample on a

188   dry matter basis; $T_{abs}I$ is the absorbance of TiO_2 at 410 nm in the ileal sample on a dry matter

189   basis; N_{il} is the concentration of a nutrient in the ileal sample on a dry matter basis; and N_f is

190   the concentration of the nutrient in the feed sample on a dry matter basis. The relationship

191   between TiO_2 absorbance at 410 nm and TiO_2 concentration in a sample was described by

192   Short et al. (1996). Statistical analyses of growth performance were performed using the

193   general linear model multivariate procedure in SPSS Statistics software, version 25.0 (IBM

194   corp., Armonk, New York, 2017). Tukey's honestly significant difference (HSD) was used for

195   multiple comparisons between dietary treatments when statistical differences ($P < 0.05$) were

196   observed. The statistical unit was the pen. Values for ADG, ADFI, and FCR were analyzed

197   according to the model $Y_i = \mu + \alpha_i + \varepsilon_{ij}$, where Y_i is the dependent variable (pen), μ represents

198   the overall sample mean, α_i the effect of the dietary treatment (i = 1, 2, 3, 4) and ε_{ij} the

199   residual error. Linear, quadratic and cubic polynomial contrasts were used to determine the

200   effects of dietary yeast protein on growth performance, digestibility, and intestinal

201   morphometry. Mortality rate (%) was calculated as MR = [(n_{t1} - n_{t2}) / n_{t1}] × 100, where n_{t1}

202   is the number of live birds at the timepoint t1, and n_{t2} the number of live birds at timepoint t2.

203   Statistical analysis of mortality rates was performed using the univariate general linear model

204   procedure in SPSS. Values are presented as means ± SD. Means and SD for litter quality

205   scores, room temperature, and relative humidity were calculated in Microsoft Excel 2016

206   using the commands "average" and "stdev.s". Differences between diet groups were

207   investigated using the general linear model procedure in SPSS statistics, considering a

208   significance level of $P < 0.05$. Mean slaughter weights were analyzed with the procedure

209   "compare means" by one-way analysis of variance in SPSS.

210

211   **RESULTS**

212   *Diets*

213   The values for the calculated composition of the diets were according to the analyzed

214   chemical composition. These are shown in Tables 1 and 2, respectively. In general, the post

215   pelleting temperatures of the pellets gradually increased in the production of starter and

216   grower diets with the addition of *C. jadinii*. Post-pelleting temperatures were 100.3, 103.7,

217   104.9 and 110.3 °C for the control, CJ10, CJ20, and CJ30 starter diets, and 92.0, 95.0, 95.0

218   and 105.0 °C for the control, CJ10, CJ20, and CJ30 grower diets, respectively. In accordance

219   with the pellet temperature, the pellet durability index increased with the addition of *C. jadinii*

220   to the diets. Pellet durability index was 92.0, 93.3, 94.6, 94.8 for the control, CJ10, CJ20, and

221   CJ30 starter diets respectively, and 87.5, 93.0, 93.4 and 95.7 for the control, CJ10, CJ20, and

222   CJ30 grower diets, respectively. Pellet durability was found to be in accordance with high-

223   quality standards.

224

225   *General health*

226   In general, the birds were healthy throughout the experiment and the mortality rate in the

227   control group was within the expected range of values for male broiler chickens in the

228   Norwegian poultry production. There was an increase in mortality with increasing levels of *C.*

229   *jadinii* in the diets, although it was not statistically significant. The overall mortality rate (d 0

230   to 30) was 4.0 *%* and the mortality rate was not affected by dietary treatment ($P = 0.168$,

231   Table 3). General health findings from the slaughter inspections are summarized in Table 4.

232   During the routine veterinarian inspection of the slaughtered birds (d 30), some of the

233   carcasses were rejected due to observed clinical signs of disease, but no statistical

234   relationships between these findings and the dietary treatments were found. There were no

235   differences in foot-pad lesions and in litter score among the dietary treatments.

