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EVOLUTION OF METHODS FOR *IN VITRO* PRODUCT DIGESTIBILITY ANALYSIS: A SYSTEMATIC REVIEW

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Abstract

The inability to reproduce certain digestive processes in vivo, high research costs and ethical aspects have led to the development of a large number of in vitro digestion models. These models allow us to take into account various factors of modeling complex multistage physiological processes occurring in the gastrointestinal tract, which makes them promising and widely used. A significant part of in vitro methods includes assessment by enzymatic digestion and are based on the calculation of nitrogen remaining after digestion in relation to the initial total nitrogen (according to the Dumas, Kjeldahl method, spectrophotometric or chromatographic method). There are also a number of titrometric methods (pH-stat), which are mainly used to assess the digestibility of feed, most successfully for aquatic animals due to the simplicity of their digestive tract. Methods for assessing the digestibility of food products by enzymatic digestion have undergone various stages of evolution (since 1947) and have been widely modified by including various enzymes (pepsin, trypsin, pancreatin, erepsin, etc.) in model systems, indices for various products have been determined on their basis (pepsin-digest-residue (PDR) index, 1956; pepsin pancreatin digest (PPD) index, 1964; pepsin digest dialysate (PDD), 1989). As a result, a single protocol was formed to study the digestibility of food — INFOGEST (2014–2019), which includes three stages of digestion (oral, gastric and intestinal). It allows researchers to accurately reproduce the conditions of the human gastrointestinal tract and is widely used by scientists around the world.

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Introduction

Nowadays, the concept of food nutritional value includes a degree of the digestibility and assimilability, the presence or generation of minor biologically active compounds or anti-alimentary factors in the digestion process in addition to the main indicators (safety, energy and biological value).

The interest to the investigation and understanding of the food digestibility processes has increased over the last decade. Today, the food product digestibility has been studied using different *in vitro* and *in vivo* models. With that, to study questions linked with diet components, new ingredients and foods, the priority is given to models that include farm animals and humans as it is possible to obtain the most accurate results with their use [1]. However, it is technically difficult to analyze the complex multi-stage process that takes place during digestion in humans or animals, and it is not always possible to carry out such investigations from the ethical and financial points of view. In this connection, *in vitro* digestion models simulating processes in the gastrointestinal tract are proposed as an alternative to *in vivo* experiments [2]. There is a real need for the development and use of *in vitro* models that allow accurate simulation of the physiological processes during digestion taking into consideration factors such as the presence of certain digestive enzymes and salts and their concentration, pH value, digestion duration. To simulate the digestion processes, static and dynamic models were developed, which common features are correct simulation of the digestion processes and digestive liquids in animals and humans [3]. The main requirements for these models are flexibility, accuracy and reproducibility; they should be a decent alternative to animal and human models and allow rapid screening of food products and ingredients [4]. *In vitro* simulation of the digestive processes approximated to the *in vivo* physiological conditions is widely used

in pharmacology, feeding and nutrition sciences, food chemistry being useful tools for studying and understanding changes, interactions as well as bioavailability and metabolites of nutrients, medicines and anti-nutritional compounds.

Over the last decade, different *in vitro* digestion models have been used worldwide to analyze structural and chemical changes occurring in food and feed matrices. For example, using *in vitro* digestion models, the digestibility of feed and feed additives including those obtained from genetically modified plants has been studied. Models with simulation of rumen liquid [5,6,7], gastric and intestinal juices of pigs and poultry [8] with the use of the isolated porcine small intestine [9] and others are widely used. These studies allow analyzing the character of digestion of different substances for particular species of animals and poultry. Methods for *in vitro* determination of the food digestibility are aimed, first of all, to detection of the digestibility of the main substances that ensure anabolic processes and synthesis of metabolically active substances in the body.

Modern *in vitro* digestion models described in the literature are static or dynamic with the use of various enzyme systems. Due to the fact that dynamic models are complex computerized systems and 89% of all publications are based on the use of static models, we examined the latter in this paper.

The aim of this paper is to present a review of static *in vitro* digestion models by analysis of the evolution of these methods with regard to the development of digestion models (parameters, protocols, guidelines) and to study a possibility of their use in the field of food analysis.

Objects and methods

Study design: systematic review according to the protocol of the PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions [10].

The strategy for the search of publications is presented in Figure 1.

Criteria for inclusion are:

1. Correspondence to the theme of the systematic review by one of four modalities: *in vitro* method, static model, product digestion, use of enzymes.
2. Original research published in the peer-reviewed journal, patent for invention.
3. Data on the *in vitro* digestion method are given.
4. Publication is devoted to the study of the digestibility of products, feedstuff, main nutrients, in particular, protein.

Criteria for exclusion are:

1. Studies that envisage the investigation of the digestibility and assimilation of nutrients *in vivo* (including the use of laboratory rodents, farm animals, poultry, humans).
2. Studies that envisage the investigation using the model of digestion in rumen and intestine of ruminants (including the use of "artificial" rumen, rumen liquid, gastric and intestinal juice of pigs and poultry).
3. Studies that envisage the investigation of absorbability by the ideal method (in the small intestine of rats, pigs; terminal method).
4. Studies based on the *in situ* approach.
5. Studies of drug metabolism *in vitro*.

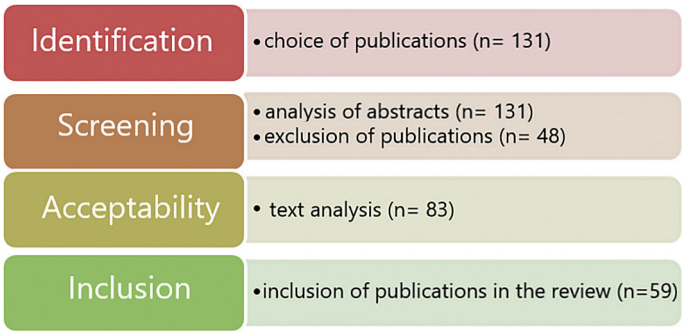

Figure 1. Strategy of selection and inclusion of publications into the systematic review

A search for relevant scientific publications was carried out using the Russian and foreign electronic databases: Web of Science, United States National Library of Medicine (pubmed.gov), Russian scientific electronic library (elibrary.ru), Russian State Public Scientific and Technical Library in Russian and English for a period of 1950 to 2021.

Titles of papers obtained in the course of search were analyzed; part of publications was excluded as inconsistent with the criteria for inclusion. Then, abstracts of selected papers were analyzed and the second exclusion was carried out. After that, the following data were selected from each publication included into the review: author(s), year of publication, country; aim and design of the research; verification of the statistical hypothesis; description of the methodology of the experiment; results. A detailed analysis of each publication included into the review was performed based on the specific elements of research questions and the aim of the review by double data extraction (two independent researchers worked on the review).

All data obtained by this way were used for analysis and systematization of the results.

