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A proposed framework to establish *in vitro–in vivo* relationships using gastric digestion models for food research†

Joanna Nadia, [a] Debashree Roy, [a] Carlos A. Montoya, [a,b] Harjinder Singh, [a] Alejandra Acevedo-Fani [a] and Gail M. Bornhorst *[a,c]

In vitro digestion methods have been utilized in food research to reduce *in vivo* studies. Although previous studies have related *in vitro* and *in vivo* data, there is no consensus on how to establish an *in vitro–in vivo* relationship (IVIVR) for food digestion. A framework that serves as a tool to evaluate the utility and limitations of *in vitro* approaches in simulating *in vivo* processes is proposed to develop IVIVRs for food digestion, with a focus on the gastric phase as the main location of food structural breakdown during digestion. The IVIVR consists of three quantitative levels (A, B, and C) and a qualitative level (D), which relate gastric digestion kinetic data on a point-to-point basis, parameters derived from gastric digestion kinetic data, *in vitro* gastric digestion parameters with *in vivo* absorption or appearance parameters, and *in vitro* and *in vivo* trends, respectively. Level A, B, and C IVIVRs can be used to statistically determine the agreement between *in vitro* and *in vivo* data. Level A and B IVIVRs can be utilized further evaluate the accuracy of the *in vitro* approach to mimic *in vivo* processes. To exemplify the utilization of this framework, case studies are provided using previously published static and dynamic gastric *in vitro* digestion data and *in vivo* animal study data. Future food digestion studies designed to establish IVIVRs should be conducted to refine and improve the current framework, and to improve *in vitro* digestion approaches to better mimic *in vivo* phenomena.

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1. Introduction

With increasing prevalence and ease of access to information about food and health, consumers are more cautious about the health impacts of the food they consume. This widens the focus of food research and development from improving the sensory experience during food consumption to the physiological responses and health benefits of foods.[1] Increasing health benefits from foods can be achieved by understanding the food digestion process, starting from the sensory perception and initiation of structural breakdown in the mouth (oral phase), further physical and biochemical digestion in the stomach (gastric phase), biochemical digestion in the small intestine (small intestinal phase) followed by nutrient absorption and dietary fiber fermentation in the distal small intestine, and the fermentation of the unabsorbed materials in the large intestine (large intestinal phase).[2–5]

The nutrient release, absorption, and bioavailability from foods have been linked to structural changes during the digestion process, highlighting the importance of food structure and its evolution after consumption on physiological outcomes.[6] *In vitro* digestion models have become a valuable tool to study the structural changes of food during digestion, which has resulted in a growing number of studies focusing on *in vitro* food digestion.[7–9] In addition to these *in vitro* studies, previous *in vivo* studies have suggested gastric digestion as a rate-limiting step to the digestion and nutrient bioavailability in the small intestine, indicating the importance of the stomach in the overall digestion processes.[10–13] As a result, numerous *in vitro* gastric digestion approaches have been developed and used extensively to predict changes of foods in the *in vivo* stomach.

Currently there are various *in vitro* gastric digestion models and protocols in the literature, which have features and parameters that are derived from *in vivo* data with the expectation to simulate physiological conditions. However, considering the complexity of physiological responses that regulate *in vivo*

[a]*Riddet Institute, Massey University, Private Bag 11222, Palmerston North, New Zealand. E-mail: gbornhorst@ucdavis.edu*
[b]*Smart Foods & Bioproducts, AgResearch, Te Ohu Rangahau Kai Facility, Palmerston North 4474, New Zealand*
[c]*Department of Biological and Agricultural Engineering, University of California, Davis, CA, USA*

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gastric digestion, the use of certain digestion parameters based on available *in vivo* data in an *in vitro* study may not guarantee an accurate simulation of *in vivo* phenomenon.[8,14–16] Therefore, it is important to identify how data generated from *in vitro* gastric digestion models are related to *in vivo* gastric digestion data, which is useful to evaluate the performance and limitations of the *in vitro* approach used.

