	Inventaris Wob-verzoek W16-09S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2015326								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel oud				x			x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1 oud				x			x	
5	Bijlage beschrijving dierproeven 2 oud				x			x	
6	Bijlage beschrijving dierproeven 3 oud				x			x	
7	Bijlage beschrijving dierproeven 4 oud				x			x	
8	DEC-advies				x		x	x	
9	Verzoek aanvulling				x		x	x	
10	Meldingsformulier aanvulling				x		x	x	
11	Projectvoorstel herzien				x			x	
12	Bijlage beschrijving dierproeven 1 herzien				x			x	
13	Bijlage beschrijving dierproeven 2 herzien				x			x	
14	Bijlage beschrijving dierproeven 3 herzien				x			x	
15	Bijlage beschrijving dierproeven 4 herzien				x			x	
16	Mail onderzoeker 3-2-2016				x		x	x	
17	Advies CCD		x						x
18	Beschikking en vergunning				x		x	x	

Centrale Commissie Dierproeven

15 DEC 2015

ADV104002015326

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.

☒ 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	Wageningen University	
Naam van de portefeuillehouder of diens gemachtigde	[redacted]	
KvK-nummer	9215846	
Straat en huisnummer	Akkermaalsbos	12
Postbus	59	
Postcode en plaats	6700 AB	Wageningen
IBAN	NL10 RABO 0397066465	
Tenaamstelling van het rekeningnummer	Wageningen UR	

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.

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☒ Dhr. ☐ Mw.
Functie	[redacted]	
Afdeling	[redacted]	
Telefoonnummer	[redacted]	
E-mailadres	[redacted]	

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters		☐ Dhr. ☐ Mw.
Functie		
Afdeling		
Telefoonnummer		
E-mailadres		

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	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☒ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ 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
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	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 - 3 - 2016
		Einddatum	1 - 3 - 2020
3.2	Wat is de titel van het project?	Assessing dietary protein quality for humans using the pig as a model	
3.3	Wat is de titel van de niet-technische samenvatting?	Bepalen van de kwaliteit van eiwitbronnen voor humane voeding	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC-WUR
		Postadres	Postbus 9101, 6700 HB Wageningen
		E-mailadres	dec@wur.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 741 Lege

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD 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.

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

5 Checklist bijlagen — via NetFTP verzonden

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel + 4 bijlagen

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☒ DEC-advies

X bestelorder WUR914590

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:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). 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.
- 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	14 - 12 - 2015
Handtekening	[redacted]

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for 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.centralecommissiedierproeven.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.	Assessing dietary protein quality for humans using the pig as a model

2 Categories

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

- ☒ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ 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.

Not only protein quantity, but also protein quality is important in maintaining essential body functions. Therefore, assessing protein quality has been identified as a critical question by international authorities (FAO, 2013). Protein nutritional quality is related to the capacity of the different food sources of protein to achieve the different functions associated to the supply of nitrogen and amino acids in the body. The

nutritional efficiency of a protein can be determined from the extent to which dietary protein nitrogen is absorbed and retained by the organism and is able to balance daily nitrogen losses. The capacity to provide an adequate profile of bioavailable indispensable amino acid is considered as a limiting factor for protein quality.
For this purpose, an amino acid scoring approach was designed, considering the capacity of a protein source ingested at the level of the mean protein requirement derived from nitrogen balance (0.66 g/kg/d for human adults) to meet indispensable amino acid needs. A refinement of this approach, the Protein Digestibility Corrected Amino Acid Score (PD-CAAS), is currently widely adopted, and corrects the content of each indispensable amino acid of the protein by the faecal digestibility of the protein in order to evaluate the bioavailable part of these amino acids in comparison to a reference amino acid profile.
The use of a single value for crude protein digestibility to correct the dietary amount of each individual amino acid for its digestibility is considered a short-coming, when there are practically important quantitative differences in digestibility between crude protein and individual dispensable and indispensable amino acids. A further inherent shortcoming of the PD-CAAS approach is that correction of digestibility is based on an estimate of crude protein digestibility determined over the total digestive tract (i.e. faecal digestibility). Thus, microbial metabolism and interconversions of nitrogenous compounds disturb the estimation of bioavailable amino acids. For protein as a whole, absorption of nitrogen over the whole digestive tract is an appropriate measure, as nitrogen absorbed in forms other than amino acids can contribute to the nitrogen economy. For the assessment of bioavailable amino acids, however, the FAO (2013), based on critical reviews of the literature, has recommended that protein quality assessment should be based on true ileal digestibility values of individual amino acids, rather than the overall (faecal) digestibility of protein. This method is referred to as the Digestible Indispensable Amino Acid Score (DIAAS). At the present time, there is a limited quantity of data on the ileal amino acid digestibility of foods as determined in humans. Where human data are lacking, the FAO (2013) recommends that true ileal amino acid digestibility values from the growing pig be used, being preferred over those obtained from the growing laboratory rat.

While accepting the need for DIAAS estimates of human foods using the ileal cannulated pig as a model, in the same report, the FAO (2013) identifies an urgent need to develop techniques that can be adopted to assess protein quality (amino acid availability) in humans directly, using minimally invasive techniques. In this way, future estimates for true ileal digestibility values would not require ileal cannulated pigs for the standard evaluation of protein quality, but could also be used in different human populations.

Reference:
Dietary protein quality evaluation in human nutrition. Report of an FAO Expert Consultation, FAO Food and Nutrition Paper 92, ISBN 978-92-5-107417-6, FAO 2013

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?

- To provide reliable DIAAS for 28 protein sources for human nutrition using the ileal cannulated pig (as indicated by the FAO, 2013 and detailed by the FAO expert working group, 2014).
- To develop a meal/plasma signature dual stable isotope-based approach using intrinsic labelled proteins for the non-invasive evaluation of protein digestibility, amino acid bioavailability and protein nutritional quality for humans.

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

Feasibility of the project:
The first objective is feasible. Although the use of ileal cannulated pigs as a model for evaluating protein quality in humans is new, it is commonly applied for evaluation of protein quality in pig nutrition. It has been applied previously at [redacted] This project is part of a larger, international research effort in which a larger number of protein sources for humans are evaluated using

the pig model. Alignment of procedures is ensured, and inter laboratory comparison is foreseen. For a limited number of protein sources (10), comparison with human ileal amino acid digestibility values is planned, following the recommendations of the FAO (2013), using two methods. The first method uses humans with a normal gastrointestinal tract and uses naso-ileal intubation to sample digesta from the terminal ileum (Deglaire et al., 2009). This method will be [redacted] at [redacted]. [redacted] This method will be [redacted] at [redacted]

The second objective concerns the development of a methodology which is minimally invasive and can be applied in healthy humans. This method, proposed by [redacted] has not been performed before. The method will be developed by [redacted] The novelty of the method is not in the techniques applied, but in the combination of different isotope labels, simultaneously administered to subjects. Validation of the isotope method against the well-established DIAAS method is required before implementation in humans, and is part of this application. Testing the isotope method in humans is planned in 2016, but is outside the scope of this application.

3.3 Relevance

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

Both protein quantity and protein quality are of importance for maintaining body functions like, the immune system, muscle mass and growth. As protein resources for human consumption worldwide are limiting, protein quality is of increasing importance. As acknowledged by the FAO (2013), there is an immediate need to replace the old standards for assessing protein quality (PD-CAAS) by methods based on ileal disappearance of amino acids, using the pig as a model. In addition, for the somewhat longer term, there is a need to develop non-invasive and easy to implement approaches and methods for the measurement of protein and amino acid digestibility and bioavailability for different protein sources in different populations.

3.4 Research strategy

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

Within the present project, we plan to develop a novel, stable isotope based technique to measure protein quality in humans, at the same time building a database on DIAAS values for 28 protein sources for humans, using a well-established methodology based on pigs. The isotope method is also developed in pigs, and it is planned to test this later in humans (outside the scope of this application). A cross-validation between methods is included in the project.

Building the database on DIAAS values for 28 human food sources will be conducted in 6 subsequent experiments. Within each experiment, 5 food sources will be tested, and a measurement of net endogenous protein losses at the terminal ileum will be included. To this end, each experiment will be conducted as a 6x6 latin square design with 6 treatments tested in all of 6 pigs in 6 subsequent periods. The 6 treatments include 5 different human protein sources and a protein free treatment for the measurement of endogenous ileal protein losses. In total, this will provide data on 30 protein sources (6 x 5). True digestibility values will be calculated by difference, and provide results that can be compared with the isotope methodology. One of the protein sources will be included in three experiments, providing the possibility to test repeatability. In addition, as this is part of a larger, international program, inter-laboratory comparison of 10 protein sources is planned. The selection oof the 28 protein sources will be based on their quantitative importance for protein supply in humans worldwide and on their contrast in estimated protein digestibility.

For the development of the isotope method (principle is described in the appendix), milk proteins will be labelled with ^{15}N and ^{2}H by intraruminal infusion of ^{15}N labelled ammoniumsulfate and ^{2}H labelled water.

Subsequently, milk will be collected and milk proteins isolated and dried. In addition to these intrinsically labelled protein sources, a universally labelled ^{13}C labelled amino acid source will be purchased (^{13}C spirulina hydrolysate, 99% enriched).

Subsequently, these intrinsically labelled protein sources will be used for the development of the isotope method. The principle of the method is based on the different dilution of labels and is further explained in the experimental protocols. Briefly, all three labels are orally provided with a normal meal to pigs in a frequently fed manner to simulate steady state conditions. Each pig will be provided with two subsequent meals. [redacted] It is hypothesized that this increase in the isotopic ratio quantitatively reflects the increase in amino acids absorbed, and can therefore be also applied to measure differences in DIAAS scores, resulting from differences in protein digestibility.
In order to develop this methodology, three assumptions need to be verified prior to an independent validation against the DIAAS method:

1) Verify that the [redacted] ratio of amino acids in the blood is proportionally related to the quantity of ^{2}H-labelled amino acids which are absorbed.
2) Verify that protein bound amino acids ([redacted]) lead to a similar response under steady state conditions (i.e. in frequently fed pigs) than a mixture of amino acids in free form (^{13}C labelled, high enrichment, low dose).
3) Verify that amino acid metabolism in the intestinal wall or liver does not change the ratio of [redacted] in amino acids.

These assumptions will be tested in pigs that are equipped with catheters in the portal and jugular veins. Assumption 3 can be tested by comparison of the ratios of [redacted] in amino acids between the diet, portal and jugular blood plasma.