Results and discussion

Over the last 40 years, more than 2500 studies with the use of digestion simulation by *in vitro* methods were published; among them, 80% of studies were published over the last 20 years [1]. More than 200 publications were examined within the framework of the paper. *In vitro* static models are described in the significant part of analyzed literature for investigating the digestibility of protein ingredients and protein feed additives. For ease of understanding, we conditionally divided widely used methods into groups, which are described in the sections below.

Methods with the use of enzyme systems

When developing these methods, the main emphasis is shifted to simulation of parameters of enzymatic cleavage that simulate processes of digestion in the stomach or both in the stomach and small intestine.

The general principle of these *in vitro* methods is the use of mono- and multi-enzyme systems; the product digestibility can be calculated as soluble nitrogen remained after digestion in relation to the initial total nitrogen in a product. In the experiment, additional stages (filtration, centrifugation or exclusion by a molecule size) can be included before analysis. Traditionally, nitrogen and protein amounts in the obtained samples are detected by the Dumas method (combustion in the atmosphere of pure oxygen), by the Kjeldahl method, spectrophotometric or chromatographic methods.

The development of these methods was initiated abroad at the end of the 1940s; with that, the peak of the research was in 1956–1985. Foreign researchers used mainly multi-enzyme systems, most often "pepsin-pancreatin" and chromatographic methods for detection of one or several bioavailable amino acids. In 1956, Sheffner A. L. et al. published the study [11], in which they determined changes in the composition of essential amino acids by *in vitro* pepsin digestion of protein products and the pepsin-digest-residue (PDR) amino acid index was described. The authors used this index to describe differences between the composition and structure of essential amino acids released as a result of pepsin digestion and the amino acid structure of the initial protein and undigested residue.

The essence of the method consisted in incubation of the analyzed product sample (contained 1 g of protein) in 30 ml of the pepsin solution (25 g) with addition of 0.1 N hydrochloric acid for 24 hours followed by assessment of the content of essential amino acids. Profiles of essential amino acids in a food product and its hydrolysate expressed as a percent of the total content of essential amino acids were compared obtaining data about essential amino acids in the unhydrolyzed protein residue. Then, results obtained for the hydrolyzate and for the residue were compared with the corresponding values for egg protein determining the geometric mean for the "egg ratio" and obtained results were multiplied by the coefficient of the relative amount of the digestible substance and residue generated by the pepsin action on the sample analyzed and egg protein.

It is not easy to calculate the PDR index; however, division of this index by the digestibility coefficient of the respective proteins gave values that predicted quite accurately the biological value of proteins under study in *in vivo* experiments [11]. In this connection, this method became quite widespread. Later on, researchers included enzymes such as pancreatin, trypsin and erepsin in addition to pepsin into the system [12], as well as antimicrobial preparations (Thiomersal) to prevent the microbial growth in the system "substrate-enzyme" and obtain more pure data.

In 1964, Akeson W. R. and Stahmann M. A. published a paper [13] introducing the concept of the pepsin pancreatin digest (PPD) index. The reaction of the enzymatic cleavage of protein samples (100 mg protein) included their incubation in the solution of pepsin (1.5 mg) with addition of 0.1 N hydrochloric acid at 37 °C for 3 hours, neutralization with 7.5 ml of 0.2 N sodium hydroxide, following addition of the pancreatin solution (type III, 4 mg in 7.5 ml of phosphate buffer with pH 8.0) into the system and incubation at 37 °C for 24 hours. Then, 10 ml of the obtained suspension were mixed with 50 ml of 1% solution of picric acid, centrifuged at 1000 G for 30 min to remove undigested protein and large peptides. The obtained supernatant in a volume of 50 ml was run through the chromatographic column with anion exchange resin (AG 2-X, 200–400 mesh with three portions of 0.02 N hydrochloric acid, 5 ml each). Obtained samples were dried by lyophilization, diluted (up to 10 ml using the buffer with pH 2.2) and amino acid analysis was carried out by the ion exchange method. The pepsin pancreatin digest (PPD) index was calculated similar to PDR.

Kennedy J. et al. [14] continued to develop the methodology and in 1989 proposed to carry out an experiment on pepsin digestion in the dialysis cell. As a result, they introduced a new index PDD (pepsin digest dialysate), which is calculated similar to the PDR index discussed above. The authors obtained the PDD values for protein ingredients such as soybean meal, gelatin, gluten, casein, egg protein, cow milk enriched with carbohydrates, protein and vitamins [14]. The following was mentioned as an advantage of using the PDD index compared to the PDR and PPD: firstly, the use of a simpler device; secondly, the use of only one enzyme; thirdly, the use of the modern equipment for analysis of amino acids; fourthly, higher reproducibility and, finally, a possibility of computerized calculation [15]. For instance, Gauthier S. F. et al. [16] proposed an *in vitro* method by the example of casein. It consisted in peptic proteolysis by pepsin (3152 units/mg protein, an enzyme: substrate ratio of 1:250) in the close system followed by hydrolysis with pancreatin (an enzyme: substrate ratio of 1:25) for 24 hours in the "digestion cell") with the continuous elimination of digested products by dialysis (the circulating buffer was 10 mM sodium-phosphate buffer, pH 7.5) [16].

In 1965, in Russia, A. A. Pokrovsky and I. D. Ertanov [17] developed a method for detection of attackability (a degree of cleavage and digestion) of proteins, which gained the widespread use later on and became the conventional routine method in many laboratories. In the experiment, a product was subjected to two-stage hydrolysis with acidic and alkaline proteases in conditions close to the natural process in the human gastrointestinal tract. An amount of the accumulated low molecular weight products of hydrolysis was determined by the Lowry color reaction [18] quantitatively expressing the mass fraction of tyrosine in hydrolysate to the mass fraction of tyrosine in protein of the initial sample in percentage terms (mg tyrosine/g protein). For comparison, proteins with the high degree of the digestibility in the human and animal bodies were used [19,20,21]. Besides the tyrosine method, accumulation of proteolysis products was also controlled by the reaction of amino acids with ninhydrin [22].

Protocol according to A. A. Pokrovsky and I. D. Ertanov is described quite accurately in [23] and consists in the following. Freshly prepared pepsin solution (concentration of 1 mg/ml: 50 ml of 0.02 N hydrochloric acid solution (pH=1.2) is mixed with 50 ml of crystalline pepsin) is added to 1g of a minced sample, thoroughly agitated and warmed up at 37 °C, holding at this temperature for 3 hours. To determine the digestibility by pepsin, proteolysis is terminated and undigested protein is precipitated by addition into the sample of 50 ml of the mixture containing the 20% trichloracetic acid solution and acetone in a ratio of 3:2, agitating and filtering after 30 min. Residues of samples after digestion by pepsin are neutralized upon agitation with 0.4 ml of 2 N sodium hydroxide; after that, 15 ml of 0.02 N sodium bicarbonate solution (pH 8.2) is added, the mixture is warmed up at 37 °C for 10 min. and 15 mg of crystalline pancreatin are added with the following incubation at 37 °C for 3 hours. A degree of attackability of proteins in the product under study is assessed by an amount of non-protein nitrogen.