236

237

238   *Growth performance*

239   The results for growth performance, water intake, and carcass weights are shown in Table 3.

240   Final live BW was lower for the birds fed the CJ20 and CJ30 diets compared with the birds

241   fed the control and CJ10 diets ($P < 0.001$) and it linearly decreased with increasing levels of

242   yeast in both the starter and the grower diets ($P < 0.001$). The BW gain was lower for birds

243   fed the CJ30 diet compared with those fed the control diet during the starter period ($P < 0.05$)

244   and was lower for birds fed the CJ20 and CJ30 diets compared with the birds fed the control

245 and CJ10 diets during the grower and overall periods ($P < 0.001$). The BW gain decreased

246 linearly with increasing levels of dietary yeast during the starter period ($P < 0.01$), grower

247 and overall periods ($P < 0.001$). Feed intake did not differ among dietary treatments during

248 the starter period, but was lower for the birds fed the CJ20 and CJ30 diets compared with

249 those fed the CJ10 and control diets during the grower period ($P < 0.001$). During the overall

250 period, feed intake was lower for birds fed the CJ30 diet compared with the control and CJ10

251 diets ($P < 0.01$). Feed intake decreased linearly with increasing levels of yeast in the diets

252 during the grower and overall period ($P < 0.001$), but not during the starter period. The FCR

253 was higher in the birds fed the CJ20 and CJ30 diets compared with those fed the control diet

254 during the starter ($P < 0.01$) and overall ($P < 0.05$) periods, whereas the FCR of birds fed the

255 CJ10 diet was similar to the birds fed the control diet during the starter period. The FCR

256 increased linearly with increasing levels of yeast in the diets during the starter ($P < 0.001$),

257 grower ($P < 0.05$) and overall ($P < 0.01$) periods. Average daily water intake did not differ

258 among dietary treatments. The carcass weights of birds fed the CJ10 diet were similar to those

259 fed the control diet ($P < 0.001$) but were lower for birds fed the CJ20 and CJ30 diets. Carcass

260 weight linearly decreased with increasing amounts of *C. jadinii* in the diet ($P < 0.001$).

261

262 ***Apparent ileal digestibility of nutrients***

263 The AID of nutrients is shown in Table 5. The AID of dry matter and crude fat was lower in

264 birds fed the CJ30 diet compared with those fed the control diet ($P < 0.05$) and decreased

265 linearly with increasing levels of yeast in the diet ($P < 0.01$). Also, the AID of carbohydrates

266 and organic matter was lower for the birds fed the CJ30 diet compared with the birds fed the

267 control diet ($P < 0.01$) and decreased linearly with increasing levels of yeast in the diets ($P <$

268 0.01). The AID of CP, starch, ash, and phosphorus did not differ among dietary treatments.

269

Gut morphometric indices

271 The results of the intestinal morphometry are shown in Table 6. Ileal VH at d 10 were similar

272 in birds fed the CJ30 diet and those fed the control diet, but lower for the birds fed the CJ10

273 and CJ20 diets ($P < 0.05$), presenting a quadratic trend ($P < 0.01$). Duodenal CD tended to

274 decrease with increasing levels of yeast in the diets ($P < 0.05$). Addition of yeast to diets led

275 to a quadratic effect on ileal absorption area ($P < 0.05$), where birds fed the control and CJ30

276 diets had the highest values for absorption area.

277

278 **DISCUSSION**

279 The present study suggests that *C. jadinii* is a potential alternative protein source that can

280 replace up to 10 % of CP from SBM in diets for broiler chickens, resulting in similar growth

281 performance and nutrient digestibility compared with birds fed control diets. The nutritional

282 composition of yeast, including protein content, may vary depending on the strain, growth

283 media and downstream processing methods (Martínez-Force and Benitez, 1995; Nasseri et al.,

284 2011; Øverland and Skrede, 2017). This further implicates that as lignocellulosic hydrolysis

285 and yeast fermentation technology is developed and optimized, the nutritional value of *C.*

286 *jadinii* in poultry diets may achieve greater potential.

287

288 ***Growth performance***

289 The effect of yeast and other microbial ingredients such as bacterial meal, on growth

290 performance of broiler chickens, seems to be dependent on the inclusion levels. Similarly, the

291 growth rate of broiler chickens was maintained when yeast produced from molasse replaced

292 15 to 30 % of total CP at the expense of SBM, whereas growth performance reduced when

293 yeast replaced 45 % of the dietary CP (Daghir and Abdul-Baki, 1977). However, Rodriguez et

294 al. (2013), reported an increase in weight gain of broiler chickens fed diets containing 20 to

295 22 % of CP from distillery-vinasse *C. jadinii* at the expense of SBM, no changes in weight

296 gain at 39 to 43 % yeast-CP inclusion level, but decreased weight gain at 59 to 65 % yeast-CP

297 inclusion level compared with the birds fed the control diets. On the contrary, feeding broiler

298 chickens with diets containing bacterial meal at the expense of SBM, replacing 68.3 %

299 (Schøyen et al., 2007a) or 33.7 % (Øverland et al., 2010) of dietary CP, increased or

300 maintained the weight gain, but reduced the feed intake, thus resulting in an improved FCR of