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 project comprises two parts: a) building a database of DIAAS scores of 28 protein sources for humans, using the ileal cannulated pig as a model; this will be performed in 6 subsequent animal experiments. b) development of an isotope methodology to assess protein quality. This will require 3 subsequent animal experiments: 1) the production of intrinsically labelled [redacted] milk protein from cow's milk; 2) evaluation of the main assumptions listed above in an experiment with pigs equipped with catheters in portal and jugular veins; 3) comparison of the developed isotope methodology to determine the isotopic signature of a protein, assessment of protein digestibility with [redacted] tracer method in plasma compared with true ileal digestibility. This will be performed using ileal cannulated pigs (for measurement of DIAAS scores) that are equipped with a catheter in the jugular vein (isotope methodology).

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.

For a description see 3.4.2. Milestones include the database of DIAAS scores of 28 human protein sources developed in part a; and a developed isotope method (part b) that can be applied for assessing protein quality in humans by the simple measurement of [redacted] ratios in amino acids in plasma sampled from the peripheral circulation. This method will be tested in humans following procedures similar to the experiment in pigs (peripheral blood only). Although this experiment is outside the scope of this application, it will be conducted in close collaboration with the research in pigs, described in this application.
Building the database (part a) and the development of the isotope method (part b) can be conducted in parallel. Within part b, three phases can be distinguished, which follow the three experiments described in 3.4.2.
Should the results of phase 2 indicate that the assumptions are not valid, phase 3 will not be conducted.

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	6 subsequent pig trials, conducted in a 6x6 latin square (6 ileal cannulated pigs, 6

	periods) each measuring DIAAS scores of 5 human protein sources, and a protein-free treatment to measure net ileal endogenous losses.
2	Production of [redacted] milk protein by directly infusing [redacted] into the rumen of a cow and production of ^{2}H-intrinsically-labelled milk protein by directly infusing [redacted] into the rumen of a cow; subsequently collecting the milk and isolating the milk proteins.
3	To develop the isotope methodology in pigs, testing the main assumptions in pigs equipped with catheters in the portal and jugular veins.
4	Validation of the isotope method against the DIAAS score method using pigs equipped with ileal cannulas and a catheter in the jugular vein; testing four ^{2}H labelled protein sources and a protein-free treatment in a 5x5 latin square design.
5	
6	
7	
8	
9	
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.centralecommissiedierproeven.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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		1	6 subsequent pig trials, conducted in a 6x6 latin square (6 ileal cannulated pigs, 6 periods) each measuring DIAAS scores of 5 human protein sources, and a protein-free treatment to measure net ileal endogenous losses. In the description below, 1 trial is explained

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.

Six pigs (and two spare pigs) will be fitted with a T cannula in the distal ileum. After a recovery and adaptation period, each pig will be subjected to all of six experimental diets in 6 periods in a 6x6 latin square design. During each period, ileal effluents will be collected. DIAAS scores will be calculated from amino acid analysis in the diet and in ileal digesta, using TiO2 as the indigestible marker. Treatments will include five protein sources and a protein-free diet. Protein losses at the terminal ileum when feeding the protein-free diet are assumed to represent the net endogenous secretions.

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

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 between protein sources. It is designed to provide reliable estimates of DIAAS scores of the selected protein sources, building up a database. The number of observations planned is following the FAO guidelines (2014). Nonetheless, data will be analysed using a generalized linear mixed model. A normal distributed error and identity link will

be assumed for DIAAS scores, but these assumptions will be verified. Pigs within period will be considered the experimental unit. Protein source and period will be included as fixed effects, and pig as random effect to account for repeated observations within pigs. Although this approach is new for the estimation of true digestible amino acids protein sources for humans, it is commonly applied for evaluation of protein sources for pig nutrition, obtaining 5 or 6 observations for each protein source to be tested, and demonstrated to allow detection of significant difference of DIAAS scores of 2-3% between protein sources. One of the protein sources will be included in three trials, allowing estimation of repeatability. In addition, for 10 protein sources that are also tested in two other institutes, inter-laboratory comparisons will be performed.

B. The animals

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

In each of the 6 subsequent pig trials, 8 growing barrows (± 25 kg BW) will be purchased. Male animals will be used to ease collection of faeces without contamination with urine. Barrows will be used as entire males will be difficult to handle at the end of the trials. Surgery will be performed on all pigs, following procedures as included in [redacted] . Slight modifications with regard to the type of cannula and location will be made to ensure alignment of procedures with other institutes within the international consortium. Two pigs will be considered as spare animals, and will be used in the case of problems with digesta collections in the other animals. The number of spare pigs is based on previous experience, needed to ensure a complete set of 6 observations for each protein source. 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. To this end, spare pigs will be treated as their peers, and randomly assigned to a dietary treatment during each period. In the event that observations from another pig cannot be used, the samples obtained from the spare pigs will be analyzed, provided that they are obtained at the desired experimental treatment. 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 Wageningen University and Research institute, 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).

C. Re-use

Will the animals be re-used?

☒ 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 recommended by the FAO committee as a model for evaluating DIAAS scores of human foods. This choice is well documented in the FAO report (2013).
Reduction: in 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.
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 from 12 to 6 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 fibre 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 anaesthesia, 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.

The FAO recommendation for using the ileal cannulated pig as a model to estimate true digestibility of protein sources for humans is recent (2013, with a report on research methods released in 2014). The research proposed is part of a larger international effort ([redacted]) using the ileal cannulated pig as a model.

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

☒ 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 dimensions of the pens are 1.3 x 1.3m, 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 be consumed by the pigs and will thus interfere with the digestibility measurements.

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.

☐ 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.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

☒ 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 inhalation anaesthesia. 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?

- Feeding a protein-free diet for a period of 7 days may increase breakdown of body proteins and concomitant feelings of discomfort.
- Prolonged individual housing.
- Fasting prior to the surgical procedure

Explain why these effects may emerge.

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

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.

☒ 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 faeces, 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.

Indicate the likely incidence.

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 (8 weeks): moderate
- Protein-free diet during 7 days: mild
- The sampling procedures (2 days during each of 6 subsequent weeks): 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

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

Keeping pigs with a T-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 > Describe the method of killing that will be used and provide justifications for this choice.
☒ Yes

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.centralecommissiedierproeven.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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: Production of ^{15}N-intrinsically-labelled milk protein by directly infusing $(^{15}NH_4)_2SO_4$ into the rumen of a cow and production of ^{2}H-intrinsically-labelled milk protein by directly infusing $^{2}H_2O$ into the rumen of a cow; subsequently collecting the milk and isolating the milk proteins

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.

This experiment will be executed to produce ^{15}N-intrinsically-labelled milk protein and ^{2}H-intrinsically-labelled milk protein. Two lactating cows will receive intraruminal infusions of $(^{15}NH_4)_2SO_4$ and another two will receive intraruminal infusion of $^{2}H_2O$. The milk will be collected and processed voor consumption in the pig experiment (serial experiment 3).

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

Stable isotopes solutions will be infused into the rumen of lactating cows fitted with a rumen cannula for 7 days. Milk will be collected and processed. Cows have been previously equipped with a rumen cannula. It is estimated that for serial experiment 3, 175 L of both ^{15}N and ^{2}H enriched milk is needed.

Two cows will receive a continuous intraruminal infusion of 100 g of [redacted], to reach an isotopic enrichment of the protein of about [redacted]. After 3 days of infusion, ^{15}N enriched milk will be collected for 4 consecutive days, and will be sent off for separation of the milk proteins.

Two cows will receive a continuous intraruminal infusion of [redacted] to reach an isotopic enrichment of the protein of about [redacted] After 3 days of infusion, ^{2}H enriched

milk will be collected for 4 consecutive days, and will be sent off for separation of milk protein, lactose and fat.

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

In line with the objective of this experiment, the number of cows required depends only on the amount of milk needed for conducting the experiment number 3, as further explained below. No statistical evaluation of the data is required.

B. The animals

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

4 Holstein Frisian dairy cows, previously fitted with a rumen cannula will be used. These fistulated cows are part of the dairy herd of [redacted]", and will be selected for this experiment, based on the desired stage of lactation.
The number of cows needed is estimated from the amount of intrinsically labelled milk proteins needed for conducting experiment number 3. It is assumed the cows are in mid lactation, thus producing about 25 L/d, assuming an efficiency of conversion of [redacted]

C. Re-use

Will the animals be re-used?

☐ 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: Alternatives to produce the desired quantity of intrinsically labelled protein sources are not available.
Reduction: the amount of milk needed is calculated carefully to be enough, but not exceeding the amount of labelled milk required for the study.
Refinement: Cows are well adapted to handling procedures. Infusion procedures will be optimized allowing the cows to stand up or lie down without noticing the equipment.

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

The cows will be carefully transferred to the tie-stall, and infusion procedures will be conducted by experienced staff. Suffering is already minimal.

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.

This experiment is solely used to produce intrinsically labelled milk for other experiments.

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

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

During the period of infusion, cows will be housed in a tie-stall barn to ensure they cannot move away from the infusion equipment. They can stand up and lie down in a normal way.

Animals will receive a grass-maize silage based roughage diet which meet their requirements. The amount of feed provided to individual animals will be adjusted according to the nutritional guidelines set by CVB standards and will be in line with the standard practice. Roughage will be available ad libitum and concentrates will be provided manually in three equal portions, given at 0600, 1400 and 2200h.

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.

☐ 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.

☐ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

☐ 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?

Apart from housing the cows in a tie-stall barn, no adverse effects on animal welfare are expected

Explain why these effects may emerge.

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

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.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

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').

Housing in the tie-stall during the infusion procedure: mild.

Cumulative level of discomfort is therefore estimated as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☒ No

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

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.

☐ Yes

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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: To develop the isotope methodology in pigs, testing the main assumptions in pigs equipped with catheters in the portal and jugular veins.

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.

Measurement principle

The measurement is based on the principle that orally provided, labelled amino acids as well as intrinsically labelled proteins, when fed under steady state conditions, will appear in the systemic circulation and that its dilution is proportional to the quantity ingested. It then follows that, when ingesting a constant quantity of a ^{13}C labelled amino acid source in time, and a variable quantity of a [redacted] of that particular amino acid ingested, hence independent of the dilution of each label by amino acid catabolism and influx into the circulation of amino acids from breakdown of body proteins. In this way, the ratio of labelled amino acids from intrinsically labelled protein sources and the ^{13}C reference amino acid will reflect the true digestibility of the amino acids in the labelled protein source, provided that equal amounts of this protein source are ingested. When all assumptions underlying this methodology are verified, the application of this technique is simple and can be applied in humans using minimally invasive techniques, i.e. analysing the isotope ratio of amino acids in systemic blood following ingestion under steady state conditions.

