Advances in Bionic Digestion Methods for Evaluating Nutritional Value of Swine Feed: Postprint

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Abstract

In vitro enzymatic biomimetic digestion technology for evaluating the nutritional value of swine feed has gained recognition in developed countries such as Denmark, the Netherlands, and France; however, this technology has yet to transcend the conventional research paradigm that employs pancreatin with irreproducible hydrolytic activity as the enzyme source for simulated digestive fluid and utilizes "Erlenmeyer flask + shaker" as fully manual testing tools. Consequently, regarding innovation in enzymatic methods, the biological background and standardization of simulated digestive fluid preparation, along with the establishment of standardized in vitro simulated digestion tools, have consistently represented the core issues and challenges in this research domain. This review synthesizes the latest domestic and international research advances in porcine simulated digestion technology and summarizes the research achievements of the State Key Laboratory of Animal Nutrition in this field.

Full Text

Advancements in Bionic Digestion Methods for Evaluating the Nutritional Value of Pig Feed

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Abstract: Bionic digestion technology based on in vitro enzymatic methods has gained recognition in developed countries such as Denmark, the Netherlands, and France for evaluating the nutritional value of pig feed. However, this technology still follows the traditional research paradigm that uses pancreatin with irreproducible hydrolytic activity as the enzyme source for simulated digestive fluids and relies on fully manual testing tools like "Erlenmeyer flasks + shaking incubators." Consequently, the biological basis and standardization of

simulated digestive fluid preparation, along with the development of standardized in vitro digestion tools, remain central challenges and critical bottlenecks in this research field. This review summarizes recent advances in simulated digestion technology for pigs both domestically and internationally, and presents research findings from the State Key Laboratory of Animal Nutrition in this domain.

Keywords: pig; simulated digestion; bionic digestion system

The biological availability of feed nutrients serves as the primary basis for establishing feeding standards and optimizing feed formulations, representing one of the most fundamental parameters in animal nutrition research. Therefore, the accurate and rapid determination of feed nutrient biological availability has long been a shared focus of industry attention.

Since the 1950s, numerous beneficial attempts have been made to simulate enzymatic reaction processes in animals for estimating digestible energy and amino acid digestibility in feed. Currently, enzymatic methods for determining the digestible energy value of pig feed have gradually gained acceptance in developed countries including Denmark, the Netherlands, and France, becoming core technologies in their new feeding standards [1-3]. Nevertheless, existing enzymatic methods still adopt the approach of Boisen et al. [4], suffering from drawbacks such as irreproducible enzyme activity in simulated digestive fluids, considerable arbitrariness and instability in the simulated digestion process, and the lack of dedicated automated testing tools. To address these issues, the State Key Laboratory of Animal Nutrition has systematically investigated the composition of porcine digestive fluids based on previous research, establishing foundational data for simulated digestive fluid preparation and achieving reproducible and standardized digestive fluid activity. Building upon this, we developed a bionic digestion system for fully automated simulation of gastric, small intestinal, and large intestinal digestion in pigs, realizing the "automation, instrumentation, and operational standardization" of bionic digestion methods. With further development and refinement, this technology promises to provide efficient research tools for evaluating the nutritional value of pig feed in China.

1. Research Progress on Simulated Digestive Fluid Preparation for Pigs Abroad

In in vitro simulated digestion of feed, some researchers have directly used intestinal fluid or pancreatic juice from livestock as digestive fluids. Examples include the pepsin-pig small intestinal fluid method established by Japanese scholar Furuya et al. [5] and the pepsin-pig duodenal fluid-ileal fluid-fecal extract method developed by Löwgren et al. [6]. Although these methods approximate in vivo conditions in terms of enzyme source and type, small intestinal fluid preparation is complex, and significant variation exists between different batches, making reproducibility difficult. Consequently, further development of these methods has been rarely reported in recent years.

