	Inventaris Wob-verzoek W17-17								
		wordt verstrekt				**weigeringsgronden**			
nr.	**Documenten 20171667**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
1	Aanvraagformulier				x		x		
2	Projectvoorstel oud				x	x		x	
3	Niet-technische samenvatting oud								
4	Bijlage dierproeven 1 oud				x	x		x	
5	Bijlage dierproeven 2 oud				x	x		x	
6	Bijlage dierproeven 3 oud				x	x		x	
7	Bijlage dierproeven 4 oud				x	x		x	
8	Bijlage dierproeven 5 oud				x	x		x	
9	Bijlage dierproeven 6 oud				x	x		x	
10	Bijlage dierproeven 7 oud				x	x		x	
11	Ontvangstbevestiging				x		x		
12	Brief verlenging termijn				x		x		
13	DEC-advies				x		x		
14	Projectvoorstel nieuw				x	x		x	
15	Niet-technische samenvatting nieuw	x							
16	Bijlage dierproeven 1 nieuw				x	x		x	
17	Bijlage dierproeven 2 nieuw				x	x		x	
18	Bijlage dierproeven 3 nieuw				x	x		x	
19	Bijlage dierproeven 4 nieuw				x	x		x	
20	Bijlage dierproeven 5 nieuw				x	x		x	
21	Bijlage dierproeven 6 nieuw				x	x		x	
22	Bijlage dierproeven 7 nieuw				x	x		x	
23	Vraag aan DEC en reactie				x		x		
24	Verzoek aanvulling aanvraag				x		x		
25	Reactie verzoek aanvulling				x		x		
26	Adviesnota CCD		x						x
27	Beschikking en vergunning				x		x		

AVD 10400 20171667

19 MEI 2017

1.

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1	Heeft u een deelnemernummer van de NVWA? Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.	[X] Ja > Vul uw deelnemernummer in	10400
		[ ] Nee > U kunt geen aanvraag doen	
1.2	Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.	Naam instelling of organisatie	
		Naam van de portefeuillehouder of diens gemachtigde	[redacted]
		KvK-nummer	9215846
1.3	Vul de gegevens van het postadres in. Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.	Straat en huisnummer	Akkermaalsbos 12
		Postbus	59
		Postcode en plaats	6700AW Wageningen
		Iban	NL10RABO0397066465
		Tenaamstelling van het rekeningnummer	Wageningen UR
1.4	Vul de gegevens in van de verantwoordelijke onderzoeker	(Titel) naam en voorletters	[redacted]
		Functie	onderzoeker
		Afdeling	[redacted]
		Telefoonnummer	[redacted]
		Email adres	[redacted]

1.5	(Optioneel) Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	(Titel) naam en voorletters	[redacted]
		Functie	onderzoeker
		Afdeling	[redacted]
		Telefoonnummer	[redacted]
		Email adres	[redacted]
1.6	(Optioneel) Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) naam en voorletters	[ ] Dhr. [ ] Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		Email adres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	[ ] Ja > Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag	
		[X] Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	[x] Nieuwe aanvraag > Ga verder met vraag 3	
		[ ] Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het Dierenwelzijn	
		Vul uw vergunde projectnummer in en ga verder met vraag 2.2	
		[ ] Wijziging op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het Dierenwelzijn	
		Vul uw vergunde projectnummer in en ga verder met vraag 2.3	
2.3	Is dit een wijziging voor een project of dierproef waar al een vergunning voor verleend is?	[ ] Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier	
		[ ] Nee > Ga verder met vraag 3	

2.3	Is dit een melding voor een project of dierproef waar al een vergunning voor is verleend?	[ ] Nee > Ga verder met vraag 3 [ ] Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1-5-2017
		einddatum	1-5-2021
3.2	Wat is de titel van het project?		To determine the relation between protein fermentation and gut health in pigs and poultry
3.3	Wat is de titel van de niet-technische samenvatting?		Het bepalen van de relatie tussen eiwitfermentatie en darmgezondheid in varkens en pluimvee
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Wageningen UR
		Postadres	Droevendaalsesteeg 4, 6708 PB Wageningen
		E-mailadres	dec@wur.nl

4 Betaalgegevens

4.1	Om welk type aanvraag gaat het?	[X] Nieuwe aanvraag Projectvergunning €	2113
		[ ] Wijziging €	
4.2	Op welke wijze wilt u dit bedrag aan de CCD	[ ] Via een eenmalige incasso	
		[X] Na ontvangst van de factuur	

voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht
[x] Projectvoorstel + 7x appendix
[x] Niet-technische samenvatting

Overige bijlagen, indien van toepassing
[ ] Melding Machtiging
[x] Bestelorder WUR 1059474

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:
- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.

Centrale Commissie
Dierproeven Postbus 20401
2500 EK Den Haag

• dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
• dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
• dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted]
Plaats	Wageningen
Datum	10-5-2017
Handtekening	[redacted]

2.

Form
Project proposal

- This form should be used to write the project propsal of animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed
- For more information on the project proposal, see our website(www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400
1.2	Provide the name of the licenced establishment.	Wageningen University
1.3	Provide the title of the project.	To determine the relation between protein fermentation and gut health in pigs and poultry

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

[X] Basic Research
[X] Translational or applied research
[] Regulatory use of routine production
[] Research into environmental protection in the interest of human or animal health or welfare dier
[] Research aimed at preserving the species subjected to procedures

[] Higher education or training
[] Forensic enquiries
[] Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Background

Gut health is an important aspect in pig and poultry production. It contributes to overall health and welfare, but is also affects productivity and resource efficiency. Gut health has been negatively related to high dietary protein concentrations. In humans, for instance, high protein intake (from red and processed meat) has been associated with an increased risk for developing colorectal cancer (Norat et al., 2005) and dietary protein intake has been associated with a (relapse in) inflammatory bowel disease (Andersen et al., 2012; Jowett et al., 2004). In young, growing livestock species like piglets and broilers, dietary proteins are essential to provide building blocks for growth. Hence, diets for young pigs and chickens traditionally contain high protein concentrations. In addition, the average use of antibiotics in pig and broiler production systems has been reduced by 5.0% and 7.4% in 2015 compared to 2014 (Autoriteit Diergeneesmiddelen, 2016), respectively, in order to minimize the risk of microbial resistance against antibiotics. At the same time, certain gastrointestinal issues (i.e. diarrhea) remain a large problem in pig and poultry production systems. Diarrhea often occurs in piglets in the post-weaning period (i.e. post-weaning diarrhea) and dietary protein has been implicated in post-weaning diarrhea (Heo et al., 2008, 2009, 2015). In broiler chickens, high dietary protein concentrations have been demonstrated to increase the moisture content of litter (Bench et al., 2016), increasing the incidence of wet litter syndrome. Wet litter can cause footpad dermatitis and subsequently can increase the incidence of hock burn and breast irritation in broilers (de Jong et al., 2014).

Protein fermentation in the hindgut is believed to be (partially) responsible for negative effects of dietary proteins on gut health. The ileal digestion of dietary protein differs between protein sources and levels (Heo et al., 2010; Salgado et al., 2002), which affects the flow of protein into the large intestine of pigs and poultry. In addition, endogenous secretions (such as mucus, enzymes and shed epithelial cells) contribute to the flow of proteins into the large intestine. In the hindgut, these proteins can be fermented by saccharo-proteolytic bacteria (such as Clostridia, *Proteus* and *E. coli*) resulting in the proliferation of these bacteria, some of which are known pathogens. In addition, proteins have a buffering capacity in the gut, which favors proliferation of pH-sensitive bacteria, which often include pathogens. Furthermore, the negative relation between protein fermentation and gut

health may be caused by protein fermentation metabolites, such as branched-chain fatty acids, ammonia, phenolic and indolic compounds, hydrogen sulfide, biogenic amines and nitric oxide and *N*-nitroso compounds. Some of these protein fermentation metabolites have potential damaging effects on gut function, but these conclusions mainly rely on tests in in vitro systems (Lin and Visek, 1991; Hughes et al., 2008; Andriamihaja et al., 2015 Beaumont et al., 2016; Leschelle et al., 2005). Such protein fermentation metabolites are produced in pigs and poultry as well, especially when feeding diets containing high protein content or a low digestible protein source (Bikker et al., 2006; Heo et al., 2010; Pieper et al., 2012, 2014; Qaisrani et al., 2014).
In the field of human nephrology, some of the organic acids produced during fermentation of proteins are well-known as uremic toxins (i.e. *p*-cresol sulfate and indoxyl sulfate), contributing to the so-called uremic syndrome, associated with malaise, poor appetite, intestinal complaints, wasting, tendency for malnutrition, sarcopenia and systemic low-grade inflammation in patients with advanced renal disease. Young piglets may be particularly susceptible to these toxic effects, because their immune system, gut and renal function – necessary for removal of these toxic organic acids – is still immature and impaired compared to the adult situation.

Aim and objectives
Despite numerous studies on the effects of high protein diet on gut health, we have a poor understanding which metabolites are responsible for the decreased gut health. A large consortium is established between research groups from 3 Dutch universities, [redacted]. In this consortium, we aim to increase our understanding of the interaction between feed utilization and animal health. We will use untargeted and targeted metabolite platforms, with which we can establish the difference in metabolites in different parts of the intestinal lumen, blood, intestine and liver tissue as influenced by ileal undigested protein. The key objectives of our consortium are:

1. To determine the changes in metabolites in feces, intestinal content, blood, liver and intestinal tissue, and correlate these changes to changes in microbial species and gut epithelium influenced by protein fermentation in pigs and poultry.
2. To elucidate the mechanisms by which metabolites cause compromised gut health (using intestinal organoids).
3. To validate whether (dietary) interventions aiming at removing harmful metabolites/microbial species in the gut will increase gut health pigs and poultry.

The novelty of this project is the application of untargeted metabolomics across samples and species and establishing the relation between (unknown) metabolites and gut health, thereby, aiming to elucidate the working mechanisms underlying the relation between protein fermentation and gut health.
It is of importance to perform this research with both pigs and poultry, because their digestive physiology differs substantially, likely resulting in differences between species in the relation between dietary protein, protein fermentation and gut health. Chickens have two stomachs, have many places where reflux of digesta occurs (i.e. between the two stomachs, from small intestine into the stomachs and from the cloaca into the colon and ceca) and the main site of fermentation are the ceca. Furthermore, the passage rate is higher in the small intestine of chickens, resulting in a lower enzymatic digestion. Therefore, the fraction of ileal undigested – but potentially fermentable – protein differs between species. Subsequently, passage of digesta into the ceca of chickens is selective (i.e. only the soluble digesta passes), whereas in pigs (and humans) the complete digesta will flow into the colon.

Projects [redacted]

Within the [redacted] program, 3 project proposals have been granted [redacted]. The first step in this consortium includes a series of 7 studies. These studies are combined in this application, because of the common project motivation, background and main objective. In addition, the leaders of these projects had a meeting with [redacted] with the objective to reduce overlap between studies. This has resulted in a reduction of the number of studies proposed and better use of experimental animals.

The 3 granted project proposals are entitled:

1) [redacted]

These 3 project proposals together contribute to the objective of determining the relation between dietary protein, protein fermentation (metabolites) and gut health in pigs and poultry and elucidating the underlying mechanisms, and are considered as sub-projects in the context of this CCD project application.

Below, the focus and design of each sub-project are described and these sub-projects will be conducted in parallel. However, there will be strong collaboration and cross-utilization of expertise and knowledge among the sub-projects within this consortium. The added value of combining these studies in this consortium is that obtained knowledge will be shared quickly between all partners. The designs of follow-up experiments will be adapted if required based on the newly obtained data.

Sub-project 1: [redacted]

When PWD occurs in practice, a reduction in dietary protein concentration is often advised and often helps to reduce PWD, indicating the involvement of dietary protein in PWD. Highly controlled experiments have been conducted to elucidate the effects of dietary proteins on different aspects of gut function and animal performance. These studies provide valuable information regarding the relation of dietary protein and animal performance. However, under experimental conditions, PWD does not always occur (Htoo et al., 2007; Nyachoti et al., 2006). It is, therefore, questionable whether these experimental (hygenic) conditions sufficiently represent the practical situation and what the contribution of protein fermentation is to PWD in practice. This sub-project is, therefore, aimed at developing knowledge regarding the relation between protein fermentation, gut function and PWD, contributing in the end to a reduction in PWD in piglets. Identifying metabolites responsible for negative effects on gut health and thereby establishing the working mechanisms underlying the relation between protein fermentation and PWD are fundamental aspects of this sub-project.

Sub-project 2: [redacted]

Poor enzymatic protein digestibility and overfeeding of protein results in the presence of undigested protein in the distal part of the small intestine as well as in the ceca and colon of broilers and pigs, respectively. In addition to these proteins not being available for utilization by the animal, this undigested protein also becomes substrate for proteolytic fermentation with a subsequent change in microbial composition, colonization of pathogenic bacteria and the production of metabolites and toxins. The first objective of this sub-project is to understand gut health better by evaluating the impact of ileal undigested protein on microbial activity, proteolytic fermentation, intestinal health and performance of piglets and broilers.
A second objective is to study transgenerational effects in poultry. A large body of evidence links maternal/fetal (mal)nutrition to the development of diseases after birth (in rodents and human; Godfrey et al., 2000, Hansen et al., 2014, Paul et al., 2016). In poultry, it is known that dietary protein fed to broiler breeders can affect broiler offspring performance (Rao et al., 2009). When low quality protein sources are fed to broiler breeders, an increase in ileal undigested protein can increase the production of (toxic) protein fermentation metabolites. To date, it is not known whether these metabolites are also transferred to the eggs and, subsequently, can affect broiler offspring gut health and performance. Therefore, this sub-project aims to determine transgenerational effects of protein fermentation metabolites in broilers.
This sub-project will provide knowledge regarding the development of protein fermentation, how this is affected when different protein sources (differing in amino acid composition) are fed and which (unknown) metabolites are related to the negative effects on gut health. In addition, metabolite profiles in broiler breeders, their eggs and offspring, have not been determined and associated before, and this could unravel potential mechanisms underlying transgenerational effects on gut health and performance.

Sub-project 3: [redacted]
Some of the organic acids produced during protein fermentation are well-known as uremic toxins (i.e. *p*-cresol sulfate and indoxyl sulfate) and might contribute to impaired gut health in piglets. In human pathology, renal transplantation provides an interesting model, because immunosuppressive drugs to prevent rejection of the transplanted kidney compromise immunological and intestinal functioning, while renal excretory function of toxic organic acids is impaired. These patients very often suffer from diarrhea, malnutrition and sarcopenia. In this sub-project, knowledge from observational cohort studies in renal transplant patients will be applied to gain insight into the relation between dietary protein, gut microbiota, protein fermentation and uremic toxin production in piglets and how this is affected by dietary interventions (i.e. vitamins, minerals and sulfate).

Research categories
The studies performed in this consortium will provide knowledge regarding the development of protein fermentation, how this is affected when different protein sources (differing in amino acid composition) are fed, and will elucidate the mechanisms by which protein fermentation negatively affects gut health. In addition, untargeted metabolomics will be applied to identify (up till now) unknown metabolites produced during protein fermentation and their relation with gut health will be determined. This research, therefore, can be considered as fundamental research.

The knowledge obtained in this consortium will contribute to a more efficient use of dietary protein and an improvement of gut function - especially during stressful periods such as the weaning period in piglets – and potentially to the reduction of therapeutic antibiotic use in pigs and poultry. Data from this project can be applied in feed formulation but can also be used to develop new products, and this research program, therefore, can be considered as translational research. The knowledge obtained with this project will be used to advice on changes in feed regimens for pigs and poultry to overcome compromised gut health.

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3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The main objective of this project is to determine the relation between dietary protein and microbiota, protein fermentation metabolites and their effect on gut health and performance of pigs and poultry.In this project we aim to reach this objective through a series of in vitro and in vivo pig and poultry studies. With our research strategy described in 3.4 we expect to reach our main project's objectives, because the scientific partners have extensive expertise in the research areas (i.e. animal nutrition, microbiology, host physiology/metabolism and metabolomics tools) required within this project. The industrial partner is a global leader in additives for animal feed, and a direct stakeholder for the utilization of newly gained knowledge.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

This project will provide insight in causal relations between protein fermentation, its end-products and gut health of pigs and poultry. This knowledge can be applied to develop nutritional solutions to prevent gut health problems and optimize gut function, which will contribute to an increase in overall animal health, thereby improving animal welfare.
In gut health problems such as post-weaning diarrhea in piglets, therapeutic antibiotics are applied due to the involvement of *E. coli* pathogens. The knowledge developed in this project will contribute to our understanding of post-weaning diarrhea and the relation with dietary protein. This knowledge can lead to nutritional solutions for post-weaning diarrhea, thereby contributing to a reduction in therepeutic antibiotic use.Increased understanding of the relations between protein fermentation (end-products) and gut health in pigs and poultry might also lead to hypotheses relevant for other species, including humans, where protein fermentation contributes to diarrhea in patients with renal disease.

3.4 Research Strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

As mentioned in 3.1, this project is part of a [redacted] program, in which 3 project proposals, included as sub-projects in this CCD project application, have been granted [redacted]. We combine the first experiment(s) of each sub-project in this CCD project application.

Sub-project 1: [redacted]
This sub-project is aimed at developing knowledge regarding the relation between protein fermentation, gut function and PWD, contributing in the end to a reduction in PWD in piglets. The first experiment in this sub-project is a large-scale on-farm piglet study (see appendix 1 "On-farm piglet

experiment") to determine associations between PWD, protein fermentation metabolites and functional gut parameters. Based on the associations following this on-farm experiment, in vitro studies are performed to determine effects of identified metabolites and fecal water extracts (from piglets from the on-farm study) on gut function, using pig intestinal organoids. These organoids can be formed from isolated crypts of the intestine, and represent an almost complete functional derivate of the actual gut, and many relevant gut functions can be modelled in vitro in an unprecedented way. In the last phase of this sub-project, a second in vivo piglet experiment is anticipated to evaluate and validate the outcome of previous in vivo and in vitro experiments. However, this will be determined at a later stage in the project, and is, therefore, out of scope for this CCD project application.

Sub-project 2: [redacted]
The objective of this sub-project is to understand gut health better by evaluating the impact of ileal undigested protein on microbial activity, proteolytic fermentation, intestinal health and performance of piglets and broilers.
The first pig experiment (see appendix 2 "Digestibility experiment with piglets") in this sub-project aims to determine the effects of a health challenge and dietary digestible crude protein level on nutrient digestion, metabolite profile and animal performance. Low and high sanitary conditions are applied to generate a health disturbance similar as in practice, resulting in differences in sub-clinical health. Secondly, a pig experiment is performed to determine the (interactive) effects of dietary fermentable fiber and crude protein on extent of fermentation, fermentation end-products, microbial colonization, digesta passage rate, and feces quality (see appendix 3 "Digestibility experiment with cannulated pigs").
The first broiler experiment in this sub-project (see appendix 4 "Protein digestibility of different feed ingredients and the metabolic fingerprints of broilers fed these diets") aims to determine which (unknown) metabolites can be correlated to hind gut protein fermentation in broilers, as well as determine the potential of different protein sources to contribute to hind gut protein fermentation. Thereafter, an experiment is performed aimed at feed strategies to reduce protein fermentation and alter the metabolic fingerprint associated with protein fermentation in broilers and evaluate the effects of these feed strategies on gut health (see appendix 5 "Feed strategies to reduce protein fermentation and alter metabolic fingerprint associated with protein fermentation in broilers").
In the experiment described in appendix 6 ("Effects of dietary protein on metabolite profiles in serum and eggs of broiler breeders"), the aim is to determine transgenerational effects of dietary protein in poultry. Broiler breeders will be fed different diets to create a contrast in cecal protein fermentation and (serum) metabolite profiles will be determined. The transfer of metabolites to eggs and offspring will be measured, as well as effects on broiler offspring.
Follow-up experiments in this sub-project include in vitro broiler organoid studies to elucidate underlying mechanisms causing compromised gut health, based on the results and metabolites identified in the previous experiments. Subsequently, final experiments aimed at (dietary) interventions to increase gut health in pigs and poultry will depend on the outcome of these initial experiments and are, therefore, out of scope for this CCD project application.

Sub-project 3: [redacted]
In this sub-project, knowledge from observational cohort studies in renal transplant patients will be applied to gain insight into the relation between dietary protein, gut microbiota, protein fermentation and uremic toxin production in piglets and how this is affected by dietary interventions (i.e. vitamins, minerals).

A combination of in vitro and in vivo approaches will be used to gain insight into how different protein sources affect gut microbiota, large intestinal protein fermentation and uremic toxin production, and how this is affected by the supplementation of vitamins, minerals and sulfate. The experiment described in appendix 7 ("Uremic toxin production during the in vitro fermentation of proteins in ileal digesta") is aimed at the establishing predictive relations between the (amino acid) composition of ileal undigested proteins and the formation of uremic toxins during their subsequent fermentation in vitro.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

This CCD project application contains 7 appendices.
In appendix 1, an on-farm piglet experiment is described, in which piglets with and without PWD are selected, killed and sampled from multiple commercial farms, to study the natural occurrence of diarrhea and the involvement of protein fermentation metabolites and microbiota.
In appendix 2, an in vivo digestibility experiment with piglets is described, with a 2x2 factorial design. Piglets are subjected to high or low sanitary conditions and a high or low dietary level of digestible crude protein and are sacrificed at 10 weeks of age for dissection to collect intestinal contents.
In appendix 3, an in vivo experiment with cannulated pigs in described, with a 3x2 factorial arrangement with dietary fermentable fiber content (feeding low fiber, feeding high fiber and feeding low fiber + infusing fiber in colon) and dietary protein (differing in structure) entering the colon as factors. Pigs are surgically fitted with a cannula in the distal ileum and a catheter in a blood vein. After recovery, dietary treatments are applied and digesta, urine and blood is collected. Feces is collected quantitatively from plastic bags which will be attached to the rear end of the pigs.

In appendix 4, an in vivo broiler experiment is described, with a 3x2 experimental design, including 3 different protein sources at 2 levels of ileal protein digestibility. Broilers are weighed at different time points, blood sampled and killed after 29 days and sampled to determine ileal crude protein and amino acid digestibility (using an indigestible marker), metabolite profiles (in serum and digesta samples) and read-outs of gut function.
In appendix 5, an in vivo broiler experiment is described, with a 2x2x2 experimental design, including factors diet structure, fiber level and protein source. Broilers are weighed at different time points, blood sampled and killed after 29 days and sampled to determine ileal crude protein and amino acid digestibility (using an indigestible marker), metabolite profiles (in serum and digesta samples), passage rate and read-outs of gut function.
In appendix 6, broiler breeders are fed diets differing in ileal protein digestion to create a contrast in cecal protein fermentation and blood is collected to determine serum metabolite profiles. Eggs are collected and sampled to determine transfer of (protein fermentation) metabolites to eggs. Eggs are incubated, hatched and broiler offspring killed to determine effects on offspring gut parameters. Broiler breeders are sacrificed to determine the effects of dietary treatments on gut parameters.In appendix 7, pigs, surgically fitted with cannulas in the terminal ileum are fed diets containing various protein sources expected to differ in amino acid composition of the undigested fraction. Ileal digesta of these pigs is harvested and used for a series of in vitro fermentation experiments to quantify the types and production rates of various uremic toxins.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points

See paragraph 3.4.1 for a description of the project, including the different appendices of each sub-project. This CCD application contains the first experiments (appendices) of each sub-project. The sub-projects are conducted in parallel and there will be strong collaboration and cross-utilization of expertise and knowledge among the sub-projects within this consortium. Within the project, experiments will be adapted if necessary based on the outcome of previous experiments (both within and between sub-projects).

Important selection points in this project include:

- Appendix 1: before the start of the experiment described in appendix 1, a pilot study will be conducted to determine/validate quick selection criteria for post-weaning diarrhea in piglets.
- Appendix 3: the selection of protein sources will depend on the data obtained in appendix 7.
- Appendix 4: dietary treatments in the experiment described in appendix 4 will be determined on in vitro digestion tests.
- Appendix 5: protein sources included in the diets described in appendix 5 will be selected based on the outcome of the experiment described in appendix 4 (both in vitro and in vivo experiment).
- Appendix 6: selection of dietary treatments for broiler breeders will be based on the outcome of the in vitro and in vivo experiment described in appendix 4.
- Appendix 7: selection of the protein sources to be included in the diets will be based on the in vitro work described in appendix 4.

A milestone in this project includes the (ileal) digestibility of a range of dietary protein sources/levels and their concomitant effect on gut health. In all sub-projects, (selected) samples of piglets and broilers will be used for metabolomics analyses. In this way, a library of metabolites will be accomplished for piglets and broilers. A milestone in this project will be the identification of metabolic fingerprints related to protein fermentation or impaired gut health in piglets and broilers.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	On-farm piglet experiment
2	Digestibility experiment with piglets
3	Digestibility experiment with cannulated pigs
4	Protein digestibility of different feed ingredients and the metabolic fingerprints of broilers fed these diets
5	Feed strategies to reduce protein fermentation and alter metabolic fingerprint associated with protein fermentation in broilers
6	Transgenerational effects of protein fermentation metabolites in poultry
7	Uremic toxin production during the in vitro fermentation of proteins in ileal digesta

3.

Format
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven.
- Meer informatie over de niet-technische samenvatting vindt u op de website www.zbo-ccd.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Het bepalen van de relatie tussen eiwitfermentatie en darmgezondheid in varkens en pluimvee
1.2	Looptijd van het project	1-5-2017 - 1-5-2021
1.3	Trefwoorden (maximaal 5)	Eiwit, fermentatie, darmgezondheid, varkens, pluimvee

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

[X] Fundamenteel onderzoek
[X] Translationeel of toegepast onderzoek
[] Wettelijk vereist onderzoek of routinematige productie
[] Onderzoek ter bescherming van het milieu in het belang van de gezondheid of het welzijn van mens of dier
[] Onderzoek gericht op het behoud van de diersoort
[] Hoger onderwijs of opleiding
[] Forensisch onderzoek
[] Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1	Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)	Eiwit is een belangrijke component in de voeding van varkens en pluimvee, omdat het de bouwstenen levert voor groei. Wanneer voereiwit niet volledig wordt verteerd in de dunne darm, komt dit in de dikke darm terecht, waar het eiwit door de micro-organismen (zoals bacteriën) wordt afgebroken. Hierbij komen stoffen vrij die mogelijk schadelijk zijn voor de darm. Het doel van dit project is de complexe relatie tussen voereiwit, micro-organismen en eiwitfermentatie en het effect daarvan op darmgezondheid in varkens en pluimvee in kaart te brengen. Hierbij ligt de nadruk op het meten van (tot nu toe onbekende) producten die gevormd worden tijdens eiwitfermentatie.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	In dit project zal kennis verkregen worden over de relatie tussen voereiwit, eiwitfermentatie (producten) en darmgezondheid in varkens en pluimvee. Deze kennis zal bijdragen aan een efficiënter gebruik van eiwit en het verbeteren van darmgezondheid in varkens en pluimvee, en kan daarmee bijdragen aan een vermindering in het gebruik van antibiotica. Gezien de grote overeenkomst in fysiologie van varkens en mensen, zal de verkregen kennis in dit project mogelijk ook bij kunnen dragen aan het verbeteren van darmgezondheid in mensen.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	In dit project worden varkens en kippen gebruikt. Het aantal varkens is geschat op 3232 en het aantal kippen op 944.

3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	In dit project zullen dieren voornamelijk voeders gevoerd krijgen die verschillen in eiwit (hoeveelheid en kwaliteit) en worden vervolgens monsters verzameld om de effecten hiervan te bepalen. Dieren zullen ongerief ondervinden als gevolg van hanteren (voor het wegen van dieren), het nemen van rectale mestmonsters en bloedmonsters. Daarnaast kan diarree ontstaan. Verder zullen dieren geëuthanaseerd worden voor het eenmalig verzamelen van darminhoud, darmwand en organen. In twee experimenten zullen 8 varkens geopereerd worden voor het plaatsen van een canule in de dunne darm om darminhoud te kunnen verzamelen, dan wel direct substraat in de darm te kunnen infuseren. In één van deze experimenten zal tijdens deze operatie ook een katheter in een bloedvat geplaatst worden om bloed te verzamelen. Deze operatie gebeurt onder narcose en daarna worden pijnstillers verstrekt en worden de varkens individueel gehuisvest.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Dit project bevat meerdere experimenten met varkens en pluimvee. Het ongerief in de pluimvee-experimenten is geschat op licht. Twee experimenten met varkens zijn geclassificeerd als licht. Twee experimenten met in totaal 16 varkens zijn geclassificeerd als matig.
3.6	Wat is de bestemming van de dieren na afloop?	Waar mogelijk zullen dieren de voedselketen ingaan. De overige dieren die worden geëuthanaseerd voor het verzamelen van monsters zullen naar de destructie gaan.

4 Drie V’s

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Waar mogelijk zullen eerst laboratoriummodellen gebruikt worden om de kwaliteit/verteerbaarheid van eiwitbronnen in te schatten. In dit project zijn het varken en de kip de doeldieren. De interacties tussen voeding, micro-organismen en het dier zijn erg complex en kunnen niet bepaald worden met een proefdiervrij model. Ook voor het meten van (tot nu toe onbekende) producten van eiwitfermentatie en het bepalen van de gevolgen voor darmgezondheid is geen proefdiervrij model beschikbaar. Wanneer (tot nu toe onbekende) producten van eiwitfermentatie geïdentificeerd worden in dierproeven zal het werkingsmechanisme van deze producten op darmfunctie getest worden op darm-organoids. Dit zijn modellen van darmweefsel die op het laboratorium gekweekt kunnen worden vanuit darmcellen.
4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Voor elk experiment is het minimale aantal dieren dat nodig is om een betrouwbaar antwoord te krijgen op de onderzoeksvraag bepaald met behulp van statistiek. Hiervoor is gebruik gemaakt van informatie uit de literatuur.