Three assumptions need to be verified prior to the application of this method. Firstly, the assumption (assumption 1) that [redacted] ratio of amino acids in the blood is proportionally related to the quantity of [redacted] which are absorbed; Secondly, the assumption (assumption 2) that using protein bound amino acids ([redacted]) with a true digestibility near 100% (milk proteins) lead to a similar response under steady state conditions as a mixture of amino acids in free form [redacted] Thirdly, the assumption (assumption 3) that amino acid metabolism (in intestinal wall or liver) does not change the ratio of [redacted] in amino acids.

Experimental design

The assumptions listed above will be tested in an experiment with pigs, equipped with catheters in the portal and jugular veins. The pigs will receive two discrete meals, divided in [redacted] Pigs will be allowed to adapt to the size of the meal for 7 days. The increase in the intake of labelled protein will lead to an increase in the ratio of labelled [redacted] labelled amino acids in the systemic circulation. It is hypothesized that this increase in the isotopic ratio quantitatively reflects the increase in amino acids absorbed. Assumption 1 will be tested by comparing the ratio of [redacted] in amino acids between the two meals; Assumption 2 will be tested by comparing the ratio of [redacted] in amino acids in portal blood. Assumption 3 will be tested by comparing the ratio of [redacted] in amino acids between diet, portal blood and jugular blood.

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

Four barrows of about 25 kg of BW + three spare pigs will be purchased. Six barrows will be surgically fitted with catheters in the portal and jugular veins. Based on previous experience, it is estimated that two spare pigs are needed to ensure catheter patency of at least 4 animals throughout the 16d experimental period. Should problems arise during or immediately after surgery, the third spare pig will be used the next day. This is needed as occasionally vein architecture does not allow placement of a catheter, and problems with catheter patency may occur immediately during recovery. Pigs will be fasted prior to the surgical procedure, which will be conducted under inhalation anaesthesia. During the day of surgery, the day preceding and three days following, antibiotics will be used to minimize infection risks. Adequate painkillers will be used during recovery. During 7 days post-surgery, pigs will be allowed to recover, and feed allowance will be gradually increased to a feeding level of 2.5 times their estimated maintenance energy requirements.
Pigs will receive each of both meals in a change-over design, with the sequence balanced across pigs. Isotope meals will be administered on days 9 and 16 post surgery. During the 7 days preceding the isotope meals, pigs will be adapted to the size of the meals, fed twice daily.

Throughout the 16 d period, pigs will be housed individually, to prevent them from disturbing catheters of other pigs, in metabolic cages on a plastic coated floor. To collect faeces 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. Portal and peripheral blood will be collected during the test days via catheters.

Test meals

A test meal will contain [redacted]

Measurements

During the experiment, the following measurements will be conducted:

- Portal and peripheral blood samples are collected on the days of test meal provision (d9 and 16), 30 minutes before feeding the first portion and at 30 minute intervals during 4.5 hours (11 samples in total per catheter, 5 mL/timepoint). Isotopic enrichment of amino acids will be analysed [redacted]) in samples collected from the portal and peripheral circulation. When steady state conditions are verified, the ratio [redacted] ratios in the amino acids within the meal, in the portal and peripheral blood sample are calculated to meet the objectives described above.

Faeces and urine are quantitatively collected during a 4 day period preceding the day of test meal provision. Faecal and urinary excretion of total N will be determined and samples will be stored for possible analysis of [redacted] in particular fractions.

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 observations required for meeting the objectives of this trial was calculated using a power analysis in SAS, for two treatments in a change-over design, one sided testing, and assuming between animal variation as described by Ten Have et al (2011), detailed below.

B. The animals

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

Seven barrows of about 25 kg BW will be purchased. Male animals will be used to ease collection of faeces without contamination with urine. The type of pig matches the pigs used for the determination of DIAAS scores in experiment 1 and 4 of this application, and is chosen in line with FAO guidelines. The number of pigs required is minimized by using a change-over design, using the pigs as their own controls. The number of observations needed was calculated using a power analysis:

The number of observations required to determine the difference between meals was determined using proc power in SAS. Estimates of variance were obtained from Ten Have et al (2011) who measured the response in portal and arterial blood following two protein meals. Data from tryptophan were selected as this is an important amino acid. The difference in arterial tryptophan concentration was selected as a representative response parameter, as this will represent the dilution in enrichment in the proposed experiment. Ten Have et al measured a difference in arterial concentrations of about 20 µmol/L following a contrast in tryptophan intake comparable to the anticipated difference between meals. The obtained standard deviation was 5, derived from a graph in this manuscript. The power analysis was conducted for detecting a difference of 20 µmol/L, one sided and at an alpha of 0.05 and a power of 0.8. The resulting number of observations required for each treatment is 4.
It is expected that applying n=4 in a change-over design (4 observations; 2 periods) is sufficient to detect a statistically significant difference; especially as the standard deviation of the ratio between isotopes within an amino acid is likely smaller than that of an arterial concentration of an amino acid. Unfortunately, estimates of variation in these parameters are not available.
Reference: Ten Have, GAM, Engelen, MPKJ, Soeters, PB, Deutz, NEP. 2012. Absence of post-prandial gut anabolism after intake of a low quality protein meal Clinical Nutrition 31 (2012) 273-282

C. Re-use

Will the animals be re-used?

☒ 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: As described in the FAO guidelines (2013), current in vitro methods are not adequate to estimate protein quality of human protein sources. The proposed method, validated against the proposed standard (DIAAS scores in pigs, see serial nr 4), is now first developed in pigs, but can be applied later using minimally invasive procedures, directly in humans, thus replacing the ileal cannulated pig as a model.
Reduction: A change over design is applied to minimize the number of pigs needed.
Refinement: The use of isotope ratio’s within each amino acid makes this procedure independent of isotope dilution originating from amino acids from protein breakdown. Such measurements would involve the measurement of a number of extra parameters, for example and notably the portal blood flow, which is more variable and would require a higher number of animals for achieving reliable estimates of amino acid fluxes. 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 anaesthesia, 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.

Assessing protein quality has been identified as a critical question by international authorities (FAO, 2014). While accepting the need for DIAAS estimates of human foods using the pig as a model, the FAO (2014) identified an urgent need to develop techniques that can be adopted to assess protein quality (amino acid availability) in humans directly, using minimally invasive techniques. This method, proposed by [redacted]

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

☒ 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 dimensions of the pens are 1.3 x 1.3m, allowing the pigs to move around freely. Walls will be smooth (covered by plexiglass) to prevent damage to catheters. Animals will be housed on a plastic coated floor. Individual housing is needed to prevent animals from damaging catheters of pen mates. Plastic bags will be attached to the rear end of the pigs to allow quantitative collection of faeces and the collection of clean urine (funnels underneath the cage). The use of bedding material is avoided as this will prevent the collection of clean urine samples. Pens will be enriched with non-destructable toys in a weekly alternating scheme as playing material, according to procedures developed at Wageningen University.

Milk proteins and isotopically labelled sources will be provided on top of a normal, commercial feed, that will be supplied slightly restricted to ensure complete consumption. Feed will be provided at 2.5 times the energy requirements for maintenance, being close to 80% of the ad libitum intake. Water is supplied in a ratio of minimum 2.5:1 relative to the feed. In addition, water is available ad libitum through drinking nipples. The protein sources will be provided on top of the feed supply.

Prior to surgery, feed allowance will be reduced and feed will be withheld (fasting) to assure and empty digestive tract. Thereafter, feed allowance will be gradually increased

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.

☐ 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.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

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

The placement of portal and jugular catheters is conducted under inhalation anaesthesia. Animals will be treated with painkillers during recovery (at least 3 days) and with antibiotics. Animals are cared for according to a standard protocol and by skilled staff.

I. Other aspects compromising the welfare of the animals

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

Apart from individual housing and the surgical procedures (fasting prior to and recovery after), no adverse effects on welfare is expected.

Explain why these effects may emerge.

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

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.

☒ 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 faeces, indicating blockage of the intestines or inflammation of the peritoneum.
- Upon losing catheter patency.
- 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 4 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% during the 16 day 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 catheters will lead to removal of the pig from the experiment, but is not likely to lead to discomfort for the pig.

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
- Attaching faecal bags (16 days): mild
- Sampling procedures (16 days): mild
- Individual housing in absence of bedding material, in a large metabolism pen (16 days): moderate

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

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

Yes, after the procedures. Prolonged housing of pigs with a portal catheter is undesirable from an animal welfare point of view. Moreover, the location of the tip of this catheter needs to be verified during autopsy. When it becomes apparent that catheter placement is impossible, the pig will be killed during anaesthesia.

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.

☒ Yes

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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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		4	Validation of the isotope method against the DIAAS score method using pigs equipped with ileal cannulas and a catheter in the jugular vein; testing four ^{2}H labelled protein sources and a protein-free treatment in a 5x5 latin square design.

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.

Five pigs (and three spare pigs) will be fitted with a T cannula in the distal ileum and with a catheter in the jugular vein. After a recovery and adaptation period, each pig will be subjected to all of five experimental diets in 5 periods in a 5x5 latin square design. During each period, ileal effluents will be collected and blood samples collected. DIAAS scores will be calculated from amino acid analysis in the diet and in ileal digesta, using TiO_2 as the indigestible marker. One of the treatments will be the feeding of a protein-free diet. Protein losses at the terminal ileum are assumed to represent the net endogenous secretions. True amino acid digestibility will be measured using the isotope methodology (see seral nr 3). Protein sources will be selected based on a difference in the estimated ileal protein digestibility, and labelled with ^{2}H.

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

Five growing barrows (+ three spare pigs), ± 25 kg BW will be fitted with a T cannula in the distal ileum, and with a catheter in the jugular vein under inhalation anaesthesia. Pigs will be fasted prior to the surgery and will be treated with adequate painkillers and antibiotics during recovery. Pigs will be allowed 14 days to recover from the surgery and to adapt to housing conditions. After recovery from surgery, ileal effluents will be collected from each pig during 5 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 will be stored at -20°C pending

analysis. This approach is selected to allow within pig comparisons of all protein sources (latin-square design), minimizing the total duration of the trial, thereby the risk of cannula complications. This procedure has been proposed by the expert working group of the FAO (2014). On day 3, the measurement, the protein sources will be replaced by sources of identical botanical origin, but intrinsically labelled with ^{2}H. Two meals, [redacted]

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.