To overcome these limitations, Boisen et al. [7] proposed using pepsin, pancreatin, and carbohydrate enzymes to simulate gastric juice, small intestinal fluid, and large intestinal fluid in pigs, respectively, and established operational procedures for estimating digestible energy through enzymatic hydrolysis of organic matter [4,8-9]. However, the deviation between in vitro and in vivo digestibility of feed organic matter varies substantially depending on feed type (ranging from 5% to 25%) [4]. The precision of estimating digestible energy (DE) values from in vitro organic matter digestibility is not higher than that achieved using neutral detergent fiber [10], and the method is not applicable for estimating DE values of all feed types [11].

Similar to Boisen et al.' s [7] technical system, Regmi et al. [12] used pepsin, pancreatin, and cellulase to simulate digestive fluids in various segments of the porcine gastrointestinal tract, though with considerable differences in parameters such as simulated digestive fluid preparation and digestion time compared to Boisen et al. [7]. Evidently, in previous in vitro simulated digestion systems using pepsin and pancreatin as enzyme sources, parameters including total enzyme activity and digestion time were determined solely based on achieving complete hydrolysis of dietary substrates [13], demonstrating considerable arbitrariness. Consequently, these in vitro simulated digestion techniques completely deviate from actual animal digestive physiology. Furthermore, pancreatin is a complex mixture of multiple digestive enzymes, with varying enzymatic composition between batches, making reproducibility of simulated intestinal fluid impossible. Thus, numerous key technical issues remain unresolved in existing feed in vitro simulated digestion technologies, both theoretically and in practical application. Since pig feed undergoes primarily enzymatic chemical digestion in the digestive tract, with gastric and intestinal fluids as the hydrolysis medium, we must first determine the physiological basis for simulated digestive fluid composition, assess differences in hydrolytic characteristics between simulated and in vivo digestive fluids, and achieve complete reproducibility in simulated digestive fluid composition. Clarifying these issues is crucial for establishing bionic digestion methods to evaluate feed nutritional value.

2.1 Static Simulated Digesters Based on Erlenmeyer Flask Closed Systems

According to porcine digestive physiology characteristics, in vitro simulated digestion device design can be categorized as static or dynamic simulation. Currently, static simulation methods using Erlenmeyer flasks as digesters (referred to as the flask method) are commonly employed for routine nutrient (energy, protein) simulated digestion [3-4,12]. In this process, 10-50 mL of digestive fluid is placed in a 100 mL Erlenmeyer flask, with a water bath shaker providing enzymatic reaction temperature and simulating gastrointestinal motility. Subsequent digestive fluid addition and pH adjustment for later digestion stages are performed manually. Analysis of pig feed digestion simulation using the flask method by scholars from various countries between 1990-2010 revealed several

issues: 1) No clear specifications exist regarding water bath shaker performance (temperature control precision, mixing intensity) or pH variation range within flask digestive fluids [13]; 2) In practice, buffer pH is first adjusted to neutral with NaOH before adding simulated small intestinal fluid to the flask, thereby diluting the intestinal fluid—contradicting the objective fact that chyme digestion in the small intestine occurs in intestinal fluid medium; 3) When using Erlenmeyer flasks as closed digesters, some feed samples always adhere to flask walls during water bath shaker rotation, preventing complete contact with digestive fluid, with adhesion varying among feed types and showing considerable differences between flasks, yet the impact of this factor on test results has rarely been reported; 4) Regarding the effect of hydrolysis products on enzymatic reactions, the flask as a simulated digester has no material exchange with the external environment. Since feed substrates and hydrolysis products coexist in the same solution, significant product inhibition occurs as the reaction progresses, markedly different from the timely absorption of hydrolysis products in vivo; 5) The flask method typically uses filtration to separate enzymatic hydrolysates from digestion residues [3-5,12], but our preliminary research found that both domestic quantitative analytical filter paper and imported filter paper (Whatman 531) exhibit weight loss after Buchner funnel vacuum filtration, making precise control of this factor' s impact on test results difficult. Considering these variable factors, Clunies et al. [14] reported relative standard deviations as high as 4.86% and 6.63% for gastric and small intestinal digestion phases, respectively, using the flask method. Evidently, traditional static flask methods suffer from core technical problems including complex operation, non-standardized tools, inadequate mixing of chyme and digestive fluid during enzymatic reactions, inhibition of enzymatic reaction rate and equilibrium by hydrolysis products, and difficulty in quantitatively separating hydrolysates from unhydrolyzed materials. Therefore, innovating pig feed nutrient simulated digestion tools based on existing technology requires addressing these issues systematically.