301 the birds. Additionally, the same strain of *C. jadinii* successfully replaced up to 40 % of the

302 CP from conventional protein sources in diets for weanling piglets, and growth performance

303 of the pigs was maintained (Cruz and Håkenåsen et al., 2019). The difference in response to

304 dietary yeast between birds and pigs could be explained by the ability of the pigs to efficiently

305 utilize nucleic acids in the yeast as components for cell-proliferation and restitution of

306 intestinal tissue (Sijben et al., 1998). Yeast contains 3 to 9 % nucleic acids (Edozien et al.,

307 1970; Castro et al., 1971; Zee and Simard, 1975), which is considerably higher compared to

308 SBM and other conventional protein sources (Mateo and Stein, 2004; Mateo et al., 2004). In

309 broiler chickens, purine nucleotides are ultimately degraded to uric acid by the enzyme

310 xanthine oxidase. Uric acid is non-enzymatically metabolized to allantoin in low amounts in

311 birds because these have a negligible uricase enzyme activity (De Boeck and Stockx, 1978;

312 Simoyi et al., 2003). Therefore, the reduction in BW gain and feed intake of birds fed diets

313 with 20 and 30 % CP from *C. jadinii* compared with birds fed the control diet in our study,

314 may be associated with the higher content of nucleic acids derived from *C. jadinii* in these

315 diets, compared with the control diet.

316 The decreased feed intake in the birds fed the yeast diets in our study could have been caused

317 by the increasing pellet hardness (Abdollahi et al., 2013). Pellet hardness increased

318 numerically with increasing amounts of yeast in the diets, assumedly due to the fine-powder-

319 form of the yeast ingredient, which may have increased friction and temperature in the feed

320   production equipment. This effect was exacerbated by the reduction of fat in the diets,

321   resultant from balancing the energy content between dietary treatments. The reduced growth

322   performance could be related to the high pellet temperature, because high conditioning

323   temperatures (> 100 °C) may additionally cause changes in protein structures, promote

324   Maillard reactions and heat damage, alter the digestibility of CP, AA, starch, and energy of

325   diets (Pahm et al., 2008; Abdollahi et al., 2010), and affect the nutritional quality of lysine,

326   cysteine, and arginine (Ljøkjel et al., 2000). All things considered, this could explain the

327   reduced feed intake and digestibility of nutrients, resulting in reduced growth performance in

328   these broiler chicks. Alternatively, reduced growth performance may be caused by the

329   immunostimulant effects of dietary yeast which causes energy to be repartitioned from growth

330   to inflammation and immune-stimulation processes, thus reducing the energy available for

331   growth (Fox et al., 2005; Grammes et al., 2013).

332   Similar to our study, feed intake of broiler chickens decreased with increasing amounts of

333   bacterial meal in diets replacing up to 33.7 % of CP (Øverland et al., 2010), which has also

334   been reported for broiler chickens fed low levels (< 2 %) of vinasse-grown *C. jadinii* at the

335   expense of SBM (Chand and Ullah Khan, 2014). Both yeast and bacterial meal are rich in

336   nucleic acids compared to conventional feed ingredients (Castro et al., 1971; Mateo and Stein,

337   2004; Mateo et al., 2004; Hellwing et al., 2007) which may increase nucleic acid content in

338   diets and can reduce feed intake in birds (Kubota and Karasawa, 1994). It was previously

339   shown that feed intake and growth in chicks may be depressed when free adenine comprises 1

340   % of the diet (Baker and Molitoris, 1974). In addition, chitin constitutes 1 to 2 % of the yeast

341   cell-wall (Kwiatkowski and Edgar, 2012) and may be associated with reduced feed intake in

342   broiler chickens by increasing gastric viscosity and thus delaying gastric emptying and

343   increasing satiety (Razdan and Pettersson, 2019). However, the measurement of the nucleic

344   acid content and chitin in yeast, feedstuffs, and diets and their effects on feed intake was not

345   the object of this study, and thus needs further investigation.

346   Interestingly, others have reported increased feed intake in broiler chicks fed microbial

347   ingredients. Feed intake increased in birds fed diets with vinasse-grown *C. jadinii* (10 to 30

348   %) compared with birds fed control diets (Rodriguez et al., 2013). Similarly, feed intake

349   increased in broiler chickens fed diets with 33.7% CP from bacterial meal compared with

350   birds fed control diets with SBM (Schøyen et al., 2007a) and linearly increased with

351   increasing levels of bacterial protein (Schøyen et al., 2007b). Increased feed intake in studies

352   with low levels of yeast (< 0.1 %) in broiler chicken diets, could be related to increased

353   palatability caused by nucleic acids in the yeast, in a similar way as it causes flavor enhancing

354   in human food. However, birds do not possess such an acute sense of taste and pellets are

355   rapidly swallowed (Owens, 2005; Owens and Mccracken, 2007). To our knowledge, no

356   studies are available relating feed intake and palatability of yeast in broiler chickens, thus this

357   requires further investigation.