Consistent with the objectives of this experiment, statistical methods will focus on predicting DIAAS scores by true amino acid digestibility calculated using the isotope method. In this analysis the potential of the true digestibility estimated by the isotope method will be tested as predictor of the DIAAS score. The mean square prediction error will be calculated, and decomposed in 3 components: i) overall bias, ii) deviation of the slope from unity, iii) random error. Variation between and within protein sources will be separated.

B. The animals

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

For this trial, 8 growing barrows (± 25 kg BW) will be purchased. Male animals will be used to ease collection of faeces without contamination with urine. Barrows will be used as entire males will be difficult to handle at the end of the trials. Surgery will be performed on all pigs, applying procedures described above. Three pigs will be considered as spare animals, and will be used in the case of problems with digesta collections or catheter patency in the other animals. 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 for the spare pig will be extended by one period. Pigs will be about 30 kg BW at the onset of the trial, and 50-60 kg BW at the end of the trial.

For determining reliable estimates for DIAAS scores, 6 observations per protein source are required (see serial nr 1). For the objective of this experiment, however, the minimum recommended number of observations for establishing reliable DIAAS scores will be used (i.e. 5, see FAO, 2014). The total number of observations available for the comparison of DIAAS scores and isotope based estimates is 25 (5 periods x 5 pigs=25 for each amino acid), as both variation between and within protein sources can be used. In addition, protein sources will be selected based on a large difference in expected DIAAS scores.

C. Re-use

Will the animals be re-used?

☒ 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 recommended by the FAO committee as a model for evaluating DIAAS scores of human foods. This choice is well documented in the FAO report (2013).
Reduction: in 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. The objective of this comparison is to establish a method that can be directly applied in humans, thus replacing the cannulated pig model.
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 from 12 to 6 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 fibre 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 anaesthesia, 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.

Assessing protein quality has been identified as a critical question by international authorities (FAO, 2013). While accepting the need for DIAAS estimates of human foods using the pig as a model, the FAO (2013) identified an urgent need to develop techniques that can be adopted to assess protein quality (amino acid availability) in humans directly, using minimally invasive techniques. This method, proposed by [redacted]

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

☒ 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 dimensions of the pens are 1.3 x 1.3m, 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. Audio-visual between pigs contact is maintained. The use of bedding material is avoided as this will be consumed by the pigs and will thus interfere with the digestibility measurements, but cage enrichment will be available (see above)

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.

☐ 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.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

☒ 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 inhalation anaesthesia. After surgery, animals will be treated with pain killers (at least 3 days) and antibiotics.

I. Other aspects compromising the welfare of the animals

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

- Feeding a protein-free diet for a period of 7 days may increase breakdown of body proteins and concomitant feelings of discomfort.
- Prolonged individual housing.
- Fasting prior to the surgical procedure

Explain why these effects may emerge.

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

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.

☒ 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 faeces, indicating blockage of the intestines.
- upon losing catheter patency.
- 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.

Indicate the likely incidence.

The likely incidence of pigs to be removed from the experiment is estimated at 25% during the 7 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 or catheters 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
- Sampling procedures (6 weeks): mild
- Feeding of a protein-free diet (7 days): mild
- Individual housing in absence of bedding material, in a large metabolism pen (8 weeks): moderate

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

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

Keeping pigs with a T-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 > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

11 december 2015

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD104002015326
2. Titel van het project: Assessing dietary protein quality for humans using the pig as a model
3. Titel van de NTS: Bepalen van de kwaliteit van eiwitbronnen voor humane voeding
4. Type aanvraag: nieuwe aanvraag projectvergunning
5. Contactgegevens DEC:
   DEC Wageningen-UR
   [redacted]
   Secretaris: dec@wur.nl
6. Adviestraject
   Ontvangen door DEC: 05-11-2015
   Aanvraag compleet: ja
   In vergadering besproken: 16-11-2015
   Anderszins behandeld:
   Termijnonderbreking(en) van 23-11-2015 tot 3-12-2015 en van 2-11-2015 tot 6-11-2015
   Aanpassing aanvraag: 3-12-2015
   Advies aan CCD: 11-12-2015
7. Eventueel horen van aanvrager: n.v.t.
8. Correspondentie met de aanvrager
   Datum vragen: 16-11-2015
   Strekking van de vragen:
   M.b.t. het projectvoorstel:
   - Toelichting op de wijze van selecteren van de 28 eiwitten; voedergrondstoffen/planten?).

   M.b.t. de appendices (voor zover van toepassing)
   - Argumentatie voor het gebruik van 1 sexe (mannelijke dieren);
   - Argumentatie voor het gebruik van gecastreerde dieren;
   - Consistentie in gebruik van pijnstillers en antibiotica toedient bij vergelijkbare experimenten;
   - Toelichting op het aantal te opereren dieren en argumentatie m.b.t. het aantal geopereerde reservedieren; suggestie om reservedieren meteen mee te laten lopen in de proef);
   - Toelichting op de noodzaak van nog 1 extra dier (ongeopereerd) in appendix 3 en op de behandelingen die dat dier ondergaat;
   - Tekstuele opmerking m.b.t. hergebruik in appendix 2;
   - Toevoegen van het vasten bij H. en K. en uitgebreidere beschrijving van de mogelijke maatregelen ter beperking van het ongerief;
   - Enkele redactionele opmerkingen

   M.b.t. de niet-technische samenvatting:
   - Toevoegen vaneen indicatie over de manier waarop het geplande experiment met mensen is gepland en de dierexperimenten van dit project op elkaar worden afgestemd (ook in het projectvoorstel)

   De antwoorden hebben geleid tot een adequate aanpassing van de aanvraag.
9. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

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 DEC-lid, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering.

C. Beoordeling (inhoud)

1. De DEC heeft vastgesteld dat het project uit wetenschappelijk oogpunt verantwoord is.
2. De DEC heeft vastgesteld dat de in de aanvraag aangekruiste doelcategorie in overeenstemming is met de hoofddoelstelling.
3. Het substantiële belang van het project wordt door de DEC onderschreven. De (toenemende) schaarste aan eiwitbronnen vergroot de noodzaak om een goed onderbouwde schatting te maken van de kwaliteit van eiwitten. Daarnaast is er behoefte aan de ontwikkeling van niet-invasieve en gemakkelijk uitvoerbare methoden voor het meten van eiwit- en aminozuurverteerbaarheid en de biobeschikbaarheid van verschillende eiwitbronnen.
4. De DEC stelt vast dat de expertise van de onderzoekers, de voorzieningen waar de experimenten uitgevoerd worden en de onderzoeksstrategie kunnen leiden tot het behalen van de doelstelling van het project. Het gebruik van een ileale canule bij varkens wordt algemeen toegepast voor de evaluatie van de eiwitkwaliteit in varkensvoer en is door de onderzoeksgroep vaker toegepast. Ook is er ruime ervaring met het werken met (isotoop)gemerkte aminozuren in varkensstudies. Door samenwerking met andere instituten wordt ontbrekende expertise aangevuld.
5. Er is sprake van de volgende bijzonderheid op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren: varkens worden tijdelijk individueel gehuisvest. Er wordt dan ook geen beddingmateriaal gebruikt. Dit wordt voldoende beargumenteerd en is noodzakelijk vanuit de doelstelling van de proef.
6. De DEC stelt vast dat een cumulatieve inschatting van ongerief realistisch is ingeschat en geclassificeerd: voor de varkens ‘matig’, alle drie de experimenten met varkens bevatten korte perioden met matig ongerief (operatie en herstel), gevolgd door een wat langere periode met matig ongerief als gevolg van individuele huisvesting; voor de koeien bestaat het uit het vaststaan tijdens de infusieperiode.
7. De DEC heeft vastgesteld dat er geen alternatieven zijn om de doelstelling van het project te realiseren. De beschikbare proefdiervrije technieken zijn niet geschikt om de kwaliteit van eiwitbronnen op een goede manier vast te stellen. Voordat de (isotopen)techniek kan worden toegepast bij mensen, moet zij worden ontwikkeld m.b.v. een diermodel. Op termijn maakt dit het gebruik van proefdieren overbodig.
8. De DEC heeft vastgesteld dat er optimaal tegemoet gekomen wordt aan de vereiste van vermindering van dierproeven. Voor het bepalen van de eiwitkwaliteit van de eiwitbronnen worden de richtlijnen van de FAO gebruikt voor het vaststellen van het aantal benodigde varkens. Het aantal koeien dat nodig is, is berekend aan de hand van de hoeveelheid melk die nodig is voor de proeven met varkens waarin de isotopenmethode wordt ontwikkeld. Voor het ontwikkelen van de isotopenmethode is aan de hand van beschikbare informatie uit literatuur geschat hoeveel varkens nodig zijn.
9. De DEC heeft vastgesteld dat het project in overeenstemming is met de vereiste van verfijning van dierproeven. Operaties zullen worden uitgevoerd onder algehele narcose. Tijdens de herstelperiode zal adequate pijnbestrijding worden toegepast. Tijdens het uitvoeren van de metingen zullen de dieren dagelijks een aantal keren gecontroleerd worden door gecertificeerde dierverzorgers. Indien wordt opgemerkt dat een dier lijdt of er sprake is van ongerief en dit niet is voorzien als onderdeel van het proefplan dan zal een dierenarts worden geraadpleegd. In overleg zal dan worden bepaald of het dier moet worden behandeld en/of uit de proef moet worden verwijderd. Dieren zullen worden voorzien van afleidingsmateriaal in de hokken en dit materiaal zal regelmatig worden vervangen om verveling tegen te gaan.
10. De Instantie voor Dierenwelzijn heeft een positief oordeel over de kwaliteit van de aanvraag uitgebracht en de DEC heeft dit in haar overweging betrokken.
11. 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

De DEC is unaniem van mening dat het doel en de haalbaarheid van het project het gebruik van proefdieren en het ongerief dat de dieren wordt aangedaan rechtvaardigt. In dit project wordt de eiwitkwaliteit van 28 eiwitbronnen voor humane consumptie bepaald in experimenten met varkens. Daarnaast wordt een nieuwe methode ontwikkeld die gebruik maakt van stabiele isotopen. Deze methode wordt ontwikkeld en getest bij varkens maar is straks eenvoudig toe te passen in mensen. Als deze methode werkt, zijn op termijn experimenten met varkens voor dit doel niet meer nodig.

E. Advies

1. Advies aan de CCD:De DEC adviseert unaniem de vergunning te verlenen.
2. Het uitgebrachte advies is gebaseerd op consensus.

