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Physiological comparability of the harmonized INFOGEST *in vitro* digestion method to *in vivo* pig digestion

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ABSTRACT

Recently, a static *in vitro* digestion (IVD) protocol was published by Minekus and coworkers (Minekus et al., 2014) within the COST INFOGEST network. The protocol, concentrating on physiological enzyme activities had the main goal to improve the comparability of experimental data between labs. The protocol was validated in several inter-laboratory studies using skim milk powder (SMP) and indeed demonstrated improved harmonization compared with previous experiments with individual IVD protocols (Egger et al., 2016). Although the enzyme activities and salt concentrations of the harmonized protocol are based on available human *in vivo* data, confirmation of the protocol's physiological relevance has been lacking until now. The main goal of the study was therefore to compare the harmonized IVD protocol with data from *in vivo* digestion. Towards this aim, an *in vivo* pig experiment with the same SMP as used for the validation of the IVD protocol was performed followed by a comparison of protein hydrolysis between *in vivo* and *in vitro* results. Protein hydrolysis at different levels was analyzed with gel electrophoresis, mass spectrometry, high performance liquid chromatography, and spectrophotometric o-phthaldialdehyde determination of free amino acids. Principle component analysis was used for graphical data comparison.

Milk proteins detected after gastric IVD corresponded to gastric and duodenal *in vivo* samples and intestinal IVD samples corresponded to distal jejunal *in vivo* samples. Peptides identified after the gastric phase of IVD, correlated with *in vivo* gastric samples ($r = 0.8$) and intestinal IVD peptides correlated best with in vivo samples collected from the median jejunum ($r = 0.57$). Free amino acids were in both systems mainly released during the intestinal phase of digestion. Protein hydrolysis in the harmonized IVD was similar to *in vivo* protein hydrolysis in pigs at the gastric and intestinal endpoints. Therefore, the harmonized static *in vitro* protocol is suited to study protein hydrolysis at these endpoints.

1. Introduction

In vitro digestion (IVD) protocols are widely used to address questions in the field of nutritional research. They are cheaper, faster and simpler to perform than *in vivo* experiments. IVD protocols have better reproducibility and fewer inter-individual variabilities and are therefore optimal for screening experiments. However, the biological relevance of the IVD protocols needs to be validated for each research question. In the past, various protocols were applied (Hur, Lim, Decker, & McClements, 2011; Kopf-Bolanz et al., 2012; McClements & Li, 2010; Picariello et al., 2010; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005; Wickham, Faulks, & Mills, 2009), making a comparison of the study results difficult. Recently, a three-phase static IVD protocol (oral, gastric and intestinal) was elaborated among experts within the COST Action INFOGEST FA1005, based on human *in vivo* data. The development of the protocol considered available physiological enzyme activities and salt concentrations, aiming at a harmonization of IVD and therefore a better comparability of the experimental results between laboratories (Minekus et al., 2014). The improvement in the comparability of results was investigated in several inter-laboratory studies, by digesting the same batch of skim milk powder (SMP). Protein hydrolysis was analyzed at the level of proteins, peptides and free amino acids (FAAs), and the researchers concluded that comparability of the IVD results had improved (Egger et al., 2016). The harmonized protocol is now used throughout the research community and has since been cited more than

Abbreviations: FAA, free amino acids; IVD, *in vitro* digestion; SMP, skim milk powder

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200 times. Although the harmonized protocol was established based on available human data, the physiological relevance of the harmonized protocol towards *in vivo* digestion was not experimentally clarified. To fill this gap in knowledge, a pig *in vivo* experiment was performed, using the exact same SMP as previously used for establishing the IVD. Due to the similarities of eating patterns and the physiology of the gastrointestinal tract to those of humans (Sciascia, Daş, & Metges, 2016), pigs were chosen as the model organism. This work focuses on comparing the gastric and intestinal endpoints of the harmonized IVD protocol (Egger et al., 2016; Minekus et al., 2014) with *in vivo* pig digestion after the gastric, duodenal and various intestinal sections.

2. Materials and methods

2.1. Chemicals and reagents

Skim milk powder (SMP, Fonterra, Auckland, New Zealand) and standard milk powder (Test-SMP, LEDOR MMP S I390, Hochdorf, Switzerland) with a similar composition were used (Supplemental Table 1). All chemicals and enzymes were from Sigma Aldrich (Buchs, Switzerland). The enzymes and bile extracts used for IVD were of porcine origin and are described in detail by Minekus et al. (Minekus et al., 2014).

2.2. Experimental diets, animals and procedures

The *in vivo* experiment was approved by the animal welfare department of the government authority (approval number 2015_04_FR; 26115). Twenty piglets were preselected from the Agroscope breeding herd two weeks after weaning according to litter, sex and body weight. The pigs were housed in one pen and fed *ad libitum* the commonly used pelleted post-weaning diet until they reached a mean body weight of 25 kg. Then, 10 pigs were selected randomly from the original group and housed in pairs in one pen until they reached a mean body weight of 40 kg. The pigs were fed *ad libitum* with a pelleted barley-soybean meal-based grower diet (Supplemental Table 1) containing 5% standard skim milk powder and 5% milk permeate to keep the gut micro-flora adapted to milk based feedstuffs.