4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	In dit project zijn het varken en de kip de doeldieren en er zijn geen alternatieve (dier)modellen beschikbaar om de complexe relatie en interacties tussen voeding, micro-organismen en het dier te bepalen.
4.4	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	• Tijdens de experimenten zullen de dieren dagelijks gecontroleerd worden door gecertificeerde en bekwame dierverzorgers. • In geval van ziekte of ongerief anders dan voorzien is in het proefplan, zal een dierenarts worden geraadpleegd en na overleg bepaald worden of een dier moet worden behandeld of uit de proef gehaald moet worden. • In de proeven waarin een canule geplaatst wordt in de darm van varkens zullen deze operaties uitgevoerd worden onder algehele narcose en pijnstilling toegediend worden na de operatie. • Het hanteren van dieren, het verzamelen van bloed of mestmonsters en het euthanaseren van dieren wordt gedaan door bekwaam personeel om zo weinig mogelijk stress te veroorzaken. • Varkens krijgen afleidingsmateriaal in de hokken en dit wordt regelmatig vervangen. • Kippen krijgen bodembedekking (behalve wanneer mest + urine verzameld wordt gedurende 4 dagen) en zitstokken in de hokken. • Dieren worden in groepen gehuisvest. Een uitzondering hierop zijn de varkens met een darmcanule (en bloedvatkatheter). Deze varkens worden individueel gehuisvest omdat de varkens anders elkaars canule/katheter kunnen beschadigen. Individueel gehuisveste varkens kunnen elkaar wel zien en horen en de varkens krijgen afleidingsmateriaal.

5 In te vullen door de CCD

Publicatie datum	

Beoordeling achteraf	

4.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure On-farm piglet experiment

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The role of protein fermentation in the occurrence of post-weaning diarrhea (PWD) is poorly understood. The relation between dietary protein and PWD has been studied under highly controlled conditions, but then often diarrhea does not occur (Htoo et al., 2007; Nyachoti et al., 2006). It is, therefore, of importance to study PWD under practical conditions. The aim of this experiment is to determine associations between PWD, protein fermentation metabolites and gut function indices in piglets kept under commercial conditions.

General design

In this experiment, piglets will be selected and sampled from commercial farms. In collaboration with and advised by veterinarians, multiple farms will be selected. Farms will be classified based on known parameters (such as average daily gain, feed efficiency, mortality, known occurrence of outbreaks PWD). At each farm, piglets with and without PWD are selected and dissected for sample collection. In total, 200 piglets will be sampled, including healthy control piglets selected on each farm.

First, piglets are identified based on clinical signs of diarrhea (or non-diarrhea for the controls). Secondly, a rectal fecal sample is collected and scored on fecal consistency. The inclusion criteria (such as fecal pH, ammonia concentration) for piglets with PWD will be determined in a pilot study (see below, A – paragraph 3). Based on these developed criteria, piglets are classified as PWD and non-PWD (controls) and, lastly, are blood-sampled and killed for sample collection. Blood-sampling is performed before killing because the method of killing might affect the (protein fermentation) metabolite profile in the blood. Collected samples at dissection include digesta, urine and intestinal tissues.

Recently, Dou et al. (2017) showed that pre-weaning microbiota diversity and composition could be related to PWD under experimental conditions. In this experiment, fecal samples will, therefore, be collected at two additional time points before dissection, in which microbiota/pathogen analyses are conducted. This will enable the investigation of causal relations between microbial composition and/or pathogens and PWD.

Differences between farms in feed composition can occur, but are generally small in Dutch piglet farming. Feed-specific data will be recorded at each farm and will be part of the farm effect in the data analysis.

Primary outcome parameters

First, analyses will be conducted in samples from all 200 piglets to quantify protein fermentation, using known protein fermentation products (i.e. *p*-cresol). Based on these results, a sub-set will be selected to obtain a representative reflection of the variation in protein fermentation in PWD piglets. In this subset, detailed laboratory analyses will be performed. Detailed analyses include microbiota composition in digesta or fecal samples, presence of pathogens causing diarrheal disease, intestinal morphology, mucosal barrier function measures and blood inflammatory cytokines. In addition, (untargeted and targeted) metabolomics analyses will be performed in feces, digesta or blood samples. Finally, multivariate analyses are performed

to determine associations between protein fermentation, PWD, pathological/functional read-outs of gut function, microbiota parameters, metabolomics analyses and immunological measures.

References

Dou, S., P. Gadonna-Widehem, V. Rome, D. Hamoudi, L. Rhazi, L. Lakhal, T. Larcher, N. Bahi-Jaber, A. Pinon-Quintana, A. Guyonvarch, I. L. E. Huërou-Luron, and L. Abdennebi-Najar. 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS One 12:e0169851.

Htoo, J. K., B. A. Araiza, W. C. Sauer, M. Rademacher, Y. Zhang, M. Cervantes, and R. T. Zijlstra. 2007. Effect of dietary protein content on ileal amino acid digestibility, growth performance, and formation of microbial metabolites in ileal and cecal digesta of early-weaned pigs. J. Anim. Sci. 85:3303-3312.

Nyachoti, C. M., F. O. Omogbenigun, M. Rademacher, and G. Blank. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. J. Anim. Sci. 84:125-134.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

In this experiment, we are studying PWD and the variation in protein fermentation under practical circumstances. Therefore, we will exclusively focus on natural occurrence of diarrhea and will not impose (dietary) treatments. The onset of PWD occurs around 3 to 4 days after weaning (advise of pig veterinarian; Kyriakis et al., 1999; Madec et al., 1998) and can remain up to 2 weeks after weaning (Kyriakis et al., 1999; Heo et al., 2008). Three to 14 days following weaning will be the window for PWD identification and subsequent dissection and sample collection at the commercial farms. Because early life microbial biomarkers could be of importance as well, fecal samples are collected in the pre-weaning period as well. In total, 3 fecal samples are collected per piglet (1 pre-weaning, 1 post-weaning and 1 for PWD identification/before dissection). It is unknown which piglets will develop diarrhea in the post-weaning period, therefore, a larger group of piglets will be sampled for feces in the pre-weaning and early post-weaning period (see also B - estimated numbers).

In this experiment, microbial composition and microbial metabolites in piglets are studied, and, therefore, individual piglets which have received antimicrobial treatments should not be included. When sows have been treated with antimicrobials, her piglets will not be included. In general, few group antimicrobial treatments are applied to piglets in the pre-weaning period. Pre-weaned piglets which have been treated individually will not be included in this study. In the post-weaning period, antimicrobial treatments (for PWD) should be withheld in the period of sample collection in the departments from which piglets will be selected. In case of diseased piglets (other than diarrhea), there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. For instance, if individual piglets should be treated for joint infections, these piglets will not be included in the experiment. Selection of piglets will be based on clinical signs of PWD and a rectal fecal sample is obtained to verify diarrhea or non-diarrhea. A maximum of 3 attempts is used per piglet per day in order to obtain a rectal fecal sample from a piglet (via manual stimulation).
Based on the fecal samples collected post-weaning, piglets will be selected, blood-sampled and killed (within 36h after fecal sampling), and, subsequently, the gastrointestinal tract will be removed and tissues and intestinal digesta collected.

Laboratory analyses as described in the previous paragraph will be conducted in the obtained samples and the data following statistical analyses will result in important associations between protein fermentation, PWD, pathological/functional read-outs of gut function, microbiota parameters, metabolomics analyses and immunological measures.

References

Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2008. Effects of feeding low protein diets to piglets on plasma urea nitrogen, faecal ammonia nitrogen, the incidence of diarrhoea and performance after weaning. Arch. Anim. Nutr. 62:343-358.
Kyriakis, S. C., V. K. Tsiloyiannis, J. Vlemmas, K. Sarris, A. C. Tsinas, C. Alexopoulos, and L. Jansegers. 1999. The effect of probiotic LSP 122 on the control of post-weaning diarrhoea syndrome of piglets. Res. Vet. Sci. 67:223-228.
Madec, F., N. Bridoux, S. Bounaix, and A. Jestin. 1998. Measurement of digestive disorders in the piglet at weaning and related risk factors. Prev. Vet. Med. 35:53-72.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

This experiment is not focused on differences in response parameters between (dietary) treatments, but is aimed at obtaining samples from piglets with and without PWD under commercial conditions. Little is known about the variation in protein fermentation during PWD under commercial conditions. We, therefore, performed power analyses with data from experiments focusing on high or low protein diets and diarrhea in weaned piglets. Using fecal score (Pieper et al., 2012a), fecal ammonia concentrations (Heo et al., 2009) and colonic ammonia concentrations (Heo et al., 2010) in the power analysis results in a requirement of 32, 22 and 32 piglets, respectively, to obtain a power of ≥0.8. We expect higher variation between animals under commercial conditions compared to experimental studies. Furthermore, variation in other read-out parameters are expected to be higher. Therefore, the variation in the power analyses with fecal and colonic ammonia concentration was multiplied with 3, which resulted in 166 and 262 animals, respectively. We, therefore, estimate to require 200 piglets for dissection. The main focus is on piglets with PWD and we expect greater variation in the PWD piglets, therefore, we anticipate to include 150 PWD piglets and 50 (non-PWD) control piglets.
In addition, we have consulted a statistician regarding numbers of animals and farms. There is variation between farms in incidence of PWD, and, therefore, we need to include multiple farms to obtain a representative sample of the Dutch piglet population. The inclusion of multiple farms allows us to include farm as an effect in the data analysis, in order to determine whether differences in the relation between PWD and protein fermentation (metabolites) occur between farms.
In addition, we will perform a pilot study before the start of this experiment, to determine the frequency and concentration of protein fermentation (end-products) in feces of piglets with diarrhea. This pilot study has been discussed with the Animal Welfare Body (work protocol "The concentration of protein fermentation end-products in faeces of piglets with post-weaning diarrhoea") and was not considered an animal experiment as referred to in the Dutch Act on Animal Experiments since the experimental procedures described in the protocol cause less pain or distress than the insertion of a needle under good veterinary practice. In short, in this pilot, fecal samples will be obtained from piglets in practice (multiple farms) with and without diarrhea. If piglets do not defecate spontanously when being handled, a fecal sample will be obtained via manual stimulation. Fecal consistency score and pH are determined in the fecal samples. Subsequently, fecal ammonia concentration is analyzed. Other protein fermentation end-products are also analyzed and correlated with ammonia to determine the appropriateness of fecal ammonia as an indicator of protein fermentation. These pilot data are required to determine the criteria for the selection of piglets with PWD in this experiment and will also be used to verify the required number of piglets, but we do not expect to require more than 200 piglets.

Data from this experiment will be analyzed using multivariate analyses. For instance, metabolomics data from piglets with and without PWD will be analyzed by partial least squares discriminant analysis. In addition, functional read-outs of gut function will be analyzed with principal component analysis and the following principal components will be subsequently related to protein fermentation indices.

References
Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2009. Feeding a diet with decreased protein content reduces indices of protein fermentation and the incidence of postweaning diarrhea in weaned pigs challenged with an enterotoxigenic strain of Escherichia coli. J. Anim. Sci. 87:2833-2843.
Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2010. Feeding a diet with a decreased protein content reduces both nitrogen content in the gastrointestinal tract and post-weaning diarrhoea, but does not affect apparent nitrogen digestibility in weaner pigs challenged with an enterotoxigenic strain of Escherichia coli. Anim. Feed. Sci. Technol. 160:148-159.Pieper, R., S. Kröger, J. F. Richter, J. Wang, L. Martin, J. Bindelle, J. K. Htoo, D. von Smolinski, W. Vahjen, J. Zentek, and A. G. van Kessel. 2012. Fermentable fiber ameliorates fermentable protein-induced changes in microbial ecology, but not the mucosal response, in the colon of piglets. J. Nutr. 142:661-667.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Piglets will be used in this experiment, because this is the target animal. This experiment is aimed at the variation present in the Dutch piglet population and, therefore, no appropriate model is available.

Piglets will be collected from multiple commercial farms. At each farm, we will select and kill piglets for sample collection. These selected piglets will include healthy control piglets (i.e. no diarrhea) and piglets with PWD. Sexe will be recorded, but is not used as a selection criterion, because it is not expected that PWD, and the relation with protein fermentation, differs between sexe. Potential differences in the ratio between male and female will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from commercial farms.
A total of 200 piglets will be sacrificed for sample collection. These 200 piglets will be selected based on criteria (i.e. fecal consistency, pH, ammonia concentration; verified in the pilot study) and in order to obtain these 200 piglets, rectal fecal samples will be collected from more piglets. In addition, to identify the presence of pathogens or microbial biomarkers before the onset of PWD, fecal samples will be collected from piglets in the pre-weaning and early post-weaning period as well. It is beforehand unknown which piglets will develop PWD. Assuming that the required 200 piglets will be obtained from 5 farms (i.e. 40 piglets per farm), with a division of 30 PWD and 10 control piglets at each farm, and a prevalence of 5% PWD on the day of selection for dissection, fecal samples in the pre-weaning / early post-weaning period should be collected from 600 piglets at each farm (i.e. 30 / 0.05 = 600 piglets/farm). This will in total be 600 x 5 = 3000 piglets from which feces will be sampled.
Selection and dissection of piglets (with and without PWD) will be between 3-14 days post-weaning, because this is the period in which the incidence of PWD is highest.

Species	Origin	Maximum number of animals	Life stage
Pigs	Mild	200	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: the piglet is the target animal in this experiment. This experiment is aimed at the variation present in the Dutch piglet population and, therefore, no appropriate model is available. Reduction: the number of animals is based on power analyses and this number will be verified with a pilot study. Refinement: the selection of piglets will be based on minimal invasive measures, i.e. clinical signs of PWD and rectal fecal samples. The attempts to obtain a fecal sample are confined to 3 at each time point. Killing of the piglets will be performed by skilled personnel to minimize stress.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals will be obtained from commercial farms. Individual sows and pre-weaning piglets are not included in this experiment when they have been treated with antibiotics. In the post-weaning period of sample collection, (group) antimicrobial treatments for PWD should be withheld, because treatments will affect microbial composition and microbial metabolite production and the development of PWD. In case of individual diseased piglets (other than diarrhea), there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. After piglets are killed and sampled this experiment is finished and potential required treatments can again be applied when advised by a veterinarian according to normal Dutch legislation. Killing and dissection of piglets is performed by skilled personnel.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature overview has been made and the literature search (using google scholar) included the key words piglets, post-weaning diarrhea, gut health, dietary protein and protein fermentation. To the best of our knowledge, associations between PWD, protein fermentation and gut function measures have not been determined in commercial settings before. In addition, this project has been peer-reviewed by 5 independent reviewers and this on-farm approach was highly encouraged by the reviewers.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

The piglets in this experiment are obtained from commercial farms and, therefore, piglets are kept under commercial conditions and not all housing conditions of Annex III of the Directive 2010/63/EU are met: - 3.1b. The piglets are checked daily by the respective farmer or a qualified person (Wod), and sick piglets will be noted. Piglets with PWD cannot be treated immediately, because our objective is to determine the mechanisms underlying diarrhea in piglets, including microbial composition and production of microbial metabolites, and antibiotic treatment will interfere with

these measurements. - 3.3b and 3.6a: no bedding material will be provided. Environmental enrichment will be as usual at each specific farm. - Table 7.3: the surface area for piglets will be according to commercial conditions.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[] No > Continue with question H.
[X] Yes > Describe this establishment.

The experiment will take place at commercial pig farms, which are not official establishments licensed by the NVWA.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Piglets will be sampled and dissected at commercial pig farms. It is of importance that the conditions are in agreement with commercial practices. Before piglet selection and dissection, the respective farm personnel will perform daily routine inspections. Fecal samples before dissection are collected by a qualified person (Wod). Killing and dissection will be performed by qualified persons.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Selected piglets will be blood-sampled and killed for sampling of tissues and intestinal digesta. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

During the (post-weaning) period of fecal sampling and until dissection, no antimicrobial treatments should be provided for PWD. This might result in a higher incidence or severity of PWD.

Explain why these effects may emerge.

Withholding antimicrobial treatments might increase the incidence or severity of PWD.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Antimicrobial treatments cannot be applied because these treatments will interfere with the measurements. No specific measures are taken to minimize adverse effects, but piglets will be inspected daily by the respective farm personnel. In case of diseased piglets (other than diarrhea), there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. At each specific farm, the day of selection and dissection will be determined following discussion with the respective farmer, to determine the day at which PWD prevalence is usually highest. Our experience is that this is usually around 4-7 days post-weaning. Assuming the onset of PWD is at 3-4 days post-weaning, this means that the period of not treating piglets is kept between 1-3 days, However, the exact window of selection and dissection will be determined with the respective farmer, but will be at maximum until day 14 post-weaning.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Antimicrobial treatments (for PWD) should be withheld in de post-weaning period, because antibiotics will interfere with the measurements. In case of sick piglets, there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. For instance, piglets showing severe lethargy can be euthanized when advised by the veterinarian.

Indicate the likely incidence.

The likely incidence of humane endpoints to occur in the post-weaning period is estimated at 3%, i.e. slightly higher than the average mortality rate in the Dutch weaned piglet population of 2.2%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort in this experiment is estimated to be mild. Experimental conditions are according to commercial settings, except that no antimicrobial treatments can be provided in the sampling period (mild). Discomfort related to rectal fecal sampling, blood sampling and killing is also estimated as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Piglets need to be killed in order to collect the required samples.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Describe the method of killing that will be used and provide justifications for this choice.

[X] Yes

5.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 2	Type of animal procedure Digestibility experiment with piglets

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The aim of this experiment is to test the effect of sanitary conditions, dietary crude protein (CP), and their interaction, on CP digestion and fermentation, fermentation end-products, and piglet performance. The experiment will be designed in a 2 x 2 factorial arrangement with sanitary conditions (high, HSC; or low, LSC) and dietary digestible CP content (high, HCP; or low, LCP) as factors. Output parameters will be ileal and total tract nutrient digestibility, feces quality (consistency score, dry matter content, and pH), body weight gain, and feed efficiency. In addition, metabolites of protein fermentation (e.g. ammonia, amines, phenols and sulfides) in feces will be measured.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

The contrast in sub-clinical health status will be according to van der Meer et al. (2016). Briefly, piglets will be obtained from a commercial nursery farm in the Netherlands. At 4 weeks of age, piglets will be transported to the experimental farm and allocated to LSC or HSC treatments. At arrival, piglets will be weighed and HSC piglets will receive antibiotic and anthelmintic treatments, whereas LSC piglets will remain untreated. HSC and LSC piglets are housed in separate rooms. HSC piglets will be housed in thoroughly cleaned and disinfected pens and a strict hygiene protocol (including showering, change of clothes, hairnet, and face mask) will be adhered when entering the HSC room [redacted]). A mixture of manure of commercial pig farms will be spread weekly in the LSC pens to increase pathogenic pressure and no hygiene protocol will be applied ([redacted] unpublished). In addition, LSC piglets will be exposed to nylon bags containing a mixture of pig manure and straw, ground to pass a 1 mm screen, as a model for dust ([redacted] unpublished).
From arrival until 6 weeks of age, piglets will receive the same weaner diets as they received in the nursery farm. From 6 weeks of age, experimental piglet diets will be fed. Within sanitary status, pens of pigs will be randomly assigned to LP or HP diets. Diets will be fed for 4 weeks.
At 10 weeks of age, piglets will be sacrificed and intestinal contents from the ileum and colon will be collected. At maximum 48 h before dissection, piglets will be housed individually and will be adapted to frequent feeding. Starting from 24h before dissection, pigs will be frequently fed (once every 6h from 24-6h prior to dissection and once every hour from 6h prior to dissection). This is required to obtain a steady-state in the gastro-intestinal tract. Our experience is that if pigs are used to meal feeding and will remain on the same diet, pigs will readily adapt to individual housing and frequent feeding.

References

van der Meer, Lammers, Jansman, Rijnen, Hendriks, and Gerrits. Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation. J Anim Sci (2016) 94:4704-4719. [redacted].

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of replicate pens per treatment has been calculated by power analysis (Proc Power; SAS 9.3) using data from a previous experiment with comparable design (with 6 piglets per pen; [redacted]). Because ileal CP is considered a main output parameter for the current experiment, this parameter is used for the power analysis. In the previous experiment, only total tract CP digestibility was measured ([redacted]). From literature (de Vries et al, 2014), it is calculated that the variation in ileal CP digestibility measurements is 3 x larger than total tract CP digestibility measurements. Assuming a residual standard deviation of 4.4 (3 x 1.47; [redacted]) and averages of ileal CP digestibility of 70, 82, 79, and 85% for the four treatments, it follows that n=9 at a power of 0.8. The standard deviation used in the power analysis is based on the study of [redacted]) where 6 piglets per pen were used. We, therefore, include 6 piglets per pen as well. This number of piglets per pen also resembles the practical situation.

References

[redacted].de Vries, Pustjens, van Rooijen, Kabel, Hendriks, and Gerrits. Effects of acid-extrusion on the degradability of maize dried distillers grain with solubles in pigs. J Anim Sci (2014) 92:5496-5506.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

In total, 216 female piglets will be used in this experiment divided over 3 batches (72 piglets per batch). Piglets will be housed with 6 piglets per pen and pen will be considered the experimental unit. The 4 dietary treatments will be tested in 3 pens each batch, resulting in 9 replicates per treatment.
Piglets will be obtained from a commercial nursery at 4 weeks of age and remain in the experimental facilities until 10 weeks of age.
Only female piglets will be used to exclude a potential effect of sexe. Because female animals generally respond more intense to immune interventions than male animals, we have chosen to use female piglets to maximize contrasts between treatments. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial farm.

Species	Origin	Maximum number of animals	Life stage

Pigs	Mild	216

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: The piglet is the target animal in this experiment. The interaction between sanitary status and protein fermentation cannot be tested with ex vivo techniques. Hence, it was chosen to test this in an in vivo experiment. Reduction: The number of piglets to be used has been based on previous observations and is minimized to the number needed to detect differences between the treatments. Contrasts between the treatments are maximized within the range observed in practical settings, to minimize the number of piglets needed. Refinement: Piglets will be group-housed for the major the part of the study and the duration of individual housing is limited to 48h to minimize discomfort. In addition, during individual housing, pigs will be able to see and hear each other, have snout contact and will receive toys as enrichment.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All animal handling procedures will be conducted by experienced staff (feeding, weighing, transport). Various, non-destructible toys will be made available to the pigs as environmental enrichment, both during group- and individual housing, and toys will be alternated regularly. Occurence of clinical health issues due to sanitary conditions is not expected based on previous studies (van der Meer et al 2014 and [redacted]

unpublished). However, in case of clinical signs of illness, there will be discussion between researchers, animal caretakers and a veterinarian to determine is further measures are required. No exceptional adverse effects of this experiment on the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature overview on this topic has been performed. To the best of our knowledge, the interactive effects of sanitary conditions and dietary protein level on protein fermentation and its end-products has not been performed previously. If newly published papers indicate novel insights in the topic that have not been taken into account but are of relevance for the proposed experimental design, experimental procedures will be reconsidered.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Housing and care is according to Annex III, except during the last 48h of the experiment, when pigs will be housed individually in pens. Individual housing during this period is needed to make sure that every animal consumes feed at each time point a meal is given (once every 6h from 24-6h prior to dissection and once every hour from 6h prior to dissection). This is required to obtain a steady-state in the gastro-intestinal tract. The floor area of the pens in which pigs will be individually housed, will be min. 2 m2, allowing the pigs to move around freely. The use of bedding material is avoided as this will be consumed by the pigs and will thus interfere with the digestibility measurements. Pigs will be able to see and hear each other and have snout contact. Animals will receive toys as enrichment.

G. Location where the animals procedures are performed

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Pigs might experience pain during killing. The potential pain is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Previous studies using the same contrasts in sanitary conditions (van der Meer et al., 2014; and [redacted]., unpublished) did induce differences in sub-clinical health status. It is possible that piglets in the low sanitary status treatment develop clinical health problems, however, the

previous studies (van der Meer et al., 2014; and [redacted]., unpublished) showed no adverse effects of the low sanitary status on animal welfare. Pigs might experience stress as a result from individual housing.

Explain why these effects may emerge.

The potential adverse effects can occur due to the sanitary conditions. Individual housing is required to feed individually and frequently to obtain a steady-state in the gastro-intestinal tract.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Occurence of clinical health issues due to sanitary conditions is not expected based on previous studies ([redacted]). In case of clinical signs of illness, there will be discussion between researchers, animal caretakers and a veterinarian to determine is further measures are required. The period of individual housing is restricted to 48h and pigs will be able to see and hear each other, have snout contact and pigs will receive toys as enrichment.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Based on previous studies using the same contrasts in sanitary conditions [redacted]), the procedures are not expected to give rise to circumstances that require humane endpoints. In the case of clinical illness in the expert opinion of the veterinarian, animals will be removed from the experiment.

Indicate the likely incidence.

Based on our previous experiments the likely incidence is estimated to be below 5%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The expected level of discomfort is classified as mild. The low sanitary conditions treatment may result in a lower sub-clinical health status. Based on previous studies using the same contrasts in sanitary conditions (van der Meer et al., 2014 and [redacted]), the level of discomfort for the LSC treatment is judged as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Pigs will be killed and dissected after the procedures. This is necessary to obtain samples for digestibility measurements.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Describe the method of killing that will be used and provide justifications for this choice.

[X] Yes

6.

Appendix
Description animal procedures

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- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 3	Type of animal procedure Digestibility experiment with cannulated pigs

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The aim of this experiment is to test the effect of fermentable fiber (FF), crude protein (CP), and their interaction, on the extent of fermentation, fermentation end-products, microbial colonization, digesta passage rate, and feces quality. The experiment will be designed in a 3 x 2 factorial arrangement with dietary FF content (low FF, high FF, low FF+FF infused in colon) and CP (two protein isolates varying in protein structure) entering the colon as factors. The selection of protein sources will depend on the data obtained in appendix 7. Diets will have low or high FF contents. Apart from affecting fermentation in the large intestine, dietary FF will affect endogenous protein losses, fermentation, and microbial biomass in the small intestine, thereby changing the substrate arriving at the colon. A third treatment where the low FF is fed and FF is infused in the colon, will be tested to separate the effects of FF in the small intestine from fermentation characteristics in the large intestine. Output parameters will be total tract nutrient digestibility, digesta retention time in the large intestine, feces quality (consistency score, dry matter content, and pH), and microbiota composition in feces. In addition, a combination of targeted and untargeted metabolomics in feces and blood samples will be used to measure metabolites of protein fermentation.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

After arrival, pigs will be allowed to acclimatize to the new environment for a minimum of 8 days. Fecal grab samples are taken and analyzed for the presence of parasites. If required pigs will receive anthelmintic treatment. During the acclimatization periods, pigs will be socialized according to a fixed protocol. Pigs will be surgically fitted with a cannula in the distal ileum and a catheter in the jugular vein. After surgery pigs are allowed to recover and adapt to the housing conditions for a minimum of 7 days before they are switched to the experimental diets. After recovery from surgery, the experimental phase will start and samples will be collected from each pig during 6 subsequent measurement periods. This approach is selected to allow within pig comparisons of the dietary treatments (Latin square design), minimizing the total duration of the experiment, thereby the risk of cannula and catheter complications. Each period will consist of 5 days adaptation to the experimental diet, followed by collection of ileal digesta during 12 hours on days 6 and 7, infusion of protein/fermentable fiber and markers to estimate mean retention time of the digesta into the colon (via the cannulas) at day 8 and urine+feces collection and blood sampling (via the catheter) at days 9-11. Such a time frame (6 periods x 11 days) is commonly used in studies with ileal cannulated growing pigs. To collect feces quantitatively, plastic bags will be attached to the rear end of the pigs as described by Van Kleef et al. (1994). Clean urine will be quantitatively collected using funnels underneath the cage.

Reference

van Kleef, Deuring, and van Leeuwen. A new method of faeces collection in the pig. Lab Anim (1994) 28: 78-79.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of replicates per treatment has been based on literature where similar output parameters were studied (Bach Knudsen et al. 2007; de Vries et al. 2016; Otto et al. 2003, Wilfart et al. 2007). For the most important parameters, power was calculated for several experimental design scenarios according to the method of Stroup (1999), using the data presented in these studies. It was concluded that a 6x6 Latin square design gave sufficient power (between 0.8 and 0.9) for the most important parameters (nutrient digestibility, SCFA concentrations in peripheral blood, large intestinal retention time).

References

Bach Knudsen, Jørgensen, and Canibe. Quantification of the absorption of nutrients derived from carbohydrate assimilation: model experiment with catheterised pigs fed on wheat- or oat-based rolls. Br J Nutr (2007) 84:449-458.
de Vries, β-Glucans and resistant starch alter the fermentation of recalcitrant fibers in growing pigs. PlosOne (2016) 11: e0167624.
Otto, Yokoyama, Hengemuehle, Bermuth, van Kempen, and Trottier. Ammonia, volatile fatty acids, phenolics, and odor offensiveness in manure from growing pigs fed diets reduced in protein concentration. J Anim Sci (2003) 81: 1754-1763.
Wilfart, Montagne, Simmins, Noblet, and van Milgen. Br J Nutr (2007) 98:54-62.
Stroup. Mixed model procedures to assess power, precision, and sample size in the design of experiments. Proc Biopharm Am Stat Assoc (1999) 15-24.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Eight barrows (approximately 25 kg) will be obtained from a commercial pig farm. Only pigs that are healthy without hernias (testicular or umbilical), damaged ears or unsound feet are selected, because these can complicate surgery. Surgery will be performed on 8 pigs. Two pigs will be considered spare animals and will be used in the case of problems with digesta collections or catheter patency. If pigs are replaced by spare pigs during the experiment, observations can be included in the statistical analyses, provided that these pigs have been used for at least two of the experimental periods. Should problems arise during the last period, the measurement period will be extended by one period. Pigs will be approximately 60-70 kg at the end of the experiment.
Barrows will be used so that urine and feces can be collected separately. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial farm.