2.2 Dynamic Simulated Digestion Devices

In response to the drawbacks of static simulated digestion devices, developed countries have increasingly focused on developing dynamic simulated digestion systems. These devices primarily consist of custom-made simulated digesters, digestive fluid secretion systems, and automatic buffer pH adjustment systems, partially achieving dynamic secretion of simulated digestive fluids and periodic changes in physicochemical parameters of digestion conditions. For example, in the 1980s, Canadian scholar Gauthier et al. [15] developed a device for simulating protein digestion and absorption in the porcine small intestine, while Mahidol University in Thailand designed a dynamic continuous dialysis system for simulating micronutrient digestion and absorption in the porcine gastrointestinal tract [16]. These two devices share similar design features: using dialysis bags to simulate intestinal wall absorption function, water baths to provide constant temperature, specialized stirrers or rotary shakers to simulate small intestinal motility, and peristaltic pumps to slowly input buffer into digesters to

simulate the physicochemical environment of mesenteric capillaries, promoting forward enzymatic reactions.

Compared with the flask method, these devices offer the advantages of minimal alteration to the digestive fluid environment by hydrolysis products and automatic separation of hydrolysis products from unhydrolyzed materials via dialysis bags. However, they suffer from low automation levels, requiring manual operation for peristaltic pump and water bath shaker control and digestive fluid injection. Additionally, the stirrer design employs vertical agitation with dialysis bags placed naturally vertically or horizontally without dedicated fixation, making thorough mixing of feed samples and digestive fluid difficult and creating challenges in transferring unhydrolyzed residues after experiments (analyzing hydrolysis product concentrations in dialysate may introduce errors from sample concentration, buffer evaporation, or volume changes). No specialized cleaning procedures exist for partially hydrolyzed products remaining in dialysis bags after simulated digestion.

With advancements in modern automatic control and human-computer interaction technologies, international development of monogastric animal simulated digestion devices has gradually progressed toward fully automated program control. In 1995, the Netherlands Organization for Applied Scientific Research (TNO) developed a porcine gastric-small intestinal digestion bionic system that could automatically simulate gastric emptying, small intestinal motility and absorption, and digestive fluid secretion through computer control. However, this system was originally designed for research on gastrointestinal microbial growth and pharmacological metabolism, and its relatively long digestive tract design easily caused chyme blockage and incomplete emptying, resulting in poor repeatability for feed nutrient digestibility determination and significant deviation from in vivo values [17]. The dynamic gastric digestion system developed by National Chung Hsing University in Taiwan could simulate postprandial changes in gastric juice pH and pepsin secretion stages to determine feed protein digestibility in the stomach [18]. This device essentially adopted Gauthier et al.' s [15] design for the gastric digester but utilized American LabView (Ver. 6.1) software to automate control of peristaltic pumps, stirrers, and pH meters via computer, essentially enabling unattended operation. However, functionally, it still could not overcome drawbacks similar to Gauthier et al.' s [15] simulated digester.

Thus, the core technology determining the functionality of dynamic gastrointestinal simulated digestion systems lies in the design of simulated digesters (reactors) and simulated digestion processes. Existing reported automatic simulated digestion devices cannot achieve fully automated quantitative determination of pig feed nutrient digestibility in terms of either functionality or automatic control technology. Therefore, in designing new fully automated bionic digestion systems, several issues require in-depth investigation: How to ensure thorough mixing of feed samples and digestive fluid while preventing chyme adhesion and drying during digestion? How to prevent errors from chyme sample

transfer when transitioning between digestion stages (e.g., gastric to small intestinal phase)? How to achieve highly reproducible separation of hydrolysis products from unhydrolyzed materials after simulated digestion while facilitating automation? How to achieve fully automated simulation of a complete digestion process through computer program control with human-computer interaction? Can simulated digestion test results be reproducible, additive, and consistent with in vivo methods? These questions remain to be thoroughly studied.

