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Application of in vitro human digestion models in the field of nutrition science

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1. SUMMARY

Simulating digestion that takes place in the gastrointestinal tract is a widely used procedure in various fields of food and nutrition science, as well as in the pharmaceutical industry, as it is less costly and labor-intensive in human clinical trials and animal experiments, while not raising ethical issues either. The process of digestion converts nutrients and bioactive compounds in foods into physiologically active compounds. In vitro digestion models have been proved to be effective tools for the complete understanding and observation of the complex transformation processes that occur during digestion. Of course, in vitro studies cannot be substitutes for in vivo experiments, but they play a key role in the pre-screening, ranking and classification of samples before in vivo studies. In this article, we show why in vitro digestion models are important, how and in what systems they can be used. Several in vitro digestion models have been developed in static, dynamic and semidynamic forms. In this summary, we present the unified static model developed within the framework of the COST INFOGEST program, a unified, convention-based protocol for in vitro digestion of food samples prepared with the involvement of more than 200 researchers from 32 countries. The application of the unified model allowed the comparison of the results of different research groups.

2. Introduction and literature review

2.1. Digestion and absorption in a nutshell

During neurohormonally regulated **[1]** digestion, food is broken down by a specific series of mechanical and biochemical processes (***Figure 1.***). Nutrients, which are bodybuilding substances and energy sources made up from macromolecules (proteins, lipids, carbohydrates), as well as vitamins, minerals and trace elements enter the blood and lymphatic circulation through the intestinal barrier through the process of absorption **[2]**.

Digestion begins in the mouth and then continues in the spaces of the gastrointestinal tract (stomach, duodenum, jejunum). 90% of the nutrients is absorbed in the small intestine (*jejunum, ileum*) and the rest is absorbed in the stomach (*ventriculus, gaster*) and the large intestine (*colon*). Absorption is aided by the circular folds of the small intestine mucosa and the submucosal layer, as well as the intestinal villi, protrusions of the absorbing epithelial cells (*microvilli*, brush border). The most important supporters of luminal digestion are enzymes found in fluids secreted by the gastrointestinal glands, which are catalysts for the degradation of macromolecules, as well as brush border enzymes in the plasma membrane of microvilli. Moving toward the small intestine, the small intestinal fluid, mixed with the pancreatic juice, neutralizes the acidic chyme (stomach content) coming from the stomach, which is needed for the further hydrolysis and absorption of nutrients.

Bile secretions released into the small intestine promote hydrolysis by pancreatic lipase through the emulsification of fats. Bile acids also play an

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important role in absorption by solubilizing digestion products in the form of micelles **[3]**.

2.2. Importance of in vitro digestion models

Digestion is a complex system of biochemical processes whose physiological conditions are very difficult to reproduce accurately. The most accurate answers to questions related to nutrition can be obtained from human studies, yet simulated *in vitro* gastrointestinal digestion is widely used in food and nutrition science, as the study of *in vivo* digestion of foods (clinical trials, animal experiments) is resource-intensive and often ethically questionable **[7, 8]**. On the other hand, the physiological processes of animal models do not always correspond to the physiological processes taking place in the human body **[9]**, and the interpretation of the results and the reproducibility of the experiments may be hampered by the large differences between the individuals participating in the experiments **[10, 11]**.

The advantages of *in vitro* methods over *in vivo* studies are that they are cost-effective, less labor-intensive, have no ethical constraints, they are faster, easier to manage, less risky and involve less responsibility **[11, 12]**. They focus only on the mechanism under study, provide controlled conditions and easy sampling possibilities, can be used for parallel measurements of a large number of samples for screening purposes. Tests can be reproduced easily by checking and standardizing experimental parameters **[11]**. They include the oral, gastric and small intestinal phases and, occasionally, the fermentation section of the colon. *In vitro* methods attempt to realistically mimic *in vivo* physiological conditions, enzymatic and microbiological digestion processes through the gastrointestinal tract, taking into account the presence and concentrations of digestive enzymes, pH, digestion time, as well as salt concentration. *In vitro* studies are not substitutes for *in vivo* experiments, but can be considered as a tool to facilitate the pre-screening, ranking and classification of a large number of samples **[13]**. Thus, with the help of pre-screening for animal feeding experiments, fewer experimental animals are needed, and in the case of experiments performed on human cells and microbiota (colon model) the absorption and the release of different metabolites can be modeled.

2.3. Applicability of in vitro digestion models

In the case of foods, the sum of the nutritional values of each component does not provide a complete picture of the true nutritional value available to the body. *In vitro* digestion provides an opportunity to test the amount of components released from the food matrix during its passage through the gastrointestinal tract using analytical methods, i.e., the bioactivity and bioaccessibility of the food, as well as the bioavailability to the body at the site of absorption **[11]**.