Indicators obtained in the control experiments (I/control — suspension of the analyzed sample in 0.02 N hydrochloric acid; II/ control solution of enzyme(s)) are subtracted from a value characterizing hydrolysis. The data are presented in% to total nitrogen.

Calculations are carried out by the equation:

$$K = A - B - C \quad (1)$$

where:

K is an increase in hydrolysis products due to the action of enzyme(s);

A is the concentration of hydrolysis products in the sample analyzed;

B is the concentration of the same products in the food product suspension, I/control;

C is the concentration of the same products in the solution of enzyme, II/ control.

Currently, many variations of the method by A. A. Pokrovsky and I. D. Ertanov have been developed and used [17]: in modification of the V. M. Gorbatov All-Russian Research Institute for Meat Industry (now Gorbatov Research Center for Food Systems) (Moscow, Russia) [24]; Moscow State University of Applied Biotechnology (now Moscow State University of Food Productions) (Moscow, Russia) [25,26]; Institute for Meat and Dairy Industry (Minsk, Republic of Belarus). Specific features of modifications were a possibility to use dialysis bags, control of pH, periodicity of sampling without stopping proteolysis. For example, the use of dialysis after each stage allows removing hydrolysis products from the sphere of the reaction to avoid inhibition of digestive enzymes by low molecular weight peptides and free amino acids [27].

The specialists of the Moscow State University of Applied Biotechnology (now Moscow State University of Food Productions) (Moscow, Russia) developed the apparatus for hydrolysis of minced product samples (on the basis of the content of about 150 g protein in it). The apparatus consists of several cells that represent a system of internal and external vessels divided by the semipermeable membrane. A sample and 15 ml of the hydrochloric acid solution (0.02 mol/l) are placed into the internal vessel of the apparatus, while 60 ml of the hydrochloric acid solution with the same concentration and then 15 mg of crystalline pepsin (the enzyme concentration is 1 mg/ml) are placed into the external vessel. With that, the internal vessel is placed into the external one so that its bottom is dipped into the solution and the levels of liquids in both vessels are equal. The experiment is performed at a temperature of 37 °C. The reaction is carried out with agitation of the liquid using a mixer (rotation frequency $1s^{-1}$) and a sample (0.1 cm^3) is taken three times at hour intervals from the internal vessel. After that, a volume of hydrochloric acid equal to a volume of the sample taken is transferred to the vessel. The biuret reagent (1 ml) is added into each sample and the control sample (1 ml of distilled water and 1 ml of the biuret reagent) is prepared. The samples are placed into cuvettes, the latter are placed into a photoelectric colorimeter and absorbance is measured at a wavelength of 540 nm. For the further hydrolysis, the liquid from the external glass is replaced with 15 ml of $NaHCO_3$ (0.02 mol/l) and pepsin digest is neutralized with 0.4 ml of the NaOH solution (2 mol/l). Then, 15 ml of the $NaHCO_3$ solution (0.02 mol/l) are added and 15 mg of crystalline trypsin are introduced after temperature equalization. The subsequent procedures were conducted in a similar way measuring absorbance of three samples by the color reaction according to the Lowry method using a photoelectric colorimeter. Data determined by a calibration graph are recalculated with account for the total volume of liquid in the external and internal vessels and, then, these values are added up. Values obtained in the control experiments are subtracted from the tyrosine concentration, which characterizes a hydrolysis degree: an enzyme solution in the first experiment and a suspension of the analyzed product in the buffer solution in the second experiment. Accumulation of hydrolysis products detected by the Lowry method is expressed in micrograms of tyrosine per 1 g of dry matter.

The modification of the Institute for Meat and Dairy Industry (Minsk, Republic of Belarus) resides in the sequential exposure of protein substances of the product under study to the proteinase system consisting in pepsin and trypsin upon continuous agitation and removal of hydrolysis products from the reaction sphere with the subsequent photometric measurement of the product color intensity with the Folin's reagent, quantitatively expressing the tyrosine concentration (μg/cm^3) due to an effect of proteolytic enzymes on a product over 6 hours with a one-hour interval.

The integration of methods developed by Russian and foreign scientists is of special scientific interest. For example, Bologa et al. [28] obtained the pepsin-pancreatic index for whey proteins using the system "pepsin-trypsin- chymotrypsin".

Table 1. Systemized data about comparison of methods for assessment of the food product digestibility by enzymatic digestion

Analytic method*		AA	AA	AA	A/AA	L	L	L
Authors		Sheffner et al., 1956 [11]	Akeson, Stahmann 1964 [13]	Kennedy et al., 1956 [14]	Gauthier et al., 1986 [16]	Pokrovsky, Ertanov, 1965 [17]	Lipatov, Yudina, Lisitsyn, 1994 [27]	Institute for Meat and Dairy Industry (2020)
Gastric enzymes	Pepsin	×	×	×	×	×	×	×
	Trypsin	–	–	–	–	–	×	×
	Temperature 37 °C	–	×	×	×	×	×	×
	pH 1.2–3	×	–	–	–	×	×	×
	30–60 min	–	–	–	×	–	–	–
	120–180 min	–	×	–	–	×	×	–
	6 hours	–	–	–	–	–	–	×
	24 hours	×	–	×	–	–	–	–
	Dialysis	–	–	×	–	–	–	–
	Termination of proteolysis	–	–	–	–	–	–	×
Intestinal enzymes	Pancreatin	–	×	–	×	×	–	–
	Temperature 37 °C	–	×	–	×	×	–	–
	pH 8	–	×	–	–	×	–	–
	< 30–60 min	–	–	–	–	×	–	–
	120–180 min	–	–	–	–	–	–	–
	24 hours	–	×	–	×	–	–	–
	Dialysis	–	–	–	×	–	–	–

Note: * AA — amino acid analysis; A — determination of nitrogen content; L — Lowry method (reaction of Folin's reagent with phenol radicals of amino acids).

Among various methods, it is worth noting the method by N. M. Savich and G. M. Zholdaspaeva [29], in which protein under study is placed on a strip of filter paper in several replications, part of samples are processed with the proteolytic enzyme (pepsin/papain) and another part is held as a control. After incubation, all samples are colored with amido black 10B; then, the complex of protein with the dye is eluted and the protein content in the obtained colored solutions is determined by the spectrophotometric method. The digestibility is assessed as a proportion of hydrolyzed protein expressed in percent of the initial quantity [29]. Today, this method is used mainly for assessment of grain raw materials.

Table 1 presents variability of the existing static *in vitro* methods for studying cleavage of analyzed products with different nature using the enzyme systems.