9

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Wageningen University
[redacted]
Akkermaalsbos 12
Postbus 59
6700 AB Wageningen

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD104002015326

Uw referentie

Bijlagen

Datum 21 januari 2016
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 14 december 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project Assessing dietary protein quality for humans using the pig as a model met aanvraagnummer AVD104002015326. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben :

Welke informatie nog nodig
Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen;

In de bijlagen 3.4.4.1 en 3.4.4.3 en 3.4.4.4 wordt het bepalen van het lichaamsgewicht van de dieren niet beschreven. In bijlage 3.4.4.1 beschrijft u het belang van het inzetten van groeiende varkens volgens advies van de FAO, omdat groei ook een indicatie is voor eiwit kwaliteit. Na chirurgie lijkt ontwikkeling van het lichaamsgewicht een belangrijke welzijnsparameter, of gewichtsverlies een criterium voor toepassen van Humane eindpunten. Kunt u aangeven of u de dieren weegt gedurende het experiment en wanneer dit het geval is kunt u dan deze parameters opnemen in de bijlagen dierproeven?
Het is zeker de bedoeling om de varkens te wegen (wekelijks), ook om de voergift te kunnen bepalen. Was ik vergeten te vermelden – Sorry! Ik heb dit nu opgenomen in de bijlagen. Evt gewichtsverlies kan ook een criterium zijn voor het toepassen van een Humaan eindpunt, al verwacht ik dat voerweigeringen hier geschikter voor zijn (want treden op voor het ontstaan van gewichtsverlies). Omdat we de varkens wekelijks wegen kan gewichtsverlies over een 7 daagse period het gevolg zijn van het tijdstip van wegen tov bv urineren. Dit kan leiden tot een verkeerde inschatting van gewichtsverlies als welzijnsparameter. Ik heb daarom gewichtsverlies over een 14 daagse periode in bijlagen 3.4.4.1, en 3.4.4.4 opgenomen als humaan eindpunt, maar niet in 3.4.4.3 omdat dat experiment te kort duurt.

In bijlage 3.4.4.2 beschrijft u bij punt C. dat de dieren worden hergebruikt, u heeft hier niet de bijbehorende vraag beantwoord "Explain why re-use is considered acceptable for this animal procedure".
Kunt u deze vraag alsnog beantwoorden?
Ik begrijp dit niet goed, want bij punt C staat achter dit kopje de volgende tekst: "The cows have been previously equipped with a rumen fistula. Re-use of the animals in this experiment is acceptable as the discomfort in previous experiments has not been considered severe, and after the last experiment, the cows have been in the normal dairy herd for a longer period of time. "
Ik heb deze tekst zo gelaten, maar hoor graag van u als er iets mis mee is.

In de NTS bij 3.1 beschrijft u de doelstellingen van het project. Hier beschrijft dat dit project wordt uitgevoerd in varkens. Hierdoor komt het noemen van de koeien in 3.3 voor de lezer onverwachts. Kunt u onder 3.1 de koeien opnemen in de NTS
Gedaan

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. 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.

Bijlage:
- formulier Melding Bijlagen via de post

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

- 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

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

1.2 Bij welke aanvraag hoort de bijlage?
Het aanvraagnummer staat in de brief of de ontvangstbevestiging.

Aanvraagnummer	

2 Bijlagen

2.1 Welke bijlagen stuurt u mee?
Vul de naam of omschrijving van de bijlage in.

☐	
☐	
☐	

3 Ondertekening

3.1 Onderteken het formulier en stuur het met alle bijlagen op naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

Centrale Commissie Dierproeven i.o.

Melding

Bijlagen via de post

- 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.zbo-ccd.nl
- Of bel met ons: 0900 28 000 28 (10 ct/min).

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager [redacted]

Postcode | Huisnummer

1.2 Bij welke aanvraag hoort de bijlage?
Het aanvraagnummer staat in de brief of de ontvangstbevestiging.

Aanvraagnummer AD V 10402015326

2 Bijlagen

2.1 Welke bijlagen stuurt u mee?
Vul de naam of omschrijving van de bijlage in.

☒ Project proposal 'Assessing dietary protein quality for humans using the pig as a model'

☐

☐

3 Ondertekening

3.1 Onderteken het formulier en stuur het met alle bijlagen op naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam [redacted]

Datum 21-12-2015

Handtekening [redacted]

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for 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.centralecommissiedierproeven.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.	Assessing dietary protein quality for humans using the pig as a model

2 Categories

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

- ☒ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ 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.

Not only protein quantity, but also protein quality is important in maintaining essential body functions. Therefore, assessing protein quality has been identified as a critical question by international authorities (FAO, 2013). Protein nutritional quality is related to the capacity of the different food sources of protein to achieve the different functions associated to the supply of nitrogen and amino acids in the body. The

nutritional efficiency of a protein can be determined from the extent to which dietary protein nitrogen is absorbed and retained by the organism and is able to balance daily nitrogen losses. The capacity to provide an adequate profile of bioavailable indispensable amino acid is considered as a limiting factor for protein quality.
For this purpose, an amino acid scoring approach was designed, considering the capacity of a protein source ingested at the level of the mean protein requirement derived from nitrogen balance (0.66 g/kg/d for human adults) to meet indispensable amino acid needs. A refinement of this approach, the Protein Digestibility Corrected Amino Acid Score (PD-CAAS), is currently widely adopted, and corrects the content of each indispensable amino acid of the protein by the faecal digestibility of the protein in order to evaluate the bioavailable part of these amino acids in comparison to a reference amino acid profile.
The use of a single value for crude protein digestibility to correct the dietary amount of each individual amino acid for its digestibility is considered a short-coming, when there are practically important quantitative differences in digestibility between crude protein and individual dispensable and indispensable amino acids. A further inherent shortcoming of the PD-CAAS approach is that correction of digestibility is based on an estimate of crude protein digestibility determined over the total digestive tract (i.e. faecal digestibility). Thus, microbial metabolism and interconversions of nitrogenous compounds disturb the estimation of bioavailable amino acids. For protein as a whole, absorption of nitrogen over the whole digestive tract is an appropriate measure, as nitrogen absorbed in forms other than amino acids can contribute to the nitrogen economy. For the assessment of bioavailable amino acids, however, the FAO (2013), based on critical reviews of the literature, has recommended that protein quality assessment should be based on true ileal digestibility values of individual amino acids, rather than the overall (faecal) digestibility of protein. This method is referred to as the Digestible Indispensable Amino Acid Score (DIAAS). At the present time, there is a limited quantity of data on the ileal amino acid digestibility of foods as determined in humans. Where human data are lacking, the FAO (2013) recommends that true ileal amino acid digestibility values from the growing pig be used, being preferred over those obtained from the growing laboratory rat.

While accepting the need for DIAAS estimates of human foods using the ileal cannulated pig as a model, in the same report, the FAO (2013) identifies an urgent need to develop techniques that can be adopted to assess protein quality (amino acid availability) in humans directly, using minimally invasive techniques. In this way, future estimates for true ileal digestibility values would not require ileal cannulated pigs for the standard evaluation of protein quality, but could also be used in different human populations.

Reference:
Dietary protein quality evaluation in human nutrition. Report of an FAO Expert Consultation, FAO Food and Nutrition Paper 92, ISBN 978-92-5-107417-6, FAO 2013

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?

- To provide reliable DIAAS for 28 protein sources for human nutrition using the ileal cannulated pig (as indicated by the FAO, 2013 and detailed by the FAO expert working group, 2014).
- To develop a meal/plasma signature dual stable isotope-based approach using intrinsic labelled proteins for the non-invasive evaluation of protein digestibility, amino acid bioavailability and protein nutritional quality for humans.

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

Feasibility of the project:
The first objective is feasible. Although the use of ileal cannulated pigs as a model for evaluating protein quality in humans is new, it is commonly applied for evaluation of protein quality in pig nutrition. It has been applied previously at [redacted]. This project is part of a larger, international research effort in which a larger number of protein sources for humans are evaluated using

the pig model. Alignment of procedures is ensured, and inter laboratory comparison is foreseen. For a limited number of protein sources (10), comparison with human ileal amino acid digestibility values is planned, following the recommendations of the FAO (2013), using two methods. The first method uses humans with a normal gastrointestinal tract and uses naso-ileal intubation to sample digesta from the terminal ileum (Deglaire et al., 2009). This method will be [redacted] at [redacted]

The second objective concerns the development of a methodology which is minimally invasive and can be applied in healthy humans. This method, proposed by [redacted] has not been performed before. The method will be developed by [redacted] The novelty of the method is not in the techniques applied, but in the combination of different isotope labels, simultaneously administered to subjects. Validation of the isotope method against the well-established DIAAS method is required before implementation in humans, and is part of this application. Testing the isotope method in humans is planned in 2016, but is outside the scope of this application.

3.3 Relevance

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

Both protein quantity and protein quality are of importance for maintaining body functions like, the immune system, muscle mass and growth. As protein resources for human consumption worldwide are limiting, protein quality is of increasing importance. As acknowledged by the FAO (2013), there is an immediate need to replace the old standards for assessing protein quality (PD-CAAS) by methods based on ileal disappearance of amino acids, using the pig as a model. In addition, for the somewhat longer term, there is a need to develop non-invasive and easy to implement approaches and methods for the measurement of protein and amino acid digestibility and bioavailability for different protein sources in different populations.

3.4 Research strategy

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

Within the present project, we plan to develop a novel, stable isotope based technique to measure protein quality in humans, at the same time building a database on DIAAS values for 28 protein sources for humans, using a well-established methodology based on pigs. The isotope method is also developed in pigs, and it is planned to test this later in humans (outside the scope of this application). A cross-validation between methods is included in the project.

Building the database on DIAAS values for 28 human food sources will be conducted in 6 subsequent experiments. Within each experiment, 5 food sources will be tested, and a measurement of net endogenous protein losses at the terminal ileum will be included. To this end, each experiment will be conducted as a 6x6 latin square design with 6 treatments tested in all of 6 pigs in 6 subsequent periods. The 6 treatments include 5 different human protein sources and a protein free treatment for the measurement of endogenous ileal protein losses. In total, this will provide data on 30 protein sources (6 x 5). True digestibility values will be calculated by difference, and provide results that can be compared with the isotope methodology. One of the protein sources will be included in three experiments, providing the possibility to test repeatability. In addition, as this is part of a larger, international program, inter-laboratory comparison of 10 protein sources is planned. The selection oof the 28 protein sources will be based on their quantitative importance for protein supply in humans worldwide and on their contrast in estimated protein digestibility.

For the development of the isotope method (principle is described in the appendix), milk proteins will be labelled with ^{15}N and ^{2}H by intraruminal infusion of ^{15}N labelled ammoniumsulfate and ^{2}H labelled water.