Using these models, we seek to answer the question whether these degraded components retain their biological activity, and if so, how this bioactive form is utilized: is it absorbed or can it be utilized in an active form.

In practice, following *in vitro* digestion, the digested material is centrifuged and/or filtered because of the presence of undigested constituents that form a colloidal dispersion (ultrafiltration), it is dialyzed and the amount of bioavailable components in the resulting supernatant is determined. However, the use of dialysis and the measurement of solubility (through centrifugation and/or filtration) can lead to different bioavailability values even for the same sample **[14, 15]**. Nor can it be generalized that higher values are obtained by dialysis or by measuring solubility **[14, 16, 17]**. It should also be taken into account that not all soluble or dialyzable components are absorbed by the body, but components that are not part of the bioavailable fraction under real conditions can also diffuse through the semipermeable membrane **[11, 18, 19]**.

Knowledge of bioavailability can be important in food development when selecting the right processing technology. Other production technologies may be more advantageous if they are selected on the basis of the bioavailability of the given food component and rather than the degree of degradation **[20]**. Studies are useful in comparing the effects of food processing, as well as in ranking different protein sources, and also in examining factors that potentially affect digestion **[19]**. *In vitro* studies can also be used to estimate the glycemic effect of foods **[19, 21, 22]**, to assess the safety of genetically modified products **[11, 23]**, or to characterize the allergenic potential of proteins **[8]**.

2.4. Characterization and grouping of in vitro digestion models

During the design of the experiments, an *in vitro* model appropriate for the purpose of the study should be selected, even the modeling of individual digestion phases may be warranted. Method selection is aided by considering the types of samples the given digestion model have been previously validated for. The majority (~89%) of digestion models (***Table 1***) are static, but models can also be dynamic or semi-dynamic **[24]**.

In **static modeling**, physical processes (chewing, shearing, mixing, hydration, variable parameters, such as time) can only be imitated to a limited extent. In contract to the dynamic model, the complexity of the gastrointestinal tract cannot be modeled statically.

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Dynamic models can be applied more efficiently in physiological studies than static ones **[23]**. They are much more complex and their operation requires a large amount of samples **[18]**. Their application is limited because their use requires more money, labor and time than static models **[18, 23]**.

Digestion is a dynamic process, with food that enters the gastrointestinal tract moving at varying rates depending on its structure, rheology and other properties, while physicochemical conditions (varying pH, ionic strength, digestive enzyme concentrations) also affecting its efficiency. These process changes are taken into account by dynamic models.

Dynamic models can also be used to study processes that depend on the reaction time (e.g., changes in bioavailability as a function of the food matrix, nutrient interactions) due to their ability to more accurately model the kinetics of the biochemical reactions taking place during digestion, since samples can be taken from the compartments of the models (which mimic the different sections of the gastrointestinal tract) as the digested material passes through them.

Semi-dynamic digestion protocols represent a trade-off between simple static models and more complex, more costly dynamic systems **[8]**. In these cases, the gastric section is modeled dynamically, which is combined with a statically modeled small intestinal phase **[8]**.

2.4.1. Brief characterization of widely used dynamic *in vitro* human digestion models

In practice, several dynamic *in vitro* digestion models have emerged, some of the most important of which are summarized in ***Table 1***. These automated systems consist of one or more units that can model the stomach alone, the stomach-small intestine tract or the stomach-small intestine-large intestine tract. The structures of the systems are different, which is illustrated here by two more complex models (presented through the processes of digestion and fermentation) (***Figures 2 and 3***).

The pioneers of simulated dynamic human digestion models are the TIM digestion models (TNO (gastro-) Intestinal Models) developed by the TNO (Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek) (***Figure 2***), which have been widely validated in the study of food digestibility, nutrient bioavailability, as well as the fate and efficacy of functional ingredients **[25]**. TIM-1 is a stomach-small intestine model, while TIM-2 models the colon. A simpler version of TIM-1 is the tiny-TIM system, which mimics the small intestine with only one unit, allowing for higher throughput **[26]**. Although the gastric compartment of the TIM-1 system meets the requirements of many *in vitro* digestion experiments, supported by the bioavailability results of nutrients, bioactive components and drugs, to answer certain specific questions (which may be related to the interaction between food and gastric behavior), a more advanced stomach model have to be developed **[27]**. The TIMagc (TIM **a**dvanced **g**astric **c**ompartment) system, a more advanced gastric model compared to the gastric section of the TIM model, consists of 3 compartments that mimic the *corpus ventriculi,* and the proximal and distal parts of the horizontal section following the stomach body (*antrum*) **[27]**.