358   In similarity to our study, some studies have shown that FCR increases with increasing levels

359   of yeast protein in the diets for broiler chickens. The FCR of broiler chicks fed diets with 20.1

360   % CP from dried sugar-cane yeast replacing SBM, was higher than the birds fed control diets

361   (Alves Longo et al., 2005). Accordingly, FCR also increased in broiler chickens fed diets with

362   39 to 65 % CP from vinasse-grown *C. jadinii* at the expense of soybean, compared with birds

363   fed the control diet (Rodríguez et al., 2011; Rodríguez et al., 2013). The higher FCR and

364   lower growth performance in birds fed the yeast-based diets in our experiment may be

365   explained by an adverse effect of yeast in the utilization of energy in broiler chicken diets

366   (Rodríguez et al., 2011). As a feed additive, yeast has shown to benefit FCR in broiler

367   chickens. Adding lower levels of *S. cerevisiae* yeast (3.5 to 10.5 g/kg) in SBM-based diets for

368　broiler chickens (Chand and Ullah-Khan, 2014) resulted in increased weight gain and

369　decreased FCR in the birds fed increasing amounts of yeast.

370　***Nutritional evaluation of diets with C. jadinii***

371　The amino acid composition of *C. jadinii* is similar to that of SBM, but lower in methionine

372　and arginine compared to SBM, thus these differences were compensated by adding synthetic

373　amino acids to the diets. A limiting factor to the present syudy could be an innacurate

374　estimation of the amino acid availability in *C. jadinii*, as the standardized-ileal-digestibility

375　values for this ingredient in broiler chickens were not known. This coud have led to a

376　potential overestimation of the amino acid availability, and thus reduced weight gain. Yeast-

377　cell walls consist of complex polysaccharide structures, resistant to digestion in several

378　species (Roelofsen and Hoette, 1951; Kihlberg, 1972; Wogan, 1975), which may reduce the

379　availability of protein in yeast (Rumsey et al., 1991). Cell walls may contain chitin, which can

380　reduce nutrient absorption, digestibility of fat and digestibility of CP in broiler chickens

381　(Schiavone et al., 2017). Although no effect of *C. jadinii* on the AID of CP and starch was

382　found, the AID of crude fat was lower in diets with yeast compared with the control diets. The

383　lower fat digestibility in diets with increasing levels of yeast might be due to an increase in

384　chitin content in the diets, derived from *C. jadinii*. Chitin present in yeast cell-walls

385　(Kwiatkowski and Edgar, 2012), is a known anti-nutritional factor which can reduce lipase

386　activity and fat absorption in the small intestine (Kobayashi et al., 2002; Razdan and

387　Pettersson, 2019) and has been associated with reduced digestibility of dry matter in broiler

388　chickens (Khempaka et al., 2006). The reduced growth performance in birds fed the *C.*

389　*jadinii*-containing diets could also be a result of a lower proportion of energy from fat was

390　available for growth. Alternatively, others document that yeast possesses antioxidant activity

391　and binds mycotoxins (Kogan and Kocher, 2007) which can improve digestive function by

392　releasing available nutrients bound to anti-nutritional factors.

393   *C. jadinii* also have a high content of minerals, which can vary from 4 to 14 % depending on

394   the utilized growth media (Rodríguez et al., 2011). The lower AID of carbohydrates in the

395   CJ30 diet might explain the lower growth performance of birds in this group compared to

396   those fed the control diet because carbohydrates are the main energy source for growth.

397   ***Gut-morphometric indices***

398   The intestinal epithelia are major sites of nutrient absorption, and to some degree, of nutrient

399   digestion, essential in the uptake of protein and amino acids necessary for growth. Intestinal

400   morphology may be positively affected by the presence of yeast in the diet, although this was

401   not the case in the present study. Previously, the inclusion of 40 % CP from *C. jadinii* in diets

402   for young pigs resulted in increased intestinal VH and VH:CD (Cruz and Håkenåsen et al.,

403   2019), which has been associated with improved gut health and increased capacity to digest

404   and absorb nutrients (Laudadio et al., 2012). Mannooligosaccharides in yeast-walls bind to

405   pathogenic bacteria such as *Salmonella* spp. and *Escherichia coli* and promote their flushing