Methods for assessing food protein digestibility based on changes in environmental pH (pH-stat, titration method)

Although this method also uses enzymes, it is based on the regression equation of calculation of changes in pH of the reaction medium that occur due to liberation of protons from cleaved peptide bonds in protein hydrolysis by digestive enzymes and has been used for many decades.

The beginning of the development of this method is considered to be the 1970s, when Maga J. A. et al [30] revealed a close interrelation with the initial rate of protein hydrolysis under the action of trypsin assessed as an indirect measure of pH in the reaction mixture over time and the digestibility of protein samples in *in vivo* experiments [28]. Later on, this method was subjected to various modifications in terms of using enzyme combinations and duration of the experiment [31]. In 1977, for example, Hsu H. W. et al. [32] published data on testing various enzyme combinations to improve the correlation coefficients between *in vitro* experiments (drop in pH) and *in vivo* protein digestibility (in rats). The result of the work was the pH-stat method, in which the multi-enzyme systems (trypsin, chymotrypsin and peptidase) were used [33]. The method validity was assessed by comparison of data with coefficients of the protein digestibility for rats. It was revealed that when using the new pH-stat method to assess 23 sources of food protein, the correlation coefficient with the apparent digestibility of protein for rats was 0.9; with that, an effect of the trypsin inhibitor, chlorogenic acid and thermal treatment on the digestibility was shown. Therefore, the authors established that the high content of ash affected the results of the digestibility of the protein under study and demonstrated that this method based on the pH decline depended on the buffering capacity of the protein analyzed [8].

The standard protocol of the *in vitro* pH-stat experiment for assessing the food protein digestibility according to Hsu H. W. et al. [32] consists in the following. Analyzed samples with the known protein content are comminuted into fine powder, which can pass through an 80-Mesh sieve (with a hole diameter of 0.18 mm). Then, a protein aqueous suspension is prepared on the basis of 6.25 mg protein/ml based on distilled water, 50 ml are taken, pH is brought to 8.0 with 0.1 N hydrochloric acid and /or sodium hydroxide and the mixture is placed into the water bath at 37 °C. The multi-enzyme solution containing (in 1 ml) 1.6 mg trypsin, 3.1 mg chymotrypsin and 1.3 mg peptidase is prepared and held in the ice bath, pH is brought to 8.0 with 0.1 N hydrochloric acid and /or sodium hydroxide. The multi-

enzyme solution (5 ml) is added to the warmed protein suspension (50 ml) and the mixture is agitated in the water bath at 37 °C. Sodium caseinate is used as a standard. pH is recorded automatically over 10 min using a recording pH meter. During the reaction, a rapid pH drop is observed, which is caused by liberation of carboxyl groups of amino acids from the protein chain by proteolytic enzymes.

The following can be determined additionally: 1) graphical dependence of the pH drop on time for each enzyme individually according to the procedure described for the multi-enzyme system; 2) an effect of the trypsin inhibitor contained in the sample analyzed on digestion by trypsin or the multi-enzyme system by addition of soybean trypsin inhibitor (23.4 mg)/ trypsin inhibitor type II-S to 50 ml of casein suspension (6.25 mg/ml) by detecting the mixture digestibility using trypsin (1.6 mg/ml) or the multi-enzyme solution according to the procedure described above; 3) buffering abilities of different protein sources by the following way: 50 ml of the protein suspension (6.25 mg/ml) are brought to pH 8, with 0.1N NaOH or HCl at 37 °C; after that; the protein suspension is slowly titrated with 0.0096 N HCl to pH 6.45 for 10 min; the buffering capacity of a protein source is determined as an amount of used acid [32,33].

To overcome the sensitivity of the pH-stat method to the buffering ability of protein samples, Pedersen B. et al. (1983) [34] revised the method considering an amount of alkali consumed in the reaction medium as an indirect indicator of the true digestibility of protein in rats. The authors maintained pH of the reaction at the constant level of 8 during titration with alkali for 10 min, which allowed improving the correlation coefficient from c 0.9 to 0.96 with the residual error of 1.29 after assessment of 30 protein samples [33]. The authors assumed that an effect of the ash content on the test results was linked with differences in the content of mineral substances mainly, calcium. The authors proposed to use two different regression equations for accurate prediction of the digestibility of protein samples from plant and animal origin. However, even proposed equations for predicting the digestibility of the protein source type are unreliable due to the fact that it is necessary to know the average dissociation of α-amino groups and the number of peptide bonds in the structure of proteins present in the analyzed ingredient when measuring a degree of protein hydrolysis by this method [8].

Subsequently, the pH-stat method developed by Hsu H. W. et al. [32] was modified by Satterlee L. D. et al. [33]. The experiment was aimed to assessment of different feed ingredients with the high protein content using three variants of enzyme mixtures and compared with the results of the true protein digestibility in male broiler chickens subjected to cecectomy (endoscopic dissection) [35]. The high correlation of the performed experiment with the lysine digestibility was revealed in male broiler chickens for the ingredients tested. However, the experiment did not show any relation with the lysine digestibility and the protein efficiency ratio in the samples of feather and meat meal of different quality.

Therefore, the pH-stat method has the following disadvantages: low accuracy of prediction of protein bioavailability (correlation with *in vitro* experiments is 0.9 to 0.96); labor intensity and complexity of the method for routine quality control of samples analyzed; reproducible results are obtained only for easily digestible sources of pure protein with known data about average dissociation of α-amino acids and the number of peptide bonds.

The systemized data and comparison of the pH-stat methods (titration method) for assessing food protein digestion as well as objects of research are presented in Table 2.

Table 2. Systemized data about evolution of the pH-stat method (titration method) for assessing food protein digestion

Authors	Experimental conditions	Analyzed objects
Maga J.A. et al [30]	Enzyme — trypsin; incubation temperature and time — 37 °C, 10 min	Sodium caseinate, defatted peanut meal, defatted cotton meal, protein concentrate of fish meal, soybean isolate
Vavak, D.L.R. [31]	Enzyme — trypsin -chymotrypsin; incubation temperature and time — 37 °C, 10 min	Purified dried grain protein
Hsu H.W. et al. [32]	Enzymes — pork trypsin (type IX, 14190 BAEE / mg protein), bovine chymotrypsin (type II, 60 U/mg powder), pork peptidase (III, 40 U/ g powder); incubation temperature and time — 37 °C, 10 min; control- sodium caseinate.	Soybean isolate; cotton meal; durum semolina; whole-grain white wheat meal, whole-lactose defatted whey; partly purified milk whey; standard caseins; soybean meal of different thermal processing (90, 70 and 20 PDI); soybean protein concentrate
Satterlee L.D. et al [33]	Enzymes — pork trypsin (type IX), pork peptidase (I), bovine a-chymotrypsin (type II), bacterial pronase P or E; incubation temperature and time — 37 °C, 20 min; control- sodium caseinate.	Soybean isolate, protein concentrate from fermented wheat, protein concentrate from corn
Pedersen B., Eggum B. O. [34]	Enzymes — pepsin (7192,100 mU/mg powder), pork trypsin (type IX, 14190 BAEE/mg protein), bovine chymotrypsin (type II, 47 U/ mg powder), pork peptidase (I, 22 U/ mg powder); Streptomyces griseus protease (4.4 U/mg powder); incubation temperature and time — 37 °C, 10 min; control- sodium caseinate.	Lyophilized beef, cod fillet, eggs