Subsequently, milk will be collected and milk proteins isolated and dried. In addition to these intrinsically labelled protein sources, a universally labelled ^{13}C labelled amino acid source will be purchased (^{13}C spirulina hydrolysate, 99% enriched).

Subsequently, these intrinsically labelled protein sources will be used for the development of the isotope method. The principle of the method is based on the different dilution of labels and is further explained in the experimental protocols. Briefly, all three labels are orally provided with a normal meal to pigs in a frequently fed manner to simulate steady state conditions. Each pig will be provided with two subsequent meals. [redacted]. It is hypothesized that this increase in the isotopic ratio quantitatively reflects the increase in amino acids absorbed, and can therefore be also applied to measure differences in DIAAS scores, resulting from differences in protein digestibility.
In order to develop this methodology, three assumptions need to be verified prior to an independent validation against the DIAAS method:

1) Verify that the [redacted] ratio of amino acids in the blood is proportionally related to the quantity of ^{2}H-labelled amino acids which are absorbed.
2) Verify that protein bound amino acids ([redacted]) lead to a similar response under steady state conditions (i.e. in frequently fed pigs) than a mixture of amino acids in free form (^{13}C labelled, high enrichment, low dose).
3) Verify that amino acid metabolism in the intestinal wall or liver does not change the ratio of [redacted] in amino acids.

These assumptions will be tested in pigs that are equipped with catheters in the portal and jugular veins. Assumption 3 can be tested by comparison of the ratios of [redacted] in amino acids between the diet, portal and jugular blood plasma.

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 project comprises two parts: a) building a database of DIAAS scores of 28 protein sources for humans, using the ileal cannulated pig as a model; this will be performed in 6 subsequent animal experiments. b) development of an isotope methodology to assess protein quality. This will require 3 subsequent animal experiments: 1) the production of intrinsically labelled [redacted] milk protein from cow's milk; 2) evaluation of the main assumptions listed above in an experiment with pigs equipped with catheters in portal and jugular veins; 3) comparison of the developed isotope methodology to determine the isotopic signature of a protein, assessment of protein digestibility with [redacted] tracer method in plasma compared with true ileal digestibility. This will be performed using ileal cannulated pigs (for measurement of DIAAS scores) that are equipped with a catheter in the jugular vein (isotope methodology).

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.

For a description see 3.4.2. Milestones include the database of DIAAS scores of 28 human protein sources developed in part a; and a developed isotope method (part b) that can be applied for assessing protein quality in humans by the simple measurement of [redacted] ratios in amino acids in plasma sampled from the peripheral circulation. This method will be tested in humans following procedures similar to the experiment in pigs (peripheral blood only). Although this experiment is outside the scope of this application, it will be conducted in close collaboration with the research in pigs, described in this application.
Building the database (part a) and the development of the isotope method (part b) can be conducted in parallel. Within part b, three phases can be distinguished, which follow the three experiments described in 3.4.2.
Should the results of phase 2 indicate that the assumptions are not valid, phase 3 will not be conducted.

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	6 subsequent pig trials, conducted in a 6x6 latin square (6 ileal cannulated pigs, 6

	periods) each measuring DIAAS scores of 5 human protein sources, and a protein-free treatment to measure net ileal endogenous losses.
2	Production of [redacted] milk protein by directly infusing ([redacted] into the rumen of a cow and production of ^{2}H-intrinsically-labelled milk protein by directly infusing [redacted] into the rumen of a cow; subsequently collecting the milk and isolating the milk proteins.
3	To develop the isotope methodology in pigs, testing the main assumptions in pigs equipped with catheters in the portal and jugular veins.
4	Validation of the isotope method against the DIAAS score method using pigs equipped with ileal cannulas and a catheter in the jugular vein; testing four ^{2}H labelled protein sources and a protein-free treatment in a 5x5 latin square design.
5	
6	
7	
8	
9	
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.centralecommissiedierproeven.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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 Type of animal procedure: 6 subsequent pig trials, conducted in a 6x6 latin square (6 ileal cannulated pigs, 6 periods) each measuring DIAAS scores of 5 human protein sources, and a protein-free treatment to measure net ileal endogenous losses. In the description below, 1 trial is explained

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.

Six pigs (and two spare pigs) will be fitted with a T cannula in the distal ileum. After a recovery and adaptation period, each pig will be subjected to all of six experimental diets in 6 periods in a 6x6 latin square design. During each period, ileal effluents will be collected. DIAAS scores will be calculated from amino acid analysis in the diet and in ileal digesta, using TiO2 as the indigestible marker. Treatments will include five protein sources and a protein-free diet. Protein losses at the terminal ileum when feeding the protein-free diet are assumed to represent the net endogenous secretions.

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

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 between protein sources. It is designed to provide reliable estimates of DIAAS scores of the selected protein sources, building up a database. The number of observations planned is following the FAO guidelines (2014). Nonetheless, data will be analysed using a generalized linear mixed model. A normal distributed error and identity link will

be assumed for DIAAS scores, but these assumptions will be verified. Pigs within period will be considered the experimental unit. Protein source and period will be included as fixed effects, and pig as random effect to account for repeated observations within pigs. Although this approach is new for the estimation of true digestible amino acids protein sources for humans, it is commonly applied for evaluation of protein sources for pig nutrition, obtaining 5 or 6 observations for each protein source to be tested, and demonstrated to allow detection of significant difference of DIAAS scores of 2-3% between protein sources. One of the protein sources will be included in three trials, allowing estimation of repeatability. In addition, for 10 protein sources that are also tested in two other institutes, inter-laboratory comparisons will be performed.

B. The animals

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

In each of the 6 subsequent pig trials, 8 growing barrows (± 25 kg BW) will be purchased. Male animals will be used to ease collection of faeces without contamination with urine. Barrows will be used as entire males will be difficult to handle at the end of the trials. Surgery will be performed on all pigs, following procedures as included in [redacted] . Slight modifications with regard to the type of cannula and location will be made to ensure alignment of procedures with other institutes within the international consortium. Two pigs will be considered as spare animals, and will be used in the case of problems with digesta collections in the other animals. The number of spare pigs is based on previous experience, needed to ensure a complete set of 6 observations for each protein source. 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. To this end, spare pigs will be treated as their peers, and randomly assigned to a dietary treatment during each period. In the event that observations from another pig cannot be used, the samples obtained from the spare pigs will be analyzed, provided that they are obtained at the desired experimental treatment. 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 Wageningen University and Research institute, 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.

C. Re-use

Will the animals be re-used?

☒ 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 recommended by the FAO committee as a model for evaluating DIAAS scores of human foods. This choice is well documented in the FAO report (2013).
Reduction: in 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.
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 from 12 to 6 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 fibre 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 anaesthesia, 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.

The FAO recommendation for using the ileal cannulated pig as a model to estimate true digestibility of protein sources for humans is recent (2013, with a report on research methods released in 2014). The research proposed is part of a larger international effort ([redacted]) using the ileal cannulated pig as a model.

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

☒ 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 dimensions of the pens are 1.3 x 1.3m, 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 be consumed by the pigs and will thus interfere with the digestibility measurements.

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.

☐ 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.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

☒ 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 inhalation anaesthesia. 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?

- Feeding a protein-free diet for a period of 7 days may increase breakdown of body proteins and concomitant feelings of discomfort.
- Prolonged individual housing.
- Fasting prior to the surgical procedure

Explain why these effects may emerge.

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

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.

☒ 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 faeces, 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 14day 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 (8 weeks): moderate
- Protein-free diet during 7 days: mild
- The sampling procedures (2 days during each of 6 subsequent weeks): 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

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

Keeping pigs with a T-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 > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: Production of ^{15}N-intrinsically-labelled milk protein by directly infusing $(^{15}NH_4)_2SO_4$ into the rumen of a cow and production of ^{2}H-intrinsically-labelled milk protein by directly infusing $^{2}H_2O$ into the rumen of a cow; subsequently collecting the milk and isolating the milk proteins

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.

This experiment will be executed to produce ^{15}N-intrinsically-labelled milk protein and ^{2}H-intrinsically-labelled milk protein. Two lactating cows will receive intraruminal infusions of $(^{15}NH_4)_2SO_4$ and another two will receive intraruminal infusion of $^{2}H_2O$. The milk will be collected and processed voor consumption in the pig experiment (serial experiment 3).

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

Stable isotopes solutions will be infused into the rumen of lactating cows fitted with a rumen cannula for 7 days. Milk will be collected and processed. Cows have been previously equipped with a rumen cannula. It is estimated that for serial experiment 3, 175 L of both ^{15}N and ^{2}H enriched milk is needed.

Two cows will receive a continuous intraruminal infusion of [redacted], to reach an isotopic enrichment of the protein of about [redacted] After 3 days of infusion, ^{15}N enriched milk will be collected for 4 consecutive days, and will be sent off for separation of the milk proteins.

Two cows will receive a continuous intraruminal infusion of [redacted] to reach an isotopic enrichment of the protein of about [redacted] After 3 days of infusion, ^{2}H enriched

milk will be collected for 4 consecutive days, and will be sent off for separation of milk protein, lactose and fat.

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

In line with the objective of this experiment, the number of cows required depends only on the amount of milk needed for conducting the experiment number 3, as further explained below. No statistical evaluation of the data is required.

B. The animals

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

4 Holstein Frisian dairy cows, previously fitted with a rumen cannula will be used. These fistulated cows are part of the dairy herd of [redacted] and will be selected for this experiment, based on the desired stage of lactation.
The number of cows needed is estimated from the amount of intrinsically labelled milk proteins needed for conducting experiment number 3. It is assumed the cows are in mid lactation, thus producing about 25 L/d, assuming an efficiency of conversion of [redacted]

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

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

The cows have been previously equipped with a rumen fistula. Re-use of the animals in this experiment is acceptable as the discomfort in previous experiments has not been considered severe, and after the last experiment, the cows have been in the normal dairy herd for a longer period of time.

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: Alternatives to produce the desired quantity of intrinsically labelled protein sources are not available.
Reduction: the amount of milk needed is calculated carefully to be enough, but not exceeding the amount of labelled milk required for the study.
Refinement: Cows are well adapted to handling procedures. Infusion procedures will be optimized allowing the cows to stand up or lie down without noticing the equipment.

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

The cows will be carefully transferred to the tie-stall, and infusion procedures will be conducted by experienced staff. Suffering is already minimal.

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.

This experiment is solely used to produce intrinsically labelled milk for other experiments.

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

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

During the period of infusion, cows will be housed in a tie-stall barn to ensure they cannot move away from the infusion equipment. They can stand up and lie down in a normal way.