406   from the gastrointestinal tract, which can balance microbial flora, prevent infections and

407   formation of toxins, and increase the availability and absorption of nutrients (Ewing and Cole,

408   1994). Beta-glucans in yeast inhibit the growth of *E. coli*, by altering membrane permeability

409   (Rahar et al., 2011) contributing to normal intestinal function. The high content of *β*-glucans

410   in yeast cell-wall (50 to 60 %) enhances the functional status of macrophages and neutrophils

411   (Kogan and Kocher, 2007), which can help to cope with infections in young birds. On the

412   other hand, increased VH may be a consequence of a greater need for digestive capacity

413   (Svihus, 2014) caused by limited amounts of available nutrients in the gastrointestinal tract.

414   The findings of peritonitis, ascites and foot-pad inflammation, and general disease in birds fed

415   diets containing *C. jadinii* (especially replacing 30 % of CP) compared with birds in the

416   control group, may indicate impaired health-status caused by an accumulation of nucleic acids

417   in the birds fed those diets, although these results did not reach statistical significance and

would require further investigation. Alternatively, the increased number of cases of foot-pad inflammation in the CJ30 group may be associated with the slightly increased litter humidity (N.S) in that group, which predisposes to bacterial proliferation and foot-pad dermatitis (Eichner et al., 2007).

CONCLUSIONS

Cyberlindnera jadinii grown on local lignocellulosic sugars successfully replaced up to 10 % CP from soybean meal in diets for broiler chickens without compromising growth performance, nutrient digestibility and intestinal-absorptive capacity, showing the potential of *C. jadinii* as a local based protein source in broiler chicken diets. Replacing SBM with levels higher than 10 % of *C. jadinii* on CP basis (20 and 30 %) however, seemed to reduce growth performance of broiler chickens. Further research is necessary to explain the mechanisms of action of *C. jadinii* on the digestion and metabolism of broiler chickens and to determine the optimal levels of inclusion of *C. jadinii* in broiler chicken diets.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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444   in the samplings, and the chemical analysis of diets and biological samples.

445

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Table 1. Ingredient composition and calculated content (g/kg, unless otherwise stated) of experimental diets fed to broiler chickens from day 1 to 30 post-hatching.

Ingredients g/kg, as is	Starter diets[1]				Grower diets[1]			
	Control	CJ10	CJ20	CJ30	Control	CJ10	CJ20	CJ30
Wheat[a]	528	536	545	553	625	631	637	643
Oats[b]	100	100	100	100	100	100	100	100
Cyberlindnera jadinii[c]	0	49	98	147	0	42	83	125
Soybean meal[d]	153	102	51	0	126	84	42	0
Fishmeal[e]	20	20	20	20	20	20	20	20
Rapeseed meal[f]	20	20	20	20	20	20	20	20
Potato protein concentrate[g]	50	50	50	50	30	30	30	30
Maize gluten meal[h]	50	50	50	50	20	20	20	20
Soy oil	43	36	30	23	25	20	15	10
Vitamin and trace-mineral mix[i]	6.4	6.3	6.3	6.3	6.2	6.3	6.3	6.3
Limestone meal	9.5	9.5	9.2	9.1	8.0	7.9	7.8	7.7
Monocalcium phosphate	6.7	7.8	8.8	9.9	4.6	5.5	6.3	7.2
Sodium bicarbonate	4.1	3.3	2.4	1.6	4.0	3.1	2.3	1.4
Sodium chloride	0.1	0.1	0.0	0.0	0.6	0.6	0.6	0.6
L-Lysine	3.8	3.3	2.9	2.5	3.8	3.4	3.0	2.6
L-Methionine	2.6	2.8	2..9	3.0	2.7	2.8	2.9	3.0
L-Arginine	1.5	2.0	2.4	2.8	1.6	1.9	2.3	2.6
L-Threonine	1.2	1.0	0.8	0.7	1.5	1.3	1.1	0.9
L-Tryptophan	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.1
Enzymes	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Titanium dioxide (TiO$_2$)	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Calculated content								
Metabolizable energy[j]	2,892	2,892	2,892	2,892	2,820	2,820	2,820	2,820
Crude protein	235	235	235	235	200	200	200	200
Crude fat	594	574	555	535	425	392	358	325
CP from *Cyberlindnera jadinii* (%)	0.0	10.0	20.0	30.0	0.0	10.0	20.0	30.0
Amino acids (g/16gN)								
Lysine	1.41	1.42	1.43	1.44	1.22	1.23	1.23	1.24
Methionine	0.68	0.69	0.71	0.72	0.60	0.61	0.62	0.63
Cysteine	0.37	0.36	0.34	0.32	0.37	0.32	0.30	0.29
Threonine	0.95	0.95	0.95	0.96	0.83	0.83	0.83	0.83
Tryptophan	0.27	0.27	0.27	0.27	0.24	0.24	0.24	0.24
Arginine	1.45	1.46	1.46	1.47	1.27	1.27	1.27	1.27