Therefore, the pH-stat test is mainly used to predict the digestibility of easily digestible sources of pure proteins with known data about average dissociation of α-amino groups and the number of peptide bonds. It is worth noting that from the beginning of the 1990s, the pH-stat method has been used only to assess feed ingredients for aquatic animals, which is linked with the simplicity of their digestive tract, in particular, the enzyme composition, as well as with the wide use of sources of easily digestible proteins, such as fish meal, for their feeding [36]. Up to now, all accumulated data obtained by the pH-stat method especially using purified enzymes extracted from the stomach and intestine of the targeted animals have been in agreement with the *in vivo* digestibility analyses on the targeted aquatic animals [37,38]. In conclusion, it is necessary to note that the average constant of casein dissociation and the number of peptide bonds are used in calculation of the degree of hydrolysis of the objects analyzed as standards to eliminate restrictions of the method [8].

It is worth noting that over the indicated period, no methods based on the changes in the pH of the medium were revealed in the national literature even in the case of studying feedstuff and feeding meal. In the Russian Federation, two state standards have been approved and used for studying the feedstuff digestibility. The first standard GOST R55987–20141 describes the method that includes incubation of a preliminary defatted and dried sample of feeding meal in the solution of pepsin in diluted hydrochloric acid at a temperature of 45 °C for 16 hours, filtration of the suspension and detection of the mass fraction of the dry residue on a filter in relation to the mass of the initial defatted and dried sample (digestibility of feeding meal) or mass fraction of nitrogen in the dry residue on a filter (protein digestibility) in relation to the mass of nitrogen in the initial defatted and dried sample. When the mass fraction of fat in feeding meal is less than 10%, the digestibility is determined without defatting. The document includes the description of differences in process temperature and duration, centrifugation regimes, expression of the result in percent. The second standard GOST 24230–802 includes the method for detection of a degree of the digestibility (dissolution) of the dry matter using enzymes pepsin and celloviridine also with the description of differences in process temperature and duration, centrifugation regimes, expression of the result in percent.

Despite a large number of *in vitro* methods for studying the product digestibility, the main of which are described in this paper, all of them have limitations and drawbacks. To solve this problem and within the framework of the investigation of wholesome properties of foods by their assessment in the digestive process, COST Action network was created in 2011. Since 2015, it has turned into the international research network uniting more than 440 scientists from 150 institutes in 45 countries of Europe, USA, Canada, Argentina, Australia, New Zealand and others [39]. Scientists from the INFOGEST network [40] are guided by particular goals such as: to harmonize conditions of *in vitro* digestion; develop a static model that would be easily adjusted and used for various objects and a large research community; have a possibility to compare the results of the investigations.

In 2019, the standardized protocol for *in vitro* investigation of the food product digestibility INFOGEST was published as a result of the large-scale work. Published for the first time in 2014, the method has acquired the status of Highly Cited Paper for agricultural sciences over the last years and was cited in journals included into Web of Science more than 650 times. The method is widely used by scientists worldwide with multiple goals for various food products and endpoints [40]. It is an easy-to-use static digestion method, in which samples of food products are subjected to sequential oral, gastric and intestinal digestion; while parameters such as electrolytes, enzymes, bile, dilution, pH and digestion duration are based on available physiological data. However, the method is not suitable for simulation of digestion kinetics [39,40]. The altered and improved digestion method INFOGEST 2.0 [41] allows avoiding problems linked with the initial method, such as inclusion of the oral phase and the use in gastric lipase. The method is widely used in assessment of endpoints that arise as a result of food product digestion by analysis of digestion products (for example, peptides/amino acids, fatty acids, simple sugars) and assessment of release of food microelements from the food matrix. The whole protocol can be completed within about 7 days including about 5 days that are necessary to determine the activity of enzymes of the gastric and intestinal digestion [42]. This protocol was also used by Russian scientists [43].

Therefore, methods for studying cleavage of different products and the creation of *in vitro* models is a dynamically developing field of knowledge (Figure 2).

Today, methods developed in the period from 1964 that include one or two digestion phases using several enzymes (pepsin/trypsin/pancreatin) as well as the recent methods that simulate the digestion processes in one, two or three sequential phases (oral, gastric and intestinal) with the use of multi-component mixtures of enzymes are applied depending on objects of a study and aims of an experiment [1].

These methods are widely used to assess the digestibility of proteins [42], lipids [44] and carbohydrates [45], allergenicity of proteins and their resistance during the digestion process [46,47,48,49], release and bioavailability of nutrients from complex and simple food matrices [50,51], to study interaction of various compounds with nutrients [50].

[9] GOST R55987–2014 "Feeds, raw material for mixed feeds. Method for determination of digestibility of feather meal in vitro". Moscow: Standartinfopm, 2020. — 11 p.

[2] GOST 24230–80 "Vegetable feeds. Method for determination of digestibility in vitro". Moscow: IPK Publishing House of Standards, 2003. — 4 p.

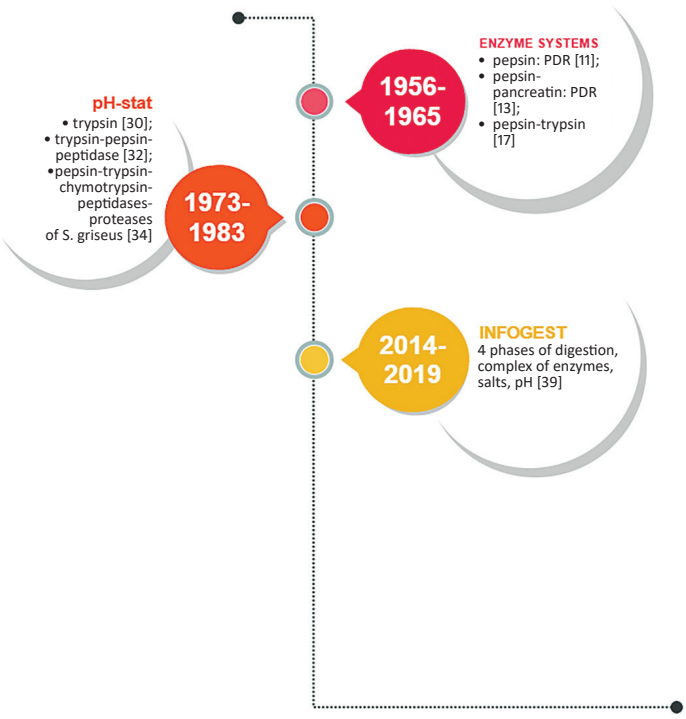

Figure 2. Evolution of *in vitro* static digestion models

The recent trend in the application of this method includes the study of the development and stability of bioactive molecules (including peptides) during digestion in the gastrointestinal tract [52,53,54], digestibility and bioavailability of phytochemical substances of plants and their metabolites with the antioxidant activities [55,56], bioavailability of pollutants to evaluate the health risks [57]; to assess viability of incapsulated beneficial microorganisms [58].