The SHIME model (Simulator of Human Intestinal Microbial Ecosystem) is a system comprising 5 reactors (stomach-small intestine, ascending, transverse and descending colon sections, ***Figure 3***), available at the Universities of Ghent (Belgium) and Wageningen (the Netherlands) **[42, 43]**. One variant is M-SHIME (Mucus-SHIME), which models the microbial colonization of the mucosa by the microbiota. Application of the SHIME model with the so-called HMI™ module (**H**ost-**M**icrobe **I**nteraction Module) allows the long-term study of the interaction of the microbiota with host cells over 48 hours **[11, 33, 44]**.

In dynamic models, in most cases, differential gastric emptying (with the exception of, for example, TIM, SHIME) for various food consistencies (solid, liquid), dynamic pH profile and experimental duration can be controlled, and the peristaltic movement in the stomach can be mimicked in some cases (TIM, HGS, DGM, SIMGI). For some dynamic models (e.g., SHIME, TIM), food must be subjected to drastic physical grinding (ultra-turrax homogenizer, blender, etc.) before *in vitro* digestion, instead of the modeling of chewing, to avoid the clogging of the tubes of the system **[32, 33]**. The disadvantage of this is that the structure of the food is not taken into account, although the viscosity and particle size of the digested material are important features that influence the release of the components to be tested during digestion **[8, 18, 33]**.

In some systems (e.g., ESIN, TIM, ARCOL, SHIME, DIDGI), dialysis has also been attempted to remove from the system water-soluble components that form colloidal dispersions with undigested, insoluble materials **[45]**. In the case of colon models (TIM-2, ARCOL, SHIME), for example, it is important to remove microbial metabolites, as they may inhibit the growth and/or further fermentation of bacteria. For TIM-1, ESIN and DIDGI, negative feedback inhibition of enzymes by reaction products is prevented by dialysis. Dialysis also affects the composition of the digested material, thereby affecting viscosity and pH.

Certain dynamic model systems can be adjusted to simulate digestive systems corresponding to that of the age (e.g., adult, infant: e.g.,. TIM-1, tiny-TIM, DIDGI, SHIME) and health status (healthy, sick, obese, suffering from IBS: e.g.,. TIM-1, TIMagc, DGM, SHIME) of the given target group. Colon simulating units in the TIM-2, SIMGI, ARCOL, Gibson, SHIME

models are usually inoculated with fecal inoculum **[11, 33, 40]**. In the case of ESIN, system units representing the small intestine made inoculation with a sample of human feces and its maintenance under anaerobic conditions possible **[33]**. The ARCOL model is the first fermentation model that maintains anaerobiosis inside the fermenter through the metabolic activity of the microbiota, without having to flush it with N_2 or CO_2, as in most *in vitro* models simulating the colon. However, the disadvantage of *in vitro* intestinal fermentation systems is that they are not capable of sufficiently realistic modeling of host effects (e.g., absorption, host microbiota interactions) unless the system is combined with *in vitro* intestinal cell line models (e.g., Caco-2 human colon cancer epithelial cells) **[33, 46]**.

2.5. Detailed presentation of the simulated human in vitro digestion model used in NAIK ÉKI, based on the common agreement proposed by different European countries

In 2011, the so-called COST INFOGEST FA1005 Action (2011-2014) titled *European Cooperation in Science and Technology: Improving health properties of food by sharing our knowledge on the digestive process, Project No. Food & Agricultural 1005* was launched, whose task was to build a network of leading European institutes, with the common goal to fully understand the digestion of foods and the degradation processes that take place in the gastrointestinal tract. Members of the network included researchers with different professional backgrounds, such as food engineers, gastroenterologists, nutrition science experts, immunologists and food professionals, so the topic could be approached from several perspectives. The Hungarian scientific community was represented in the COST Infogest Action by staff members of our institute (Dr. Éva Gelencsér, Dr. Krisztina Takács, Emőke Néméthné Dr. Szerdahelyi, Dr. András Nagy). The main objective of the project was to characterize raw materials and processed foods for better nutrient utilization, and to assess how the occurrence and stability of beneficial food components in the gastrointestinal tract varies depending on the processing method and the nature of the food matrix. In addition, the task included the establishment of *in vitro, in vivo, in silico* digestion models, their comparison, and the study of health effects (e.g., allergies).

In order to achieve the objectives of the project, and in the possession of up-to-date expertise regarding human digestion, to explore the relationship between *in vitro* and *in vivo* measurements, it was necessary to harmonize and standardize published *in vitro* digestion models **[11, 47]**. Different model systems used different conditions, which made it impossible to compare the results obtained in different digestion studies, and this could lead to contradictory conclusions **[23, 24]**.

A solution to this is presented by the so-called INFOGEST *in vitro* human digestion model standardized on the basis of the combined versions of several *in vitro* digestion protocols **[23, 45]**, which models the digestion of healthy adults through the oral-stomach-small intestine phases **[48]**.