[1]Control diet (Control); diets with 10, 20, and 30 *%* crude protein (CP) from *C. jadinii* (CJ10, CJ20, and CJ30).
[a]Wheat (g/kg): dry matter (DM) 859, CP 98, crude fat 20, ash 14, starch 570, crude fiber 18.
[b]Oats (g/kg): DM 861, CP 92, crude fat 57, ash 30, starch 425, crude fiber 95.
[c]Dried inactivated *C. jadinii*: DM 970 g/kg, CP 470 g/kg, crude fat 16 g/kg, ash 78 g/kg, gross energy 4756 kcal/kg; essential amino acid content in grams per 16g N: Arg 24.4, His 8.5, Ile 21.6, Leu 31.6, Lys 30.6, Met 5.2, Phe 18.4, Thr 25.6, Val 25.9, Trp 6.2.
[d]Non-GMO soybean meal (g/kg): DM 877, CP 457, crude fat (Soxhlet with HCL hydrolysis) 23, ash 57, starch (including simple sugars) < 10, crude fiber (Fibertec) 77.
[e]Fishmeal (g/kg): DM 930, CP 701, crude fat 86, ash 167, starch < 10, crude fiber 7.
[f]Rapeseed meal (g/kg): DM 910, CP 356, crude fat 113, ash 61 starch 14, crude fiber 104.
[g]Potato protein concentrate, Cargil, Denmark (g/kg) DM 914, CP 725, crude fat 30, ash 20; gross energy 21.8 MJ/kg.
[h]Maize gluten meal (g/kg): DM 940, CP 616, crude fat 38, ash 33, starch 160, crude fiber 14.
[i]Vitamin-trace mineral premix, provided per 1 kg of diet: vitamin A 9600 IU; dl-α-tocopheryl acetate 100 mg; cholecalciferol 5000 IU; menadione 6 mg; thiamin 3.9 mg; riboflavin 7.4 mg; pantothenic acid 59 mg; niacin 20 mg; pyridoxine 12 mg; biotin 0.4 mg; cyanocobalamin 20 µg; betaine 1.1 g; selenium 0.29 mg; Fe (FeSO$_4$) 67 mg; Mn (MnO) 127 mg; Zn (ZnO) 60 mg; Cu(CuSO$_4$) 11 mg; I (Ca [IO$_3$]) 1.28 mg.
[j]Apparent metabolizable energy, values in kilocalorie per kilogram, calculated based on Centraal Veevoederbureau (2005).

Table 2. Analyzed chemical composition of experimental diets

Composition, g/kg	Diets[a]							
	Starter				Grower			
	Control	CJ10	CJ20	CJ30	Control	CJ10	CJ20	CJ30
Dry matter	911	913	919	923	893	900	902	908
Ash	53	49	50	51	42	44	45	44
Crude protein[b]	258	258	262	259	232	233	233	231
Starch	371	389	401	403	403	423	440	442
Crude fat	55	55	48	45	48	43	41	36
Crude fiber	35	34	33	32	33	34	30	32
Phosphorus	6.0	6.6	7.2	7.6	5.6	5.2	6.3	6.8
Potassium	8.3	8.5	8.6	8.9	7.6	7.8	7.9	8.2
Calcium	9.9	8.3	7.9	8.1	6.2	6.1	6.8	6.4
Sodium	2.0	1.7	1.4	1.2	1.6	1.6	1.5	1.4
Essential amino acids								
Arginine	14.0	13.3	13.1	12.9	12.3	12.0	11.7	11.1
Histidine	5.4	5.3	5.0	4.8	4.8	4.6	4.3	4.0
Isoleucine	9.2	9.2	8.9	8.8	7.4	7.2	6.9	6.8
Leucine	18.5	18.6	18.8	18.1	15.5	15.3	15.1	14.0
Lysine	13.7	13.3	12.8	12.3	12.0	11.7	11.2	10.7
Methionine	6.0	6.0	6.2	6.6	5.9	5.9	5.6	5.7
Phenylalanine	11.3	11.3	10.8	10.6	9.3	9.0	8.6	8.2
Threonine	9.0	9.0	9.3	9.1	8.2	7.9	7.9	7.4
Valine	8.4	8.5	8.5	9.0	7.5	7.4	7.5	7.4
Tryptophan	2.7	2.5	2.6	2.7	2.5	2.2	2.4	2.4
Non-essential. amino acids								
Alanine	9.6	9.6	10.0	10.2	7.7	7.8	8.1	8.0
Asparagine	19.8	19.0	18.5	16.8	16.0	15.1	14.6	13.5
Glycine	9.0	8.3	8.2	8.1	7.4	7.2	7.1	6.9
Glutamate	49.0	48.7	48.3	47.3	46.0	45.2	44.0	42.1
Cysteine	3.3	3.4	3.1	2.9	3.1	2.8	2.7	2.6
Tyrosine	7.0	6.8	6.9	7.0	4.2	4.2	4.2	4.4
Proline	15.1	13.6	14.7	14.7	13.7	13.0	13.5	13.2
Serine	10.0	10.0	10.2	9.7	8.9	8.5	8.5	8.0
Total amino acids	218.5	213.9	213.3	209.1	189.7	184.6	181.5	173.8