Conclusion

1. It was established that *in vitro* digestion models are promising and alternative options of *in vivo* experiments to simulate complex multi-stage physiological processes in the gastrointestinal tract that allow taking into account such factors as the presence and concentration of digestive enzymes, pH values in the gastric and intestinal phase, digestion duration, salt concentration and so on.
2. It was found that the used means for studying *in vitro* the food product digestibility include the assessment methods using enzymatic digestion that can be based on the calculation of nitrogen remained after digestion in relation to the initial total nitrogen (by the Dumas method, Kjeldahl method, spectrophotometric or chromatographic methods) and include additional stages such as filtration, centrifugation or exclusion by a molecule size, as well as methods (pH-stat, titration method) that are based on changes in pH of the reaction medium (occurring due to liberation of protons from cleaved peptide bonds in protein hydrolysis by digestive enzymes) and depend on the buffering capacity of the protein under study, which are mainly used for prediction of the digestibility of easily digestible sources of pure proteins with known data about average dissociation of α-amino groups and the number of peptide bonds.
3. It was revealed that the pH-stat methods are mainly used to assess the digestibility of feedstuff for aquatic animals, which is linked with the simplicity of their digestive tract, in particular, the enzyme composition, as well as with the wide use of sources of easily digestible proteins for their feeding, while methods for assessment of the digestibility by enzymatic digestion are widely used also for analysis of different food product types.
4. It was established that methods for assessment of the food product digestibility by enzymatic digestion have undergone different stages of evolution (since 1947) and have been used in the wide modification by inclusion of various enzymes (pepsin, trypsin, pancreatin, erepsin, etc.) as well as antimicrobial preparations into model systems, which allowed introducing concepts of pepsin-digest-residue (PDR) index (1956); pepsin pancreatin digest (PPD) index (1964); pepsin digest dialysate (PDD) (1989). Among these indices, the latter has several advantages compared to the other above mentioned indices such as the use of a simpler device, only one enzyme, modern equipment for analysis of amino acids, higher reproducibility and a possibility of computerized calculation. At the same time, the development of methods based on changes in pH of the medium began from 1973 when a close interrelation with the initial rate of protein hydrolysis under the action of trypsin was revealed with the following analysis of various combinations of enzymes (1977) and the study of a possibility to overcome method sensitivity to the buffering ability of protein samples (1983) and comparison with the results of the true protein digestibility in farm animals (1984).
5. Currently, in the Russian Federation and Republic of Belarus, several methods for *in vitro* detection of the food product digestibility have been developed and used, which allow detailed assessment of quality indicators of these products: with the use of mono- and multi-enzyme systems, the modified apparatus of MGUPB, the model system of the Institute for Meat and Dairy Industry and others, which characteristic features are a possibility to use dialysis bags, control of pH, parameters of proteolysis termination and precipitation of undigested protein. In addition, several state standards for studying the feedstuff digestibility are in force (GOST R55987–2014 and GOST 24230–80), which methods are based on incubation of a preliminarily defatted and dried sample in the solution of pepsin in diluted hydrochloric acid as well as detection of a degree of the digestibility of the dry matter using enzymes pepsin and celloviridine.
6. The INFOGEST (2019) method allows quite accurate reproduction of the conditions in the gastrointestinal

tract due to the sequential use of digestive enzymes in the physiological concentrations; simulation of a suitable environment for the action of enzymes (temperature, pH, presence of co-factors), duration of each phase that is close to the physiological (for humans) and removal of digestive products. In this connection it is widely used by scientists when studying food products.