Species	Origin	Maximum number of animals	Life stage
Pigs	Moderate	8	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: The pig is the target animal in this experiment. Although fermentation can be tested in vitro, the complex interactive effects of dietary protein and fermentable fiber with passage rate and bacterial colonization on fermentative processes cannot be tested with ex vivo techniques. Hence, it was chosen to test this in an in vivo experiment. Reduction: A Latin square design is used to minimize the number of pigs needed. Refinement: Before surgery pigs will be socialized for a minimum of 5 days (according to a socialization protocol), so that they are well adapted to handling procedures after surgery and during sampling.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Surgical procedures will be conducted under complete anesthesia, and adequate analgesia are used during recovery. No exceptional adverse effects of this experiment on the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature overview on this topic has been performed. To the best of our knowledge, the interactive effects of fermentable fiber and dietary protein structure on the combination of protein fermentation, its end-products and passage rate and digestibility have not been performed previously. If newly published papers indicate novel insights in the topic that have not been taken into account but are of relevance for the proposed experimental design, experimental procedures will be reconsidered.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Pigs will be housed individually in metabolism pens during the recovery phase and during the experimental period. The floor area of the pens will be min. 2 m2, allowing the pigs to move around freely. Walls will be smooth to prevent damage to cannulas. Animals will be housed on a plastic coated floor. Individual housing is needed to prevent animals from damaging cannulas and catheters of pen mates. Pigs will be able to see and hear each

other. The use of bedding material is avoided as this will be consumed by the pigs and will thus interfere with the digestibility measurements. Animals will receive toys as enrichment, which are regularly changed.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[X] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Surgical procedures will be performed under anesthesia. After surgery, animals will be treated with analgesia for at least 3 days and with antibiotics. The potential pain experienced during killing is minor and short-term, and, therefore, no pain relieving methods are applied.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

I. Other aspects compromising the welfare of the animals

Individual housing, fasting prior to surgery and recovery from surgical procedures may have adverse effects on animal welfare. Infection and inflammation due to the presence of the cannula and/or catheter may occur.

Explain why these effects may emerge.

These adverse effects may emerge because they are part of the experimental procedures.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Infection and inflammation will be prevented or minimised by providing antibiotics after the surgical procedures and whenever required based on clinical signs of infection. Long-term effects of antibiotic treatment on the microbiota are minimised by administering the antibiotics intramusculair/in the wound (and not orally) and by allowing a recovery period of (a minimum of) 7 days and an adaptation period of 5 days to the diet. Adverse effects on animal welfare due to individual housing are minimised by providing toys as environmental enrichment and pigs will be able to see and hear each other.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

The following humane endpoints will apply. Pigs will be euthanized in the following situations: • During recovery from surgery, a pig does not start eating within 2 days and subsequently produce feces, indicating blockage of the intestines. • A cannula is lost and cannot be placed back. • A pig has fever during 5 successive days, is not responding to treatments proposed by a veterinarian, and shows signs of infection and inflammation. • A pig has feed refusals exceeding 20% of the amount of feed offered for a period exceeding 7 days. • In the expert judgement of the veterinarian, future observations on a pig will not provide reliable results.

Indicate the likely incidence.

The likely incidence of pigs to be removed from the experiment is estimated at 25%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The level of discomfort is expected as follows: • Surgical procedures: moderate • Sampling procedures (9.5 weeks): mild • Individual housing in the absence of bedding material in large metabolism pens (11 weeks): moderate. The cumulative discomfort in this experiment is estimated at moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Pigs will be killed after the procedures. Keeping pigs with a cannula is complicated. Moreover, the location of the cannula and tip of catheter need to be verified during autopsy.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Describe the method of killing that will be used and provide justifications for this choice.

[X] Yes

7.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 4	Type of animal procedure Protein digestibility of different feed ingredients and the metabolic fingerprints of broilers fed these diets

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The aim of this experiment is to determine which unknown metabolites can be correlated to hind gut protein fermentation in broilers, as well as determine the potential of different protein sources to contribute to hind gut protein fermentation. Protein fermentation is likely involved in the occurrence of gut health problems and of diarrhea.

Experimental diets
Broilers will be fed, from hatch to 29 days of age, different experimental diets in a 3x2 complete randomized block design. The diets will consist of one of 3 different protein sources (from ingredients currently used in poultry feed), at 2 levels of ileal protein digestibility. In vitro tests (in vitro pre-digestion, to simulate gastric and enteric digestion, followed by gas production method, to simulate hind gut fermentation) will be used prior to the feed trial to determine which protein sources will be added into the experimental diets and which method(s) of creating poorer digestible proteins will be used.

Read-out parameters
The following measurements will be recorded:

- performance measurements (feed intake, water intake, weight gain)
- litter and cloaca scores
- ileal & fecal crude protein and amino acids digestibility (marker method)
- Di-animo-pimelic acid (DAPA), indicator of microbial biomass, measured in ileal and cecal digesta
- untargeted metabolomics and biogenic amines targeted metabolomics (using serum, cecal and colon digesta)

- intestinal lesion scores
- relative organ weights (proventiculus, gizzard, duodenum, jujenum, ileum, ceca, colon, pancreas and liver)
- cecal digesta pH
- gut leakage (using serum marker: fluorescein isothiocyanate (FITC)-Dextran)
- villus height and crypt depth
- microbiota population in ceca

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Individual tags:

On day 0 all birds are given individual neck labels. This is necessary to allow us to link metabolic data from individual birds to their growth performance and individual gut health measures. Secondly, tagging is needed to guarantee we can separate birds if they accidentally mix at a young age.

Weighing & cloaca scores:
Individual weighing is done on day 0, 7, 14, 21 and 28. Simultaneously, a cloaca score is given each chick (1 minute/bird/time point). This is the most practical and least stressful method as they are only handled once per time point.

Fecal collection:

During the last 4 days of the trial broilers are placed on slats to allow for fecal collection. Prior to these last 4 days broilers will be housed on comfortable SoftCell bedding.

Blood sampling:
Prior to the dissection a single blood sample is taken from each broiler for metabolomics measurements. Blood samples are taken by an experienced technician.

Dissection:
Organs and digesta for the broilers will be collected for the above mentioned read-out parameters. Broilers are fasted 3 hours and then allowed to feed for 3 hours prior to euthanasia (on day 29), to ensure the presence of sufficient digesta in the different parts of the GIT (de Vries et al., 2014). Particularly for the determination of CP, amino-acids and markers in ileal digesta, large quantities of digesta are needed.

Reference
de Vries, S., Kwakkel, R.P., Pustjens, A.M., Kabel, M.A., Hendriks, W.H. and Gerrits W.J.J., 2014, Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with Cr2O3 and cobalt-ethylenediamine tetraacetic acid in broiler chickens. Poultry Science 93:2010–2017

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

We will use an in vitro digestibility and fermentation trial prior to this trial to select feed ingredients and by doing so less experimental diets are included in this research. Selection of ingredients will be based on the contrast in hind gut protein fermentation that can be created. We will determine this by measuring a simulated ileal protein digestion and the soluble fraction that is left after digestion. As it is known that in poultry fractional separation occurs and that mostly the soluble components enter the ceca (de Vries et al., 2014), which is the main site of fermentation. The availability of protein for cecal bacteria in the undigested soluble fraction is then measured using the gas production technique (Cone et al., 2005). Both measures (digestibility and fermentibility) are used as selection criteria.

Sample size determination:

There are no metabolomics data available to use for sample size determination. Pre-cecal CP digestibility is our second most important read-out parameter. We also consider growth performance important measurements.

We consider a difference in digestibility of 2.5% between treatments to be relevant. We would like to detect differences with a significance level α = 5% and a power β = 80%.
In previous trials using male broilers, a standard deviation for ileal CP digestibility of 2.53% (Qaisrani et al., 2015) and 1.78% (personal communication) was found. This standard deviation is based on including 8 male broilers per pen. Based on this we assume σ = 2.15%.

Calculation of the number of replicate pens:

n = { [Z(α/2) + Zß]^2 } / [(Δ/σ)^2]

Following formula will be used to determine sample size:

Using prerequisites, following values can be obtained from the Standard Normal Curves Areas table (Ott and Longnecker, 2010) or calculated:
Z0.05/2=1.96
Z0.80 = 0.85
Δ = 2.5
σ = 2.15

Thus:
n = (1.96 + 0.85)^2 / (2.5 / 2.15)^2 = 5.84

It can be deduced from this statistical power analysis that 6 repetitions are required per treatment group.

Number of birds per pen:

The variation used for the power analysis is based on previous studies (Qaisrani et al., 2015 and recent unpublished data) in which 8 male broilers per pen were used. Reducing the number of birds per pen may increase the variation between the pens, and will result in a higher number of pens required to obtain a minimal power of 80%.

For ileal CP and amino acid digestibility analysis we require at least 5 grams of digesta dry matter (personal communication). We should be able to obtain 1.14 g dry matter form the terminal ileum of a broiler (Kluth et al., 2005). Based on this information we can assume we will need to pool digesta of at least 5 birds and also in a previous trial (unpublished data) pooled digesta of 5 birds was enough for CP and amino acid analysis. Hence 8 birds should be sufficient for ileal digestibility measurements. In addition, 8 male broilers per pen will allow the detection of differences in growth performance between treatments.

In total 6 (3 diets x 2 degrees of protein digestibility) x 6 (replicate pens) x 8 (animals per pen) = 288 birds are required.

References

Cone, J.W., Jongbloed, A.W., Van Gelder, A.H. and de Lange L., 2005, Estimation of protein fermentation in the large intestine of pigs using a gas production technique. Ani. Feed Sci. Tech. 123-124:463-472Kluth, H., Mehlhorn, K. and Rodehutscord, M., 2005, Studies on the intestine section to be sampled in broiler studies on precaecal amino acid digestibility. Archives of Animal Nutrition, 59(4):271-279
Ott, R.L. and Longnecker, M., 2010. An Introduction to Statistical Methods and Data Analysis. Brooks/Cole Cengage Learning, ISBN-13: 978-0-495-01758-5

Qaisrani, S.N., van Krimpen, M.M., Kwakkel, R.P., Verstegen, M.W.A. and Hendriks, W.H., 2015, Diet structure, butyric acid, and fermentable carbohydrates influence growth performance, gut morphology, and cecal fermentation characteristics in broilers. Poultry Science 94:2152–2164

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Animals used will be *Gallus gallus domesticus* (domestic chickens), fast growing strain (Ross 308), males and will be obtained from a commercial hatchery. The species chosen is the target species of the study.

Gender related differences are not of interest in this study. To maintain maximum accuracy of measurements it is necessary to use birds with similar genetics and a similar gender. Since absorption of nutrients and metabolism are highly different between male and female chicks, the experiment is done in one sexe to decrease the variation in measurements. Males are preferred over females as they have a higher metabolic rate and feed intake, which should allow for more sample collection (digesta) per individual. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial hatchery.

Species	Origin	Maximum number of animals	Life stage
Gallus gallus domesticus, Ross 308	Mild.	288	

C. Re-use

Will the animals be re-used?

C. Re-use

[X] No, continue with question D.

[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No

[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement Broilers are the species of interest. The present animal experiment cannot be replaced by an experiment using another animal species due to the specificities of the avian digestive tract. A mechanistical modelling approach is excluded due to the complexity of interactions and modulators (largely still unknown) involved in protein fermentation. To our knowledge, such a model does not exist, let alone a model which can simulate the effects of protein fermentation on gut health and performance. As a consequence, this experiment has to be conducted with chickens. Reduction There are in vitro tests that aim to predict ileal protein digestibility and can be used for studying hind gut fermentation. These can, however, not replace the animal model, since they lack the interaction with the gut wall. Especially in these experiments where the effect on (gut-) health is studied, this interaction is highly relevant. In vitro tests will be used to get an indication of the order of possible protein fermentation, as described above. These in vitro tests will be used to determine feed ingredients prior to this trial, reducing the number of experimental groups required. Furthermore, we strive to collect as many samples (organs, blood, digesta) as possible from each individual chick, instead of using different chicks for different measurements. The number of replicates is determined using a sample size determination formula to ensure a minimal number of replicates is used while maintaining statistical power. Refinement We choose measurements that can be performed after killing of the birds and we reduced the time broilers spend on slats to just the last 4 days. Killing, blood sampling and neck labeling will be performed by experienced technicians.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Birds will be group housed in floor pens with SoftCell bedding (except during the last four days when they are group housed on slats without SoftCell bedding) and have perches. As mentioned above, invasive measurements during life other than a single blood sampling have been avoided. To our knowledge there are no environmental disadvantages to this trial.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

An extensive literature review has been conducted and very little is known about the mechanisms involved in protein fermentation and how this affects the animal's health. Also to the knowledge of the authors metabolic profiles related to protein fermentation in broilers have never been recorded. Knowledge of these metabolic profiles is expected to be very important in the improvement of gut health in farm animals and hence the reduction of antibiotic use.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Housing and care is according to Annex III, except during the last four days when birds will be housed on slats, which is needed for fecal collection. The perches will remain in the pens in this period.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Minor pain could be induced by neck labeling, blood sampling and euthanasia. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Diarrhea might occur in the groups fed lower digestible proteins.

Explain why these effects may emerge.

Birds are fed lower digestible proteins as it is part of the aim of this study to investigate hind gut protein fermentation (which results from undigested proteins passing the ileum).

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Other than lower dietary digestible protein in some of the treatment groups, all diets will be formulated to meet broiler requirements, with a similar fiber level. Fresh bedding will be provided after regular litter scores. Drinking nipples (checked daily) will be kept clean to avoid water spillage.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

There is a very small chance of severe weight loss due to diarrhea. If a bird loses 10% or more of its body weight compared to the previous weighing, it is removed from the trial and killed according to a method listed in Annex IV of Directive 2010/63/EU. If a bird shows clinical signs of sickness (diarrhea, inactivity, lack of appetite), there will be discussion between researchers, animal caretakers and, if required, with a veterinarian to determine if further measures are required.

Indicate the likely incidence.

The likely incidence of this we expect to be very low (below 5%), as all diets will be formulated with sufficient nutrients for growth, although broilers fed the lower digestible proteins are expected to have a lower growth response.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort is classified as mild.

End of experiment

L. Method of killing

L. Method of killing

Will the animals be killed during or after the procedures?

[] No

[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

This is required for the collection of ileal and cecal contents and organ samples.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Describe the method of killing that will be used and provide justifications for this choice.

[X] Yes

8.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 5	Type of animal procedure Feed strategies to reduce protein fermentation and alter metabolic fingerprint associated with protein fermentation in broilers

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The experiment described in appendix 4 should have given insight in the metabolic fingerprint of broilers associated with pre-cecal poorly digestible protein, which is expected to result in high hind gut protein fermentation. The aim of this experiment is to determine the effects some feed strategies (composition and processing) have on the metabolic fingerprint, protein fermentation associated metabolites and gut health of broilers.

Experimental diets

Broilers will be fed from hatch to 29 days of age, different experimental diets in a 2x2x2 design. Included factors are diet structure (particle size), fiber level and protein source. Fibers are known to be the preferred energy source of microbiota in the gut. In the presence of fibers, undigested proteins are not used as an energy source by the microbiota but can be used as a nitrogen source for microbial growth. Such a fiber feed strategy might reduce the production of (harmful) protein fermentation metabolites. A coarse dietary structure is expected to reduce passage rate and increase peristalsis and anti-peristalsis and therefore improve ileal protein digestibility. Further details of the diets will be determined based on the results of appendix 4 and the in vitro test (Boisson two-step, to simulate gastric and enteric digestion, followed by gas production method, to simulate hind gut fermentation). The contrast in dietary protein will be based on ileal crude protein and amino acid digestibility measures.

Read-out parameters

The following measurements will be recorded:

- Performance measurements (feed intake, water intake, weight gain)
- Untargeted and targeted metabolomics (using serum, cecal and colon digesta, we will be targeting metabolites that have been found to be associated with hind gut protein fermentation in experiment 1)
- Litter and cloaca scores
- Di-animo-pimelic acid (DAPA), indicator of microbial biomass, measured in ileal and cecal digesta
- Intestinal lesion scores
- Relative organ weights (proventiculus, gizzard, duodenum, jejenum, ileum, ceca, colon, pancreas and liver)
- Cecal digesta pH, gut leakage (using serum marker)
- Villus height and crypt depth
- Ileal and colon amino acid digestibility (marker method)
- Microbiota population in the ceca
- Passage rate

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Individual tags:

On day 0 all birds are given individual neck labels. This is necessary to allow us to link metabolic data from individual birds to their growth performance. Secondly, tagging is needed to guarantee we can separate birds if they accidentally mix at a young age.

Weighing & cloaca scores:
Individual weighing is done on day 0, 7, 14, 21 and 28. Simultaneously, a cloaca score is given each chick (1 minute/bird/time point). This is the most practical and least stressful method as they are only handled once per time point.

Passage rate measurements:

5 broilers per pen will be orally dosed with 3 gel capsules containing 150 mg titanium (Ti) oxide at day 24. At each time point (30, 90, 180, 270 and 360 min. after oral dosage), one bird per pen is euthanized, after which the contents of the different gastro-intestinal segments are collected.

Fecal collection:
During the last 4 days of the trial broilers are placed on slats to allow for fecal collection. Prior to these last 4 days broilers will be housed on comfortable SoftCell bedding.

Blood sampling:
Prior to the dissection a single blood sample is taken from each broiler for metabolomics measurements. Blood samples are taken by an experienced technician.

Dissection:
Organs and digesta for the broilers will be collected for the above mentioned read-out parameters. Broilers are fasted 3 hours and then allowed to feed for 3 hours prior to euthanasia (on day 29), to ensure the presence of sufficient digesta in the different parts of the GIT (de Vries et al., 2014). Particularly for the determination of CP, amino-acids and markers in ileal digesta, large quantities of digesta are needed.

Reference: de Vries, S., Kwakkel, R.P., Pustjens, A.M., Kabel, M.A., Hendriks, W.H. and Gerrits W.J.J., 2014, Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with Cr2O3 and cobalt-ethylenediamine tetraacetic acid in broiler chickens. Poultry Science 93:2010–2017

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Number of replicate pens:

There are no metabolomics data available to use for sample size determination. Pre-cecal CP digestibility is our second most important read-out parameter. We consider a difference in digestibility of 2.5% between treatments to be relevant. We would like to detect differences with a significance level α = 5% and a power β = 80%. In previous trials a standard deviation for ileal CP digestibility in male broilers of 2.53% (Qaisrani et al., 2015) and 1.78% (personal communication) was found. Based on this we assume σ = 2.15%.

Calculation of the number of replicate pens:

$n = \{ [Z(\alpha/2) + Z\beta]^2 \} / [(\Delta/\sigma)^2]$

Following formula will be used to determine sample size:

Using prerequisites, following values can be obtained from the Standard Normal Curves Areas table (Ott and Longnecker, 2010) or calculated:

$Z_{0.05/2}=1.96$

$Z_{0.80} = 0.85$

$\Delta = 2.5$

$\sigma = 2.15$

Thus:

$n = (1.96 + 0.85)^2 / (2.5 / 2.15)^2 = 5.84$

It can be deduced from this statistical power analysis that 6 repetitions are required per treatment group.

Number of birds per pen:

For ileal CP and amino acid digestibility analysis we require at least 5 grams of digesta dry matter. We should be able to obtain 1.14 g dry matter form the terminal ileum of a broiler (Kluth et al., 2005). Based on this information we can assume we will need to pool digesta of 5 birds.

For the passage rate measurements we will require 5 birds per pen which will be killed at different time points (van Krimpen et al., 2011).

We require different broilers to be used for digestibility and passage rate measurements because both measurements require total collection of digesta from gastrointestinal segments.
An additional broiler should be included in case of unexpected mortality.

Hence, 5+1+5 = 11 birds per pen are required. We know from previous trials (Qaisrani et al., 2015 and recent unpublished data) for significant measurement for FCR at least 8 male birds per pen are required. Hence 11 male birds should be sufficient also for FCR measurement.

In total 8 (treatments) x 6 (replicate pens) x 11 (animals per pen) = 528 birds are required.

References:

Qaisrani, S.N., van Krimpen, M.M., Kwakkel, R.P., Verstegen, M.W.A. and Hendriks, W.H., 2015, Diet structure, butyric acid, and fermentable carbohydrates influence growth performance, gut morphology, and cecal fermentation characteristics in broilers. Poultry Science 94:2152–2164

Ott, R.L. and Longnecker, M., 2010. An Introduction to Statistical Methods and Data Analysis. Brooks/Cole Cengage Learning, ISBN-13: 978-0-495-01758-5

Kluth, H., Mehlhorn, K. and Rodehutscord, M., 2005, Studies on the intestine section to be sampled in broiler studies on precaecal amino acid digestibility. Archives of Animal Nutrition, 59(4):271-279
Van Krimpen, M.M., Kwakkel, R.P., Van Der Peet-Schwering, C.M.C., Den Hartog, L.A. & Verstegen, M.W.A. (2011) Effects of dietary energy concentration, nonstarch polysaccharide concentration, and particle sizes of nonstarch polysaccharides on digesta mean retention time and gut development in laying hens. British Poultry Science 52:6.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Animals used will be *Gallus gallus domesticus* (domestic chicken), fast growing strain (Ross 308), males, ages from 0 days to slaughter age and will be obtained from a commercial hatchery. The species chosen is the target species of the study.

Gender related differences are not of interest in this study. To maintain maximum accuracy of measurements it is necessary to use birds with similar genetics and a similar gender. Since absorption of nutrients and metabolism are highly different between male and female chicks, the experiment is done in one sexe to decrease the variation in measurements. Males are preferred over females as they have a higher metabolic rate and feed intake, which should allow for more sample collection (digesta) per individual. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial hatchery.

Species	Origin	Maximum number of animals	Life stage
Gallus gallus domesticus, Ross 308	Mild	528	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement Broilers are the species of interest. The present animal experiment cannot be replaced by an experiment using another animal species due to the specificities of the avian digestive tract. A mechanistic modelling approach is excluded due to the complexity of interactions and modulators (largely still unknown) involved in protein fermentation. To our knowledge, such a model does not exist, let alone a model which can simulate the effects of protein fermentation on gut health and performance. As a consequence, this experiment has to be conducted with chickens. Reduction There are in vitro tests that aim to predict ileal protein digestibility and can be used for studying hind gut fermentation. These can, however, not replace the animal model, since they lack the interaction with the gut wall. Especially in these experiments where the effect on (gut-) health is studied, this interaction is highly relevant. Furthermore, we strive to collect as many samples (organs, blood, digesta) as possible from each individual chick, instead of using multiple birds for multiple measurements, reducing the number of birds required. Passage rate measurements, however, requires killing of birds a specific time points and use of all the digesta from all gastrointestinal tract segments, therefore these birds cannot be used for ileal digestibility analysis. The number of replicates is determined using a sample size determination formula to ensure a minimal number of replicates are used while maintaining statistical power. Refinement We choose measurements that can be performed after killing. Blood sampling and neck labling will be performed by experienced technicians.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Birds will be group housed throughout the trial, in floor pens with SoftCell bedding and have perches. During the last four days, when birds are housed on slats, perches will still be available. As mentioned above, invasive measurements during life, other than a single blood sampling, have been avoided. To our knowledge there are no environmental disadvantages to this trial.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

An extensive literature review has been conducted and very little is known about the mechanisms involved in protein fermentation and how this affects the animal's health. This experiment is a continuation of appendix 4 in which we aim to discover metabolic profiles related to protein fermentation. In this experiment we will examine how feeding strategies change these profiles and potentially, identify mechanisms underlying gut health. This knowledge is expected to be very important in the improvement of gut health in chickens and hence the reduction of antibiotic use.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Housing and care is according to Annex III, except during the last four days when birds will be housed on slats without bedding material, which is needed for fecal collection. The perches will remain in the pens in this period.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Minor pain could be induced by neck labeling, blood sampling and euthanasia. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Diarrhea might occur in the groups fed lower digestible proteins, lower fiber levels and pellets with fine particles.

Explain why these effects may emerge.

Birds are fed diet contrasting in protein digestibility, fiber content and structure as it is part of the aim of this study to investigate how these contrasts in feed affect hind gut protein fermentation and gut health.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Other than these contrasts, all diets will be formulated to meet broiler requirements. Fresh bedding will be provided after regular litter scores. Drinking nipples (checked daily) will be kept clean to avoid water spillage.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

There is a very small chance of severe weight loss due to diarrhea. If a bird loses 10% or more of its body weight compared to the previous weighing, it is removed from the trial and killed according to a method listed in Annex IV of Directive 2010/63/EU. If a bird shows clinical signs of sickness (diarrhea, inactivity, lack of appetite), there will be discussion between researchers, animal caretakers and, if required, with a veterinarian to determine if further measures are required.

Indicate the likely incidence.

The likely incidence of this we expect to be very low (below 5%), as all diets will be formulated with sufficient nutrients for growth, although broilers fed the lower digestible proteins are expected to have a lower growth response.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort is classified as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

This is required for the collection of ileal and cecal contents and organ samples.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Describe the method of killing that will be used and provide justifications for this choice.

[X] Yes

8.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 6	Type of animal procedure Transgenerational effects of protein fermentation metabolites in poultry

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Dietary protein fed to broiler breeders can affect broiler offspring performance (Rao et al., 2009). To date, the mechanisms by which broiler offspring is affected by maternal feeding is not known. We hypothesize that metabolites produced upon protein fermentation in the ceca of broiler breeders are absorbed and transferred to the eggs, which may cause imprinting in the broiler offspring decreasing intestine function.

General design

Thirty-two female broiler breeders will be group-housed and divided over two dietary treatments, differing in ileal protein digestibility to obtain a contrast in protein entering the ceca. Protein sources will be selected based on the outcome (i.e. ileal protein digestibility) of in vitro and in vivo broiler experiments described in appendix 4. These diets will be fed to broiler breeders for 5 weeks and, blood samples will be collected to determine (protein fermentation) metabolite profiles. In addition, broiler breeders will be weighed at the start and end of the feeding trial. Eggs will be collected from each individual broiler breeder in the last week(s) of the trial (i.e. week 4-5). Thereafter, broiler breeders are killed for sample collection to determine effects of dietary treatments on the gut.

Two eggs per broiler breeder are used to determine the metabolic profile in the eggs. Three eggs per broiler breeder are incubated, hatched and broiler offspring killed for sample collection (intestine, blood, liver).

Primary outcome parameters

Untargeted metabolomics and targeted (protein fermentation products) metabolomics will be performed in blood samples of broiler breeders, in the eggs (yolk and/or albumen) and in samples from the broiler offspring to determine changes in metabolites. Intestinal tissue samples of broiler breeders are used for morphology to determine the effects of the dietary treatment on the gut.

Samples obtained from broiler offspring at dissection (i.e. blood, organs, intestinal tissue) will be analysed on metabolite profiles and functional read-outs (i.e. morphology of intestinal samples, DNA methylation and histone modifications in intestine or liver samples). Metabolomics profiles of broiler breeders and broiler offspring are subsequently correlated with these gut parameters to establish potential pathways that are differentially activated/silenced by certain metabolites affecting gut health.

Reference

Rao, K., J. Xie, X. Yang, L. Chen, R. Grossmann, and R. Zhao. 2009. Maternal low-protein diet programmes offspring growth in association with alterations in yolk leptin deposition and gene expression in yolk-sac membrane, hypothalamus and muscle of developing Langshan chicken embryos. Br. J. Nutr. 102:848-857.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Broiler breeders will be fed diets differing in ileal protein digestibility to determine transgenerational effects of protein fermentation metabolites.

Broiler breeders
Individual identification
Breeders will be marked with spray (not red) to enable individual identification of breeders within a pen.

Body weight
Broiler breeders will be weighed at the start and end of the feeding trial to determine potential differences in body weight between treatments. Individual birds will be placed in a crate and weighed on a weighing scale. Feed intake (per pen) will be recorded as well.

Blood sampling
At the start and end of the feeding trial, blood samples will be obtained from each broiler breeder to determine shifts in metabolite profiles due to the dietary treatments. Birds will be fixated by the animal caretaker and a blood sample will be taken from the wing vein by an experienced technician.

Artificial insemination
In order to obtain fertilized eggs, broiler breeders are inseminated. Semen will be obtained from multiple roosters from a commercial breeder company. The female broiler breeders will be inseminated twice a week in the first week and once a week in the weeks thereafter, with pooled semen.

Eggs
Eggs are collected in the last experimental week(s). During collection of eggs, breeders are observed continuously during that morning/day, in order to know which egg belongs to which breeder. This is required to link the egg metabolite profile to the breeder metabolite profile and still have group-housing. If there is uncertainty regarding which broiler breeder laid the egg, broiler breeders within the pen will be palpated to check the presence/absence of an egg.
Egg samples (yolk and/or albumen) are used for metabolomics analyses to determine the transfer of (protein fermentation) metabolites from broiler breeders to the eggs. The yolk sac metabolome will be analysed to understand the changes in available nutrients for the developing embryo.

Broiler offspring
Settable eggs will be incubated and hatched. A maximum of 96 day-old broiler chicks will be killed for sample collection (intestine, blood, liver).

This approach allows us to evaluate the effects of protein fermentation on gut morphology and metabolite profile of broiler breeders and, subsequently, evaluate if these broiler breeder metabolite profiles are correlated with the egg metabolite profiles, and with the broiler offspring metabolite profiles and functional read-outs of gut health.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Protein sources are selected based on the outcome of the in vitro tests and in vivo broiler experiment described in appendix 4, in order to obtain a contrast in ileal protein digestion between dietary treatments.
We chose artificial insemination instead of natural fertilization, because variation between broiler breeders in different pens might increase with natural fertilization due to differences in vitality between males. Furthermore, minimal required numbers of broiler breeders for primary outcome parameters are estimated based on previous experiments and power analysis (see next paragraph).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Ross 308 broiler breeder females will be obtained from a commercial breeding company. Ross 308 is the most commonly used breed in North-Western Europe. Broiler breeders will be obtained between 30-40 weeks of age, in order to perform the experiment in peak production.