[a]Control diet based on soybean meal, wheat, and oats (Control); diets with 10, 20 and 30 % crude protein from *C. jadinii* (CJ10, CJ20, and CJ30).

[b]Pregl-Dumas (N × 6.25).

632 **Table 3.** Effects of increasing dietary level of *Cyberlindnera jadinii* yeast protein on the growth
633 performance of broiler chickens during the starter (d 0 to 10), grower (d 11 to 30) and overall (d 0 to
634 30) periods[1]

Item, g/bird	Diet[2]				SEM[3]	*P*-value	Linear
	Control	CJ10	CJ20	CJ30			
Start BW	42.1	42.2	41.6	41.8	0.38	0.647	0.378
Final live BW#	1,987[a]	1,935[a]	1,824[b]	1,699[b]	36.11	< 0.001	< 0.001
BW Gain							
Starter period	246[a]	239[ab]	226[ab]	188[b]	13.67	0.036	0.007
Grower period	1,699[a]	1,653[a]	1,555[b]	1,469[b]	24.89	< 0.001	< 0.001
Overall	1,944[a]	1,892[a]	1,782[b]	1,657[b]	36.14	< 0.001	< 0.001
Feed intake							
Starter period	275	288	296	254	12.31	0.124	0.334*
Grower period	2,310[a]	2,257[a]	2,150[b]	2,075[b]	32.61	< 0.001	< 0.001
Overall	2,586[a]	2,546[ab]	2,446[ab]	2,329[b]	38.94	0.001	< 0.001
FCR[4]							
Starter period	1.12[b]	1.21[ab]	1.32[a]	1.36[a]	0.04	0.004	< 0.001
Grower period	1.36	1.37	1.38	1.41	0.02	0.183	0.038
Overall	1.33[b]	1.35[ab]	1.37[ab]	1.41[a]	0.02	0.027	0.004
Water intake[4] (ml/bird grower)	3,742	3,887	3,577	3,402	145.82	0.248	0.111
Water: feed intake (grower)	1.57	1.60	1.54	1.57	0.04	0.791	0.820
Slaughter weight●	1,225[a]	1,188[a]	1,115[b]	1,030[c]	10.57	< 0.001	< 0.001
Mortality rate (%)¤	2.0	3.6	4.4	5.6	1.79	0.561	0.168

635 [1]Values are presented as least-square means, (n = 20).

636 [2]Control diet based on soybean meal, wheat, and oats (Control); diets with 10, 20 and 30 *%* crude protein from

637 *C. jadinii* (CJ10 CJ20, and CJ30).

638 [3]SEM, pooled standard error of the means.

639 [4]FCR, feed conversion ratio (gram feed per gram gain).

640 [5]Water consumption calculated from days 8 to 28 (ml/bird); n = 10.

641 [a-c]Values in the same row with different superscripts differ (P < 0.05).

642 *Quadratic trend (P < 0.05).

643 ●Individual slaughter weight was registered at the slaughterhouse, eviscerated.

644 #Final live weight is calculated based on the growth curve for each pen.

645 ¤Mortality rate per dietary treatment is calculated as the number of deaths divided by total birds in each

646 treatment.