REFERENCES

1. Lucas-González, R., Viuda-Martos, M., Pérez-Alvarez, J. A., Fernández-López, J. (2018). In vitro digestion models suitable for foods: Opportunities for new fields of application and challenges. *Food Research International*, 107, 423–436. https://doi.org/10.1016/j.foodres.2018.02.055
2. Bohn, T., Carriere, F., Day, L., Deglaire, A., Egger, L., Freitas, D. et al. (2018). Correlation between in vitro and in vivo data on food digestion. What can we predict with static in vitro digestion models? *Critical Reviews in Food Science Nutrition*, 58(13), 2239–2261. https://doi.org/10.1080/10408398.2017.1315362
3. Fedulova, L.V., Vasilevskaya, E.R., Kotenkova, E.A., Kalinova, E.A. (2018). Algorithm of in vitro assessment for products containing bioactive substances. *Vsyo o myase,* 6, 47–49. https://doi.org/10.21323/2071–2499–2018–6–47–49 (In Russian)
4. Shani-Levi, C., Alvito, P., Andrés, A., Assunçao, R., Barberá, R., Blanquet-Diot, S. et al. (2017). Extending in vitro digestion models to specific human populations: Perspectives, practical tools and bio-relevant information. *Trends in Food Science and Technology*, 60, 52–63. https://doi.org/10.1016/j.tifs.2016.10.017
5. Cottyn, B.G., De Boever, J.L., Vanacker, J.M. (1990). The estimation of nutritive value of dairy cattle feed. *Archiv für Tierernaehrung*, 40(10), 969–980. https://doi.org/10.1080/17450399009428448
6. Duskaev, G.K., Levakhin, G.I., Nurzhanov, B.S., Rysayev, A.F., Meshcheryakov, A.G. (2016). Results of researches on digestibility in vitro and in situ of developed feed additives. *Herald of Beef Cattle Breeding*, 4(96), 126–131. (In Russian)
7. Hristov, A.N., Bannink, A., Crompton, L.A., Huhtanen, P., Kreuzer, M., McGee, M. et al. (2019). Invited review: Nitrogen in ruminant nutrition: A review of measurement techniques. *Journal of Dairy Science*, 102(7), 5811–5852. https://doi.org/10.3168/jds.2018–15829
8. Bryan, D.D.S.L., Classen, H.L. (2020). In vitro methods of assessing protein quality for poultry. *Animals*, 10(4), Article 551. https://doi.org/10.3390/ani10040551
9. Golovko, E.N., Omarov, M.O., Slesareva, O.A. (2012). Correction of digestibility of protein for pigs at level of terminal ileum. *Processimg of the North Caucasus Scientific Research Institute of Animal Husbandry*, 1(1), 75–80. (In Russian)
10. Liberati, A., Altman, D.G., Tetzlaff, J., Mulrow, C., Gøtzsche, P.C., Ioannidis, J.P.A. et al. (2009). The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *Journal of Clinical Epidemology*, 62(10), e1-e34. https://doi.org/10.1016/j.jclinepi.2009.06.006
11. Eckfeldt, G.A., Sheffner, A.L., Spector, H. (1956). The pepsin-digest-residue (PDR) amino acid index of net protein utilization. *The Journal of Nutrition*, 60(1), 105–120. https://doi.org/10.1093/jn/60.1.105
12. Raghunath, M., Rao, B.S.N. (1984). Relationship between relative protein value and somein vitro indices of protein quality. *Journal of Biosciences*, 6(5), 655–661. https://doi.org/10.1007/BF02702707
13. Akeson, W.R., Stahmann, M.A. (1964). A Pepsin Pancreatin Digest index of protein quality evaluation. *The Journal of Nutrition*, 83(3), 257–261. https://doi.org/10.1093/jn/83.3.257
14. Kennedy, J.F., Noy, R.J., Stead, J.A., White, C.A. (1989). A new rapid enzyme digestion method for predicting in vitro protein quality (PDD index). *Food Chemistry*, 32(4), 277–295. https://doi.org/10.1016/0308–8146(89)90087–3
15. Owusu-Apenten, R. (2002). Food protein analysis: quantitative effects on processing (1st ed.). New York: CRC Press, 2002. https://doi.org/10.1201/9780203910580
16. Gauthier, S.F., Vachon C., Savoie L. (1986). Enzymatic conditions of an in vitro method to study protein digestion. *Journal of Food Science*, 51(4), 960–964. https://doi.org/10.1111/j.1365–2621.1986.tb11208.x
17. Pokrovskiy, A.A., Erganov, I.D. (1965). The attackability of food proteins by proteolytic enzymes in vitro. *Problems of Nutrition*, 3, 38–44. (In Russian)
18. Vasilevskaya, E.R., Kotenkova, E.A., Lukinova, E.A., Kalinova, E.A. (2017) Research methodology of *Sus scrofa* tissue extracts protein-peptide components. *Theory and practice of meat processing*, 2(3), 79–85. https://doi.org/10.21323/2414–438X–2017–2–3–79–85
19. Slonimskii, G.L., Braudom E. E., Ertanov, I.D. et al. (1970). The atackability of proteins in the composition of new foods by proteolytic enzymes. *Problems of Nutrition*, 6, 25–31. (In Russian)
20. Volova, T.G., Barashkov, V.A. (2010). Characteristics of proteins synthesized by hydrogen-oxidizing microorganisms. *Applied Biochemistry and Microbiology*, 46(6), 574–579. https://doi.org/10.1134/S0003683810060037
21. Dorikhovich, A.N., Ostrik, A.S., Tsyrik, N.A. (1987). Exploded cereals are a new type of non-traditional raw materials in confectionery production. *Food Industry*, 2, 34–35. (In Russian)
22. Kovalev, N. I., Ivanov, E. L., Stepanov, V. N. (1974). The effect of culinary processing on the vitamin content and trace elements in seeds of leguminous plants. [Vliianie kulinarnoi obrabotki na soderzhanie vitaminov i mikroelementov v semenakh bobovykh]. *Die Nahrung*, 18(5), 511.
23. Tsyladze, E.A., Egiazaryan, S.A. (1975). Digestibility of meat proteins of herbivorous fish by proteolytic enzymes. *Trudy VNIRO*, 105, 128–130. (In Russian)
24. Krylova, V.B., Gustova, T.V. (2016). Aspects of the destructive changes in the main nutrients of canned meat in pieces «stewed beef of the top grade» under the non-normative temperature and humidity conditions of storage. *Theory and practice of meat processing*, 1(3), 21–34. https://doi.org/10.21323/2414–438X-2016–1–3–21–34 (In Russian)
25. Rudakova, T. (2017). Enzymetic method for determination of biological value of dairy products with grain ingredients for baby nutrition. *Grain Products and Mixed Fodder's*. https://doi.org/10.15673/gpmf.v17i2.525 (In Ukrainian)
26. Antipova, L.V., Glotova, I.A., Rogov, I.A. (2004). Methods of research of meat and meat products. Moscow: Kolos, 376. (In Russian)
27. Lipatov, N.N., Yudina, S.B., Lisitsyn, A.B. (1994). Improved device and methodology for determining the digestibility of proteins "in vitro". *Problems of Nutrition*, 4, 43–44. (In Russian)
28. Bologa, M.K., Sprinchan, E.G., Bologa, A.M. (2008). Isolation of lactulose product and protein-mineral concentrate. *Electronic processing of materials*, 5, 79–84. (In Russian)
29. Savich, N.M., Zholdaspaeva, G.M. (1993) Proteolysis of prolamine proteins in grain and its significance in biochemistry and biotechnology. Biotechnology in crop breeding. Almaty: Izd. Kazahskoj akad. sel'skohozyajstvennyh nauk. 133. (In Russian)
30. Maga, J.A., Lorenz, K., Onayemi, O.I. (1973). Digestive acceptability of proteins as measured by the initial rate of in vitro proteolysis. *Journal of Food Science*, 38(1), 173–174. https://doi.org/10.1111/j.1365–2621.1973.tb02806.x
31. Peiretti P. G. (2020). In Vitro Digestibility in Animal Nutritional Studies. MDPI: Basel, Switzerland. 378. https://doi.org/10.3390/books978–3–03936–460–2.
32. Hsu, H.W., Vavak, D.L., Satterlee, L.D., Miller, G.A. (1977). A multienzyme technique for estimating protein digestibility. *Journal of Food Science*, 42(5), 1269–1273. https://doi.org/10.1111/j.1365–2621.1977.tb14476.x
33. Satterlee, L.D., Kendrick, J.G., Marshall, H.F., Jewell, D.K., Ali, R.A., Heckman, M.M. et al. (1982). In vitro assay for predicting protein efficiency ratio as measured by rat bioassay: collaborative study. *Journal of the Association of Official Analytical Chemists*, 65(4), 798–809. https://doi.org/10.1093/jaoac/65.4.798
34. Pedersen, B., Eggum, B.O. (1983). Prediction of protein digestibility by an in vitro enzymatic pH-stat procedure. *Zeitschrift Für Tierphysiologie Tierernährung Und Futtermittelkunde*, 49 (1–5), 265–277. https://doi.org/10.1111/j.1439–0396.1983.tb00808.x
35. Clunies, M., Leeson, S. (1984). In vitro estimation of dry matter and crude protein digestibility. *Poultry Science*, 63(1), 89–96. https://doi.org/10.3382/ps.0630089
36. Tibbetts, S.M., Milley, J.E., Ross, N.W., Verreth, J.A.J., Lall, S.P. (2011). In vitro pH-Stat protein hydrolysis of feed ingredients for Atlantic cod, Gadus morhua. 1. Development of the method.