The primary outcome parameters for this study are the metabolite profiles in the serum samples and eggs of broiler breeder females. To the best of our knowledge, metabolite profiles have not been determined in serum or eggs from broiler breeders fed different diets to induce protein fermentation. In the study from Pieper et al. (2012), metabolite profiles were determined in colon digesta and urine in weaned piglets fed diets differing in fermentable protein and total dietary fiber level (2x2 design). Using 8 piglets per treatment, they were able to find metabolite clusters associated with the diet and identify several metabolites that differed between treatments.
A power analysis we also conducted (using the glmpower procedure of SAS 9.3), using blood uric acid levels as influenced by dietary crude protein level reported by Corzo et al. (2005). Plasma uric acid levels averaged 6.04 and 3.82 mg/dL for broilers fed high (212 g/kg) or low (173 g/kg) crude protein level, respectively, with an average SD of 1.32. Using these values and setting the power at ≥ 0.8, this requires a total of 14 animals.
We expect larger variation in metabolite profiles, because we also apply untargeted metabolomics (instead of targeted as applied in the study of Pieper et al., 2012) and we also use eggs. We, therefore, estimate to require 16 broiler breeders per dietary treatment.
Two eggs are collected from each broiler breeder to determine metabolite profiles in the eggs. Additionally, we require two eggs for incubation and hatching to determine the effects on gut parameters in the broiler offspring. To account for unsettable eggs (~9%; van Emous et al., 2013) and a hatchability of settable eggs of 84% (van Emous et al., 2015), we collect/incubate 3 eggs per broiler breeder. These eggs are collected in one week, in order to minimize variation due to different storage times before incubation.

In total this experiment will include 32 broiler breeders and at max 96 broilers.

References

Corzo, A., C. A. Fritts, M. T. Kidd, and B. J. Kerr. 2005. Response of broiler chicks to essential and non-essential amino acid supplementation of low crude protein diets. Anim. Feed. Sci. Technol. 118:319-327.
Pieper, R., K. Neumann, S. Kröger, J. F. Richter, J. Wang, L. Martin, J. Bindelle, J. K. Htoo, W. Vahjen, A. G. van Kessel, and J. Zentek. 2012. Influence of fermentable carbohydrates or protein on large intestinal and urinary metabolomic profiles in piglets. J. Anim. Sci. 90:34-36.
van Emous, R. A., R. P. Kwakkel, M. M. van Krimpen, and W. H. Hendriks. 2013. Effects of growth patterns and dietary crude protein levels during rearing on body composition and performance in broiler breeder females during the rearing and laying period. Poultry Sci. 92:2091-2100.
van Emous, R. A., R. P. Kwakkel, M. M. van Krimpen, H. van den Brand, and W. H. Hendriks. 2015. Effects of growth patterns and dietary protein levels during rearing of broiler breeders on fertility, hatchability, embryonic mortality, and offspring performance. Poultry Sci. 94:681-691.

Species	Origin	Maximum number of animals	Life stage
Ross 308 broiler breeder females	Mild	32	
Ross 308 broiler offspring	Mild	96	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: the broiler breeders are the target animal in this experiment. There are no appropriate models available to study the (protein fermentation) metabolites produced by broiler breeders, the transfer of these metabolites to the egg and the effects on the gut of the broiler offspring. Reduction: artificial insemination is used instead of natural fertilization in order to minimize variation due to differences in vitality between males. In addition, required numbers of animals are estimated based on literature and power analysis. Refinement: broiler breeders will be checked twice daily by qualified personnel on general health and behavior. In case of disease, this will be discussed between the researcher, animal caretaker, veterinarian and/or animal welfare officer to determine appropriate actions. Broiler breeders will be group-housed and will be provided with sufficient feeding- and drinking places, perches and bedding material. In addition, laying nests will be provided to the broiler breeders. Skilled personnel will handle the animals in order to minimize stress during procedures such as weighing and blood sampling. In addition, the light in the stable will be dimmed prior to catching the birds.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Birds are group-housed, provided with sufficient bedding material and will have perches. Birds will be checked twice daily on general health and behavior. Climate conditions will be checked twice daily as well and immediately adjusted if required. Dimming the light before handling the birds (for weighing, blood sampling) allows the birds to become calm. Fresh bedding material will be provided regularly, to minimize the risk of wet litter and ammonia emission.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature survey has been conducted using google scholar and key words including broiler, broiler breeder, dietary protein, protein fermentation, transgenerational and metabolic programming. Especially in rodent models, there is substantial evidence linking maternal nutrition to offspring health and disease. The mechanisms underlying such transgenerational effects remain largely unknown. In poultry, very little data are available on this topic. To the best of our knowledge, (serum) metabolome profiles in broiler breeders in relation to dietary protein and transfer of (protein fermentation) metabolites to eggs have not been determined before.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Minor pain can be experienced during blood sampling and killing. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

There is a very small chance that broiler breeders might develop diarrhea due to the dietary treatments.

Explain why these effects may emerge.

A diet inducing protein fermentation is part of the experimental design. This diet is required to study (transgenerational) effects of protein fermentation metabolites.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Fresh bedding material will be provided regularly, to minimize the risk of wet litter (due to diarrhea) and subsequent effects on legs and skin of the broiler breeders.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Body weight loss due to diarrhea is possible in broiler breeders fed a protein source of low digestibility. If broiler breeders show clinical signs of diarrhea/sickness, birds will be weighed and birds are removed from the trial in case of 10% body weight loss (compared to the start weight). Injuries such as a broken wing or leg can occur similarly as in practice. If these injuries occur, these will be noticed during daily inspections. Birds are removed from the trial and killed according to a method listed in Annex IV of Directive 2010/63/EU.

Indicate the likely incidence.

The likely incidence is estimated at 1%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort in this experiment is estimated to be mild. This is based on weighing (mild), blood sampling (mild) and artificial insemination (mild) of broiler breeders and killing of broiler breeders and offspring.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Broiler breeders and broiler offspring are killed to collect samples (i.e. intestinal tissues).

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

10.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 7	Type of animal procedure Uremic toxin production during the in vitro fermentation of proteins in ileal digesta

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Eight pigs will be fitted with a cannula in the distal ileum. After a recovery and adaptation period, pigs will be subjected to eight experimental diets in 8 periods in a latin-square design. These diets will differ in dietary protein source and/or structure (i.e. amino acid composition). These proteins will be selected based on data from literature and from the results of the in vitro study described in appendix 4. During each period, ileal effluents will be collected and pooled by diet for further in vitro analysis. As a secondary objective fecal samples and clean urine samples will be analyzed for uremic toxins, the end-products of protein fermentation.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Eight growing barrows, ± 25 kg BW will be fitted with a cannula in the distal ileum under inhalation anesthesia. Pigs will be fasted prior to the surgery and will be treated with adequate painkillers and antibiotics during recovery. Pigs will be allowed to recover from the surgery and to adapt to housing conditions for a minimum of 7 days. After recovery from surgery, ileal effluents will be collected from each pig during 8 subsequent measurement periods. Each period will consist of a 5 day adaptation period, followed by collection of ileal digesta during 12 hours on days 5 and 7. Ileal digesta will be pooled per pig over the experimental period and diet will be stored at -20°C pending analysis. This approach is selected to ensure the ileal digesta for further in vitro studies is originating from the same pigs. The latin-square approach minimizes the number of pigs needed for this study, and has also been proposed by the expert working group of the FAO (2014). Feces will be sampled by rectal sampling during day 3, 4 and 6 and urine samples will be collected from funnels mounted underneath the metabolism cage.

Reference:
Research approaches and methods for evaluating the protein quality of human foods; Report of a FAO Expert Working Group, ISBN 978-92-5-108695-7, FAO 2014

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

This experiment is not designed to detect statistically significant differences in the composition of ileal digesta among protein sources. It is designed to provide representative samples of ileal digesta of the selected protein sources, with the objective to further study the fermentation of the undigested proteins in these samples. Hence, ileal digesta harvested from the pigs will be pooled by dietary treatment for the analysis of amino acid composition and will be the basis for a series of in vitro studies into the production of uremic toxins during fermentation (not described in this application). In addition, the effects of the protein sources on fecal characteristics and urinary excretion of uremic toxins will be analysed following a normal latin-square design.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Eight growing barrows (± 25 kg BW) will be purchased from a commercial pig farm. Only pigs that are healthy without hernias (testicular or umbilical), damaged ears or unsound feet are selected, because these can complicate surgery. Male animals will be used to ease collection of feces without contamination with urine. Barrows will be used as entire males will be difficult to handle at the end of the trials. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial farm. No spare pigs will be used, but in case no samples can be collected from two pigs for at least two periods, an extra collection period will be added. Pigs will be about 30 kg BW at the onset of the trial, and 60-70 kg BW at the end of the trial. This is a comparable BW range as used previously at our facilities, and well within the range of BW, maintaining healthy pigs and functional cannulas used in published literature. The use of growing pigs for this research is recommended by the FAO (2013). Body weight will be determined upon arrival of the pigs, and after surgery weekly until the end of the last experimental period.

Species	Origin	Maximum number of animals	Life stage
Pigs	Moderate	8	

C. Re-use

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: In this experiment, the connection with protein fermentation in the renal transplant cohort of the [redacted] is important. Obtaining ileal effluents from humans is complicated, and the pig is recommended by the FAO committee as a model for evaluating Digestible Indispensable Amino Acid Scores (DIAAS) of human foods. This choice is well documented in the FAO report (2013). Reduction: The protein sources used in this experiment will be selected based on the in vitro (small intestinal) digestion study described in appendix 4, to ensure a contrast in amino acid composition of ileal digesta large enough for this study. Using a latin-square design, within pig variability can be separated from the variation between protein sources, hence minimizing the number of pigs to be used for the study of uremic toxin excretion, and that the pooled ileal digesta to be used for subsequent in vitro fermentation studies originate from the same pigs. Refinement: after careful consideration, the length of the adaptation period, depending on the number of days the animal needs for adapting to new diets between experimental periods, was reduced from 12 to 5 days. In this way, every period within each trial lasts 7 days instead of 14 days, reducing the total duration of each trial to 8 weeks, which is often used for evaluating effects of fibrous diets. This decision fits within the procedures proposed by the FAO (2014), and is based on the notion that adaptation of small intestinal passage rates and digestive secretions to different protein sources is much quicker than the adaptation of the colon microbiota to changes in fiber sources. Although a straw bedding is not possible because it influences the measurements, cage enrichment will be varied weekly. Various, non-destructible toys will be made available to the pigs, in a weekly alternating schedule, following a protocol developed at Wageningen University. Audio-visual contact between pigs is maintained.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Surgical procedures will be conducted under complete anesthesia, and adequate painkillers are used during recovery.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature search has been performed in Scopus (13-4-2017), using the search terms "uremic toxin", "protein fermentation". Although the production of uremic toxin following protein fermentation is well documented (particularly in relation to kidney patients), no efforts have been published predicting the composition of uremic toxins from variation in the amino acid composition of proteins flowing into the colon. No literature sources were found with similar objectives as described in this appendix.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Pigs will be housed individually in metabolism pens during the recovery phase and during the experimental periods. The floor area of the pens will be min. 2 m2, allowing the pigs to move around freely. Walls will be smooth (covered by plexiglass) to prevent damage to cannulas. Animals will be housed on a plastic coated floor. Individual housing is needed to prevent animals from damaging cannulas of pen mates, but audio-visual contact will be possible. The use of bedding material is avoided as this will prevent the collection of clean urine samples from funnels mounted underneath the cage.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

G. Location where the animals procedures are performed

[X] No > Continue with question H.

[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.

[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[X] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Surgical procedure will be performed under anesthesia. After surgery, animals will be treated with painkillers (at least 3 days) and with antibiotics.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Individual housing without bedding material, fasting prior to surgery and recovery from surgical procedures may have adverse effects on animal welfare. Infection and inflammation due to the presence of the cannula and/or catheter may occur. Digestive problems (i.e.diarrhea) with changes in the diet are not expected based on previous experience and because there is an adapatation period to each diet.

Explain why these effects may emerge.

These adverse effects may emerge because they are part of the experimental procedures.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Infection and inflammation will be prevented or minimised by providing antibiotics after the surgical procedures and whenever required based on clinical signs of infection. Adverse effects on animal welfare due to individual housing are minimised by providing toys as environmental enrichment and pigs will be able to see and hear each other.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

The following human endpoints will apply. Pigs will be euthanized should one of the following conditions apply: • during the recovery from surgery, a pig does not start eating within 2 days, and subsequently produce feces, indicating blockage of the intestines or inflammation of the peritoneum. •a cannula is lost and it cannot be placed back into the distal ileum immediately. • a pig has a fever during 5 successive days, not responding to medical treatments proposed by a veterinarian, and signs of infection and inflammation. • a pig has feed refusals exceeding 20% of the amount of feed offered for a period exceeding 7 days. • in the expert judgement of the veterinarian, future observations on a pig will not provide reliable results. • a pig suffers from body weight loss during a 14 day period

Indicate the likely incidence.

The likely incidence of pigs to be removed from the experiment is estimated at 25% during the 8 week duration of the trial. The major portion of this 25% is expected to occur during the first two days following surgery. In addition, technical failure of cannulas will lead to removal of the pig from the experiment. If this leads to discomfort of the pig, it will be for a very short period of time.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The level of discomfort is expected to be as listed below: • Surgical procedure (including fasting): moderate • Individual housing in absence of bedding material, in a large metabolism pen (10 weeks): moderate • The sampling procedures of ileal digesta (2 days during each of 8 subsequent weeks): mild • Rectal fecal sampling during 3 days (when ileal digesta are not collected): mild. Hence the cumulative discomfort in this trial is estimated at moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Keeping pigs with a cannula in the distal ileum after the experiment is finished is undesirable from an animal welfare point of view.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

Centrale Commissie Dierproeven

11.

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Onze referentie
Aanvraagnummer
AVD1040020171667

Bijlagen
2

Datum 10 mei 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted],

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 10 mei 2017. Het gaat om uw project "To determine the telation between protein fermentation and gut health in pigs and poultry". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD1040020171667. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Datum:
10 mei 2017
Aanvraagnummer:
AVD1040020171667

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
10 mei 2017
Aanvraagnummer:
AVD1040020171667

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10400
Naam instelling of organisatie:	Wageningen University & Research
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	9215846
Straat en huisnummer:	Akkermaalsbos 12
Postbus:	59
Postcode en plaats:	6700 AW WAGENINGEN
IBAN:	NL10RABO0397066465
Tenaamstelling van het rekeningnummer:	Wageningen UR

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	Onderzoeker
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 mei 2017
Geplande einddatum:	1 mei 2021
Titel project:	To determine the telation between protein fermentation and gut health in pigs and poultry
Titel niet-technische samenvatting:	Het bepalen van de relatie tussen eiwitfermentatie en darmgezondheid in varkens en pluimvee
Naam DEC:	DEC Wageningen UR
Postadres DEC:	Droevendaalsesteeg 4 6708 PB Wageningen
E-mailadres DEC:	dec@wur.nl

Betaalgegevens

De leges bedragen:	€ 2.113,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Wageningen
Datum:	10 mei 2017

Centrale Commissie Dierproeven

12.

> Retouradres Postbus 20401 2500 EK Den Haag

Wageningen University & Research
[redacted]
Postbus 59
6700 AW WAGENINGEN

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD1040020171667

Datum 26 juni 2017
Betreft Vervolg aanvraag projectvergunning Dierproeven

Geachte [redacted],

Op 10 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "To determine the relation between protein fermentation and gut health in pigs and poultry" met aanvraagnummer AVD1040020171667. Wij gaan uw aanvraag beoordelen. In deze brief leest u wanneer u een beslissing kunt verwachten.

Complexe aanvraag
Tijdens de beoordeling van uw aanvraag blijkt dit project dermate complex te zijn dat meer tijd nodig is om de aanvraag volledig te beoordelen. Als wij nog informatie nodig hebben dan ontvangt u daarover bericht. U krijgt binnen vijfenvijftig werkdagen nadat uw aanvraag compleet is, een beslissing op uw aanvraag. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

13.

WAGENINGEN UR
For quality of life

Postbus 65 | 8200 AB Lelystad

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Wageningen
University & Research

DATUM
25 juli 2017

ONDERWERP
aanvraag projectvergunning
AVD1040020171667

ONS KENMERK
AVD1040020171667

POSTADRES
Postbus 65
8200 AB Lelystad

BEZOEKADRES
Houtribweg 39
8221 RA Lelystad

INTERNET
www.wur.nl

KvK NUMMER
09098104

CONTACTPERSOON
ing. I.E. Leushuis-Kappers

TELEFOON
+31 (0)320-238170

E-MAIL
DEC@wur.nl

Geachte CCD,

Onderstaand het advies dat de DEC-WUR heeft gegeven aangaande het project ""

A. Algemene gegevens over de procedure

1. Aanvraagnummer: **AVD1040020171667**
2. Titel van het project: To determine the relation between protein fermentation and gut health in pigs and poultry
3. Titel van de NTS: Het bepalen van de relatie tussen eiwitfermentatie en darmgezondheid in varkens en pluimvee
4. Type aanvraag: nieuwe aanvraag projectvergunning
5. Contactgegevens DEC:
   DEC-WUR
   0317-481516
   Secretaris: dec@wur.nl
6. Adviestraject
   Ontvangen door DEC: 10-05-2017
   Aanvraag compleet: ja
   In vergadering besproken: 15-05-2017 en 17-07-2017
   Anderszins behandeld:
   Termijnonderbrekingen van 16-05-2017 tot 16-06-2017, van 20-06-2017 tot 10-07-2017, van 19-07-2017 tot 20-07-2017
   Besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen: 26-06-2017
   Aanpassing aanvraag: 16-06-2017 en 20-07-2017
   Advies aan CCD:
7. De Instantie voor Dierenwelzijn heeft een positief oordeel over de kwaliteit van de aanvraag uitgebracht en de DEC heeft dit in haar overweging betrokken.

8. Eventueel horen van aanvrager
   Datum: 19-06-2017 (DEC-vergadering)
   Plaats: Wageningen
   Aantal aanwezige DEC-leden: 7
   Aanwezige (namens) aanvrager: Myrthe Gilbert
   Gestelde vragen *en antwoorden*
   - De centrale vraag van de DEC had betrekking op de samenhang van de typen dierproef onderling en in relatie tot de geformuleerde onderzoeksdoelen. De onderzoekster heeft dit mondeling toegelicht. De vragen van de DEC zijn vervolgens schriftelijk aan haar voorgelegd (zie 9).

   Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag (zie 9)

9. Correspondentie met de aanvrager
   Datum vragen: 17-05-2017, 20-06-2017 en 17-07-2017
   Gestelde vragen:
   De DEC is niet tot een ethische beoordeling van uw aanvraag gekomen, omdat de samenhang tussen de verschillende onderdelen van het project haar niet duidelijk was en zij daarom de afweging van het doel vs. het ongerief voor de proefdieren niet kan maken. Zij verzoekt u dan ook de aanvraag opnieuw in te dienen en hierin overzichtelijker aan te geven:
   (a) wat het doel is van de verschillende typen dierproef en hoe deze onderling samenhangen, mede in relatie tot het (STW-)programma en de doelen daarvan, o.a. waar het gaat om de humane toepassing, waar u naar verwijst.
   (b) Daarnaast verzoekt ze u hierbij, voor zover mogelijk een chronologie aan te brengen en voor zover van toepassing go-no-go-momenten in te passen.
   (c) Bovendien verzoekt zij u duidelijker te beargumenteren, waarom u varkens en pluimvee onderbrengt in één project.
   De DEC verzoekt u de samenhang tevens te verduidelijken m.b.v. een grafische weergave.
   De DEC zal deze aanvraag dan opnieuw behandelen in haar vergadering van 19 juni a.s. Vooruitlopend hierop heeft zij een aantal vragen geïdentificeerd m.b.t. de ingediende documenten (niet uitputtend):
   M.b.t. de appendices:
   - De DEC verzoekt u, voor zover van toepassing a) te vermelden, op welke ondergrond de varkens worden gehuisvest, als er geen beddingmateriaal wordt verstrekt en b) hoe het klimaat wordt verzorgd voor de varkens die zonder bedding individueel worden gehuisvest (en dus geen warmte bij elkaar of van bedding kunnen zoeken).
   - Bovendien verzoekt ze u bij appendix 3 voor de volledigheid toe te voegen, dat het hoge percentage (25%), waarvoor u verwacht dat de humane eindpunten worden bereikt vooral een gevolg is van (technische) problemen m.b.t. de bruikbaarheid van de canule en dus niet van onacceptabel ongerief.
   - Tot slot verzoekt ze u aan te geven, hoe de rectale mestmonsters worden genomen, hoe de manuele stimulering plaatsvindt (met het oog op mogelijk ongerief).

   Datum antwoord: 16-06-2017 (hernieuwde aanvraag t.b.v. DEC)
   Gestelde vragen DEC 19-06-2017:
   De DEC is nog niet tot een ethische beoordeling van uw aanvraag gekomen, omdat de samenhang tussen de verschillende onderdelen van het project haar nog niet duidelijk was en zij daarom de afweging van het doel vs. het ongerief voor de proefdieren niet kan maken. Zij verzoekt u dan ook de aanvraag opnieuw in te dienen en hierin de opmerkingen en suggesties te verwerken, die tijdens een mondeling overleg met u zijn besproken.
   Samengevat gaat het om de volgende opmerkingen:
   (a) De DEC verzoekt u in het projectvoorstel de relevantie van diverse typen dierproeven scherper te verwoorden met het oog op het beantwoorden van (een deel van) de geformuleerde hoofdvragen van het project (m.n. appendix 2 t.a.v. sanitary conditions en appendix 7, o.a. welke rol vitamines en mineralen hierin spelen); Zij verzoekt u dit tevens in de betreffende appendix (in de inleiding) uitgebreider te verwoorden;
   (b) De DEC verzoekt u de grafische weergave, die u hebt toegevoegd ook inhoudelijk in te vullen, aangezien het nog steeds niet duidelijk is, hoe

de verschillende delen daarvan passen in het project en de appendices (daarvoor verwijst u naar de tekst, waardoor het nog steeds een zoekplaatje is: wordt er bijv. in alle typen dierpoef naar microbioom gekeken?). Tevens verzoekt zij u de chronologie van de diverse delen en de onderlinge afhankelijkheid ervan duidelijker weer te geven (bijv. app. 4 en 7).
(c) Daarnaast verzoekt ze u in het projectplan voor zover van toepassing go-no-go-momenten in te passen.
(d) Tevens verzoekt ze u ook aan te geven, hoe u uit de veelheid aan correlaties die u m.b.v. metabolomics vindt de relevante correlaties selecteert.
(e) Bovendien verzoekt ze u de keuze voor de verschillende voercomponenten uitgebreider te onderbouwen (waarom richt u zich op eiwitten en vezels en niet op andere nutritionele oplossingen bijv. vetten/ koolhydraten?).
Daarnaast verzoekt ze u de vragen m.b.t. de appendices te beantwoorden, die eerder al zijn gesteld:

- De DEC verzoekt u, voor zover van toepassing a) te vermelden, op welke ondergrond de varkens worden gehuisvest, als er geen beddingmateriaal wordt verstrekt en b) hoe het klimaat wordt verzorgd voor de varkens die zonder bedding individueel worden gehuisvest (en dus geen warmte bij elkaar of van bedding kunnen zoeken).
- Bovendien verzoekt ze u bij appendix 3 en 7 voor de volledigheid toe te voegen, dat het hoge percentage (25%), waarvoor u verwacht dat de humane eindpunten worden bereikt vooral een gevolg is van (technische) problemen m.b.t. de bruikbaarheid van de canule en dus niet van onacceptabel ongerief.
- Tot slot verzoekt ze u aan te geven, hoe de rectale mestmonsters worden genomen, hoe de manuele stimulering plaatsvindt (met het oog op mogelijk ongerief).

Aanvullend hierop verzoekt de DEC u:

- bij appendix 2 de opmerking, dat de dieren antibiotica krijgen toegediend te verwijderen;
- de (gewijzigde) aantallen dieren in app. 4 en 5 consistent te vermelden;
- in app. 3 de formulering dat dieren worden behandeld met analgesie te corrigeren (analgetica).

De aanvraag is opnieuw door de plenaire DEC worden beoordeeld.
Daaruit zijn onderstaande vragen geformuleerd en op 19-07-2017 gesteld aan de onderzoeker. De *antwoorden van de onderzoeker zijn:*

- De HEP's zijn niet concreet en moeten scherper geformuleerd worden. De DEC doet de suggestie om de dieren te wegen en dit als vroeg kenmerk op te nemen.
  Het lichaamsgewicht aan het begin van de proef meenemen voor het bepalen van een HEP heeft – behalve voor appendix 6 – geen meerwaarde omdat het om groeiende dieren gaat. In de appendices waarin lichaamsgewicht regelmatig bepaald wordt (appendix 2 – 5 en 7 voor varkens), hebben we lichaamsgewichtsverlies toegevoegd als HEP bij J2 wanneer dit nog niet vermeld was. In de appendices voor pluimvee (4, 5 en 6) was lichaamsgewichtsverlies al meegenomen als HEP.
- In bijlage 1 zijn de aantallen dieren niet navolgbaar. Op blz. 5 wordt gesproken over 32, 22, 32 dieren. Hoe verhoudt zich dat tot 162 en 262 op blz. 6?
  Op pagina 5 wordt inderdaad eerst gesproken over 32, 22 en 32 dieren wanneer we mestscore, ammoniakconcentratie in de mest en ammoniakconcentratie in het colon, gevonden in de literatuur, als variabele gebruiken in de power analyse. Echter, verwachten we een veel hogere variatie in de beschreven proef (waarbij we biggen uit de praktijk verzamelen) t.o.v. de variatie gerapporteerd in de literatuur onder experimentele omstandigheden. Daarom hebben we de variatie met 3 vermenigvuldigd in de poweranalyse (gebruikmakend van de concentratie ammoniak in mest en colon uit de literatuur), wat resulteerde in 166 en 262 dieren. Ik realiseer me dat dit verwarrend is, daarom heb ik de tekst in appendix 1 - A3 iets aangepast.

B. Beoordeling (adviesvraag en behandeling)

1. De DEC heeft vastgesteld dat het project vergunningplichtig is (dierproeven in de zin der wet).
2. De aanvraag is een nieuwe aanvraag..
3. De DEC is competent om over de aanvraag te adviseren vanuit het oogpunt van onafhankelijkheid, onpartijdigheid en beschikbare expertises.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de behandeling van de aanvraag en het opstellen van het advies. Licht toe waarom.

C. Beoordeling (inhoud)

1. De DEC heeft vastgesteld dat de aanvraag toetsbaar is en voldoende samenhang heeft. De bijlagen hebben deels een samenhang en deels een overlap, maar dragen allen bij het directe en uiteindelijk doel van het project. De subdoelen die gesteld zijn, zijn deels tijdafhankelijk. De go/no-go momenten aan het einde van experiment 7 zijn verdedigbaar: het is niet zinvol om tussen experimenten een eerdere go/no-go-beslissing te nemen. Gezien de doelstelling is het logisch dat beide diersoorten (varkens, pluimvee) in de experimenten opgenomen zijn.
2. De DEC heeft geen tegenstrijdige wetgeving, gericht op de gezondheid en welzijn van het dier of het voortbestaan van de soort, gesignaleerd die het uitvoeren van de proef in de weg kan staan (bv. Wet Dieren en Wet Natuurbescherming).
3. De DEC heeft vastgesteld dat de in de aanvraag aangekruiste doelcategorieën in overeenstemming zijn met de hoofddoelstellingen.

Belangen en waarden

4. Het directe doel is het in kaart brengen van de complexe relatie tussen voereiwit, micro-organismen en eiwitfermentatiemetabolieten en het effect daarvan op darmgezondheid en groei van varkens en pluimvee.
   Het uiteindelijke doel is een efficiënter gebruik van eiwitbronnen waardoor:
   - de darmgezondheid in varkens en pluimvee verbetert
   - antibioticagebruik vermindert
   - inzicht ontstaat in de werkingsmechanisme die model kunnen staan voor andere diersoorten en de mens
5. De belanghebbenden in het project en hun morele waarden zijn:
   - Proefdieren: aantasting van welzijn en integriteit. De meeste proefdieren zullen licht ongerief ondervinden. Voor de dieren die geopereerd worden is het ongerief matig.
   - Doeldieren: met name de dieren in de conventionele veehouderij zullen een belang hebben bij een verbeterde gezondheid/welzijn. De dieren in de extensieve veehouderij vallen buiten de scoop van dit project, hoewel een optimale benutting ook voor deze laatste groep dieren van belang is.
   - Veehouder: economisch belang door een verbeterde gezondheid en voederconversie en een lager antibioticagebruik.
   - Onderzoeker/CRO: economisch belang, wetenschappelijk belang.
   - Voerfabrikant: economische belang. Door de grote markt voor additieven kan dit een redelijk groot belang zijn.
   - Milieu/maatschappij: Een lager antibioticagebruik kan tot minder antibioticaresistentie leiden. De DEC acht dit, in dit project, een gering belang.
   - Mens: gezondheidsbelang, enerzijds door een lagere antibioticaresistentie, anderzijds door het translationele aspect van het onderzoek.
6. Voor zover de DEC dat kan inschatten is er geen aanleiding voor de DEC om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken.

Proefopzet en haalbaarheid

7. De DEC heeft vastgesteld dat de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven, afgaande op het geschreven voorstel en het oordeel van de IvD, voldoende gewaarborgd zijn.
8. Er is veel aandacht besteed aan de opzet per experiment en een logische volgorde in de experimenten. Figuur 1 in de projectbeschrijving geeft een duidelijk overzicht van de samenhang van de experimenten in het project. De uitleesparameters richten zich met name op darmgezondheid. De aantallen dieren zijn per experiment statistisch en op basis van literatuur onderbouwd. De DEC heeft vastgesteld dat het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstelling. De gekozen strategie en experimentele aanpak kan in

de ogen van de DEC leiden tot het behalen van de doelstelling(en) binnen het kader van het project.