The harmonized protocol takes into account the advantages and disadvantages of the digestion models used so far, in order to be able to approach real conditions as closely as possible (***Figure 4***).

The developed INFOGEST model took into account, among other things, the fact that the activity of enzymes purchased from different companies or available from different batches may be different, thus, the results obtained by enzymatic degradation may also differ (which was also proved by the results of proficiency tests). A novelty of the protocol is the enzymatic degradation takes place under physiologically appropriate conditions, with a given activity. The amount of enzyme required for the assay can be determined by standard activity measurements, thereby improving reproducibility and the comparability of the results **[50]**.

It was also taken into account that different pH, mineral content, ionic strength, digestion time and enzyme affect the measured enzyme activity, which may cause differences in the results. The effect of other parameters was also investigated, such as the amount/presence of phospholipids, individual enzymes (stomach lipase) and emulsifiers, or mixtures of thereof (e.g., pancreatin, bile salts), as well as the food to digestive juices ratio, and these parameters were also standardized.

In practical application, reaction times of 2 minutes in the mouth, 2 hours in the stomach and also 2 hours in the small intestine has been recommended at 37 °C. Appropriate physiological conditions are ensured by artificial digestive juices consisting of an electrolyte stock solution, enzymes, $CaCl_2$ and water. The simulated saliva has a pH of 7.0, the simulated gastric fluid has a pH of 3.0 and the simulated small intestine fluid has a pH of 7.0. Simulated digestive juices are added to the mouth/stomach/small intestine contents in a constant ratio (50:50 v/v) during each digestion phase, and the activity of the digestive enzymes and the concentration of the bile extract used in the small intestinal fluid (10 mM) are also standardized according to the assumed physiological conditions (e.g., alpha-amylase 75 U/ml, pepsin 2000 U/ml, pancreatin 100 TAME U/ml). During the small intestinal phase, the amount of pancreatin is usually determined by trypsin activity **[45]**. If, based on the objectives, lipid or carbohydrate digestion testing is of greater importance or enzyme activity needs to be regulated precisely, individual enzymes (trypsin, chymotrypsin, α-amilase, lipase and colipase) may be used instead of pancreatin **[18, 23, 45]**. The disadvantage of using individual enzymes

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is that pancreatic proteases such as elastase and carboxypeptidase, which may play a key role in the study of certain bioactive peptides **[19]**, are omitted during the implementation of the protocol **[51]**.

It should be noted that the INFOGEST protocol does not model the degradation process by brush border membrane hydrolases **[48]**. The reason for this is that these enzymes are not commercially available and we do not have sufficient knowledge of their operation **[8, 23]**. Nowadays, however, studies have been published that used porcine jejunum brush border enzymes in addition to those steps required by the INFOGEST protocol **[48, 52, 53]**. Some research groups have replaced brush border glycosidases with fungal amyloglucosidases **[54, 55, 56]**.

The shortcomings of the INFOGEST method are revealed during the analysis of the various food components. For example, it is necessary for lipophilic components (e.g., carotenoids, plant sterols), among other things, to perform method development that ensures the extraction of lipophilic components embedded in micelles, since cells actually take up lipophilic components arranged in micelles. This way, their destiny can be traced and their quantity can be measured reliably **[13, 24, 57]**. So far, little is known about the effect of factors that influence micelle formation (mixing intensity of bile salts, lipase, lipids and digested material), standardization is not yet possible **[19, 23]**.

When modeling the small intestine, the presence of light and/or oxygen may affect the phytochemicals (e.g., carotenoids, polyphenols) and micronutrients (e.g., iron ions) in the sample **[18, 23, 58]**. The intestinal microbiota also plays a role in the bioactivation and metabolism of phytochemicals **[18, 23]**. For example, gastrointestinal bacteria affect the chemical binding of mercury (the occurrence of the more dangerous methyl mercury) and its bioavailability **[59, 60]**. A better understanding of the effects of light, oxygen and the microbiota may help to establish *in vitro - in vivo* correlations, as well as standardization in the case of specific studies that may be influenced by these factors **[23]**.

During the measurement of bioavailability and prediction studies, it is recommended to separate the different components from the undigested fraction based on the INFOGEST model, which can be achieved by solubility (centrifugation and/or filtration) or dialyzability studies **[19, 23, 45]**. Incorporating dialysis into an *in vitro* digestion model would be important not only for the measurement of bioavailability, but also because, on the one hand, products that may inhibit digestive enzymes will accumulate **[18]** and, on the other hand, it is necessary if the digested material is to be used for cell culture based **[61]** or microbiological **[62]** studies. The parameters of the dialysis used in *in vitro* digestion models are very diverse, their standardization is also necessary.

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