3.1 Composition of In Vivo Porcine Digestive Fluids

As previously mentioned, porcine simulated digestion generally proceeds sequentially through three phases: gastric → small intestinal → large intestinal digestion. Therefore, determining the composition of digestive fluids at each stage—namely, digestive enzyme activities and buffer ion concentrations—is the biological foundation for preparing simulated digestive fluids. The porcine stomach primarily functions in food storage, controlling chyme emptying, and digesting dietary proteins via pepsin under acidic conditions [19].

According to Chiang et al. [18], pepsin activity in growing pig gastric chyme varies between 240-865.4 U/g, with average pepsin activity in porcine gastric juice calculated at 737.5 U/mL based on chyme moisture content. Fujita et al. [20] reported porcine gastric juice contains Na^+ 80.6 mmol/L, K^+ 6.0 mmol/L, Cl^- 134.2 mmol/L, with pH 2.0. Since current international standard methods for pepsin assay and ion concentration detection align with those employed in the aforementioned studies, our research group adopted these parameters as the composition for simulated gastric fluid in growing pigs.

The small intestine of finishing pigs, measuring 16-21 m in length, is the primary site for feed nutrient digestion and absorption. Small intestinal digestive enzymes originate mainly from pancreatic secretions of amylase, protease, and lipase, plus minor contributions from intestinal glands secreting intestinal peptidases and disaccharidases [21]. Based on the principle that digestive activity is highest in intestinal fluid at 1.5-1.8 m from the pylorus in growing pigs [22], jejunal cannulas installed at this location revealed that under normal nutritional levels, dietary protein source and level, as well as feed type, had no significant effects on the activities of four major digestive enzymes (amylase, trypsin, chymotrypsin, lipase) and ion concentrations in jejunal fluid [23-24]. Simple and canonical correlation analyses further demonstrated no significant correlation between variations in major digestive enzyme activities in growing pig jejunal fluid and feed nutrient digestibility among individuals [25]. Based on these findings, porcine jejunal fluid composition was determined as: amylase 221.4 U/mL, trypsin 69.1 U/mL, chymotrypsin 8.7 U/mL, lipase 3.3 U/mL, Na^+ 89.9 mmol/L, K^+ 15.0 mmol/L, Cl^- 116.7 mmol/L, pH 6.44.

The porcine large intestine comprises the cecum, colon, and rectum, where incompletely digested chyme from the small intestine undergoes hydrolysis by

microbial cellulase and short-chain fatty acid production through microbial metabolism [21]. Addressing porcine large intestinal digestion characteristics, our research group investigated compositional differences in cecal fluid of growing pigs under three dietary fiber levels. Results indicated that dietary fiber level had no significant effect on fiber enzyme activity or pH in cecal fluid but significantly influenced concentrations of some ions (K^+, Cl^-) [26]. Based on these findings and considering the tolerance of regenerated cellulose dialysis bags to cellulase, porcine large intestinal fluid composition was determined as: cellulase 0.04 U/mL, Na^+ 93.2 mmol/L, K^+ 11.1 mmol/L, Cl^- 25.1 mmol/L, pH 6.42.

Based on the above parameters for in vivo porcine digestive fluid composition, our research group prepared simulated digestive fluids using reagent-grade digestive enzymes according to the principle of equal major digestive enzyme activities. The simulation effect was verified for the primary digestion environment—simulated small intestine. Results demonstrated that the hydrolytic capacity of simulated intestinal fluid on corn, wheat bran, soybean meal, cottonseed meal, and four diets reached over 94.8% of in vivo intestinal fluid values, with correlation coefficients above 0.95 [27]. Subsequently, Wang Yuming [24] established methods for purifying major digestive enzymes from porcine jejunal fluid and developed a method for preparing simulated porcine intestinal fluid based on purified digestive enzyme powders supplemented with small amounts of reagent enzymes, further narrowing the compositional gap between simulated and in vivo intestinal fluids. However, the hydrolytic capacity of simulated gastric and large intestinal fluids requires further validation.