Welzijn dieren

9. Er is sprake van de volgende bijzonderheid op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren: De keuze hiervoor is realistisch ingeschat en geclassificeerd.
   ☐ Locatie: buiten instelling vergunninghouder (10g)
10. De dieren worden niet gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen om bijlage III van richtlijn 2010/63/EU. De dieren worden soms op roosters met rubbermatten of op een tenderfootbodem gehuisvest. Dieren die voorzien zijn van een canule worden individueel gehuisvest. De onderbouwing hiervoor is valide in de ogen van de DEC.
11. De DEC stelt vast dat een cumulatieve inschatting van ongerief als "licht" resp. "matig" voor elk dier realistisch is ingeschat en geclassificeerd. Ongerief in de experimenten zal bestaan uit: monstername, eventueel optreden van diarree, inzetten en hebben van een darmcanule en/of bloedvatkatheter, euthanasie en individuele huisvesting.
12. Naast ongerief is er voor de meeste dieren geen sprake van aantasting van integriteit van het dier anders dan als gevolg van de proefbehandelingen. Voor de gecanuleerde/gekatheteriseerde dieren is wel sprake van integriteitsaantasting. Dit is te rechtvaardigen omdat dit leidt tot zuiverder resultaten en minder ongerief voor de dieren.
13. De DEC heeft vastgesteld dat, na aanpassing door de onderzoeker, de criteria voor humane eindpunten goed zijn gedefinieerd en dat goed is ingeschat welk percentage van de dieren een humaan eindpunt zal bereiken.

3 V's

14. De DEC heeft vastgesteld dat de onderzoeker voldoende aannemelijk heeft gemaakt dat er geen alternatieven zijn om de doelstelling van het project te realiseren. De complexe relatie tussen voereiwit en fermenteerbare vezels versus microben en darmwand kan nog niet in vitro bestudeerd worden.
15. De DEC heeft vastgesteld dat de onderzoeker voldoende aannemelijk heeft gemaakt dat er optimaal tegemoet gekomen wordt aan de vereiste van vermindering van dierproeven.
16. De DEC heeft vastgesteld dat het project in overeenstemming is met de vereiste van verfijning van dierproeven. De DEC ziet geen extra mogelijkheden voor verfijning, anders dan die de onderzoeker nu toepast.
17. Er is geen sprake van wettelijk verplicht onderzoek; de vraag over duplicatie is niet van toepassing.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. De dieren worden niet van beide geslachten in gelijke mate ingezet in de proeven. Daar waar gevraagd wordt om 1 sekse is dit helder onderbouwd. De andere sekse wordt niet geëuthanaseerd maar ingezet in de reguliere mesterij. De DEC heeft vastgesteld dat de aanvrager in voldoende mate wetenschappelijk heeft onderbouwd waarom dit noodzakelijk is. Er is geen sprake van surplusdieren.
19. Een deel van de dieren wordt gedood in het kader van het project om met name darmmateriaal te kunnen onderzoeken. De dieren worden gedood volgens een passende methode die vermeld staat in bijlage IV van richtlijn 2010/63/EU.
20. Dieren die niet gedood worden in het kader van het onderzoek worden afgemest conform de praktijk.

NTS

21. De NTS is naar het oordeel van de DEC een evenwichtige weergave van het project, begrijpelijk geformuleerd en voldoet aan de vereisten in de herziene Wod Art. 10.a.1.7.

D. Ethische afweging

D. Ethische afweging

1. De centrale morele vraag van het project is: Rechtvaardigt onderzoek naar de relatie tussen voereiwit, micro-organismen en eiwitfermentatie en het effect daarvan op de darmgezondheid van varkens en pluimvee het gering tot matig ongerief dat 3232 varkens en 884 kippen ondervinden tijdens dit onderzoek?

2. In de afweging heeft de DEC geconstateerd dat het hier gaat om een aanvraag met voldoende samenhang. In haar afweging heeft de DEC meegewogen dat dit project leidt tot meer inzicht in de relatie voereiwit/micro-organisme/eiwitfermentatie. Dit draagt bij aan de waarde van kennis. De DEC waardeert dit als een reëel belang. Wanneer het project zijn uiteindelijke doel bereikt, zal de kennis die verkregen kan worden, een bijdrage leveren aan een betere gezondheid en welzijn van dieren in de praktijk. De DEC waardeert deze waarden van gezondheid en welzijn als substantiële waarden. Hierbij geldt echter de kanttekening dat de DEC de bijdrage van deze kennis aan deze waarden heeft beoordeeld in het licht van de relatieve meerwaarde van dit onderzoek de opzichte van andere maatregelen ter bevordering van het dierenwelzijn. Op basis hiervan ziet de DEC dit als een reëel belang.
   De economische belangen voor de veehouder, CRO/onderzoeker en industrie schat de DEC in als beperkt. Daarnaast kan het project bijdragen aan de waarde van volksgezondheid aangezien het een bijdrage kan leveren aan de reductie van antibioticagebruik. Gezien de relatief beperkte bijdrage van de gegevens van dit onderzoek op dit punt waardeert de DEC dit als een beperkt belang.
   Tot slot zijn de waarden van de proefdieren in het geding. De aantasting van de waarden van de proefdieren als gevolg van de handelingen binnen het project is voor het grootste deel van de dieren gering. Een klein deel van de dieren heeft een matige aantasting.
3. Op basis van bovenstaande overwegingen is de DEC van mening dat het ethisch verantwoord is om onderzoek te doen naar de relatie tussen voereiwit, micro-organismen en eiwitfermentatiemetabolieten en het effect daarvan op darmgezondheid en groei van varkens en pluimvee met maximaal matigongerief voor maximaal 3232 varkens en 884 kippen. De DEC ziet in dit stadium geen mogelijkheden op het terrein van vervanging, vermindering van het aantal dieren en verfijning van de aanvraag.
   De centrale morele vraag kan met "ja" beantwoord worden.

E. Advies

1. Advies aan de CCD:
   - De DEC adviseert de vergunning te verlenen
2. Het uitgebrachte advies is gebaseerd op consensus.
3. Onderstaande knelpunt is naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies:
   - De DEC is zich terdege bewust van de gevolgen die de huidige intensieve veehouderij met zich meebrengt en zij neemt dat mee in haar ethische afweging. De DEC is van mening dat het welzijn van de dieren in de intensieve veehouderij onder druk staat en dat onderzoek een bijdrage moet leveren aan het verbeteren van de leefomstandigheden van de dieren. In het voorliggende project is voor de DEC voldoende aanwijzing dat de kennis gebruikt kan en zal worden om het welzijn (inclusief gezondheid) van dieren te verbeteren. Bovendien heeft kennis van een optimale benutting ook meerwaarde voor dieren in de extensieve veehouderij. De DEC ziet dit projecten juist als een stap naar mogelijke verbetering binnen de huidige veehouderij. De discussie over de wenselijkheid van die veehouderij-omstandigheden zal in een ander gremium dan de DEC gevoerd moeten worden.

Met vriendelijke groet,

ing. I.E. Leushuis-Kappers
secretaris DEC WUR

14.

Form
Project proposal

- This form should be used to write the project propsal of animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed
- For more information on the project proposal, see our website(www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400
1.2	Provide the name of the licenced establishment.	Wageningen University
1.3	Provide the title of the project.	To determine the relation between protein fermentation and gut health in pigs and poultry

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

[X] Basic Research
[X] Translational or applied research
[] Regulatory use of routine production
[] Research into environmental protection in the interest of human or animal health or welfare dier
[] Research aimed at preserving the species subjected to procedures

[] Higher education or training
[] Forensic enquiries
[] Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

A partnership program was set up between [redacted] The objective, formulated in this program, was to increase our understanding of the relation between dietary protein, protein fermentation, and gut health, to improve gut health and reduce digestive disorders in pigs and poultry. The research proposals finally granted (5 PhD students and 1 Post-doctoral researcher) were developed independently. Some overlap between these proposals was noted by [redacted]. Together it was decided to form a consortium with the university partners, [redacted]. The consortium aims to answer the questions posed in the individual proposals. Furthermore, animal experiments with overlap were combined between the granted projects, which has resulted in a reduction in the number of experimental animals within the consortium. Within the consortium, the coherent experiments contributing to the same objective were combined to form one large project. Across these coherent experiments, metabolomics and microbiomics analyses will be conducted, with the objective to elucidate the general mechanisms underlying compromised gut health. This has led to the current application, which contains seven studies to be performed within the framework of the consortium. In a later stage, another application may follow to address the remaining questions. These remaining questions depend on the outcome of the experiments described in this application, and follow-up studies are, therefore, out of scope for this application. Go-no-go decisions will follow after these initial 7 studies. The integrated metabolomics approach allows us to generate hypotheses for the follow-up studies and with bioinformatic approaches we will prioritize the relevant correlations to be studied in the follow-up experiments.

Background

Compromised gut health and related intestinal disorders (i.e. diarrhea) are a large problem in pig and poultry production systems. For example, diarrhea in piglets in the post-weaning period (i.e. post-weaning diarrhea, PWD) and wet litter (WL) in broiler houses are often reported causes of increased mortality and impaired health, welfare, and performance (Madec et al., 1998; Hermans et al., 2006; de Jong et al., 2014; Dunlop et al.,

2016). In pig and poultry production, antibiotic treatments to (partially) overcome these gut health problems are often applied. With the societal pressure to reduce therapeutic use of antibiotics - to minimize the risk of microbial resistance against antibiotics - controlling gut health becomes even more important in pig and poultry production.
Although, PWD and WL are multifactorial problems, dietary factors play an important role in their onset (Madec et al., 1998; Hermans et al., 2006; Dunlop et al., 2016). Particularly, high protein levels have been associated with increased risk for PWD (Heo et al., 2008, 2009, 2015) or WL (Bench et al., 2016). The underlying mechanisms by which dietary protein causes intestinal problems remain, however, largely unknown. Protein fermentation in the hindgut is believed to be (partially) responsible for negative effects of dietary proteins on gut health. The ileal digestion of dietary protein differs between protein sources and levels (Heo et al., 2010; Salgado et al., 2002), which affects the flow of protein into the large intestine of pigs and poultry. In addition, endogenous secretions (such as mucus, enzymes and shed epithelial cells) contribute to the flow of proteins into the large intestine. In the hindgut, these proteins can be fermented by saccharo-proteolytic bacteria (such as Clostridia, Proteus and E. coli) resulting in the proliferation of these bacteria, some of which are known pathogens. In addition, proteins have a buffering capacity in the gut, which favors proliferation of pH-sensitive bacteria, which often include pathogens. Furthermore, the negative relation between protein fermentation and gut health may be caused by protein fermentation metabolites, such as branched-chain fatty acids, ammonia, phenolic and indolic compounds, hydrogen sulfide, biogenic amines and nitric oxide and N-nitroso compounds. Some of these protein fermentation metabolites have potential damaging effects on gut function, but these conclusions mainly rely on tests in *in vitro* systems (Lin and Visek, 1991; Hughes et al., 2008; Andriamihaja et al., 2015 Beaumont et al., 2016; Leschelle et al., 2005) and often contradictory results were reported. Such protein fermentation metabolites are produced in pigs and poultry as well, especially when feeding diets containing high protein content or a low digestible protein source (Bikker et al., 2006; Heo et al., 2010; Pieper et al., 2012, 2014; Qaisrani et al., 2014). A causative relation between protein fermentation metabolites and gut health, can however not be deduced from these studies and the available information does not suffice for the development of nutritional interventions to improve gut health. In order to develop nutritional solutions that aim to improve gut health in pigs and poultry, the relation between dietary protein and microbiota, protein fermentation metabolites, and gut health, needs to be studied in more detail.

One potential nutritional solution for reducing (detrimental effects of) protein fermentation (metabolites) is the inclusion of fermentable fiber. The ratio between protein and fermentable fiber plays an essential role in the dynamics of protein fermentation (Le Gall et al., 2007) and some studies have reported a reduction in the intestinal concentration of protein fermentation end-products, such as ammonia, amines and phenolic compounds, in pigs fed increased levels of fermentable fiber (Bikker et al., 2006; Pieper et al., 2012, 2014). To identify potential fiber sources that can be included in such nutritional solutions, the interaction between various fiber types and protein fermentation is studied in this project.

Environmental conditions can affect the animals' microbiota composition. It has been consistently demonstrated that pigs kept under low sanitary conditions (resulting in a lower sub-clinical health status of the herd) have a reduced nitrogen digestibility compared with pigs kept under high sanitary conditions (Kampman-van der Hoek et al., 2016; van der Meer et al., 2016). This effect is likely mediated through the microbiome. It illustrates that steering of protein fermentation in the gut is dependent on the (environmental, sanitary) status under which the pigs are kept. It is, therefore, of importance to evaluate the interactive effects of sanitary status (as a model for pig farms with a reduced health status) and dietary protein content on protein fermentation (metabolites), gut health and performance in pigs.

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3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The main objective of the consortium is to investigate the relation between dietary protein and microbiota, protein fermentation metabolites, and their effect on gut health and performance of pigs and poultry. We aim to reach this objective through a series of *in vitro* and *in vivo* pig and poultry studies (described in 3.4). The specific key research questions to be studied are:

1. How are dietary proteins, microbiota, and protein fermentation (metabolites) related to intestinal health and PWD in piglets?
2. How do sanitary conditions affect the relation between dietary proteins, digestion, protein fermentation (metabolites) and performance in pigs?
3. How are dietary proteins, microbiota, and protein fermentation (metabolites) related to intestinal health and WL in broilers?
4. What (dietary) interventions can reduce or remove harmful metabolites/microbial species in the gut thereby increasing gut health in pigs and poultry?
5. Are protein fermentation metabolites transferred from broiler breeders to their eggs and is there a relation between protein fermentation in broiler breeders and intestinal function of their offspring?
6. Is there a relation between protein source, microbiota, protein fermentation, and uremic toxin production in piglets?

We expect to reach our main project's objective, because the scientific partners have extensive expertise in the research areas (i.e. animal nutrition, microbiology, host physiology/metabolism and metabolomics tools) required within this project. The industrial partner is a global leader in additives for animal feed, and a direct stakeholder for the utilization of newly gained knowledge.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

This project will provide insight in causal relations between protein fermentation, its end-products and gut health of pigs and poultry. This knowledge can be applied to develop nutritional solutions to prevent gut health problems and optimize gut function, which will contribute to an increase in overall animal health, thereby improving animal welfare and reducing the need for therapeutic use of antibiotics. Increased understanding of the relations between protein fermentation (end-products) and gut health in pigs and poultry might also lead to hypotheses relevant for other species, including humans, where protein fermentation contributes to diarrhea in patients with renal disease.

3.4 Research Strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

The central problem to be tackled is compromised gut health in pigs and poultry, leading to digestive disorders as PWD and WL. Gut health is a multifactorial concept, depending on intrinsic factors of the individual and extrinsic factors, such as diet (e.g. protein and fiber contents) and environment (e.g. sanitary status). Protein fermentation is believed to play an important role in gut health, and understanding its mode of action is pivotal to the development of nutritional solutions. There are various ways to steer protein fermentation, e.g. by the choice of the dietary protein sources and their inclusion level, or by the choice of dietary fiber sources and their inclusion level. It is proposed that many deleterious effects commonly attributed to protein fermentation are mediated through the metabolome, and to a lesser extent microbiome. The seven studies included in this project all investigate the relation between dietary protein/protein fermentation and gut health, but each study has its own focus. An overview of the seven appendices of this application is provided in figure 1.

We will use untargeted and targeted metabolite platforms, with which we can establish the difference in metabolites in different parts of the intestinal lumen, blood, intestine and liver tissue as influenced by ileal undigested protein. The novelty of this project is the application of untargeted metabolomics across samples and species to establish associations between (unknown) metabolites and gut health. In subsequent research, causal relationships between protein fermentation and gut health can be established. It is of importance to perform this research with both pigs and poultry, because their digestive physiology differs substantially, likely resulting in differences between species in the relation between dietary protein, protein fermentation, and gut health. Ileal digestibility of protein is lower in poultry than in pigs, resulting in a larger and different fraction of proteins that enters the large intestine. Furthermore, only soluble (or solubilized) proteins will be fermented in chickens. Hence, the type of proteins to be studied, and the potential fermentation metabolites to be formed, will differ between pigs and poultry. However, there is also overlap in the microbial species present in gut of pigs and poultry and in the metabolites produced following protein fermentation. Mechanisms by which these common metabolites affect the gut epithelium might be similar across species. Therefore, in this consortium, metabolomics and microbiomics data from all animal experiments are combined to elucidate common mechanisms by which metabolites affect gut health.

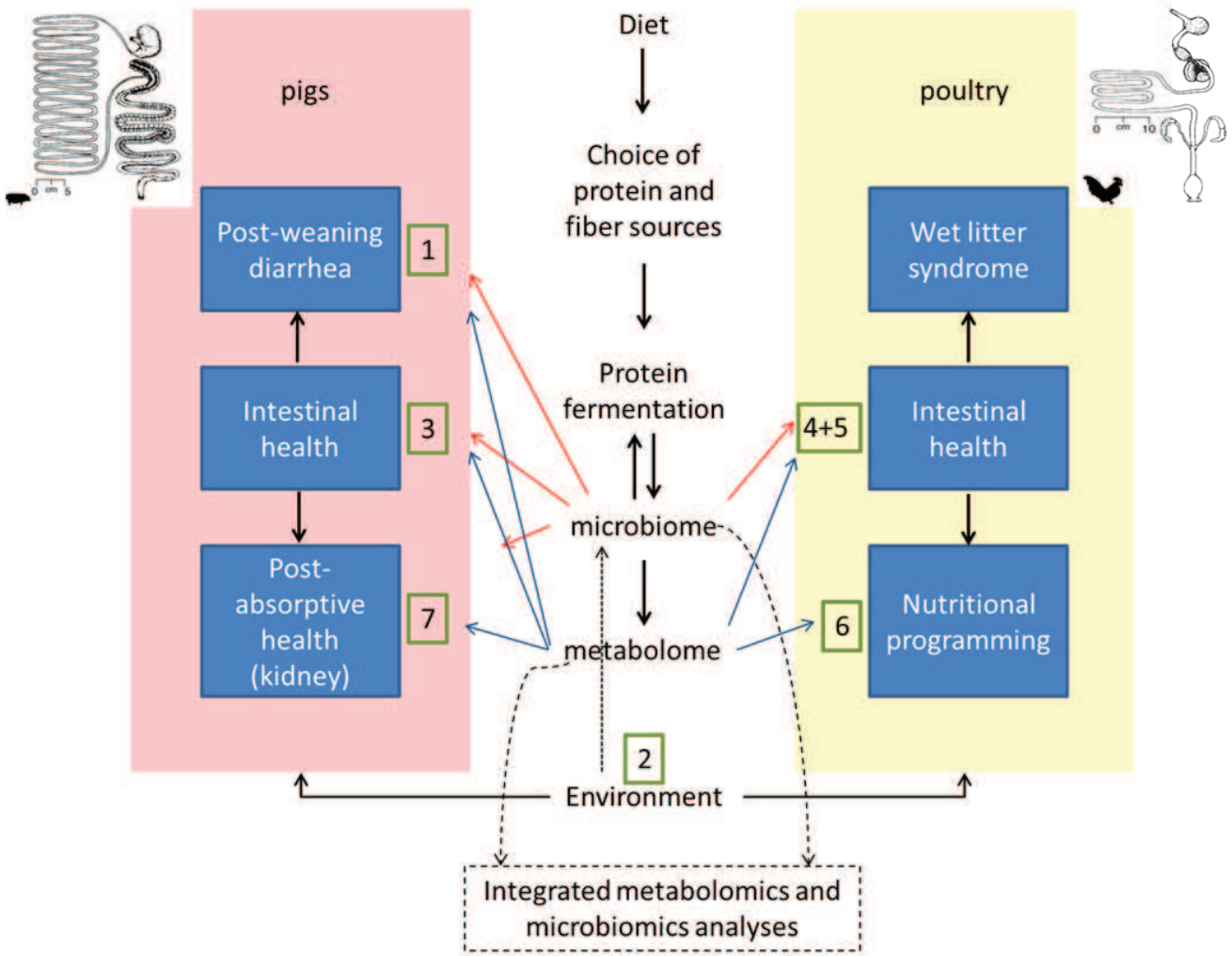

Figure 1. Overview of the research strategy. Green boxes indicate the main focus of the individual experiments, where the numbers correspond with appendix numbers. The red arrows indicate the appendices in which microbiome measurements are included and the blue arrows indicate the appendices in which metabolomics analyses are included. The metabolomics and microbiomics data will be integrated using bioinformatics tools. Appendix 1 is required to answer research question 1. Appendix 2 is required to answer research question 2. Appendix 3 and 5 are required to answer research question 4. Appendix 4 is required to answer research question 3. Appendix 6 is required to answer research question 5. Appendix 7 is required to answer research question 6.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Seven appendices are included in this CCD-application to answer the research questions as described in paragraph 3.2. These seven appendices are the first coherent experiments originating from one of three sub-projects described below.

Sub-project 1: [redacted]
This sub-project targets the relation between protein fermentation, gut function, and PWD, with the aim to reduce PWD in piglets. The first experiment in this sub-project is a large-scale on-farm piglet study (appendix 1 "On-farm piglet experiment") to determine associations between PWD, protein fermentation metabolites, and functional gut parameters in order to answer research question 1. Based on the associations established in this on-farm experiment, *in vitro* studies will be performed to assess effects of identified metabolites and fecal water extracts (from piglets from the on-farm study) on gut function, using pig intestinal organoids. These organoids can be formed from isolated crypts of the intestine, and represent an almost complete functional derivate of the actual gut. Hence, many relevant gut functions can be modelled *in vitro* in an unprecedented way. In the last phase of this sub-project, a second *in vivo* piglet experiment is anticipated. The design of this experiment will be based on the outcome of the "on-farm piglet experiment" (appendix 1) and *in vitro* experiments and is out of scope for this CCD project application.

Sub-project 2: [redacted]
This sub-project aims to study the effects of indigestible dietary proteins on microbial activity, proteolytic fermentation, intestinal health, and performance of pigs and broilers.
In the first experiment (appendix 2 "Digestibility experiment with piglets") the effect of indigestible dietary protein levels on nutrient digestion, metabolite profile, and piglet performance and the interaction with sanitary conditions are studied in order to answer research question 2. Good and sub-optimal sanitary conditions are applied to generate a health disturbance similar as in practice, resulting in differences in sub-clinical health. In a second pig experiment the (interactive) effects of dietary fermentable fibers and indigestible protein on the extent of fermentation, fermentation end-products, microbial colonization, digesta passage rate, and feces quality will be studied (appendix 3 "Digestibility experiment with cannulated pigs") in order to answer research question 4.
The first broiler experiment in this sub-project (appendix 4 "Digestibility experiment with broilers") aims to determine the contribution of different protein sources and levels to hind gut protein fermentation in broilers and determine its effects on gut health, protein fermentation metabolites and performance in order to answer research question 3. A second experiment with broilers will be performed (see appendix 5 "Experiment with broilers to evaluate feed strategies to reduce protein fermentation") to evaluate feed strategies that can potentially reduce protein fermentation (metabolites) and improve gut health of broilers in order to answer research question 3.
A second objective of this sub-project is to study transgenerational effects in poultry. A large body of evidence links maternal/fetal (mal)nutrition to the development of diseases after birth (in rodents and human; Godfrey et al., 2000, Hansen et al., 2014, Paul et al., 2016). In poultry, it is known

that dietary protein fed to broiler breeders can affect broiler offspring performance (Rao et al., 2009). When low quality protein sources are fed to broiler breeders, an increase in ileal undigested protein can increase the production of (toxic) protein fermentation metabolites. To date, it is not known whether these metabolites are also transferred to the eggs and, subsequently, can affect broiler offspring gut health and performance. In the experiment described in appendix 6 ("Effects of dietary protein on metabolite profiles in serum and eggs of broiler breeders"), transgenerational effects of dietary protein in poultry will be studied in order to answer research question 5. Broiler breeders will be fed different diets to create a contrast in cecal protein fermentation and (serum) metabolite profiles will be determined. The transfer of metabolites to eggs and offspring will be measured, as well as effects on broiler offspring.
Follow-up experiments in this sub-project include *in vitro* broiler organoid studies to elucidate underlying mechanisms causing compromised gut health, based on the results and metabolites identified in the previous experiments. Subsequently, follow-up experiments aimed at (dietary) interventions to increase gut health in pigs and poultry are anticipated. The design of these experiments will depend on the outcome of the initial experiments, and are out of scope for this CCD project application.

Sub-project 3: [redacted]

A negative association between protein fermentation and health has also been proposed in humans. In renal transplants, some of the organic acids produced during fermentation of proteins, the uremic toxins (i.e. p-cresol sulfate and indoxyl sulfate), are well-known to contribute to the so-called uremic syndrome. This syndrome is associated with malaise, poor appetite, intestinal complaints, wasting, tendency for malnutrition, sarcopenia and systemic low-grade inflammation in patients with advanced renal disease. Young piglets may be particularly susceptible to the toxic effects of this group of metabolites of protein fermentation, because their immune system, gut and renal function – necessary for removal of these toxic organic acids – is still immature and impaired compared to the adult situation.
In this sub-project, knowledge from observational cohort studies in human renal transplant patients on the relation between dietary protein, gut microbiota, protein fermentation, and uremic toxin production and how these are affected by dietary interventions, will be used to gain insight in the relation between protein fermentation (metabolites) and gut health in piglets. Vice versa, using piglets as a model, mechanistic understanding of the deleterious effects of protein fermentation can be achieved that is important for these patients.
A combination of *in vitro* and *in vivo* approaches will be used to gain insight into how different protein sources (e.g. mucus, indigestible sulphur containing feed proteins) affect gut microbiota, large intestinal protein fermentation, and uremic toxin production. The experiment described in appendix 7 ("Uremic toxin production during the in *vitro* fermentation of proteins in ileal digesta of pigs") is aimed at establishing predictive relations between the (amino acid) composition of ileal undigested proteins and the formation of uremic toxins during their subsequent fermentation *in vitro* in order to answer research question 6.
The availability of minerals (ca and mg) and vitamins (riboflavin, pantothenic acid, vitamin C) have been proven influential in the health of renal transplants. Follow-up experiments in this sub-project could, therefore, include vitamins or mineral supplementation as a nutritional strategy to reduce harmful effects of protein fermentation metabolites on post-absorptive health. The design of such a follow-up experiment will be based on the outcomes of appendix 7 and is, therefore, out of scope for this CCD project application.

References

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3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points

The sub-projects are conducted in parallel and there will be strong collaboration and cross-utilization of expertise and knowledge among the sub-projects within this consortium. The first experimental activities planned and the selection points are schematically presented in figure 2. Based on the outcome of previous experiments, experiments will be adapted if necessary (both within and between sub-projects).
Important selection points in this project include:

- Appendix 1: before the start of the experiment described in appendix 1, a pilot study will be conducted to determine/validate quick selection criteria for post-weaning diarrhea in piglets.
- Appendix 3: the selection of protein sources will depend on the data obtained in appendix 7.
- Appendix 4: dietary treatments in the experiment described in appendix 4 will be determined on in vitro digestion tests.
- Appendix 5: protein sources included in the diets described in appendix 5 will be selected based on the outcome of the experiment described in appendix 4 (both in vitro and in vivo experiment).
- Appendix 6: selection of dietary treatments for broiler breeders will be based on the outcome of the in vitro and in vivo experiment described in appendix 4.
- Appendix 7: selection of the protein sources to be included in the diets will be based on the in vitro work described in appendix 4.

In all sub-projects, (selected) samples of piglets and broilers will be used for metabolomics analyses. In this way, a library of metabolites will be accomplished for piglets and broilers. A milestone in this project will be the identification of metabolic fingerprints related to protein fermentation or impaired gut health in pigs and broilers. The integrated metabolomics and microbiomics approach across all studies allows us to generate hypotheses for the follow-up studies.

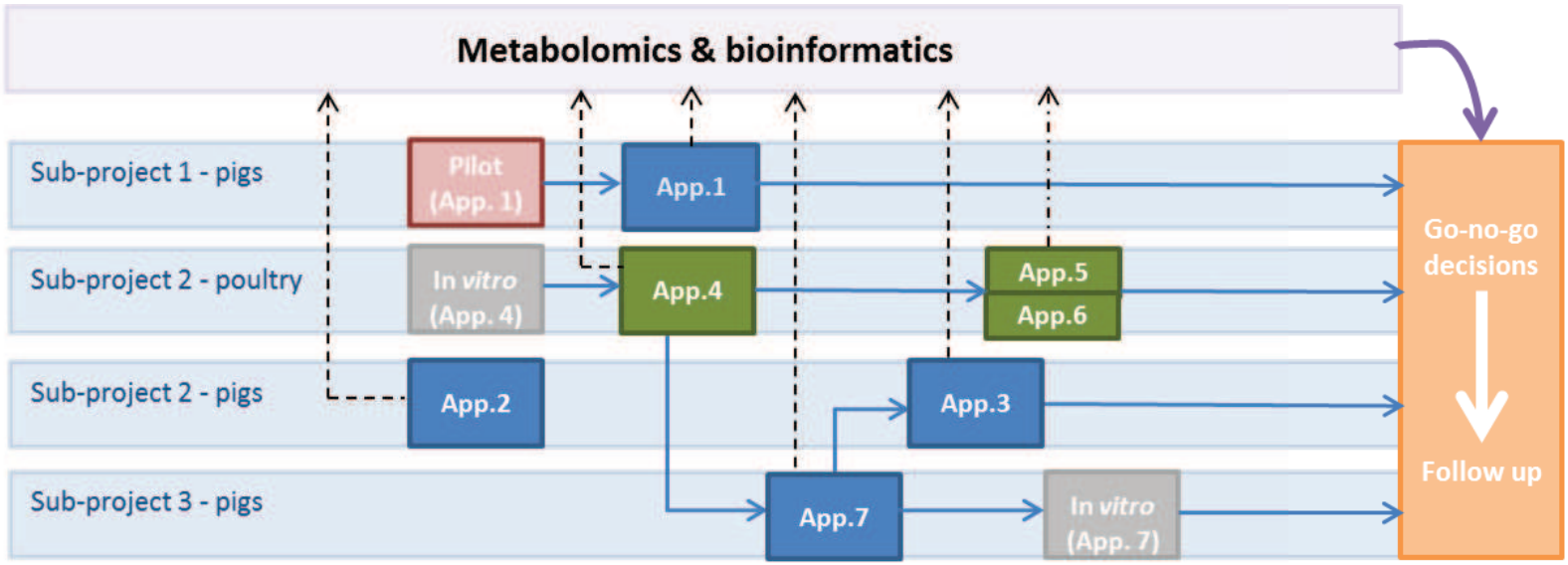

Figure 2. Overview of planned activities and selection points in the sub-projects. Boxes indicate individual experiments (red=pilot, blue= *in vivo* pig, grey=*in vitro*, green= *in vivo* broiler) or follow-up activities (orange). App. indicates the appendix in which the experimental procedures have been described. Blue arrows indicate selection points. Black arrows with dashed lines represent the flow of inputs for the integrated metabolomics approach by using bioinformatics tools.