Animals will receive a grass-maize silage based roughage diet which meet their requirements. The amount of feed provided to individual animals will be adjusted according to the nutritional guidelines set by CVB standards and will be in line with the standard practice. Roughage will be available ad libitum and concentrates will be provided manually in three equal portions, given at 0600, 1400 and 2200h.

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.

☐ 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.

☐ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

☐ 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?

Apart from housing the cows in a tie-stall barn, no adverse effects on animal welfare are expected

Explain why these effects may emerge.

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

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.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

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').

Housing in the tie-stall during the infusion procedure: mild.

Cumulative level of discomfort is therefore estimated as mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☒ No

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

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.

☐ Yes

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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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: To develop the isotope methodology in pigs, testing the main assumptions in pigs equipped with catheters in the portal and jugular veins.

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.

Measurement principle

The measurement is based on the principle that orally provided, labelled amino acids as well as intrinsically labelled proteins, when fed under steady state conditions, will appear in the systemic circulation and that its dilution is proportional to the quantity ingested. It then follows that, when ingesting a constant quantity of a ^{13}C labelled amino acid source in time, and a variable quantity of a [redacted] of that particular amino acid ingested, hence independent of the dilution of each label by amino acid catabolism and influx into the circulation of amino acids from breakdown of body proteins. In this way, the ratio of labelled amino acids from intrinsically labelled protein sources and the ^{13}C reference amino acid will reflect the true digestibility of the amino acids in the labelled protein source, provided that equal amounts of this protein source are ingested. When all assumptions underlying this methodology are verified, the application of this technique is simple and can be applied in humans using minimally invasive techniques, i.e. analysing the isotope ratio of amino acids in systemic blood following ingestion under steady state conditions.

Three assumptions need to be verified prior to the application of this method. Firstly, the assumption (assumption 1) that [redacted] ratio of amino acids in the blood is proportionally related to the quantity of [redacted] which are absorbed; Secondly, the assumption (assumption 2) that using protein bound amino acids ([redacted]) with a true digestibility near 100% (milk proteins) lead to a similar response under steady state conditions as a mixture of amino acids in free form ([redacted]). Thirdly, the assumption (assumption 3) that amino acid metabolism (in intestinal wall or liver) does not change the ratio of [redacted] in amino acids.

Experimental design

The assumptions listed above will be tested in an experiment with pigs, equipped with catheters in the portal and jugular veins. The pigs will receive two discrete meals, divided in [redacted]. Pigs will be allowed to adapt to the size of the meal for 7 days. The increase in the intake of labelled protein will lead to an increase in the ratio of labelled [redacted] labelled amino acids in the systemic circulation. It is hypothesized that this increase in the isotopic ratio quantitatively reflects the increase in amino acids absorbed. Assumption 1 will be tested by comparing the ratio of [redacted] in amino acids between the two meals; Assumption 2 will be tested by comparing the ratio of [redacted] in amino acids in portal blood. Assumption 3 will be tested by comparing the ratio of [redacted] in amino acids between diet, portal blood and jugular blood.

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

Four barrows of about 25 kg of BW + three spare pigs will be purchased. Six barrows will be surgically fitted with catheters in the portal and jugular veins. Based on previous experience, it is estimated that two spare pigs are needed to ensure catheter patency of at least 4 animals throughout the 16d experimental period. Should problems arise during or immediately after surgery, the third spare pig will be used the next day. This is needed as occasionally vein architecture does not allow placement of a catheter, and problems with catheter patency may occur immediately during recovery. Pigs will be fasted prior to the surgical procedure, which will be conducted under inhalation anaesthesia. During the day of surgery, the day preceding and three days following, antibiotics will be used to minimize infection risks. Adequate painkillers will be used during recovery. During 7 days post-surgery, pigs will be allowed to recover, and feed allowance will be gradually increased to a feeding level of 2.5 times their estimated maintenance energy requirements.
Pigs will receive each of both meals in a change-over design, with the sequence balanced across pigs. Isotope meals will be administered on days 9 and 16 post surgery. During the 7 days preceding the isotope meals, pigs will be adapted to the size of the meals, fed twice daily. Body weight of the pigs will be determined upon arrival and weekly after surgery until the end of the experimental period.

Throughout the 16 d period, pigs will be housed individually, to prevent them from disturbing catheters of other pigs, in metabolic cages on a plastic coated floor. To collect faeces 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. Portal and peripheral blood will be collected during the test days via catheters.

Test meals

A test meal will contain [redacted]

Measurements

During the experiment, the following measurements will be conducted:

- Portal and peripheral blood samples are collected on the days of test meal provision (d9 and 16), 30 minutes before feeding the first portion and at 30 minute intervals during 4.5 hours (11 samples in total per catheter, 5 mL/timepoint). Isotopic enrichment of amino acids will be analysed ([redacted]) in samples collected from the portal and peripheral circulation. When steady state conditions are verified, the ratio [redacted] ratios in the amino acids within the meal, in the portal and peripheral blood sample are calculated to meet the objectives described above.

Faeces and urine are quantitatively collected during a 4 day period preceding the day of test meal provision. Faecal and urinary excretion of total N will be determined and samples will be stored for possible analysis of [redacted] in particular fractions.

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 observations required for meeting the objectives of this trial was calculated using a power analysis in SAS, for two treatments in a change-over design, one sided testing, and assuming between animal variation as described by Ten Have et al (2011), detailed below.

B. The animals

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

Seven barrows of about 25 kg BW will be purchased. Male animals will be used to ease collection of faeces without contamination with urine. The type of pig matches the pigs used for the determination of DIAAS scores in experiment 1 and 4 of this application, and is chosen in line with FAO guidelines. The number of pigs required is minimized by using a change-over design, using the pigs as their own controls. The number of observations needed was calculated using a power analysis:

The number of observations required to determine the difference between meals was determined using proc power in SAS. Estimates of variance were obtained from Ten Have et al (2011) who measured the response in portal and arterial blood following two protein meals. Data from tryptophan were selected as this is an important amino acid. The difference in arterial tryptophan concentration was selected as a representative response parameter, as this will represent the dilution in enrichment in the proposed experiment. Ten Have et al measured a difference in arterial concentrations of about 20 µmol/L following a contrast in tryptophan intake comparable to the anticipated difference between meals. The obtained standard deviation was 5, derived from a graph in this manuscript. The power analysis was conducted for detecting a difference of 20 µmol/L, one sided and at an alpha of 0.05 and a power of 0.8. The resulting number of observations required for each treatment is 4.
It is expected that applying n=4 in a change-over design (4 observations; 2 periods) is sufficient to detect a statistically significant difference; especially as the standard deviation of the ratio between isotopes within an amino acid is likely smaller than that of an arterial concentration of an amino acid. Unfortunately, estimates of variation in these parameters are not available.
Reference: Ten Have, GAM, Engelen, MPKJ, Soeters, PB, Deutz, NEP. 2012. Absence of post-prandial gut anabolism after intake of a low quality protein meal Clinical Nutrition 31 (2012) 273-282

C. Re-use

Will the animals be re-used?

☒ 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: As described in the FAO guidelines (2013), current in vitro methods are not adequate to estimate protein quality of human protein sources. The proposed method, validated against the proposed standard (DIAAS scores in pigs, see serial nr 4), is now first developed in pigs, but can be applied later using minimally invasive procedures, directly in humans, thus replacing the ileal cannulated pig as a model.
Reduction: A change over design is applied to minimize the number of pigs needed.
Refinement: The use of isotope ratio's within each amino acid makes this procedure independent of isotope dilution originating from amino acids from protein breakdown. Such measurements would involve the measurement of a number of extra parameters, for example and notably the portal blood flow, which is more variable and would require a higher number of animals for achieving reliable estimates of amino acid fluxes. 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 anaesthesia, 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.

Assessing protein quality has been identified as a critical question by international authorities (FAO, 2014). While accepting the need for DIAAS estimates of human foods using the pig as a model, the FAO (2014) identified an urgent need to develop techniques that can be adopted to assess protein quality (amino acid availability) in humans directly, using minimally invasive techniques. This method, proposed by [redacted]

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

☒ 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 dimensions of the pens are 1.3 x 1.3m, allowing the pigs to move around freely. Walls will be smooth (covered by plexiglass) to prevent damage to catheters. Animals will be housed on a plastic coated floor. Individual housing is needed to prevent animals from damaging catheters of pen mates. Plastic bags will be attached to the rear end of the pigs to allow quantitative collection of faeces and the collection of clean urine (funnels underneath the cage). The use of bedding material is avoided as this will prevent the collection of clean urine samples. Pens will be enriched with non-destructable toys in a weekly alternating scheme as playing material, according to procedures developed at Wageningen University.

Milk proteins and isotopically labelled sources will be provided on top of a normal, commercial feed, that will be supplied slightly restricted to ensure complete consumption. Feed will be provided at 2.5 times the energy requirements for maintenance, being close to 80% of the ad libitum intake. Water is supplied in a ratio of minimum 2.5:1 relative to the feed. In addition, water is available ad libitum through drinking nipples. The protein sources will be provided on top of the feed supply.

Prior to surgery, feed allowance will be reduced and feed will be withheld (fasting) to assure and empty digestive tract. Thereafter, feed allowance will be gradually increased

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.

☐ 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.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

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

The placement of portal and jugular catheters is conducted under inhalation anaesthesia. Animals will be treated with painkillers during recovery (at least 3 days) and with antibiotics. Animals are cared for according to a standard protocol and by skilled staff.

I. Other aspects compromising the welfare of the animals

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

Apart from individual housing and the surgical procedures (fasting prior to and recovery after), no adverse effects on welfare is expected.

Explain why these effects may emerge.

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

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.

☒ 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 faeces, indicating blockage of the intestines or inflammation of the peritoneum.
- Upon losing catheter patency.
- 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 4 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% during the 16 day 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 catheters will lead to removal of the pig from the experiment, but is not likely to lead to discomfort for the pig.

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
- Attaching faecal bags (16 days): mild
- Sampling procedures (16 days): mild
- Individual housing in absence of bedding material, in a large metabolism pen (16 days): moderate

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

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

Yes, after the procedures. Prolonged housing of pigs with a portal catheter is undesirable from an animal welfare point of view. Moreover, the location of the tip of this catheter needs to be verified during autopsy. When it becomes apparent that catheter placement is impossible, the pig will be killed during anaesthesia.

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.

☒ Yes

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.centralecommissiedierproeven.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 serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		4	Validation of the isotope method against the DIAAS score method using pigs equipped with ileal cannulas and a catheter in the jugular vein; testing four ^{2}H labelled protein sources and a protein-free treatment in a 5x5 latin square design.