3.2 Development of Bionic Digestion Devices

The simulated digester serves as the site for feed bionic digestion [Figure 1: see original paper], consisting of a transparent glass tube, dialysis tube, buffer outlet, buffer inlet, digestive fluid infusion tube (with one-way valve), standard ground joint (No. 19), and rubber stopper. Before assembly, dialysis bags undergo deglycerination, desulfurization, and metal element removal, then are inserted through the ground joint at one end of the digestion tube and passed through completely. Both ends of the dialysis tube are everted at the standard ground joint, covering over 15 mm of the outer diameter and secured with rubber bands. One end is sealed with a rubber stopper, feed samples (1-2 g) and 20 mL simulated gastric fluid are added, and finally sealed with a rubber stopper containing the digestive fluid addition tube [28].

The bionic digestion system comprises simulated digesters, single-channel and multi-channel peristaltic pumps, non-contact solenoid valves, constant-temperature shakers, refrigeration systems, constant-temperature water baths, decompression devices, host computers, and control software, assembled into a fully automated bionic digestion instrument (Model: SDS-2). Each bionic digestion system contains two sets of digestion devices as shown in [Figure 2: see original paper], enabling determination of two samples per run,

with five replicate measurements obtained for each sample. In this system, the 39°C enzymatic reaction temperature is controlled by two components: a constant-temperature shaker controlling the air temperature outside the simulated digester and a water bath maintaining buffer temperature to control temperature inside the dialysis tube, keeping temperature variation within 0.5°C. The shaker precisely controls rotation at 180 r/min via electromagnetic induction for thorough mixing of digestive fluid and feed.

Before porcine bionic digestion operation, five digestion tubes for each sample determination are fixed on a dedicated rack with outlets facing upward and inlets downward, connected sequentially via silicone tubing between outlets and inlets of simulated digesters. The rack is then positioned in the constant-temperature shaker, with the inlet of the first digestion tube and outlet of the fifth tube connected to the bionic digestion system' s water inlet (peristaltic pump) and outlet (decompression device), respectively. Digestive fluid addition tubes are connected to multi-channel peristaltic pumps via quick connectors.

Digestion process parameters are programmed in the control software, including 33 items: temperature, mixing intensity, preheating duration, digestion duration for stomach, small intestine, and large intestine, solution drainage duration, cleaning liquid volume, cleaning time, cleaning frequency, and digestive fluid infusion time and volume. After preheating completion, the entire bionic digestion process begins automatically. Gastric digestion phase: solenoid valves 1 and 2 open automatically, gastric buffer is pumped into simulated digesters via peristaltic pump (11, [Figure 2: see original paper]) and circulates for 4 hours. Upon completion, solenoid valves 1 and 2 close automatically, followed by automatic drainage to remove gastric buffer outside dialysis tubes in simulated digesters. The drainage program includes automatic opening of solenoid valves 9 and 10, with peristaltic pump (11, [Figure 2: see original paper]) reversing to pump residual fluid into waste bottles. Then, three cleaning cycles run automatically to remove gastric digestion products. Each cleaning cycle includes pumping 1,500 mL deionized water into cleaning bottles via peristaltic pump (12, [Figure 2: see original paper]), automatic opening of solenoid valves 7 and 8, pumping deionized water into simulated digesters via peristaltic pump (11, [Figure 2: see original paper]) for 40 minutes of circulation, followed by another drainage program.