The aim of the pilot (app. 1) is to determine inclusion criteria for post-weaning diarrhea in piglets kept under commercial conditions. The aim of app. 1 is to determine associations between post-weaning diarrhea, protein fermentation metabolites and gut function indices in piglets kept under commercial conditions.
App. 2 aims to evaluate the interactive effects of sanitary status (as a model for pig farms with a reduced health status) and dietary protein content on protein fermentation (metabolites), gut health and performance in pigs. The aim of app. 3 is to test the potential of fermentable fiber for reducing (detrimental effects of) protein fermentation (metabolites) in pigs.
The aim of the in vitro work (app. 4) is to screen protein sources on their potential ileal digestion and hind gut fermentation in order to select protein sources for in vivo experiments. The aim of app. 4 is to determine the contribution of different protein sources and levels to hind gut protein fermentation in broilers and determine its effects on gut health and performance. Appendix 5 aims to determine if potential nutritional strategies, such as including fermentable fiber and providing a coarse diet structure, can affect protein fermentation (metabolites) and improve gut health of broilers. The aim of app. 6 is to determine if protein fermentation metabolites are transferred from broiler breeders to their eggs and can affect broiler offspring intestinal function.
Appendix 7 aims to establish predictive relations between the (amino acid) composition of ileal undigested proteins in pigs and the formation of uremic toxins (a specific group of protein fermentation metabolites) during their subsequent fermentation in vitro (in vitro – app. 7).

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	On-farm piglet experiment
2	Digestibility experiment with piglets
3	Digestibility experiment with cannulated pigs
4	Digestibility experiment with broilers
5	Experiment with broilers to evaluate feed strategies to reduce protein fermentation
6	Transgenerational effects of protein fermentation metabolites in poultry
7	Uremic toxin production during the in vitro fermentation of proteins in ileal digesta of pigs

15.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure On-farm piglet experiment

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The role of protein fermentation in the occurrence of post-weaning diarrhea (PWD) is poorly understood. The relation between dietary protein and PWD has been studied under highly controlled conditions, but then often diarrhea does not occur (Htoo et al., 2007; Nyachoti et al., 2006). It is, therefore, of importance to study PWD under practical conditions. The aim of this experiment is to determine associations between PWD, protein fermentation metabolites and gut function indices in piglets kept under commercial conditions.

General design

In this experiment, piglets will be selected and sampled from commercial farms. In collaboration with and advised by veterinarians, multiple farms will be selected. Farms will be classified based on known parameters (such as average daily gain, feed efficiency, mortality, known occurrence of outbreaks PWD). At each farm, piglets with and without PWD are selected and dissected for sample collection. In total, 200 piglets will be sampled, including healthy control piglets selected on each farm.

First, piglets are identified based on clinical signs of diarrhea (or non-diarrhea for the controls). Secondly, a rectal fecal sample is collected and scored on fecal consistency. The inclusion criteria (such as fecal pH, ammonia concentration) for piglets with PWD will be determined in a pilot study (see below, A – paragraph 3). Based on these developed criteria, piglets are classified as PWD and non-PWD (controls) and, lastly, are blood-sampled and killed for sample collection. Blood-sampling is performed before killing because the method of killing might affect the (protein fermentation) metabolite profile in the blood. Collected samples at dissection include digesta, urine and intestinal tissues.

Recently, Dou et al. (2017) showed that pre-weaning microbiota diversity and composition could be related to PWD under experimental conditions. In this experiment, fecal samples will, therefore, be collected at two additional time points before dissection, in which microbiota/pathogen analyses are conducted. This will enable the investigation of causal relations between microbial composition and/or pathogens and PWD.

Differences between farms in feed composition can occur, but are generally small in Dutch piglet farming. Feed-specific data will be recorded at each farm and will be part of the farm effect in the data analysis.

Primary outcome parameters

First, analyses will be conducted in samples from all 200 piglets to quantify protein fermentation, using known protein fermentation products (e.g. *p*-cresol). Based on these results, a sub-set will be selected to obtain a representative reflection of the variation in protein fermentation in PWD piglets. In this subset, detailed laboratory analyses will be performed. Detailed analyses include microbiota composition in digesta or fecal samples, presence of pathogens causing diarrheal disease, intestinal morphology, mucosal barrier function measures and blood inflammatory cytokines. In addition, (untargeted and targeted) metabolomics analyses will be performed in feces, digesta or blood samples. Finally, multivariate analyses are performed

to determine associations between protein fermentation, PWD, pathological/functional read-outs of gut function, microbiota parameters, metabolomics analyses and immunological measures.

References

Dou, S., P. Gadonna-Widehem, V. Rome, D. Hamoudi, L. Rhazi, L. Lakhal, T. Larcher, N. Bahi-Jaber, A. Pinon-Quintana, A. Guyonvarch, I. L. E. Huërou-Luron, and L. Abdennebi-Najar. 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS One 12:e0169851.

Htoo, J. K., B. A. Araiza, W. C. Sauer, M. Rademacher, Y. Zhang, M. Cervantes, and R. T. Zijlstra. 2007. Effect of dietary protein content on ileal amino acid digestibility, growth performance, and formation of microbial metabolites in ileal and cecal digesta of early-weaned pigs. J. Anim. Sci. 85:3303-3312.

Nyachoti, C. M., F. O. Omogbenigun, M. Rademacher, and G. Blank. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. J. Anim. Sci. 84:125-134.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

In this experiment, we are studying PWD and the variation in protein fermentation under practical circumstances. Therefore, we will exclusively focus on natural occurrence of diarrhea and will not impose (dietary) treatments. The onset of PWD occurs around 3 to 4 days after weaning (advise of pig veterinarian; Kyriakis et al., 1999; Madec et al., 1998) and can remain up to 2 weeks after weaning (Kyriakis et al., 1999; Heo et al., 2008). Three to 14 days following weaning will be the window for PWD identification and subsequent dissection and sample collection at the commercial farms. Because early life microbial biomarkers could be of importance as well, fecal samples are collected in the pre-weaning period as well. In total, 3 fecal samples are collected per piglet (1 pre-weaning, 1 post-weaning and 1 for PWD identification/before dissection). It is unknown which piglets will develop diarrhea in the post-weaning period, therefore, a larger group of piglets will be sampled for feces in the pre-weaning and early post-weaning period (see also B - estimated numbers).

In this experiment, microbial composition and microbial metabolites in piglets are studied, and, therefore, individual piglets which have received antimicrobial treatments should not be included. When sows have been treated with antimicrobials, her piglets will not be included. In general, few group antimicrobial treatments are applied to piglets in the pre-weaning period. Pre-weaned piglets which have been treated individually will not be included in this study. In the post-weaning period, antimicrobial treatments (for PWD) should be withheld in the period of sample collection in the departments from which piglets will be selected. In case of diseased piglets (other than diarrhea), there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. For instance, if individual piglets should be treated for joint infections, these piglets will not be included in the experiment. Selection of piglets will be based on clinical signs of PWD and a (rectal) fecal sample is obtained to verify diarrhea or non-diarrhea. The piglets will be carefully handled. If a piglet does not defecate during handling, the fecal sample is collected via manual stimulation. One person holds the piglet and a second person puts on a glove (new glove for each piglet), adds water on his/her little finger, carefully enters the anus and gently stimulates the rectal ceiling (max. of 2 minutes) to stimulate defecation. A maximum of 3 attempts is used per piglet per day in order to obtain a rectal fecal sample from a piglet. Based on the fecal samples collected post-weaning, piglets will be selected, blood-sampled and killed (within 36h after fecal sampling), and, subsequently, the gastrointestinal tract will be removed and tissues and intestinal digesta collected.

Laboratory analyses as described in the previous paragraph will be conducted in the obtained samples and the data following statistical analyses will result in important associations between protein fermentation, PWD, pathological/functional read-outs of gut function, microbiota parameters, metabolomics analyses and immunological measures.

References

Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2008. Effects of feeding low protein diets to piglets on plasma urea nitrogen, faecal ammonia nitrogen, the incidence of diarrhoea and performance after weaning. Arch. Anim. Nutr. 62:343-358.
Kyriakis, S. C., V. K. Tsiloyiannis, J. Vlemmas, K. Sarris, A. C. Tsinas, C. Alexopoulos, and L. Jansegers. 1999. The effect of probiotic LSP 122 on the control of post-weaning diarrhoea syndrome of piglets. Res. Vet. Sci. 67:223-228.
Madec, F., N. Bridoux, S. Bounaix, and A. Jestin. 1998. Measurement of digestive disorders in the piglet at weaning and related risk factors. Prev. Vet. Med. 35:53-72.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

This experiment is not focused on differences in response parameters between (dietary) treatments, but is aimed at obtaining samples from piglets with and without PWD under commercial conditions. Little is known about the variation in protein fermentation during PWD under commercial conditions. We, therefore, used data from experiments focusing on high or low protein diets and diarrhea in weaned piglets for power analyses. We used fecal ammonia concentrations (Heo et al., 2009) and colonic ammonia concentrations (Heo et al., 2010). We expect higher variation between animals under commercial conditions compared to experimental studies. Furthermore, variation in other read-out parameters in this experiment are expected to be higher. Therefore, the variation in the power analyses with fecal and colonic ammonia concentration was multiplied with 3. This resulted in 166 and 262 animals, when using fecal and colonic ammonia concentration, respectively, in order to obtain a power of ≥0.8. We, therefore, estimate to require 200 piglets for dissection. The main focus is on piglets with PWD and we expect greater variation in the PWD piglets, therefore, we anticipate to include 150 PWD piglets and 50 (non-PWD) control piglets.
In addition, we have consulted a statistician regarding numbers of animals and farms. There is variation between farms in incidence of PWD, and, therefore, we need to include multiple farms to obtain a representative sample of the Dutch piglet population. The inclusion of multiple farms allows us to include farm as an effect in the data analysis, in order to determine whether differences in the relation between PWD and protein fermentation (metabolites) occur between farms.
In addition, we will perform a pilot study before the start of this experiment, to determine the frequency and concentration of protein fermentation (end-products) in feces of piglets with diarrhea. This pilot study has been discussed with the Animal Welfare Body (work protocol "The concentration of protein fermentation end-products in faeces of piglets with post-weaning diarrhoea") and was not considered an animal experiment as referred to in the Dutch Act on Animal Experiments since the experimental procedures described in the protocol cause less pain or distress than the insertion of a needle under good veterinary practice. In short, in this pilot, fecal samples will be obtained from piglets in practice (multiple farms) with and without diarrhea. If piglets do not defecate spontanously when being handled, a fecal sample will be obtained via manual stimulation. Fecal consistency score and pH are determined in the fecal samples. Subsequently, fecal ammonia concentration is analyzed. Other protein fermentation end-products are also analyzed and correlated with ammonia to determine the appropriateness of fecal ammonia as an indicator of protein fermentation. These pilot data are required to determine the criteria for the selection of piglets with PWD in this experiment and will also be used to verify the required number of piglets, but we do not expect to require more than 200 piglets.

Data from this experiment will be analyzed using multivariate analyses. For instance, metabolomics data from piglets with and without PWD will be analyzed by partial least squares discriminant analysis. In addition, functional read-outs of gut function will be analyzed with principal component analysis and the following principal components will be subsequently related to protein fermentation indices.

References
Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2009. Feeding a diet with decreased protein content reduces indices of protein fermentation and the incidence of postweaning diarrhea in weaned pigs challenged with an enterotoxigenic strain of Escherichia coli. J. Anim. Sci. 87:2833-2843.
Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2010. Feeding a diet with a decreased protein content reduces both nitrogen content in the gastrointestinal tract and post-weaning diarrhoea, but does not affect apparent nitrogen digestibility in weaner pigs challenged with an enterotoxigenic strain of Escherichia coli. Anim. Feed. Sci. Technol. 160:148-159.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Piglets will be used in this experiment, because this is the target animal. This experiment is aimed at the variation present in the Dutch piglet population and, therefore, no appropriate model is available.

Piglets will be collected from multiple commercial farms. At each farm, we will select and kill piglets for sample collection. These selected piglets will include healthy control piglets (i.e. no diarrhea) and piglets with PWD. Sexe will be recorded, but is not used as a selection criterion, because it is not expected that PWD, and the relation with protein fermentation, differs between sexe. Potential differences in the ratio between male and female will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from commercial farms.
A total of 200 piglets will be sacrificed for sample collection. These 200 piglets will be selected based on criteria (i.e. fecal consistency, pH, ammonia concentration; verified in the pilot study) and in order to obtain these 200 piglets, rectal fecal samples will be collected from more piglets. In addition, to identify the presence of pathogens or microbial biomarkers before the onset of PWD, fecal samples will be collected from piglets in the pre-weaning and early post-weaning period as well. It is beforehand unknown which piglets will develop PWD. Assuming that the required 200 piglets will be obtained from 5 farms (i.e. 40 piglets per farm), with a division of 30 PWD and 10 control piglets at each farm, and a prevalence of 5% PWD on the day of selection for dissection, fecal samples in the pre-weaning / early post-weaning period should be collected from 600 piglets at each farm (i.e. 30 / 0.05 = 600 piglets/farm). This will in total be 600 x 5 = 3000 piglets from which feces will be sampled.
Selection and dissection of piglets (with and without PWD) will be between 3-14 days post-weaning, because this is the period in which the incidence of PWD is highest.

Species	Origin	Maximum number of animals	Life stage
Pigs	Mild	200	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: the piglet is the target animal in this experiment. This experiment is aimed at the variation present in the Dutch piglet population and, therefore, no appropriate model is available. Reduction: the number of animals is based on power analyses and this number will be verified with a pilot study. Refinement: the selection of piglets will be based on minimal invasive measures, i.e. clinical signs of PWD and rectal fecal samples. The attempts to obtain a fecal sample are confined to 3 at each time point. Killing of the piglets will be performed by skilled personnel to minimize stress.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals will be obtained from commercial farms. Individual sows and pre-weaning piglets are not included in this experiment when they have been treated with antibiotics. In the post-weaning period of sample collection, (group) antimicrobial treatments for PWD should be withheld, because treatments will affect microbial composition and microbial metabolite production and the development of PWD. In case of individual diseased piglets

(other than diarrhea), there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. After piglets are killed and sampled this experiment is finished and potential required treatments can again be applied when advised by a veterinarian according to normal Dutch legislation. Killing and dissection of piglets is performed by skilled personnel.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature overview has been made and the literature search (using google scholar) included the key words piglets, post-weaning diarrhea, gut health, dietary protein and protein fermentation. To the best of our knowledge, associations between PWD, protein fermentation and gut function measures have not been determined in commercial settings before. In addition, this project has been peer-reviewed by 5 independent reviewers and this on-farm approach was highly encouraged by the reviewers.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

The piglets in this experiment are obtained from commercial farms and, therefore, piglets are kept under commercial conditions and not all housing conditions of Annex III of the Directive 2010/63/EU are met: - 3.1b. The piglets are checked daily by the respective farmer or a qualified person (Wod), and sick piglets will be noted. Piglets with PWD cannot be treated immediately, because our objective is to determine the mechanisms underlying diarrhea in piglets, including microbial composition and production of microbial metabolites, and antibiotic treatment will interfere with these measurements. - 3.3b and 3.6a: no bedding material will be provided. Environmental enrichment will be as usual at each specific farm. - Table 7.3: the surface area for piglets will be according to commercial conditions.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[] No > Continue with question H.
[X] Yes > Describe this establishment.

The experiment will take place at commercial pig farms, which are not official establishments licensed by the NVWA.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Piglets will be sampled and dissected at commercial pig farms. It is of importance that the conditions are in agreement with commercial practices. Before piglet selection and dissection, the respective farm personnel will perform daily routine inspections. Fecal samples before dissection are collected by a qualified person (Wod). Killing and dissection will be performed by qualified persons.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Selected piglets will be blood-sampled and killed for sampling of tissues and intestinal digesta. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

During the (post-weaning) period of fecal sampling and until dissection, no antimicrobial treatments should be provided for PWD. This might result in a higher incidence or severity of PWD.

Explain why these effects may emerge.

Withholding antimicrobial treatments might increase the incidence or severity of PWD.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Antimicrobial treatments cannot be applied because these treatments will interfere with the measurements. No specific measures are taken to minimize adverse effects, but piglets will be inspected daily by the respective farm personnel. In case of diseased piglets (other than diarrhea), there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. At each specific farm, the day of selection and dissection will be determined following discussion with the respective farmer, to determine the day at which PWD prevalence is usually highest. Our experience is that this is usually around 4-7 days post-weaning. Assuming the onset of PWD is at 3-4 days post-weaning, this means that the period of not treating piglets is kept between 1-3 days, However, the exact window of selection and dissection will be determined with the respective farmer, but will be at maximum until day 14 post-weaning.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Antimicrobial treatments (for PWD) should be withheld in de post-weaning period, because antibiotics will interfere with the measurements. In case of sick piglets, there will be discussion between researchers, the respective farmer and, if required, with a veterinarian to determine if further measures are required. For instance, piglets showing severe lethargy can be euthanized when advised by the veterinarian.

Indicate the likely incidence.

The likely incidence of humane endpoints to occur in the post-weaning period is estimated at 3%, i.e. slightly higher than the average mortality rate in the Dutch weaned piglet population of 2.2%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort in this experiment is estimated to be mild. Experimental conditions are according to commercial settings, except that no antimicrobial treatments can be provided in the sampling period (mild). Discomfort related to rectal fecal sampling, blood sampling and killing is also estimated as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Piglets need to be killed in order to collect the required samples.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

16.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 2	Type of animal procedure Digestibility experiment with piglets

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Environmental conditions can affect the animals' microbiota composition. It has been consistently demonstrated that pigs kept under low sanitary conditions (resulting in a lower sub-clinical health status of the herd) have a reduced nitrogen digestibility compared with pigs kept under high sanitary conditions (Kampman-van der Hoek et al., 2016; van der Meer et al., 2016). This effect is likely mediated through the microbiome. It illustrates that steering of protein fermentation in the gut is dependent on the (environmental, sanitary) status under which the pigs are kept. The aim of this experiment is, therefore, to test the effect of sanitary conditions (as a model for pig farms with a reduced health status), dietary crude protein (CP), and their interaction, on CP digestion and fermentation, fermentation end-products, and piglet performance. The experiment will be designed in a 2 x 2 factorial arrangement with sanitary conditions (high, HSC; or low, LSC) and dietary digestible CP content (high, HCP; or low, LCP) as factors. Output parameters will be ileal and total tract nutrient digestibility, feces quality (consistency score, dry matter content, and pH), body weight gain, and feed efficiency. In addition, metabolites of protein fermentation (e.g. ammonia, amines, phenols and sulfides) in feces will be measured.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

The contrast in sub-clinical health status will be according to van der Meer et al. (2016). Briefly, piglets will be obtained from a commercial nursery farm in the Netherlands. At 4 weeks of age, piglets will be transported to the experimental farm and allocated to LSC or HSC treatments. At arrival, piglets will be weighed and HSC piglets will receive anthelmintic treatments, whereas LSC piglets will remain untreated. HSC and LSC piglets are housed in separate rooms. HSC piglets will be housed in thoroughly cleaned and disinfected pens and a strict hygiene protocol (including showering, change of clothes, hairnet, and face mask) will be adhered when entering the HSC room (unpublished data). A mixture of manure of commercial pig farms will be spread weekly in the LSC pens to increase pathogenic pressure and no hygiene protocol will be applied (unpublished data). In addition, LSC piglets will be exposed to nylon bags containing a mixture of pig manure and straw, ground to pass a 1 mm screen, as a model for dust (unpublished data).
From arrival until 6 weeks of age, piglets will receive the same weaner diets as they received in the nursery farm. At 6 weeks of age piglets will be weighed and feeding of experimental piglet diets will start. Within sanitary status, pens of pigs will be randomly assigned to LP or HP diets. Diets will be fed for 4 weeks and piglets will be weighed every two weeks.
At 10 weeks of age, piglets will be sacrificed and intestinal contents from the ileum and colon will be collected. At maximum 48 h before dissection, piglets will be housed individually and will be adapted to frequent feeding. Starting from 24h before dissection, pigs will be frequently fed (once every

6h from 24-6h prior to dissection and once every hour from 6h prior to dissection). This is required to obtain a steady-state in the gastro-intestinal tract. Our experience is that if pigs are used to meal feeding and will remain on the same diet, pigs will readily adapt to individual housing and frequent feeding.

References
van der Meer, Lammers, Jansman, Rijnen, Hendriks, and Gerrits. Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation. J Anim Sci (2016) 94:4704-4719.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of replicate pens per treatment has been calculated by power analysis (Proc Power; SAS 9.3) using data from a previous experiment with comparable design (with 6 piglets per pen; unpublished data). Because ileal CP is considered a main output parameter for the current experiment, this parameter is used for the power analysis. In the previous experiment, only total tract CP digestibility was measured (unpublished data). From literature (de Vries et al, 2014), it is calculated that the variation in ileal CP digestibility measurements is 3 x larger than total tract CP digestibility measurements. Assuming a residual standard deviation of 4.4 (3 x 1.47; unpublished data) and averages of ileal CP digestibility of 70, 82, 79, and 85% for the four treatments, it follows that n=9 at a power of 0.8. The standard deviation used in the power analysis is based on a previous study (unpublished data) where 6 piglets per pen were used. We, therefore, include 6 piglets per pen as well. This number of piglets per pen also resembles the practical situation.

References
de Vries, Pustjens, van Rooijen, Kabel, Hendriks, and Gerrits. Effects of acid-extrusion on the degradability of maize dried distillers grain with solubles in pigs. J Anim Sci (2014) 92:5496-5506.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

In total, 216 female piglets will be used in this experiment divided over 3 batches (72 piglets per batch). Piglets will be housed with 6 piglets per pen and pen will be considered the experimental unit. The 4 dietary treatments will be tested in 3 pens each batch, resulting in 9 replicates per treatment.
Piglets will be obtained from a commercial nursery at 4 weeks of age and remain in the experimental facilities until 10 weeks of age.
Only female piglets will be used to exclude a potential effect of sexe. Because female animals generally respond more intense to immune

interventions than male animals, we have chosen to use female piglets to maximize contrasts between treatments. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial farm.

Species	Origin	Maximum number of animals	Life stage
Pigs	Mild	216	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: The piglet is the target animal in this experiment. The interaction between sanitary status and protein fermentation cannot be tested with ex vivo techniques. Hence, it was chosen to test this in an in vivo experiment. Reduction: The number of piglets to be used has been based on previous observations and is minimized to the number needed to detect differences between the treatments. Contrasts between the treatments are maximized within the range observed in practical settings, to minimize the number of piglets needed. Refinement: Piglets will be group-housed for the major the part of the study and the duration of individual housing is limited to 48h to minimize discomfort. In addition, during individual housing, pigs will be able to see and hear each other, have snout contact and will receive toys as enrichment.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All animal handling procedures will be conducted by experienced staff (feeding, weighing, transport). Various, non-destructible toys will be made available to the pigs as environmental enrichment, both during group- and individual housing, and toys will be alternated regularly. Occurence of clinical health issues due to sanitary conditions is not expected based on previous studies (van der Meer et al 2014 and unpublished data). However, in case of clinical signs of illness, there will be discussion between researchers, animal caretakers and a veterinarian to determine is further measures are required. No exceptional adverse effects of this experiment on the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature overview on this topic has been performed. To the best of our knowledge, the interactive effects of sanitary conditions and dietary protein level on protein fermentation and its end-products has not been performed previously. If newly published papers indicate novel insights in the topic that have not been taken into account but are of relevance for the proposed experimental design, experimental procedures will be reconsidered.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Housing and care is according to Annex III, except during the last 48h of the experiment, when pigs will be housed individually in pens. Individual housing during this period is needed to make sure that every animal consumes feed at each time point a meal is given (once every 6h from 24-6h prior to dissection and once every hour from 6h prior to dissection). This is required to obtain a steady-state in the gastro-intestinal tract. The pigs will be housed on partially-slatted floor pens with rubber mats in temperature-controlled rooms. The floor area of the pens in which pigs will be individually housed, will be min. 2 m2, allowing the pigs to move around freely. The use of bedding material is avoided as this will be consumed by the pigs and will thus interfere with the digestibility measurements. Rubber mats will be removed 3 days before individual housing, because can pigs

consume pieces of rubber mat as well. During individual housing, pigs will be able to see and hear each other and have snout contact. Animals will receive toys as enrichment.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Pigs might experience pain during killing. The potential pain is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Previous studies using the same contrasts in sanitary conditions (van der Meer et al., 2014 and unpublished data) did induce differences in sub-clinical health status. It is possible that piglets in the low sanitary status treatment develop clinical health problems, however, the previous studies (van der Meer et al., 2014 and unpublished data) showed no adverse effects of the low sanitary status on animal welfare. Pigs might experience stress as a result from individual housing.

Explain why these effects may emerge.

The potential adverse effects can occur due to the sanitary conditions. Individual housing is required to feed individually and frequently to obtain a steady-state in the gastro-intestinal tract.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Occurence of clinical health issues due to sanitary conditions is not expected based on previous studies (van der Meer et al., 2014 and unpublished data). In case of clinical signs of illness, there will be discussion between researchers, animal caretakers and a veterinarian to determine is further measures are required. The period of individual housing is restricted to 48h and pigs will be able to see and hear each other, have snout contact and pigs will receive toys as enrichment.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Based on previous studies using the same contrasts in sanitary conditions (van der Meer et al., 2014 and unpublished data), the procedures are not expected to give rise to circumstances that require humane endpoints. In the case of clinical illness in the expert opinion of the veterinarian, animals will be removed from the experiment. In addition, animals are removed from the experiment in case of body weight loss (>10% compared to previous weighing).

Indicate the likely incidence.

Based on our previous experiments the likely incidence is estimated to be below 5%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The expected level of discomfort is classified as mild. The low sanitary conditions treatment may result in a lower sub-clinical health status. Based on previous studies using the same contrasts in sanitary conditions (van der Meer et al., 2014 and unpublished data), the level of discomfort for the LSC treatment is judged as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Pigs will be killed and dissected after the procedures. This is necessary to obtain samples for digestibility measurements.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

17.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 3	Type of animal procedure Digestibility experiment with cannulated pigs

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The ratio between protein and fermentable fiber plays an essential role in the dynamics of protein fermentation (Le Gall et al., 2007). The inclusion of fermentable fiber in the diet is, therefore, a potential nutritional solution for reducing (detrimental effects of) protein fermentation (metabolites). The aim of this experiment is, therefore, to test the effect of fermentable fiber (FF), crude protein (CP), and their interaction, on the extent of fermentation, fermentation end-products, microbial colonization, digesta passage rate, and feces quality. The experiment will be designed in a 3 x 2 factorial arrangement with dietary FF content (low FF, high FF, low FF+FF infused in colon) and CP (two protein isolates varying in protein structure) entering the colon as factors. The selection of protein sources will depend on the data obtained in appendix 7 and literature. Diets will have low or high FF contents. Apart from affecting fermentation in the large intestine, dietary FF will affect endogenous protein losses, fermentation, and microbial biomass in the small intestine, thereby changing the substrate arriving at the colon. A third treatment where the low FF is fed and FF is infused in the colon, will be tested to separate the effects of FF in the small intestine from fermentation characteristics in the large intestine. Output parameters will be total tract nutrient digestibility, digesta retention time in the large intestine, feces quality (consistency score, dry matter content, and pH), and microbiota composition in feces. In addition, a combination of targeted and untargeted metabolomics in feces and blood samples will be used to measure metabolites of protein fermentation.

Reference: Le Gall M, Quillien L, Seve B, Guéguen J, Lalles JP. Weaned piglets display low gastrointestinal digestion of pea (L.) lectin and pea albumin 2. JAS. 2007; 85:2972-2981.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

After arrival, pigs will be allowed to acclimatize to the new environment for a minimum of 8 days. Fecal grab samples are taken and analyzed for the presence of parasites. If required pigs will receive anthelmintic treatment. During the acclimatization periods, pigs will be socialized according to a fixed protocol. Pigs will be surgically fitted with a cannula in the distal ileum and a catheter in the jugular vein. After surgery pigs are allowed to recover and adapt to the housing conditions for a minimum of 7 days before they are switched to the experimental diets.
Immediately after surgery, pigs are kept on a soft pad under a heating lamp until pigs can regulate their body temperature again. Rectal temperature is regularly monitored, for at least 3 days after surgery. Body weight will be determined upon arrival of the pigs, and after surgery weekly until the end of the last experimental period.

After recovery from surgery, the experimental phase will start and samples will be collected from each pig during 6 subsequent measurement periods. This approach is selected to allow within pig comparisons of the dietary treatments (Latin square design), minimizing the total duration of the experiment, thereby the risk of cannula and catheter complications. Each period will consist of 5 days adaptation to the experimental diet, followed by collection of ileal digesta during 12 hours on days 6 and 7, infusion of protein/fermentable fiber and markers to estimate mean retention time of the digesta into the colon (via the cannulas) at day 8 and urine+feces collection and blood sampling (via the catheter) at days 9-11. Such a time frame (6 periods x 11 days) is commonly used in studies with ileal cannulated growing pigs. To collect feces quantitatively, plastic bags will be attached to the rear end of the pigs as described by Van Kleef et al. (1994). Clean urine will be quantitatively collected using funnels underneath the cage.