647

648 **Table 4.** Carcasses (%) rejected during the slaughter procedure inspection and litter quality scores of broiler
649 chickens fed a control diet and diets with increasing dietary levels of *Cyberlindnera jadinii* yeast

	Diet[1]			
	Control	CJ10	CJ20	CJ30
Total inspected (*n*)	225	221	218	215
Total rejected (%)[a]	2.22	5.43	4.13	6.51
General disease	0.44	2.71	1.83	2.79
Footpad lesions	0.00	0.00	0.00	0.93
Ascites	0.44	1.36	0.92	1.40
Peritonitis	0.00	0.90	0.46	1.40
Litter quality score[b]				
Day 11	1.0	1.0	1.0	1.0
Day 18	1.7	1.8	1.8	1.8
Day 25	2.1	2.3	2.3	2.3
Day 28	2.5	2.6	2.6	2.6

650 [1]Control diet based on soybean meal, wheat, and oats (Control); diets with 10, 20 and 30 % crude protein from

651 *C. jadinii* (CJ10 CJ20, and CJ30).

652 [a]Calculated as the number of cases per number of inspected birds.

653 [b]Classified on a scale from 1 to 5 according to visual and tactile humidity and consistency (1 = completely dry, 5

654 = very wet).

655

656 **Table 5.** Effects of increasing dietary levels of *Cyberlindnera jadinii* on the apparent ileal
657 digestibility of nutrients in broiler chickens[1]

AID, *%*	Diet[2]				SEM[3]	*P*-value	Linear
	Control	CJ10	CJ20	CJ30			
Dry matter	75.4[a]	73.0[ab]	71.3[ab]	69.3[b]	1.09	0.008	0.001
Crude protein[4]	81.0	79.4	78.1	76.5	1.56	0.252	0.050
Starch	97.3	97.6	99.2	99.0	0.78	0.255	0.074
Crude fat	89.1[a]	87.6[ab]	83.7[ab]	82.0[b]	1.57	0.019	0.002
Ash	52.4	52.9	50.8	46.7	2.59	0.352	0.116
Phosphorus	59.0	55.5	59.9	49.1	3.26	0.124	0.104
OM[5]	76.5[a]	74.1[ab]	72.4[ab]	70.4[b]	1.07	0.007	0.001
CHO[6]	73.6[a]	70.9 [ab]	69.3[ab]	67.4[b]	1.04	0.004	< 0.001

658 [1]Results are given as estimated marginal means (*n* = 10 birds per diet).

659 [2]Control diet (Control); diets with 10, 20 and 30 *%* crude protein (CP) from *C. jadinii* (CJ10, CJ20 and CJ30);

660 [3]SEM, pooled standard error of the means.

661 [4]Pregl Dumas, N × 6.25.

662 [5]Calculated as organic matter = dry matter − ash

663 [6]Calculated as carbohydrates = dry matter − ash − CP − crude fat

664 [a-b]Means within a row with different superscripts differ (*P* < 0.05).

665

666

667 **Table 6.** Effects of increasing dietary levels of *Cyberlindnera jadinii*, on the intestinal morphometry of broiler
668 chickens[1]

Morphometry, *μm*	Diets[2]				SEM[3]	*P*-value			
	Control	CJ10	CJ20	CJ30		Overall	Control vs. yeast	Linear	Quad.
Duodenum									
VH	1,242	1,343	1,268	1,212	76.7	0.656	0.753	0.639	0.313
CD	141	124	109	115	8.8	0.083	0.052	0.024	0.215
VH:CD	9.64	11.45	12.23	11.62	1.08	0.398	0.123	0.179	0.271
Absorption area[4]	201.8	207.6	186.1	160.1	16.5	0.191	0.356	0.058	0.344
Jejunum									
VH	558	637	593	661	45.6	0.407	0.174	0.202	0.903
CD	88	89	85	96	8.5	0.833	0.797	0.588	0.603
VH:CD	6.63	7.46	7.30	7.34	0.43	0.548	0.081	0.316	0.367
Absorption area[4]	56.1	63.6	62.2	65.2	6.46	0.783	0.363	0.382	0.736
Ileum									
VH	314[a]	264[b]	259[b]	316[a]	16.1	0.019	0.111	0.975	0.002
CD	52	50	46	52	2.5	0.334	0.318	0.643	0.127
VH:CD	6.31	5.50	5.88	6.29	0.35	0.316	0.439	0.840	0.089
Absorption area[4]	19.5	17.7	16.4	21.3	1.27	0.053	0.530	0.472	0.014

669 [1]VH, villus height; CD, crypt depth. Results are given as least-square means of two-to-five
670 observations per gut segment per animal, *n* = 10 birds per diet.

671 [2]Control diet (Control); diets with 10, 20, and 30 *%* crude protein (CP) from *Cyberlindnera jadinii*
672 (CJ10, CJ20, and CJ30).

673 [3]SEM, pooled standard error of the means.

674 [4]Calculated as VH × villus width, expressed as $\mu m^2 \times 10^{-3}$.

675

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