The SMP digestion study started 96 h (T-96) before the pigs were euthanized (T0). At T-96, the two pigs were moved into separate pens. Each pen (1.6 m^2 concrete floor and 1.9 m^2 slotted floor, without bedding or consumable playing material) allowed individual feeding and *ad libitum* access to water *via* a nipple drinker. The pigs were adapted to drink a diluted skim milk powder with a similar product to the SMP (Supplemental Table 1, 250 g Test-SMP and 500 g H_2O) by feeding them individually twice a day for a maximum of 30 min.

At T-14, the pelleted feed was withdrawn, and at T-6.5, the pigs were moved to individual pens to assess each pig's SMP consumption individually. The same batch of SMP (Supplemental Table 1, SMP) used to validate the harmonized IVD protocol (Egger et al., 2016) was used for the subsequent feedings. Each meal consisted of 250 g of SMP which was diluted in 500 mL H_2O and was fed for 30 min three times at T-6.0, T-3.0 and T-1.5 (Fig. 1). The feeding of 750 g SMP over a period of 14 h represents about 50% of the daily feed intake by a 40 kg pig. At T-1.5, access to water was stopped. In between these 30 min feeding periods, the animals were kept in pairs. At T0, the animals were euthanized in pairs with anesthetization using CO_2 followed by exsanguination at the on-site Agroscope research abattoir.

2.3. In vivo sample collection

After the pigs were euthanized, samples were immediately collected from the digestive tract. The digestive tract was excised and dissected into six sections: the stomach (S), duodenum (D, 0–30 cm after the stomach), proximal jejunum (I1, 50–150 cm jejunum), median jejunum (I2, 200–300 cm jejunum), distal jejunum (I3, the last part of the jejunum) and ileum (I4). Intestinal contents between the collected segments were not collected in order to obtain clear differences between sampling segments. The total contents of each section were collected and homogenized. The stomach contents were separated into a solid (S, solid) and a liquid (S, liquid) phase and homogenized (Polytron, Kriens, Switzerland). The pH was measured, and the samples were split into aliquots and snap frozen in liquid nitrogen. To one aliquot of *in vivo* samples, sample buffer 6 × (Tris–HCl 350 mM, pH 6.8, SDS 10%, DTT 100 mM, glycerol 50%) was added at a dilution of 1:6 and snap frozen immediately thereafter.

2.4. In vitro digestion

The same batch of SMP as the one tested *in vivo* was digested *in vitro* using the harmonized protocol (Egger et al., 2016; INFOGEST, 2014; Minekus et al., 2014). Briefly, the SMP (5 mL of a 1/10 dilution of SMP in H_2O, w:v) was mixed with 5 mL of simulated saliva (pH 7) without amylase for 2 min. Then the SMP was mixed with 10 mL of simulated gastric juice (pH 3) containing pepsin (2000 U/mL of digesta) for 120 min. Subsequently, 20 mL of simulated intestinal juice (pH 7) containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mmol/L in the total digesta) were added and incubated for 120 min. The oral, gastric and intestinal steps were performed at 37 °C under constant gentle mixing on a rotating wheel. The enzyme activities and the bile concentration were measured according to the assays described in the harmonized protocol (Minekus et al., 2014). Digestion was stopped after the gastric phase by adjusting the pH to 7 with NaOH (1 M) and by the addition of 4-(2-Aminoethyl)benzensulfonylfluorid (AEBSF, trade mark Pefabloc®, 5 mM, Roche, Basel, Switzerland) for the intestinal phase, immediately followed by snap freezing (liquid nitrogen).

2.5. In vivo and in vitro sample preparation and gel electrophoresis

The collected *in vivo* samples from each section were defrosted. A pooled sample for each section was generated by mixing equal volumes of the individual samples. Equal volumes of individual and pooled samples, as well as a sample of SMP (1:400, mixed with sample buffer),

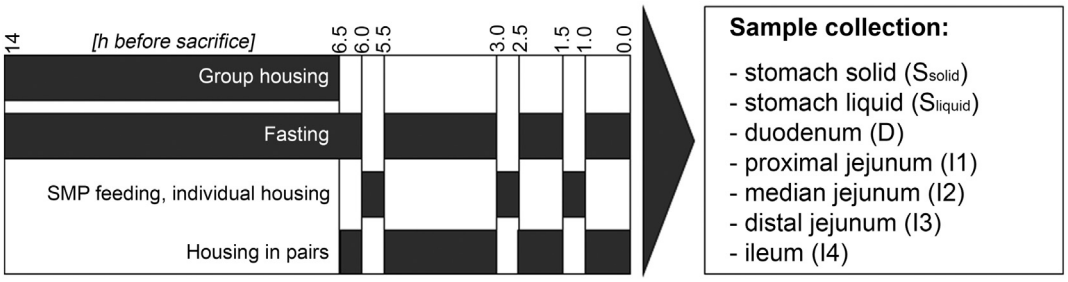

Fig. 1. Schematic drawing of the pig trial. Timeline of the pig trial starting from 14 h before sacrifice. Grey bars indicate the different phases of the trial, sample collection at time zero is indicated with sampling sections.