Reference
van Kleef, Deuring, and van Leeuwen. A new method of faeces collection in the pig. Lab Anim (1994) 28: 78-79.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of replicates per treatment has been based on literature where similar output parameters were studied (Bach Knudsen et al. 2007; de Vries et al. 2016; Otto et al. 2003, Wilfart et al. 2007). For the most important parameters, power was calculated for several experimental design scenarios according to the method of Stroup (1999), using the data presented in these studies. It was concluded that a 6x6 Latin square design gave sufficient power (between 0.8 and 0.9) for the most important parameters (nutrient digestibility, SCFA concentrations in peripheral blood, large intestinal retention time).

References

Bach Knudsen, Jørgensen, and Canibe. Quantification of the absorption of nutrients derived from carbohydrate assimilation: model experiment with catheterised pigs fed on wheat- or oat-based rolls. Br J Nutr (2007) 84:449-458.
de Vries, β-Glucans and resistant starch alter the fermentation of recalcitrant fibers in growing pigs. PlosOne (2016) 11: e0167624.
Otto, Yokoyama, Hengemuehle, Bermuth, van Kempen, and Trottier. Ammonia, volatile fatty acids, phenolics, and odor offensiveness in manure from growing pigs fed diets reduced in protein concentration. J Anim Sci (2003) 81: 1754-1763.
Wilfart, Montagne, Simmins, Noblet, and van Milgen. Br J Nutr (2007) 98:54-62.
Stroup. Mixed model procedures to assess power, precision, and sample size in the design of experiments. Proc Biopharm Am Stat Assoc (1999) 15-24.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Eight barrows (approximately 25 kg) will be obtained from a commercial pig farm. Only pigs that are healthy without hernias (testicular or umbilical), damaged ears or unsound feet are selected, because these can complicate surgery. Surgery will be performed on 8 pigs. Two pigs will be considered spare animals and will be used in the case of problems with digesta collections or catheter patency. If pigs are replaced by spare pigs during the experiment, observations can be included in the statistical analyses, provided that these pigs have been used for at least two of the experimental periods. Should problems arise during the last period, the measurement period will be extended by one period. Pigs will be approximately 60-70 kg at the end of the experiment.
Barrows will be used so that urine and feces can be collected separately. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial farm.

Species	Origin	Maximum number of animals	Life stage
Pigs	Moderate	8	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: The pig is the target animal in this experiment. Although fermentation can be tested in vitro, the complex interactive effects of dietary protein and fermentable fiber with passage rate and bacterial colonization on fermentative processes cannot be tested with ex vivo techniques. Hence, it was chosen to test this in an in vivo experiment. Reduction: A Latin square design is used to minimize the number of pigs needed. Refinement: Before surgery pigs will be socialized for a minimum of 5 days (according to a socialization protocol), so that they are well adapted to handling procedures after surgery and during sampling.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Surgical procedures will be conducted under complete anesthesia, and adequate analgesics are used during recovery. No exceptional adverse effects of this experiment on the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature overview on this topic has been performed. To the best of our knowledge, the interactive effects of fermentable fiber and dietary protein structure on the combination of protein fermentation, its end-products and passage rate and digestibility have not been performed previously. If newly published papers indicate novel insights in the topic that have not been taken into account but are of relevance for the proposed experimental design, experimental procedures will be reconsidered.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Pigs will be housed individually in metabolism pens during the recovery phase and during the experimental period in temperature controlled rooms. The floor area of the pens will be min. 2 m2, allowing the pigs to move around freely. Walls will be smooth to prevent damage to cannulas. Animals will be housed on a plastic coated floor. Individual housing is needed to prevent animals from damaging cannulas and catheters of pen mates. Pigs will be able to see and hear each other. The use of bedding material is avoided as this will be consumed by the pigs and will thus interfere with the digestibility measurements. Animals will receive toys as enrichment, which are regularly changed.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.

G. Location where the animals procedures are performed

[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.

[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[X] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Surgical procedures will be performed under anesthesia. After surgery, animals will be treated with analgesics for at least 3 days and with antibiotics. The potential pain experienced during killing is minor and short-term, and, therefore, no pain relieving methods are applied.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Individual housing, fasting prior to surgery and recovery from surgical procedures may have adverse effects on animal welfare. Infection and inflammation due to the presence of the cannula and/or catheter may occur.

Explain why these effects may emerge.

These adverse effects may emerge because they are part of the experimental procedures.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Infection and inflammation will be prevented or minimised by providing antibiotics after the surgical procedures and whenever required based on clinical signs of infection. Long-term effects of antibiotic treatment on the microbiota are minimised by administering the antibiotics intramusculair/in the wound (and not orally) and by allowing a recovery period of (a minimum of) 7 days and an adaptation period of 5 days to the diet. Adverse effects on animal welfare due to individual housing are minimised by providing toys as environmental enrichment and pigs will be able to see and hear each other.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Pigs will be euthanized should one of the following conditions apply: • During recovery from surgery, a pig does not start eating within 2 days and subsequently produce feces, indicating blockage of the intestines. • A cannula is lost and cannot be placed back. • A pig has fever during 5 successive days, is not responding to treatments proposed by a veterinarian, and shows signs of infection and inflammation. • A pig has feed refusals exceeding 20% of the amount of feed offered for a period exceeding 7 days. • In the expert judgement of the veterinarian, future observations on a pig will not provide reliable results. • A pig suffers from body weight loss (>10%) during a 14 day period.

Indicate the likely incidence.

The likely incidence of pigs to be removed from the experiment is estimated at 25% (20% due to technical problems with the cannula).

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The level of discomfort is expected as follows: • Surgical procedures: moderate • Sampling procedures (9.5 weeks): mild • Individual housing in the absence of bedding material in large metabolism pens (11 weeks): moderate. The cumulative discomfort in this experiment is estimated at moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Pigs will be killed after the procedures. Keeping pigs with a cannula is complicated. Moreover, the location of the cannula and tip of catheter need to be verified during autopsy.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

18.

Appendix
Description animal procedures

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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 4	Type of animal procedure Digestibility experiment with broilers

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Protein fermentation is likely involved in the occurrence of gut health problems and diarrhea. The aim of this experiment is to determine the contribution of different protein sources and levels to hind gut protein fermentation in broilers and determine its effects on gut health, protein fermentation metabolites and performance.

Experimental diets

Broilers will be fed, from hatch to 29 days of age, different experimental diets in a 3x2 complete randomized block design. The diets will consist of one of 3 different protein sources (from ingredients currently used in poultry feed), at 2 levels of ileal protein digestibility. In vitro tests (in vitro pre-digestion, to simulate gastric and enteric digestion, followed by gas production method, to simulate hind gut fermentation) will be used prior to the feed trial to determine which protein sources will be added into the experimental diets and which method(s) of creating poorer digestible proteins will be used.

Read-out parameters

The following measurements will be recorded:

- performance measurements (feed intake, water intake, weight gain)
- litter and cloaca scores
- ileal & fecal crude protein and amino acids digestibility (marker method)
- Di-animo-pimelic acid (DAPA), indicator of microbial biomass, measured in ileal and cecal digesta
- untargeted metabolomics and biogenic amines targeted metabolomics (using serum, cecal and colon digesta)

- intestinal lesion scores
- relative organ weights (proventiculus, gizzard, duodenum, jujenum, ileum, ceca, colon, pancreas and liver)
- cecal digesta pH
- gut leakage (using serum marker: fluorescein isothiocyanate (FITC)-Dextran)
- villus height and crypt depth
- microbiota population in ceca

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Individual tags:

On day 0 all birds are given individual neck labels. This is necessary to allow us to link metabolic data from individual birds to their growth performance and individual gut health measures. Secondly, tagging is needed to guarantee we can separate birds if they accidentally mix at a young age.

Weighing & cloaca scores:
Individual weighing is done on day 0, 7, 14, 21 and 28. Simultaneously, a cloaca score is given each chick (1 minute/bird/time point). This is the most practical and least stressful method as they are only handled once per time point.

Fecal collection:
During the last 4 days of the trial broilers are placed on slats to allow fecal collection. Prior to these last 4 days broilers will be housed on comfortable SoftCell bedding.

Blood sampling:
Prior to the dissection a single blood sample is taken from each broiler for metabolomics measurements. Blood samples are taken by an experienced technician.

Dissection:
Organs and digesta for the broilers will be collected for the above mentioned read-out parameters. Broilers are fasted 3 hours and then allowed to feed for 3 hours prior to euthanasia (on day 29), to ensure the presence of sufficient digesta in the different parts of the GIT (de Vries et al., 2014). Particularly for the determination of CP, amino-acids and markers in ileal digesta, large quantities of digesta are needed.

Reference
de Vries, S., Kwakkel, R.P., Pustjens, A.M., Kabel, M.A., Hendriks, W.H. and Gerrits W.J.J., 2014, Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with Cr2O3 and cobalt-ethylenediamine tetraacetic acid in broiler chickens. Poultry Science 93:2010–2017

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

We will use an in vitro digestibility and fermentation trial prior to this trial to select feed ingredients and by doing so less experimental diets are included in this research. Selection of ingredients will be based on the contrast in hind gut protein fermentation that can be created. We will determine this by measuring a simulated ileal protein digestion and the soluble fraction that is left after digestion. As it is known that in poultry fractional separation occurs and that mostly the soluble components enter the ceca (de Vries et al., 2014), which is the main site of fermentation. The availability of protein for cecal bacteria in the undigested soluble fraction is then measured using the gas production technique (Cone et al., 2005). Both measures (digestibility and fermentibility) are used as selection criteria.

Sample size determination:

There are no metabolomics data available to use for sample size determination. Pre-cecal CP digestibility is our second most important read-out parameter. We also consider growth performance important measurements.

We consider a difference in digestibility of 2.5% between treatments to be relevant. We would like to detect differences with a significance level α = 5% and a power β = 80%.
In previous trials using male broilers, a standard deviation for ileal CP digestibility of 2.53% (Qaisrani et al., 2015) and 1.78% (personal communication) was found. This standard deviation is based on including 8 male broilers per pen. Based on this we assume σ = 2.15%.

Calculation of the number of replicate pens:

$n = \{ [Z(\alpha/2) + Z\beta]^2 \} / [(\Delta/\sigma)^2]$

Following formula will be used to determine sample size:

Using prerequisites, following values can be obtained from the Standard Normal Curves Areas table (Ott and Longnecker, 2010) or calculated:
$Z_{0.05/2}=1.96$
$Z_{0.80} = 0.85$
$\Delta = 2.5$
$\sigma = 2.15$

Thus:
$n = (1.96 + 0.85)^2 / (2.5 / 2.15)^2 = 5.84$

It can be deduced from this statistical power analysis that 6 repetitions are required per treatment group.

Number of birds per pen:

The variation used for the power analysis is based on previous studies (Qaisrani et al., 2015 and recent unpublished data) in which 8 male broilers per pen were used. However, for digestibility analysis fewer broilers are required. For ileal CP and amino acid digestibility analysis we require at least 5 grams of digesta dry matter. We should be able to obtain 1.14 g dry matter from the terminal ileum of a broiler (Kluth et al., 2005). Based on this information we assume we need to pool digesta of at least 5 birds and also in a previous trial (unpublished data) pooled digesta of 5 birds was enough for CP and amino acid analysis.

However, reducing the number of birds per pen may increase the variation between the pens, and will result in a higher number of pens required to obtain a minimal power of 80%. We do not have data from which we can calculate the effect of reducing the number of birds per pen on the statistical power. We assume, however, that increasing the number of replicates will increase the power while reducing the number of birds. So, instead of using 6 replicates with 8 birds per pen (48 birds per treatment), we will use 5 birds per pen with 8 replicates (40 birds per treatment). Additionally, we expect a maximum mortality in the first week of 4%. To be able to deal with this 2 additional birds per treatment should be added. If necessary these extra birds will be reallocated to a pen within treatment if mortality occurs in the first week.
In total 6 (3 diets x 2 degrees of protein digestibility) x 8 (replicate pens) x 5 (animals per pen) + 12 extra birds (2 per treatment) = 252 birds are required.

References

Cone, J.W., Jongbloed, A.W., Van Gelder, A.H. and de Lange L., 2005, Estimation of protein fermentation in the large intestine of pigs using a gas production technique. Ani. Feed Sci. Tech. 123-124:463-472
Kluth, H., Mehlhorn, K. and Rodehutscord, M., 2005, Studies on the intestine section to be sampled in broiler studies on precaecal amino acid digestibility. Archives of Animal Nutrition, 59(4):271-279
Ott, R.L. and Longnecker, M., 2010. An Introduction to Statistical Methods and Data Analysis. Brooks/Cole Cengage Learning, ISBN-13: 978-0-495-01758-5

Qaisrani, S.N., van Krimpen, M.M., Kwakkel, R.P., Verstegen, M.W.A. and Hendriks, W.H., 2015, Diet structure, butyric acid, and fermentable carbohydrates influence growth performance, gut morphology, and cecal fermentation characteristics in broilers. Poultry Science 94:2152–2164

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Animals used will be *Gallus gallus domesticus* (domestic chickens), fast growing strain (Ross 308), males and will be obtained from a commercial hatchery. The species chosen is the target species of the study.

Gender related differences are not of interest in this study. To maintain maximum accuracy of measurements it is necessary to use birds with similar genetics and a similar gender. Since absorption of nutrients and metabolism are highly different between male and female chicks, the experiment is done in one sexe to decrease the variation in measurements. Males are preferred over females as they have a higher metabolic rate and feed intake, which should allow for more sample collection (digesta) per individual. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial hatchery.

Species	Origin	Maximum number of animals	Life stage

Gallus gallus domesticus, Ross 308 Mild. 252

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement Broilers are the species of interest. The present animal experiment cannot be replaced by an experiment using another animal species due to the specificities of the avian digestive tract. A mechanistical modelling approach is excluded due to the complexity of interactions and modulators (largely still unknown) involved in protein fermentation. To our knowledge, such a model does not exist, let alone a model which can simulate the effects of protein fermentation on gut health and performance. As a consequence, this experiment has to be conducted with chickens. Reduction There are in vitro tests that aim to predict ileal protein digestibility and can be used for studying hind gut fermentation. These can, however, not replace the animal model, since they lack the interaction with the gut wall. Especially in these experiments where the effect on (gut-) health is studied, this interaction is highly relevant. In vitro tests will be used to get an indication of the order of possible protein fermentation, as described above. These in vitro tests will be used to determine feed ingredients prior to this trial, reducing the number of experimental groups required. Furthermore, we strive to collect as many samples (organs, blood, digesta) as possible from each individual chick, instead of using different chicks for different measurements. The number of replicates is determined using a sample size determination formula to ensure a minimal number of replicates is used while maintaining statistical power. Refinement We choose measurements that can be performed after killing of the birds and we reduced the time broilers spend on slats to just the last 4 days. Killing, blood sampling and neck labeling will be performed by experienced technicians.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Birds will be group housed in floor pens with SoftCell bedding (except during the last four days when they are group housed on slats without SoftCell bedding) and have perches. As mentioned above, invasive measurements during life other than a single blood sampling have been avoided. To our knowledge there are no environmental disadvantages to this trial.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

An extensive literature review has been conducted and very little is known about the mechanisms involved in protein fermentation and how this affects the animal's health. Also to the knowledge of the authors metabolic profiles related to protein fermentation in broilers have never been recorded. Knowledge of these metabolic profiles is expected to be very important in the improvement of gut health in farm animals and hence the reduction of antibiotic use.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Housing and care is according to Annex III, except during the last four days when birds will be housed on slats, which is needed for fecal collection. The perches will remain in the pens in this period.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Minor pain could be induced by neck labeling, blood sampling and euthanasia. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Diarrhea might occur in the groups fed lower digestible proteins.

Explain why these effects may emerge.

Birds are fed lower digestible proteins as it is part of the aim of this study to investigate hind gut protein fermentation (which results from undigested proteins passing the ileum).

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Other than lower dietary digestible protein in some of the treatment groups, all diets will be formulated to meet broiler requirements, with a similar fiber level. Fresh bedding will be provided after regular litter scores. Drinking nipples (checked daily) will be kept clean to avoid water spillage.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

There is a very small chance of severe weight loss due to diarrhea. If a bird loses 10% or more of its body weight compared to the previous weighing, it is removed from the trial and killed according to a method listed in Annex IV of Directive 2010/63/EU. If a bird shows clinical signs of sickness (diarrhea, inactivity, lack of appetite), there will be discussion between researchers, animal caretakers and, if required, with a veterinarian to determine if further measures are required.

Indicate the likely incidence.

The likely incidence of this we expect to be very low (below 5%), as all diets will be formulated with sufficient nutrients for growth, although broilers fed the lower digestible proteins are expected to have a lower growth response.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort is classified as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

This is required for the collection of ileal and cecal contents and organ samples.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

19.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 5	Type of animal procedure Experiment with broilers to evaluate feed strategies to reduce protein fermentation

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The experiment described in appendix 4 should have given insight into the contribution of different protein sources and levels to hind gut protein fermentation (metabolites) and its effects on gut health and performance in broilers. The aim of this experiment is to determine if potential nutritional strategies, such as including fermentable fiber and providing a coarse diet structure, can affect protein fermentation (metabolites) and improve gut health of broilers.

Experimental diets

Broilers will be fed from hatch to 29 days of age, different experimental diets in a 2x2x2 design. Included factors are diet structure (particle size), fiber level and protein source. Fibers are known to be the preferred energy source of microbiota in the gut. In the presence of fibers, undigested proteins are not used as an energy source by the microbiota but can be used as a nitrogen source for microbial growth. Such a fiber feed strategy might reduce the production of (harmful) protein fermentation metabolites. A coarse dietary structure is expected to reduce passage rate and increase peristalsis and anti-peristalsis and therefore improve ileal protein digestibility. Further details of the diets will be determined based on the results of appendix 4 and the in vitro test (Boisson two-step, to simulate gastric and enteric digestion, followed by gas production method, to simulate hind gut fermentation). The contrast in dietary protein will be based on ileal crude protein and amino acid digestibility measures.

Read-out parameters

The following measurements will be recorded:

- Performance measurements (feed intake, water intake, weight gain)
- Untargeted and targeted metabolomics (using serum, cecal and colon digesta, we will be targeting metabolites that have been found to be associated with hind gut protein fermentation in experiment 1)
- Litter and cloaca scores
- Di-animo-pimelic acid (DAPA), indicator of microbial biomass, measured in ileal and cecal digesta
- Intestinal lesion scores
- Relative organ weights (proventiculus, gizzard, duodenum, jejenum, ileum, ceca, colon, pancreas and liver)
- Cecal digesta pH, gut leakage (using serum marker)
- Villus height and crypt depth

- Ileal and colon amino acid digestibility (marker method)
- Microbiota population in the ceca
- Passage rate

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Individual tags:

On day 0 all birds are given individual neck labels. This is necessary to allow us to link metabolic data from individual birds to their growth performance. Secondly, tagging is needed to guarantee we can separate birds if they accidentally mix at a young age.

Weighing & cloaca scores:
Individual weighing is done on day 0, 7, 14, 21 and 28. Simultaneously, a cloaca score is given to each chick (1 minute/bird/time point). This is the most practical and least stressful method as they are only handled once per time point.

Passage rate measurements:

5 broilers per pen will be orally dosed with 3 gel capsules containing an insoluble and soluble indigestible marker. At each time point (30, 90, 180, 270 and 360 min. after oral dosage), one bird per pen is euthanized, after which the contents of the different gastro-intestinal segments are collected.

Fecal collection:
During the last 4 days of the trial broilers are placed on slats to allow fecal collection. Prior to these last 4 days broilers will be housed on comfortable SoftCell bedding.

Blood sampling:
Prior to the dissection a single blood sample is taken from each broiler for metabolomics measurements. Blood samples are taken by an experienced technician.

Dissection:

Organs and digesta for the broilers will be collected for the above mentioned read-out parameters. Broilers are fasted 3 hours and then allowed to feed for 3 hours prior to euthanasia (on day 29), to ensure the presence of sufficient digesta in the different parts of the GIT (de Vries et al., 2014). Particularly for the determination of CP, amino-acids and markers in ileal digesta, large quantities of digesta are needed.

Reference: de Vries, S., Kwakkel, R.P., Pustjens, A.M., Kabel, M.A., Hendriks, W.H. and Gerrits W.J.J., 2014, Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with Cr2O3 and cobalt-ethylenediamine tetraacetic acid in broiler chickens. Poultry Science 93:2010–2017

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Number of replicate pens:

There are no metabolomics data available to use for sample size determination. Pre-cecal CP digestibility is our second most important read-out parameter. We consider a difference in digestibility of 2.5% between treatments to be relevant. We would like to detect differences with a significance level α = 5% and a power β = 80%. In previous trials a standard deviation for ileal CP digestibility in male broilers of 2.53% (Qaisrani et al., 2015) and 1.78% (personal communication) was found. Based on this we assume σ = 2.15%.

Calculation of the number of replicate pens:
n = { [Z(α/2) + Zβ]^2 } / [(Δ/σ)^2]

Following formula will be used to determine sample size:

Using prerequisites, following values can be obtained from the Standard Normal Curves Areas table (Ott and Longnecker, 2010) or calculated:
Z0.05/2=1.96
Z0.80 = 0.85
Δ = 2.5
σ = 2.15

Thus:
n = (1.96 + 0.85)^2 / (2.5 / 2.15)^2 = 5.84

It can be deduced from this statistical power analysis that 6 repetitions are required per treatment group.

Number of birds per pen:
For ileal CP and amino acid digestibility analysis we require at least 5 grams of digesta dry matter. We should be able to obtain 1.14 g dry matter form the terminal ileum of a broiler (Kluth et al., 2005). Based on this information we can assume we will need to pool digesta of 5 birds.

For the passage rate measurements we will require 5 birds per pen which will be killed at different time points (van Krimpen et al., 2011).

We require different broilers to be used for digestibility and passage rate measurements because both measurements require total collection of digesta from gastrointestinal segments.
All 10 birds per pen will be needed in one of the two dissections. Therefore additional broilers are needed to deal with unexpected mortality. We expect a maximum mortality in the first week of 4%. To be able to deal with this, 3 additional birds per treatment should be added.

In total 8 (treatments) x 6 (replicate pens) x 10 (animals per pen) + 24 (3 extra birds per treatment) = 504 birds are required.

References:

Qaisrani, S.N., van Krimpen, M.M., Kwakkel, R.P., Verstegen, M.W.A. and Hendriks, W.H., 2015, Diet structure, butyric acid, and fermentable carbohydrates influence growth performance, gut morphology, and cecal fermentation characteristics in broilers. Poultry Science 94:2152–2164

Ott, R.L. and Longnecker, M., 2010. An Introduction to Statistical Methods and Data Analysis. Brooks/Cole Cengage Learning, ISBN-13: 978-0-495-01758-5

Kluth, H., Mehlhorn, K. and Rodehutscord, M., 2005, Studies on the intestine section to be sampled in broiler studies on precaecal amino acid digestibility. Archives of Animal Nutrition, 59(4):271-279

Van Krimpen, M.M., Kwakkel, R.P., Van Der Peet-Schwering, C.M.C., Den Hartog, L.A. & Verstegen, M.W.A. (2011) Effects of dietary energy concentration, nonstarch polysaccharide concentration, and particle sizes of nonstarch polysaccharides on digesta mean retention time and gut development in laying hens. British Poultry Science 52:6.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Animals used will be *Gallus gallus domesticus* (domestic chicken), fast growing strain (Ross 308), males, ages from 0 days to slaughter age and will be obtained from a commercial hatchery. The species chosen is the target species of the study.

Gender related differences are not of interest in this study. To maintain maximum accuracy of measurements it is necessary to use birds with similar genetics and a similar gender. Since absorption of nutrients and metabolism are highly different between male and female chicks, the experiment is done in one sexe to decrease the variation in measurements. Males are preferred over females as they have a higher metabolic rate and feed intake, which should allow for more sample collection (digesta) per individual. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will be obtained from a commercial hatchery.

Species	Origin	Maximum number of animals	Life stage
Gallus gallus domesticus, Ross 308	Mild	504	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

C. Re-use

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement Broilers are the species of interest. The present animal experiment cannot be replaced by an experiment using another animal species due to the specificities of the avian digestive tract. A mechanistic modelling approach is excluded due to the complexity of interactions and modulators (largely still unknown) involved in protein fermentation. To our knowledge, such a model does not exist, let alone a model which can simulate the effects of protein fermentation on gut health and performance. As a consequence, this experiment has to be conducted with chickens. Reduction There are in vitro tests that aim to predict ileal protein digestibility and can be used for studying hind gut fermentation. These can, however, not replace the animal model, since they lack the interaction with the gut wall. Especially in these experiments where the effect on (gut-) health is studied, this interaction is highly relevant. Furthermore, we strive to collect as many samples (organs, blood, digesta) as possible from each individual chick, instead of using multiple birds for multiple measurements, reducing the number of birds required. Passage rate measurements, however, requires killing of birds a specific time points and use of all the digesta from all gastrointestinal tract segments, therefore these birds cannot be used for ileal digestibility analysis. The number of replicates is determined using a sample size determination formula to ensure a minimal number of replicates are used while maintaining statistical power. Refinement We choose measurements that can be performed after killing. Blood sampling and neck labling will be performed by experienced technicians.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Birds will be group housed throughout the trial, in floor pens with SoftCell bedding and have perches. During the last four days, when birds are housed on slats, perches will still be available. As mentioned above, invasive measurements during life, other than a single blood sampling, have been avoided. To our knowledge there are no environmental disadvantages to this trial.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

An extensive literature review has been conducted and very little is known about the mechanisms involved in protein fermentation and how this affects the animal's health. This experiment is a continuation of appendix 4 in which we aim to discover metabolic profiles related to protein fermentation. In this experiment we will examine how feeding strategies change these profiles and potentially, identify mechanisms underlying gut health. This knowledge is expected to be very important in the improvement of gut health in chickens and hence the reduction of antibiotic use.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Housing and care is according to Annex III, except during the last four days when birds will be housed on slats without bedding material, which is needed for fecal collection. The perches will remain in the pens in this period.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

Minor pain could be induced by neck labeling, blood sampling and euthanasia. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Diarrhea might occur in the groups fed lower digestible proteins, lower fiber levels and pellets with fine particles.

Explain why these effects may emerge.

Birds are fed diet contrasting in protein digestibility, fiber content and structure as it is part of the aim of this study to investigate how these contrasts in feed affect hind gut protein fermentation and gut health.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Other than these contrasts, all diets will be formulated to meet broiler requirements. Fresh bedding will be provided after regular litter scores. Drinking nipples (checked daily) will be kept clean to avoid water spillage.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

There is a very small chance of severe weight loss due to diarrhea. If a bird loses 10% or more of its body weight compared to the previous weighing, it is removed from the trial and killed according to a method listed in Annex IV of Directive 2010/63/EU. If a bird shows clinical signs of sickness (diarrhea, inactivity, lack of appetite), there will be discussion between researchers, animal caretakers and, if required, with a veterinarian to determine if further measures are required.

Indicate the likely incidence.

The likely incidence of this we expect to be very low (below 5%), as all diets will be formulated with sufficient nutrients for growth, although broilers fed the lower digestible proteins are expected to have a lower growth response.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort is classified as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.

L. Method of killing

[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

This is required for the collection of ileal and cecal contents and organ samples.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

20.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 6	Type of animal procedure Transgenerational effects of protein fermentation metabolites in poultry

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Dietary protein fed to broiler breeders can affect broiler offspring performance (Rao et al., 2009). To date, the mechanisms by which broiler offspring is affected by maternal feeding is not known. We hypothesize that metabolites produced upon protein fermentation in the ceca of broiler breeders are absorbed and transferred to the eggs, which may cause imprinting in the broiler offspring decreasing intestine function. If maternal nutrition indeed affects intestine function in the offspring, maternal feeding could be used as a nutritional strategy to improve gut health in the offspring.

General design

Thirty-two female broiler breeders will be group-housed and divided over two dietary treatments, differing in ileal protein digestibility to obtain a contrast in protein entering the ceca. Protein sources will be selected based on the outcome (i.e. ileal protein digestibility) of in vitro and in vivo broiler experiments described in appendix 4. These diets will be fed to broiler breeders for 5 weeks and, blood samples will be collected to determine (protein fermentation) metabolite profiles. In addition, broiler breeders will be weighed at the start and end of the feeding trial. Eggs will be collected from each individual broiler breeder in the last week(s) of the trial (i.e. week 4-5). Thereafter, broiler breeders are killed for sample collection to determine effects of dietary treatments on the gut.

Two eggs per broiler breeder are used to determine the metabolic profile in the eggs. Three eggs per broiler breeder are incubated, hatched and broiler offspring killed for sample collection (intestine, blood, liver).

Primary outcome parameters

Untargeted metabolomics and targeted (protein fermentation products) metabolomics will be performed in blood samples of broiler breeders, in the eggs (yolk and/or albumen) and in samples from the broiler offspring to determine changes in metabolites. Intestinal tissue samples of broiler breeders are used for morphology to determine the effects of the dietary treatment on the gut.

Samples obtained from broiler offspring at dissection (i.e. blood, organs, intestinal tissue) will be analysed on metabolite profiles and functional read-outs (i.e. morphology of intestinal samples, DNA methylation and histone modifications in intestine or liver samples). Metabolomics profiles of broiler breeders and broiler offspring are subsequently correlated with these gut parameters to establish potential pathways that are differentially activated/silenced by certain metabolites affecting gut health.