Various quantitative and qualitative methods to identify relationships between *in vitro* and *in vivo* food digestion data, or *in vitro–in vivo* relationships (IVIVR), have been reported in the literature as an attempt to validate *in vitro* digestion models.[8,17–22] However, there has been no consensus on how an IVIVR should be established in food research. There are no clear guidelines on how an IVIVR can be utilized to interpret the limitations of *in vitro* digestion, as well as to improve *in vitro* approaches to better mimic physiological outcomes. Guidelines on IVIVR establishment for food research would be useful in the translation of *in vitro* food digestion data to certain health benefits or physiological effects, which is likely the future direction of food digestion research. Although currently the use of *in vitro* studies for food labelling to support food nutrition- and health-related claims has not been regulated, the increasing use of *in vitro* digestion models may be accompanied with regulatory requirements in the future to inform consumers.[17,23] This suggests the need for establishing a framework for developing IVIVRs for food digestion research that focuses on the gastric phase as the main location of structural transformation during digestion. Such a framework will be useful to determine specific relationships between *in vitro* digestion methods with *in vivo* digestion processes, as well as to identify limitations in mimicking the complex physiological aspects of *in vivo* digestion processes *in vitro*.

The closest framework to an IVIVR for food digestion was the food breakdown classification system (FBCS) framework proposed by Bornhorst *et al.*[17] In the FBCS framework, the likelihood of similarity between *in vitro* and *in vivo* gastric digestion of solid foods was classified according to the initial hardness and rate of softening of foods during *in vitro* gastric digestion. However, foods are not always consumed in solid form and a relationship to *in vivo* data was not directly established in that work. There is an opportunity to develop a framework to relate *in vitro* and *in vivo* data that can be applied to any type of food without restrictions on physical form, and without being limited by a specific digestion model or method, which will help researchers in food area to evaluate and improve their *in vitro* digestion approaches. Here, an IVIVR framework is proposed for food digestion with a focus on the gastric phase. The framework was adapted from the *in vitro–in vivo* correlation practices in the pharmaceutical field.[24] Case studies are provided using previously published *in vitro* and *in vivo* data to demonstrate the application of the IVIVR framework, selected works from the literature are reviewed, a description is given as to how they would fit within this proposed framework, and challenges and opportunities in utilizing the IVIVR framework for future studies are identified.

2. *In vitro–in vivo* correlation (IVIVC) in the pharmaceutical field

In vitro studies (in the form of drug release testing using a standard apparatus) are commonly used by the pharmaceutical industry as a tool to study and predict the *in vivo* performance of pharmaceutical products, due to their less time- and cost-intensive nature compared to clinical or animal studies.[25] The utilization of *in vitro* studies to predict the physiological outcome of oral drugs in the pharmaceutical field is clearly regulated, where a meaningful relationship between *in vitro* dissolution and *in vivo* absorption behavior of a dosage form must be established prior to using *in vitro* testing as a surrogate for an *in vivo* study.[24,26] Such relationship between *in vitro* dissolution and *in vivo* absorption data in pharmaceutical products is known as an *in vitro–in vivo* correlation (IVIVC).[27]

An IVIVC is defined by United States Food and Drug Administration (FDA) as "a predictive mathematical model describing the relationship between an *in vitro* property of an extended-release dosage form and a relevant *in vivo* response".[24] In establishing a successful IVIVC in the pharmaceutical field, the *in vitro* and *in vivo* studies must be appropriately designed and constructed.[28] It is recommended to use three or more formulations that represent slow-, medium-, and fast-release rates to gather *in vitro* and *in vivo* kinetic data to define an IVIVC, although a minimum of two formulations with contrasting release rates (*e.g.*, highest and lowest release rate) can also be used.[29,30] The *in vivo* response, which is generally obtained as peripheral plasma concentration over time, must be mathematically transformed to *in vivo* release or absorption data prior to correlating with *in vitro* data.[26,28]

An IVIVC model is generally established using linear regression analysis between *in vivo* and *in vitro* data. The model is then validated (*i.e.*, checked for its accuracy in estimating *in vivo* values) by applying it to predict *in vivo* plasma concentration using *in vitro* dissolution data for either the same formulation (internal validation) or other formulations with different release rates (external validation). The prediction error of the model is calculated as the difference between the measured *in vivo* values with the predicted values based on the model. The model is considered validated if the mean absolute percent error (MAPE) across all formulations tested does not exceed 10%, and the prediction error for individual formulations does not exceed 15%.[25,31] After model development and validation are completed, the IVIVC model can be used to predict the *in vivo* profile using the *in vitro* dissolution profile of drug formulations with similar dissolution or release mechanisms.[31,32] A validated IVIVC can be used to request a biowaiver, *i.e.*, an exemption to avoid *in vivo* bioavailability and/or bioequivalence studies for drug products with similar release mechanisms from a drug regulatory agency during drug development.[25,27] Having a validated IVIVC is especially useful during production scale-up and changes in the drug manufacturing process after approval by pharmaceutical regu-

latory agencies (post-approval changes), which ultimately reduces the cost and time of the drug to market.[31–33]