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.

Five pigs (and three spare pigs) will be fitted with a T cannula in the distal ileum and with a catheter in the jugular vein. After a recovery and adaptation period, each pig will be subjected to all of five experimental diets in 5 periods in a 5x5 latin square design. During each period, ileal effluents will be collected and blood samples collected. DIAAS scores will be calculated from amino acid analysis in the diet and in ileal digesta, using TiO_2 as the indigestible marker. One of the treatments will be the feeding of a protein-free diet. Protein losses at the terminal ileum are assumed to represent the net endogenous secretions. True amino acid digestibility will be measured using the isotope methodology (see seral nr 3). Protein sources will be selected based on a difference in the estimated ileal protein digestibility, and labelled with ^{2}H.

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

Five growing barrows (+ three spare pigs), ± 25 kg BW will be fitted with a T cannula in the distal ileum, and with a catheter in the jugular vein under inhalation anaesthesia. Pigs will be fasted prior to the surgery and will be treated with adequate painkillers and antibiotics during recovery. Pigs will be allowed 14 days to recover from the surgery and to adapt to housing conditions. After recovery from surgery, ileal effluents will be collected from each pig during 5 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 will be stored at -20°C pending

analysis. This approach is selected to allow within pig comparisons of all protein sources (latin-square design), minimizing the total duration of the trial, thereby the risk of cannula complications. This procedure has been proposed by the expert working group of the FAO (2014). On day 3, the measurement, the protein sources will be replaced by sources of identical botanical origin, but intrinsically labelled with ^{2}H. [redacted]

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.

Consistent with the objectives of this experiment, statistical methods will focus on predicting DIAAS scores by true amino acid digestibility calculated using the isotope method. In this analysis the potential of the true digestibility estimated by the isotope method will be tested as predictor of the DIAAS score. The mean square prediction error will be calculated, and decomposed in 3 components: i) overall bias, ii) deviation of the slope from unity, iii) random error. Variation between and within protein sources will be separated.

B. The animals

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

For this trial, 8 growing barrows (± 25 kg BW) will be purchased. Male animals will be used to ease collection of faeces without contamination with urine. Barrows will be used as entire males will be difficult to handle at the end of the trials. Surgery will be performed on all pigs, applying procedures described above. Three pigs will be considered as spare animals, and will be used in the case of problems with digesta collections or catheter patency in the other animals. 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 for the spare pig will be extended by one period. Pigs will be about 30 kg BW at the onset of the trial, and 50-60 kg BW at the end of the trial. Body weight will be determined upon arrival and after surgery weekly until the end of the last experimental period.

For determining reliable estimates for DIAAS scores, 6 observations per protein source are required (see serial nr 1). For the objective of this experiment, however, the minimum recommended number of observations for establishing reliable DIAAS scores will be used (i.e. 5, see FAO, 2014). The total number of observations available for the comparison of DIAAS scores and isotope based estimates is 25 (5 periods x 5 pigs=25 for each amino acid), as both variation between and within protein sources can be used. In addition, protein sources will be selected based on a large difference in expected DIAAS scores.

C. Re-use

Will the animals be re-used?

☒ 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 recommended by the FAO committee as a model for evaluating DIAAS scores of human foods. This choice is well documented in the FAO report (2013).

Reduction: in 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. The objective of this comparison is to establish a method that can be directly applied in humans, thus replacing the cannulated pig model.
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 from 12 to 6 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 fibre 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 anaesthesia, 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.

Assessing protein quality has been identified as a critical question by international authorities (FAO, 2013). While accepting the need for DIAAS estimates of human foods using the pig as a model, the FAO (2013) identified an urgent need to develop techniques that can be adopted to assess protein quality (amino acid availability) in humans directly, using minimally invasive techniques. This method, proposed by [redacted]

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

☒ 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 dimensions of the pens are 1.3 x 1.3m, 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. Audio-visual between pigs contact is maintained. The use of bedding material is avoided as this will be consumed by the pigs and will thus interfere with the digestibility measurements, but cage enrichment will be available (see above)

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.

☐ 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.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

☒ 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 inhalation anaesthesia. After surgery, animals will be treated with pain killers (at least 3 days) and antibiotics.

I. Other aspects compromising the welfare of the animals

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

- Feeding a protein-free diet for a period of 7 days may increase breakdown of body proteins and concomitant feelings of discomfort.
- Prolonged individual housing.
- Fasting prior to the surgical procedure

Explain why these effects may emerge.

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

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.

☒ 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 faeces, indicating blockage of the intestines.
- upon losing catheter patency.
- 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.
- a pig shows body weight loss in a 14d period.

Indicate the likely incidence.

The likely incidence of pigs to be removed from the experiment is estimated at 25% during the 7 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 or catheters 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
- Sampling procedures (6 weeks): mild
- Feeding of a protein-free diet (7 days): mild
- Individual housing in absence of bedding material, in a large metabolism pen (8 weeks): moderate

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

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

Keeping pigs with a T-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 > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Van: [redacted]
Verzonden: woensdag 3 februari 2016 9:03
Aan: Vergunningenloket
CC: [redacted]
Onderwerp: RE: vragen bij AVD104002015326

Beste [redacted]

Hierbij mijn reactie op onderstaande vraag:
Het protocol zoals gepubliceerd door de FAO schrijft voor dat elke proefdier zijn eigen eiwitvrije controle dient te hebben. De reden daarvoor is dat van al het eiwit dat de dikke darm instroomt, afhankelijk van de verstrekte eiwitbron, globaal tussen de 50 en 100% van endogene oorsprong is (mucus, epitheelcellen, microbiele biomassa, enzymen). Het is kwantitatief daarom nogal een belangrijke eiwitstroom, waar ook tussen-dier variatie op zit. Ik zie daarom geen mogelijkheden om uitkomsten voor de schatting van endogene eiwitverliezen van dieren of groepen te combineren zonder verlies van precisie van de schatting van de ware eiwitverteerbaarheid van eiwitbronnen.

Als er nog vragen zijn dan hoor ik het graag,

Vriendelijke groet,

[redacted]

Van: Info-zbo [mailto:info@zbo-ccd.nl]
Verzonden: dinsdag 2 februari 2016 9:26
Aan: [redacted]
CC: [redacted]
Onderwerp: RE: vragen bij AVD104002015326

Geachte [redacted]

U heeft een aanvraag voor projectvergunning ingediend bij de CCD. Het gaat om het project AVD104002015326 getiteld: Assessing dietary protein quality for humans using the pig as a model. De CCD heeft uw aanvraag besproken. U heeft al een aantal vragen beantwoord, hartelijk dank hiervoor, in aanvulling hierop heeft de CCD nog een vraag over het inzetten van de groepen met eiwitvrij dieet. Is het noodzakelijk om in elke proefgroep een dergelijke controle groep in te zetten of zijn er mogelijkheden om uitkomsten van deze groep te combineren.

Met vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (let op: nieuw emailadres!)

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Wageningen University
[redacted]
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T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD104002015326

Uw referentie

Bijlagen
1

Datum 09 FEB. 2016
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 14 december 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Assessing dietary protein quality for humans using the pig as a model" met aanvraagnummer AVD104002015326. Wij hebben uw aanvraag beoordeeld.

Op 21 januari 2016 hebben wij u nog een aantal vragen gesteld om aanvullende informatie. U heeft deze op 21 januari 2016 beantwoord en naar aanleiding van deze aanvullende informatie de documenten van uw aanvraag aangepast. Op 2 februari 2016 hebben wij u nogmaals een vraag gesteld, deze heeft u op 9 februari 2016 voldoende beantwoord. Dit antwoord heeft niet tot aanpassing van de documenten geleid.

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. U kunt met uw project "Assessing dietary protein quality for humans using the pig as a model" starten. De vergunning wordt afgegeven van 1 maart 2016 tot 1 maart 2020.
Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-WUR gevoegd. Dit advies is opgesteld op 11 december 2015. Op 21 januari 2016 hebben wij de DEC een vraag gesteld, deze vraag is door de DEC beantwoord op 21 januari 2016. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. Om te voldoen aan datgene wat voortvloeit uit artikel 10a. van de wet worden aan meerjarige projecten twee algemene voorwaarden gesteld. Dit 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 gegevens 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).

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Wageningen universiteit
Adres:	Postbus 59
Postcode en woonplaats:	6700 AW WAGENINGEN
Deelnemersnummer:	10400

deze projectvergunning voor het tijdvak 1 maart 2016 tot 1 maart 2020, voor het project "Assessing dietary protein quality for humans using the pig as a model" met aanvraagnummer AVD104002015326, volgens advies van Dierexperimentencommissie DEC-WUR.
De functie van de verantwoordelijk onderzoeker is [redacted]
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 14 december 2015;
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 21 januari 2016;
   b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 21 januari 2016;
   c. Advies van Dierexperimentencommissie dd 11 december 2015, ontvangen op 14 december 2015;
   d. De aanvullingen op uw aanvraag, ontvangen op 21 januari 2016 en 9 februari 2016.

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst
Bijlage 3.4.4.1 6 subsequent pig trials, conducted in a 6x6 latin square (6 ileal cannulated pigs, 6 periods) each measuring DIAAS scores of 5 human protein sources, and a protein-free treatment to measure net ileal endogenous losses. In the description below, 1 trial is explained	Varkens (Sus scrofa domesticus) / barrows 25 kg	48	Matig
Bijlage 3.4.4.2 Production of 15N-intrinsically-labelled milk protein by directly infusing (15NH4)2SO4 into the rumen of a cow and production of 2H-intrinsically-labelled milk protein by directly infusing 2H2O into the rumen of a cow; subsequently collecting the milk and isolating the milk proteins	Runderen (Bos taurus) / Holstein Frisian Pensfistel koeien	4	Licht
Bijlage 3.4.4.3 To develop the isotope methodology in pigs, testing the main assumptions in pigs equipped with catheters in the portal and jugular veins.	Varkens (Sus scrofa domesticus) / barrows 25 kg	7	Matig
Bijlage 3.4.4.4 Validation of the isotope method against the DIAAS score method using pigs equipped with ileal cannulas and a catheter in the jugular vein; testing four 2H labelled protein sources and a protein-free treatment in a 5x5 latin square design.	Varkens (Sus scrofa domesticus) / barrows 25 kg	8	Matig

Voorwaarden

Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat eventuele go/no go beslissingen worden genomen met instemming van de IvD.

In artikel 10, lid 1a 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 overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

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 van Economische Zaken 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 13c 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 13d 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.