Aquaculture, 319(3–4), 398–406. https://doi.org/10.1016/j.aquaculture.2011.07.013
37. Mirzakhani, M. K., Kenari, A.A., Motamedzadegan, A. (2018). Prediction of apparent protein digestibility by in vitro pH-stat degree of protein hydrolysis with species-specific enzymes for Siberian sturgeon (Acipenser baeri, Brandt 1869). *Aquaculture*, 496, 73–78. https://doi.org/10.1016/j.aquaculture.2018.07.014
38. Wang, R., Mohammadi, M., Mahboubi, A., Taherzadeh, M.J. (2021). In-vitro digestion models: a critical review for human and fish and a protocol for in-vitro digestion in fish. *Bioengineered*, 12(1), 3040–3064. https://doi.org/10.1080/21655979.2021.1940769
39. Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Balance, S. et al. (2019) INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols,* 14(4), 991–1014. https://doi.org/10.1038/s41596–018–0119–1
40. Colombo, R., Ferron, L., Frosi, I., Papetti, A. (2021). Advances in static in vitro digestion models after COST action Infogest consensus protocol. *Food and Function*. 12(17), 7619–7636. https://doi.org/10.1039/D1FO01089A
41. Ariëns, R.M.C., Bastiaan-Net, S., van de Berg-Somhorst D.B.P.M., El Bachrioui, K., Boudewijn, A., van den Dool R. T.M. et al. (2021). Comparing nutritional and digestibility aspects of sustainable proteins using the INFOGEST digestion protocol. *Journal of Functional Foods*, 87, Article 104748. https://doi.org/10.1016/j.jff.2021.104748
42. Mansilla, W.D., Marinangeli, C.P.F., Cargo-Froom, C., Franczyk, A., House, J.D., Elango, R. et al. (2020). Comparison of methodologies used to define the protein quality of human foods and support regulatory claims. *Applied Physiology, Nutrition, and Metabolism*, 45(9), 917–926. https://doi.org/10.1139/apnm-2019–0757
43. Vasilevskaya, E.R., Akhremko, A.G., Gryzlova, A.S., Ivanova, E.A. (2019). In vitro methodology for digestion and absorption of food proteins study. *Vsyo o myase*, 6, 42–44. https://doi.org/10.21323/2071–2499–2019–6–42–44 (In Russian)
44. Giang, T.M., Gaucel, S., Brestaz, P., Anton, M., Meynier, A., Trelea I. C. et al. (2016). Dynamic modeling of in vitro lipid digestion: Individual fatty acid release and bioaccessibility kinetics. *Food Chemistry*, 194, 1180–1188. https://doi.org/10.1016/j.foodchem.2015.08.125
45. Elia, M, Cummings, J.H. (2007). Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates. *European Journal of Clinical Nutrition*, 61, S40-S74. https://doi.org/10.1038/sj.ejcn.1602938
46. Astwood, J.D., Leach, J.N., Fuchs, R.L. (1996). Stability of food allergens to digestion in vitro. *Nature Biotechnology*, 14(10), 1269–1273. https://doi.org/10.1038/nbt1096–1269
47. Fu, T.-J. (2002). Digestion stability as a criterion for protein allergenicity assessment. *Annals of the New York Academy of Sciences*, 964(1), 99–110. https://doi.org/10.1111/j.1749–6632.2002.tb04135.x
48. Ladics, G.S., Selgrade, M.K. (2009). Identifying food proteins with allergenic potential: evolution of approaches to safety assessment and research to provide additional tools. *Regulatory Toxicology and Pharmacology*, 54(3SUPPL), S2-S6. https://doi.org/10.1016/j.yrtph.2008.10.010
49. Fernandez, A., Mills, E.N.C., Koning, F., Moreno, F.J. (2019). Safety assessment of immune-mediated adverse reactions to novel food proteins. *Trends in Biotechnology*, 37(8), 796–800. https://doi.org/10.1016/j.tibtech.2019.03.010
50. Norton, J.E., Espinosa, Gonzalez Espinosa, Y., Watson, R.L., Spyropoulos, F., Norton, I.T. (2015). Functional food microstructures for macronutrient release and delivery. *Food and Function*, 6(3), 663–678. https://doi.org/10.1039/c4fo00965g
51. Silva, V., Jayasinghe, M.A., Senadheera, S.A., Ranaweera, K.K.D.S. (2020). Determination of macronutrient compositions in selected, frequently consumed cereals, cereal based foods, legumes and pulses prepared according to common culinary methods in Sri Lanka. *Journal of Food Science and Technology*, 57(3), 816–820. https://doi.org/10.1007/s13197–019–04085-x
52. Capriotti, A.L., Cavaliere, C., Piovesana, S., Samperi, R., Laganà, A. (2016). Recent trends in the analysis of bioactive peptides in milk and dairy products. *Analytical and Bioanalytical Chemistry*, 408(11), 2677–2685. https://doi.org/10.1007/s00216–016–9303–8
53. Bechaux, J., Gatellier, P., Le Page, J.-F., Drillet, Y., Sante-Lhoutellier, V. (2019). A comprehensive review of bioactive peptides obtained from animal byproducts and their applications. *Food and Function*, 10(10), 6244–6266. https://doi.org/10.1039/C9FO01546A
54. Wojtunik-Kulesza, K., Oniszczuk, A., Oniszczuk, T., Combrzyński, M., Nowakowska, D., Matwijczuk, A. (2020). Influence of in vitro digestion on composition, bioaccessibility and antioxidant activity of food polyphenols — A non-systematic review. *Nutrients*, 12(5), Article 1401. https://doi.org/10.3390/nu12051401
55. Tarascou, I., Souquet, J.M., Mazauric, J.P., Carrillo, S., Coq, S., Canon, F. et al. (2010). The hidden face of food phenolic composition. *Archives of Biochemistry and Biophysics*, 501(1), 16–22. https://doi.org/10.1016/j.abb.2010.03.018
56. Delimont, N.M., Rosenkranz, S.K., Haub, M.D., Lindshield, B.L. (2017). Salivary proline-rich protein may reduce tannin-iron chelation: a systematic narrative review. *Nutrition and Metabolism*, 14(1), Article 47. https://doi.org/10.1186/s12986–017–0197-z
57. Cui, X.-Y., Xiang, P., He, R.-W., Juhasz, A., Ma, L.Q. (2016). Advances in in vitro methods to evaluate oral bioaccessibility of PAHs and PBDEs in environmental matrices. *Chemosphere*, 150, 378–389. https://doi.org/10.1016/j.chemosphere.2016.02.041
58. Audisio, M.C. (2017) Gram-positive bacteria with probiotic potential for the Apis mellifera L. honey bee: the experience in the northwest of Argentina. *Probiotics and Antimicrobial Proteins*, 9(1), 22–31. https://doi.org/10.1007/s12602–016–9231–0

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