Reference

Rao, K., J. Xie, X. Yang, L. Chen, R. Grossmann, and R. Zhao. 2009. Maternal low-protein diet programmes offspring growth in association with

alterations in yolk leptin deposition and gene expression in yolk-sac membrane, hypothalamus and muscle of developing Langshan chicken embryos. Br. J. Nutr. 102:848-857.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Broiler breeders will be fed diets differing in ileal protein digestibility to determine transgenerational effects of protein fermentation metabolites.

Broiler breeders

Individual identification

Breeders will be marked with spray (not red) to enable individual identification of breeders within a pen.

Body weight

Broiler breeders will be weighed at the start and end of the feeding trial to determine potential differences in body weight between treatments. Individual birds will be placed in a crate and weighed on a weighing scale. Feed intake (per pen) will be recorded as well.

Blood sampling

At the start and end of the feeding trial, blood samples will be obtained from each broiler breeder to determine shifts in metabolite profiles due to the dietary treatments. Birds will be fixated by the animal caretaker and a blood sample will be taken from the wing vein by an experienced technician.

Artificial insemination

In order to obtain fertilized eggs, broiler breeders are inseminated. Semen will be obtained from multiple roosters from a commercial breeder company. The female broiler breeders will be inseminated twice a week in the first week and once a week in the weeks thereafter, with pooled semen.

Eggs

Eggs are collected in the last experimental week(s). During collection of eggs, breeders are observed continuously during that morning/day, in order to know which egg belongs to which breeder. This is required to link the egg metabolite profile to the breeder metabolite profile and still have group-housing. If there is uncertainty regarding which broiler breeder laid the egg, broiler breeders within the pen will be palpated to check the presence/absence of an egg.

Egg samples (yolk and/or albumen) are used for metabolomics analyses to determine the transfer of (protein fermentation) metabolites from broiler breeders to the eggs. The yolk sac metabolome will be analysed to understand the changes in available nutrients for the developing embryo.

Broiler offspring
Settable eggs will be incubated and hatched. A maximum of 96 day-old broiler chicks will be killed for sample collection (intestine, blood, liver). This approach allows us to evaluate the effects of protein fermentation on gut morphology and metabolite profile of broiler breeders and, subsequently, evaluate if these broiler breeder metabolite profiles are correlated with the egg metabolite profiles, and with the broiler offspring metabolite profiles and functional read-outs of gut health.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Protein sources are selected based on the outcome of the in vitro tests and in vivo broiler experiment described in appendix 4, in order to obtain a contrast in ileal protein digestion between dietary treatments.
We chose artificial insemination instead of natural fertilization, because variation between broiler breeders in different pens might increase with natural fertilization due to differences in vitality between males. Furthermore, minimal required numbers of broiler breeders for primary outcome parameters are estimated based on previous experiments and power analysis (see next paragraph).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Ross 308 broiler breeder females will be obtained from a commercial breeding company. Ross 308 is the most commonly used breed in North-Western Europe. Broiler breeders will be obtained between 30-40 weeks of age, in order to perform the experiment in peak production.

The primary outcome parameters for this study are the metabolite profiles in the serum samples and eggs of broiler breeder females. To the best of our knowledge, metabolite profiles have not been determined in serum or eggs from broiler breeders fed different diets to induce protein fermentation. In the study from Pieper et al. (2012), metabolite profiles were determined in colon digesta and urine in weaned piglets fed diets differing in fermentable protein and total dietary fiber level (2x2 design). Using 8 piglets per treatment, they were able to find metabolite clusters associated with the diet and identify several metabolites that differed between treatments.
A power analysis we also conducted (using the glmpower procedure of SAS 9.3), using blood uric acid levels as influenced by dietary crude protein level reported by Corzo et al. (2005). Plasma uric acid levels averaged 6.04 and 3.82 mg/dL for broilers fed high (212 g/kg) or low (173 g/kg) crude protein level, respectively, with an average SD of 1.32. Using these values and setting the power at ≥ 0.8, this requires a total of 14 animals.
We expect larger variation in metabolite profiles, because we also apply untargeted metabolomics (instead of targeted as applied in the study of Pieper et al., 2012) and we also use eggs. We, therefore, estimate to require 16 broiler breeders per dietary treatment.
Two eggs are collected from each broiler breeder to determine metabolite profiles in the eggs. Additionally, we require two eggs for incubation and hatching to determine the effects on gut parameters in the broiler offspring. To account for unsettable eggs (~9%; van Emous et al., 2013) and a

hatchability of settable eggs of 84% (van Emous et al., 2015), we collect/incubate 3 eggs per broiler breeder. These eggs are collected in one week, in order to minimize variation due to different storage times before incubation.
In total this experiment will include 32 broiler breeders and at max 96 broilers.

References

Corzo, A., C. A. Fritts, M. T. Kidd, and B. J. Kerr. 2005. Response of broiler chicks to essential and non-essential amino acid supplementation of low crude protein diets. Anim. Feed. Sci. Technol. 118:319-327.
Pieper, R., K. Neumann, S. Kröger, J. F. Richter, J. Wang, L. Martin, J. Bindelle, J. K. Htoo, W. Vahjen, A. G. van Kessel, and J. Zentek. 2012. Influence of fermentable carbohydrates or protein on large intestinal and urinary metabolomic profiles in piglets. J. Anim. Sci. 90:34-36.
van Emous, R. A., R. P. Kwakkel, M. M. van Krimpen, and W. H. Hendriks. 2013. Effects of growth patterns and dietary crude protein levels during rearing on body composition and performance in broiler breeder females during the rearing and laying period. Poultry Sci. 92:2091-2100.
van Emous, R. A., R. P. Kwakkel, M. M. van Krimpen, H. van den Brand, and W. H. Hendriks. 2015. Effects of growth patterns and dietary protein levels during rearing of broiler breeders on fertility, hatchability, embryonic mortality, and offspring performance. Poultry Sci. 94:681-691.

Species	Origin	Maximum number of animals	Life stage
Ross 308 broiler breeder females	Mild	32	
Ross 308 broiler offspring	Mild	96	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: the broiler breeders are the target animal in this experiment. There are no appropriate models available to study the (protein fermentation) metabolites produced by broiler breeders, the transfer of these metabolites to the egg and the effects on the gut of the broiler offspring. Reduction: artificial insemination is used instead of natural fertilization in order to minimize variation due to differences in vitality between males. In addition, required numbers of animals are estimated based on literature and power analysis. Refinement: broiler breeders will be checked twice daily by qualified personnel on general health and behavior. In case of disease, this will be discussed between the researcher, animal caretaker, veterinarian and/or animal welfare officer to determine appropriate actions. Broiler breeders will be group-housed and will be provided with sufficient feeding- and drinking places, perches and bedding material. In addition, laying nests will be provided to the broiler breeders. Skilled personnel will handle the animals in order to minimize stress during procedures such as weighing and blood sampling. In addition, the light in the stable will be dimmed prior to catching the birds.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Birds are group-housed, provided with sufficient bedding material and will have perches. Birds will be checked twice daily on general health and behavior. Climate conditions will be checked twice daily as well and immediately adjusted if required. Dimming the light before handling the birds (for weighing, blood sampling) allows the birds to become calm. Fresh bedding material will be provided regularly, to minimize the risk of wet litter and ammonia emission.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature survey has been conducted using google scholar and key words including broiler, broiler breeder, dietary protein, protein fermentation, transgenerational and metabolic programming. Especially in rodent models, there is substantial evidence linking maternal nutrition to offspring health and disease. The mechanisms underlying such transgenerational effects remain largely unknown. In poultry, very little data are available on this topic. To the best of our knowledge, (serum) metabolome profiles in broiler breeders in relation to dietary protein and transfer of (protein fermentation) metabolites to eggs have not been determined before.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

H. Pain and pain relief

Minor pain can be experienced during blood sampling and killing. The potential pain described is minor and short-term, and, therefore, no pain relieving methods are applied.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

There is a very small chance that broiler breeders might develop diarrhea due to the dietary treatments.

Explain why these effects may emerge.

A diet inducing protein fermentation is part of the experimental design. This diet is required to study (transgenerational) effects of protein fermentation metabolites.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Fresh bedding material will be provided regularly, to minimize the risk of wet litter (due to diarrhea) and subsequent effects on legs and skin of the broiler breeders.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Body weight loss due to diarrhea is possible in broiler breeders fed a protein source of low digestibility. If broiler breeders show clinical signs of diarrhea/sickness, birds will be weighed and birds are removed from the trial in case of 10% body weight loss (compared to the start weight). Injuries such as a broken wing or leg can occur similarly as in practice. If these injuries occur, these will be noticed during daily inspections. Birds are removed from the trial and killed according to a method listed in Annex IV of Directive 2010/63/EU.

Indicate the likely incidence.

The likely incidence is estimated at 1%.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort in this experiment is estimated to be mild. This is based on weighing (mild), blood sampling (mild) and artificial insemination (mild) of broiler breeders and killing of broiler breeders and offspring.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Broiler breeders and broiler offspring are killed to collect samples (i.e. intestinal tissues).

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

21.

Appendix
Description animal procedures

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- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 7	Type of animal procedure Uremic toxin production during the in vitro fermentation of proteins in ileal digesta of pigs

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

During fermentation of proteins, organic acids are produced. Some of these organic acids are known as uremic toxins (i.e. p-cresol sulfate and indoxyl sulfate) in human renal transplant patients. These uremic toxins are also produced during protein fermentation in the hindgut of piglets and might be detrimental for post-absorptive health, thereby reducing overall piglet health and performance. The first step is to determine which uremic toxins can be produced during fermentation of different proteins. This experiment is, therefore, aimed at establishing predictive relations between the (amino acid) composition of ileal undigested proteins and the formation of uremic toxins (i.e. a group of protein fermentation metabolites) during their subsequent fermentation in vitro.
Eight pigs will be fitted with a cannula in the distal ileum. After a recovery and adaptation period, pigs will be subjected to eight experimental diets in 8 periods in a latin-square design. These diets will differ in dietary protein source and/or structure (i.e. amino acid composition). These proteins will be selected based on data from literature and from the results of the in vitro study described in appendix 4. During each period, ileal effluents will be collected and pooled by diet for further in vitro analysis. As a secondary objective fecal samples and clean urine samples will be analyzed for uremic toxins, the end-products of protein fermentation.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

After arrival, pigs will be allowed to acclimatize to the new environment for a minimum of 8 days. During the acclimatization periods, pigs will be socialized according to a fixed protocol.
Eight growing barrows, ± 25 kg BW will be fitted with a cannula in the distal ileum under inhalation anesthesia. Pigs will be fasted prior to the surgery and will be treated with adequate painkillers and antibiotics during recovery. Pigs will be allowed to recover from the surgery and to adapt to housing conditions for a minimum of 7 days. Immediately after surgery, pigs are kept on a soft pad under a heating lamp until pigs can regulate their body temperature again. Rectal temperature is regularly monitored, for at least 3 days after surgery. After recovery from surgery, ileal effluents will be collected from each pig during 8 subsequent measurement periods. Each period will consist of a 5 day adaptation period, followed by collection of ileal digesta during 12 hours on days 5 and 7. Ileal digesta will be pooled per pig over the experimental period and diet will be stored at -20°C pending analysis. This approach is selected to ensure the ileal digesta for further in vitro studies is originating from the same pigs. The latin-square approach minimizes the number of pigs needed for this study, and has also been proposed by the expert working group of the FAO (2014). Feces will be sampled during day 3, 4 and 6 and urine samples will be collected from funnels mounted underneath the metabolism cage. To collect feces quantitatively, plastic bags will be attached to the rear end of the pigs as described by Van Kleef et al. (1994).

References:
- Research approaches and methods for evaluating the protein quality of human foods; Report of a FAO Expert Working Group, ISBN 978-92-5-108695-7, FAO 2014
- van Kleef, Deuring, and van Leeuwen. A new method of faeces collection in the pig. Lab Anim (1994) 28: 78-79

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

This experiment is not designed to detect statistically significant differences in the composition of ileal digesta among protein sources. It is designed to provide representative samples of ileal digesta of the selected protein sources, with the objective to further study the fermentation of the undigested proteins in these samples. Hence, ileal digesta harvested from the pigs will be pooled by dietary treatment for the analysis of amino acid composition and will be the basis for a series of in vitro studies into the production of uremic toxins during fermentation (not described in this application). In addition, the effects of the protein sources on fecal characteristics and urinary excretion of uremic toxins will be analysed following a normal latin-square design.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Eight growing barrows (± 25 kg BW) will be purchased from a commercial pig farm.
Only pigs that are healthy without hernias (testicular or umbilical), damaged ears or unsound feet are selected, because these can complicate surgery. Male animals will be used to ease collection of feces without contamination with urine. Barrows will be used as entire males will be difficult to handle at the end of the trials. The choice for one sexe will not lead to a surplus in breeding stock of experimental animals, because animals will

be obtained from a commercial farm. No spare pigs will be used, but in case no samples can be collected from two pigs for at least two periods, an extra collection period will be added. Pigs will be about 30 kg BW at the onset of the trial, and 60-70 kg BW at the end of the trial. This is a comparable BW range as used previously at our facilities, and well within the range of BW, maintaining healthy pigs and functional cannulas used in published literature. The use of growing pigs for this research is recommended by the FAO (2013). Body weight will be determined upon arrival of the pigs, and after surgery weekly until the end of the last experimental period.

Reference:
Dietary protein quality evaluation in human nutrition; Report of an FAO Expert Consultation, ISBN 978-92-5-107417-6, FAO 2013

Species	Origin	Maximum number of animals	Life stage
Pigs	Moderate	8	

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: In this experiment, the connection with protein fermentation in the renal transplant cohort of the [redacted] is important. Obtaining ileal effluents from humans is complicated, and the pig is recommended by the FAO committee as a model for evaluating Digestible Indispensable Amino Acid Scores (DIAAS) of human foods. This choice is well documented in the FAO report (2013). Reduction: The protein sources used in this experiment will be selected based on the in vitro (small intestinal) digestion study described in appendix 4, to ensure a contrast in amino acid composition of ileal digesta large enough for this study. Using a latin-square design, within pig variability can be separated from the variation between protein sources, hence minimizing the number of pigs to be used for the study of uremic toxin excretion, and that the pooled ileal digesta to be used for subsequent in vitro fermentation studies originate from the same pigs. Refinement: after careful consideration, the length of the adaptation period, depending on the number of days the animal needs for adapting to new diets between experimental periods, was reduced from 12 to 5 days. In this way, every period within each trial lasts 7 days instead of 14 days, reducing the total duration of each trial to 8 weeks, which is often used for evaluating effects of fibrous diets. This decision fits within the procedures proposed by the FAO (2014), and is based on the notion that adaptation of small intestinal passage rates and digestive secretions to different protein sources is much quicker than the adaptation of the colon microbiota to changes in fiber sources. Although a straw bedding is not possible because it influences the measurements, cage enrichment will be varied weekly. Various, non-destructible toys will be made available to the pigs, in a weekly alternating schedule, following a protocol developed at Wageningen University. Audio-visual contact between pigs is maintained. Before surgery pigs will be socialized for a minimum of 5 days (according to a socialization protocol), so that they are well adapted to handling procedures after surgery and during sampling. References: - Research approaches and methods for evaluating the protein quality of human foods; Report of a FAO Expert Working Group, ISBN 978-92-5- 108695-7, FAO 2014 - Dietary protein quality evaluation in human nutrition; Report of an FAO Expert Consultation, ISBN 978-92-5-107417-6, FAO 2013

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Surgical procedures will be conducted under complete anesthesia, and adequate painkillers are used during recovery.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature search has been performed in Scopus (13-4-2017), using the search terms “uremic toxin”, “protein fermentation”. Although the production of uremic toxin following protein fermentation is well documented (particularly in relation to kidney patients), no efforts have been published predicting the composition of uremic toxins from variation in the amino acid composition of proteins flowing into the colon. No literature sources were found with similar objectives as described in this appendix.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Pigs will be housed individually in metabolism pens during the recovery phase and during the experimental periods in temperature controlled rooms. The floor area of the pens will be min. 2 m2, allowing the pigs to move around freely. Walls will be smooth (covered by plexiglass) to prevent damage to cannulas. Animals will be housed on a plastic coated floor. Individual housing is needed to prevent animals from damaging cannulas of pen mates, but audio-visual contact will be possible. The use of bedding material is avoided as this will prevent the collection of clean urine samples from funnels mounted underneath the cage.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[X] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Surgical procedure will be performed under anesthesia. After surgery, animals will be treated with painkillers (at least 3 days) and with antibiotics.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Individual housing without bedding material, fasting prior to surgery and recovery from surgical procedures may have adverse effects on animal welfare. Infection and inflammation due to the presence of the cannula and/or catheter may occur. Digestive problems (i.e.diarrhea) with changes in the diet are not expected based on previous experience and because there is an adapatation period to each diet.

Explain why these effects may emerge.

These adverse effects may emerge because they are part of the experimental procedures.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Infection and inflammation will be prevented or minimised by providing antibiotics after the surgical procedures and whenever required based on clinical signs of infection. Adverse effects on animal welfare due to individual housing are minimised by providing toys as environmental enrichment and pigs will be able to see and hear each other.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Pigs will be euthanized should one of the following conditions apply: • during the recovery from surgery, a pig does not start eating within 2 days, and subsequently produce feces, indicating blockage of the intestines or inflammation of the peritoneum. • a cannula is lost and it cannot be placed back into the distal ileum immediately. • a pig has a fever during 5 successive days, not responding to medical treatments proposed by a veterinarian, and signs of infection and inflammation. • a pig has feed refusals exceeding 20% of the amount of feed offered for a period exceeding 7 days. • in the expert judgement of the veterinarian, future observations on a pig will not provide reliable results. • a pig suffers from body weight loss (>10%) during a 14 day period

Indicate the likely incidence.

The likely incidence of pigs to be removed from the experiment is estimated at 25% during the 8 week duration of the trial (20% due to technical problems with the cannula). The major portion of this 25% is expected to occur during the first two days following surgery. In addition, technical failure of cannulas will lead to removal of the pig from the experiment. If this leads to discomfort of the pig, it will be for a very short period of time.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The level of discomfort is expected to be as listed below: • Surgical procedure (including fasting): moderate • Individual housing in absence of bedding material, in a large metabolism pen (10 weeks): moderate • The sampling procedures of ileal digesta (2 days during each of 8 subsequent weeks): mild • Attaching plastic bag for collecting feces (when ileal digesta are not collected): mild. Hence the cumulative discomfort in this trial is estimated at moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

Keeping pigs with a cannula in the distal ileum after the experiment is finished is undesirable from an animal welfare point of view.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

22.

[redacted]

Van: DEC WUR <dec@wur.nl>
Verzonden: donderdag 7 september 2017 8:37
Aan: 'info@zbo-ccd.nl'
Onderwerp: RE: Verzoek aanvullende informatie projectvergunningsaanvraag AVD1040020171667

Categorieën: Dossier: [redacted]

Beste [redacted]

Excuses, die zin is er per abuis in blijven staan. Voor het opstellen van een advies maak ik gebruik van een standaard document en B4 wordt daar uit gehaald indien niet van toepassing. Dat had in dit geval ook moeten gebeuren. Er waren geen DEC-leden betrokken bij het project; alle aanwezige DEC-leden waren betrokken bij de behandeling van de aanvraag en het opstellen van het advies.
Zal ik een aangepast advies opsturen of is deze mail voldoende?

Met vriendelijke groeten,

[redacted]

[redacted]

From: info@zbo-ccd.nl [mailto:info@zbo-ccd.nl]
Sent: woensdag 6 september 2017 14:14
To: DEC WUR
Subject: Verzoek aanvullende informatie projectvergunningsaanvraag AVD1040020171667

Geachte DEC Wageningen UR,

Op 10-05-2017 hebben wij een aanvraag voor een projectvergunning dierproeven ontvangen waarover uw DEC advies heeft uitgebracht. Het gaat om het project 'To determine the telation between protein

fermentation and gut health in pigs and poultry' met aanvraagnummer AVD1040020171667.

In uw advies geeft u onder B4 aan: Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de behandeling van de aanvraag en het opstellen van het advies. Licht toe waarom. Kunt u aangeven of er sprake is geweest van uitsluiting van leden, en indien dit het geval is hoeveel leden er waren uitgesloten en waarom?

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,

[redacted]
Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl

.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl

Centrale Commissie Dierproeven

23.

> Retouradres Postbus 20401 2500 EK Den Haag

Wageningen University & Research
[redacted]
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6700 AW WAGENINGEN

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD1040020171667

Datum 8 september 2017
Betreft aanvraag projectvergunning Dierproeven

Geachte [redacted],

Op 10 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "To determine the telation between protein fermentation and gut health in pigs and poultry" met aanvraagnummer AVD1040020171667. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

Welke informatie nog nodig
Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen:

Onduidelijkheden
In Bijlage Dierproeven 3.4.4.2 geeft u onder H aan dat de dieren pijn kunnen ondervinden bij het doden en niet verdoofd worden. Kunt u toelichten welke dodingsmethode u toepast, of het doden volgens praktijkomstandigheden gebeurd en of de methode benoemd staat in bijlage IV van richtlijn 2010/63/EU?

Leges
De leges die u verschuldigd bent zijn nog niet door ons ontvangen of de betaling is nog niet verwerkt. Uw aanvraag is niet compleet als de leges niet zijn ontvangen.
Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen omdat de gegevens onvolledig of onduidelijk zijn.

Datum:
8 september 2017
Aanvraagnummer:
AVD1040020171667

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Stuurt u het per post op, gebruik dan het formulier dat u bij deze brief krijgt.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Centrale Commissie Dierproeven

Melding bijlagen

U wilt één of meerdere bijlagen naar ons versturen? Voeg altijd deze Melding Bijlagen toe.
Wij weten dan welke documenten van u zijn en hoeveel documenten u opstuurt.
Meer informatie vindt u op www.centralecommissiedierproeven.nl
Of bel met ons: 0900 28 000 28 (10 ct/min).

1 Uw Gegevens

Naam instelling: Wageningen University & Research

Adres: .....

Postcode en plaats: .....

Aanvraagnummer: AVD1040020171667

2 Bijlagen
Welke bijlagen stuurt u mee?
Vink de bijlagen aan of vul de naam of omschrijving in.

O Projectvoorstel

O Beschrijving Dierproeven

O Niet-technische samenvatting

O Melding Machtiging

O Aanvraagformulier

O .....

O .....

O .....

Datum:
8 september 2017
Aanvraagnummer:
AVD1040020171667

3 Ondertekening

Naam: .....

Datum: ..... - ..... - .....

Handtekening: .....

Onderteken het formulier en stuur het met alle bijlagen op naar:
Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Gevraagde informatie

In de brief van 8 september 2017 heeft de CCD om aanvullende informatie gevraagd in aanvraagnummer AVD1040020171667. Hieronder vindt u de gevraagde informatie.

In Bijlage Dierproeven 3.4.4.2 zal een overdosis anesthesie met voorafgaande sedatie gebruikt worden als dodingsmethode (zoals genoemd in bijlage IV van richtlijn 2010/63/EU). Aangezien ook bij verdoving (via injectie) kortdurende pijn ervaren zou kunnen worden door het dier, hebben we bij H aangegeven dat pijn kan voorkomen tijdens het doden.
De complete procedure voor dissectie voor het verzamelen van ileale digesta is als volgt:
1. Twee dagen voor dissectie worden varkens individueel gehuisvest.
2. Vanaf 24 tot 6 uur voor dissectie worden de varkens iedere 6 uur gevoerd.
3. Vanaf 6 uur voor dissectie worden de varkens ieder uur gevoerd.
4. Tien minuten voor dissectie worden de varkens verdoofd middels intraveneuze injectie. Hierna zijn de varkens ontspannen, stil en met de ogen bijna helemaal dicht.
5. De varkens worden overgebracht naar een andere ruimte, waar de buikholte wordt geopend en het maagdarmpakket wordt verwijderd onder anesthesie.
6. Direct na het verwijderen van het maagdarmpakket worden de varkens gedood met een overdosis anesthesie.

Myrthe Gilbert, PhD
[redacted]

WAGENINGEN
UNIVERSITY & RESEARCH

From: Info-zbo [mailto:info@zbo-ccd.nl]
Sent: vrijdag 8 september 2017 11:56
To: Vergunningenloket <vergunningen@wur.nl>
Cc: [redacted]
Subject: Aanhouden AVD1040020171667

Geachte meneer, mevrouw,
Op 10 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "To determine the telation between protein fermentation and gut health in pigs and poultry" met aanvraagnummer AVD1040020171667. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In bijgaande brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op.
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen.

Als er nog vragen zijn, dan hoor ik dat graag.

Met vriendelijke groeten,
Namens de Centrale Commissie Dierproeven

[redacted]

www.centralecommissiedierproeven.nl
Postbus 20401 | 2500 EK | Den Haag
T: 0900 – 28 000 28 (10 ct/min)
E: info@zbo-ccd.nl

25.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Wageningen University & Research
[redacted]
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6700 AW WAGENINGEN

Centrale Commissie Dierproeven
Postbus 20401
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centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD1040020171667
Bijlagen
1

Datum 18 september 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 10 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "To determine the telation between protein fermentation and gut health in pigs and poultry" met aanvraagnummer AVD1040020171667. Wij hebben uw aanvraag beoordeeld.

Op 12 september 2017 heeft u uw aanvraag aangevuld. Dit betrof de dodingsmethode van Bijlage Dierproeven 3.4.4.2.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
De algemene voorwaarde(n) zijn opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.

U kunt met uw project "To determine the telation between protein fermentation and gut health in pigs and poultry" starten. De vergunning wordt afgegeven van 18 september 2017 tot en met 1 mei 2021.

Overige wettelijke bepalingen blijven van kracht.

Hierbij wijzen wij u erop dat de locatie waar u de dierproeven wilt uitvoeren, VOORAF aangemeld moet zijn bij NVWA. Dit kan door een e-mail te sturen aan CHD@nvwa.nl met de gegevens van de vergunninghouder en gegevens van de nieuwe locatie.

Uit de memorie van toelichting bij de dierproevenregeling:
2.1.1 Direct melden van wijzigingen met betrekking tot de instellingsvergunning
Na verlening van een instellingsvergunning zal de vergunninghouder wijzigingen die betrekking hebben op de instellingsvergunning onverwijld door moet geven aan de Minister. Het gaat daarbij om wijzigingen in één van de personen die op grond van artikel 6, derde lid, van de wet in de instellingsvergunning worden vermeld, en elke significante wijziging van de structuur of de werking van een inrichting die het dierenwelzijn negatief kan beïnvloeden. Hierbij moet worden gedacht aan gevallen waarbij op basis van de gewijzigde situatie mogelijk anders op een aanvraag om een instellingsvergunning zou zijn beslist, dan wel wijzigingen die bekend moeten zijn bij de toezichthouder met het oog op inspecties. Hieronder valt in ieder geval elke nieuwe locatie waar proefdieren worden gehuisvest of dierproeven worden verricht, grootschalige verbouwingen op deze locaties en reorganisaties. De vergunninghouder geeft deze wijzigingen door aan de Minister. De wijzigingen moeten worden doorgegeven zodra de vergunninghouder hier redelijkerwijs van op de hoogte kan zijn.

Procedure
Wij hebben advies gevraagd bij de Dierexperimentencommissie DEC Wageningen UR. Dit advies is opgesteld op 25 juli 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Wij hebben de DEC om aanvullende informatie gevraagd. Op 7 september 2017 heeft de DEC gereageerd op onze vragen. Dit betrof de eventuele betrokkenheid van DEC-leden bij de beoordeling van de aanvraag. Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie nemen wij over, inclusief de daaraan ten grondslag liggende motivering. Er worden aanvullende algemene voorwaarde(n) gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt

tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Centrale Commissie Dierproeven
namens deze:

[redacted]

Algemeen Secretaris

Bijlagen:
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Wageningen University & Research
Adres:	Postbus 59
Postcode en plaats:	6700 AW WAGENINGEN
Deelnemersnummer:	10400

deze projectvergunning voor het tijdvak 18 september 2017 tot en met 1 mei 2021, voor het project "To determine the telation between protein fermentation and gut health in pigs and poultry" met aanvraagnummer AVD1040020171667, volgens advies van Dierexperimentencommissie DEC Wageningen UR. Er worden aanvullende algemene voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is Onderzoeker.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 10 mei 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 25 juli 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 25 juli 2017;
c Advies van dierexperimentencommissie d.d. 25 juli 2017, ontvangen op 25 juli 2017.
d De aanvullingen op uw aanvraag, ontvangen op 12 september 2017

Aanvraagnummer:
AVD1040020171667

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 On-farm piglet experiment				
	Varkens (Sus scrofa domesticus) /	200	Licht	
3.4.4.2 Digestibility experiment with piglets				
	Varkens (Sus scrofa domesticus) /	216	Licht	
3.4.4.3 Digestibility experiment with cannulated pigs				
	Varkens (Sus scrofa domesticus) /	8	Matig	
3.4.4.4 Digestibility experiment with broilers				
	Kippen /	252	Licht	
3.4.4.5 Experiment with broilers to evaluate feed strategies to reduce protein fermentation				
	Kippen /	504	Licht	
3.4.4.6 Transgenerational effects of protein fermentation metabolites in poultry				32 broiler breeder females 96 broiler offspring
	Kippen /	128	Licht	
3.4.4.7 Uremic toxin production during the in vitro fermentation of proteins in ileal digesta of pigs				
	Varkens (Sus scrofa domesticus) /	8	Matig	

Aanvraagnummer:
AVD1040020171667

Voorwaarden
Op grond van artikel 10a1 lid 2 van de Wet op de dierproeven zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat go/no go momenten worden afgestemd met de IvD.

In artikel 10, lid 1 sub a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in afstemming met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarde wijzigen of intrekken.

Aanvraagnummer:
AVD1040020171667

Weergave wet- en regelgeving

Dit project en wijzigingen
Volgens artikel 10c van de Wet op de Dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn

kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13d volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13c is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.
De Minister heeft vrijstelling ontheffing verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.

Locatie
De vergunning wordt verleend voor een project waarbij dierproeven geheel of gedeeltelijk worden verricht buiten een inrichting van een gebruiker (artikel 10g van de wet).