After gastric bionic digestion, small intestinal bionic digestion begins: solenoid valves 3 and 4 open automatically, small intestinal buffer is pumped into simulated digesters via peristaltic pump (11, [Figure 2: see original paper]) and circulates for 30 minutes, then concentrated simulated small intestinal fluid is automatically pumped into dialysis tubes via multi-channel peristaltic pump (13, [Figure 2: see original paper]). Small intestinal buffer continues circulating for 16 hours. Upon completion, solenoid valves 3 and 4 close automatically, followed sequentially by drainage and three cleaning cycles. After small intestinal bionic digestion, large intestinal bionic digestion begins: solenoid valves 5 and 6 open automatically, large intestinal buffer is pumped into simulated di-

gesters via peristaltic pump (11, [Figure 2: see original paper]) and circulates for 30 minutes, then concentrated simulated large intestinal fluid is automatically pumped into dialysis tubes via multi-channel peristaltic pump (14, [Figure 2: see original paper]). Large intestinal buffer continues circulating for 3.5 hours. Upon completion, solenoid valves 5 and 6 close automatically, followed sequentially by drainage and six cleaning cycles, completing the entire bionic digestion process. Since bionic digestion of feed fat is difficult to achieve, defatting of dried undigested residues is performed using anhydrous ethanol [28].

3.3 Validation of Bionic Digestion Method for Simulating Pig Feed Nutrient Digestion

To optimize the measurement precision of the bionic digestion system, we investigated influencing factors in the testing process and variation sources of measurement results, determining optimal conditions: sample grinding fineness of 60 mesh (passing 0.3 mm sieve), six cleaning cycles for digestion residues, and 90 days of effective use for preprocessed dialysis bags [29]. Among all steps in bionic digestion, variation in dry matter mass of undigested residues constitutes the primary cause of result variation [30]. Simulated digestive fluid reagent kits stored at 4°C or room temperature for up to 10 months do not affect test results [31].

Based on these parameters, we validated the reproducibility and additivity of the bionic digestion method. Results showed that in two-phase gastric-small intestinal digestion, within-batch, between-batch, and total coefficients of variation for dry matter digestibility and enzymatic hydrolysate energy values of corn, soybean meal, cottonseed meal, and wheat bran were all below 1.40% in the same bionic digestion system. Between different bionic digestion systems, within-instrument, between-instrument, and total coefficients of variation for these four feed ingredients were all below 1.64% [28,32]. In three-phase gastric-small intestinal-large intestinal digestion, coefficients of variation for control parameters (buffer flow rate, cleaning liquid input volume, simulated digestive fluid input volume, temperature control throughout the process, mixing intensity, etc.) across six laboratories were within 11%, while coefficients of variation for enzymatic hydrolysate energy values of corn and soybean meal were controlled within 1.7%. Additivity testing using eight diets formulated from corn, soybean meal, cottonseed meal, and wheat bran demonstrated that measured and theoretical values of dietary dry matter digestibility and enzymatic hydrolysate energy values conformed to the linear function Y=X, indicating satisfactory additivity [33]. These results demonstrate that the bionic digestion system meets basic requirements for quantitative analysis in terms of reproducibility and additivity.

To further investigate the relationship between bionic digestion and biological methods, correlation analysis using 19 pig diets revealed a correlation coefficient of 0.87 between enzymatic hydrolysate energy values determined by bionic digestion and digestible energy values by biological methods, with residual stan-

dard deviation of estimation not exceeding 146.3 J/g and absolute deviation within 250.8 J/g. Additionally, using ten cottonseed meal sources with different origins, digestible amino acid contents determined by bionic digestion showed significant correlation with in vivo values for all amino acids except methionine [24]. These findings indicate that bionic digestion represents a potential technique for estimating digestible energy values and digestible amino acid contents in pig feed.

Standardization of bionic digestion methods may be achieved through simultaneous advances in simulated digestive fluid standardization and fully automated bionic digestion instrument development. Currently, the bionic digestion method has been promoted and used in over 30 institutions in China with significant economic benefits, though some issues have emerged during application. Our research team is conducting systematic work on optimizing porcine in vivo enzyme extraction methods, improving large intestinal simulated digestion effects, and refining production processes for bionic digestion systems (instruments), achieving expected progress. Subsequent work will address user feedback to optimize bionic digestion methods while ensuring fundamental principles of reproducibility and additivity, providing technical support for future revisions of China' s pig feed ingredient standards and feeding standards.

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Note: Figure translations are in progress. See original paper for figures.