Based on their ability to predict the complete profile of plasma drug levels over time, quantitative IVIVCs are divided into three main categories (Table 1): Level A, Level B, and Level C. A subcategory of Level C known as multiple Level C is also possible.[24,31] Level A is considered the highest correlation, as it directly relates every data point of an *in vitro* (*i.e.*, fraction of drug dissolved) and *in vivo* (*i.e.*, fraction of drug absorbed) measurement over time, showing that the *in vitro* approach reflects the kinetics of the *in vivo* process for that drug. Level A correlations can be used to support biowaivers in the case of changes in the manufacturing setup that may affect the drug performance.[26] A significant 1 : 1 correlation is desired in Level A, which is indicated by the slope and correlation coefficient (r) that are both close to 1. During the process of correlation development, the *in vitro* dissolution conditions may be adjusted to obtain a 1 : 1 *in vitro–in vitro* correlation.[24,28] Although the term "correlation" in an IVIVC specifies linear relationships between the *in vitro* and *in vivo* data, non-linear correlations, while uncommon, may also be appropriate for comparison.[24]

Level B correlates analogous parameters between *in vitro* and *in vivo* data derived from data reduction through mathematical modeling of the data, such as half-dissolution or half-disappearance time (Table 1). Each data point in the Level B IVIVC plot corresponds to a formulation with a specific *in vitro* and *in vivo* value. Consequently, it does not uniquely reflect the actual *in vivo* plasma level curve or *in vitro* dissolution curve as there are various possibilities of kinetics that have the same half-dissolution or half-disappearance time.[27] Establishing a Level B correlation requires *in vitro* and *in vivo* data from at least three formulations with different release rates. Due to data reduction to derive the analogous *in vitro* and *in vivo* parameters that does not a provide a point-to-point correlation, a Level B correlation is less predictive than Level A and has limited application in the pharmaceutical industry, including to support biowaivers. However, a Level B correlation is crucial if the dissolution rate of the drug limits the absorption process.[28,31]

Level C correlations utilize data from multiple formulations, where each formulation contributes to one data point in the IVIVC plot. In a Level C correlation, the amount of drug dissolved *in vitro* at a specific time point (*e.g.*, $t_{60\%}$ [the time to dissolve 60% of the drug]) is correlated with one or more *in vivo* pharmacokinetic parameters (*e.g.*, C_{max} [maximum plasma concentration] or AUC [area under the curve of the absorption data]). When only examined at one *in vitro* time point, a Level C correlation cannot be used to support biowaivers since it does not reflect the complete shape of the plasma profile, but it may be useful in early stages of drug formulation development. However, if a correlation is found between *in vivo* pharmacokinetic parameter(s) with *in vitro* drug dissolution kinetics at 3 or more time points that cover the beginning, middle, and end of the dissolution profile (*e.g.*, *in vivo* C_{max} with *in vitro* $t_{20\%}$, $t_{60\%}$, and $t_{80\%}$), a multiple Level C correlation is present. This multiple Level C correlation can be used to justify biowaivers due to its equivalence to a Level A correlation.[28,31]

In addition to the three quantitative IVIVC categories, there is a qualitative correlation known as Level D. Level D cannot be used to justify a biowaiver as it correlates non-parametric rank order between *in vitro* dissolution parameters and *in vivo* pharmacokinetic parameters. However, it can be used to aid the development of a formulation or processing procedure.[30]

Although the IVIVC concept has been well-defined and established in the pharmaceutical area, it is noteworthy that not all drug formulations can exhibit an IVIVC. The *in vitro* aqueous solubility and *in vivo* intestinal permeability of a drug formulation, known as the Biopharmaceutics Classification System (BCS), determine the likelihood of establishing an IVIVC. There are four classes of drugs according to the BCS, where classes with high solubility and/or permeability have a higher likelihood for establishing an IVIVC, while a class with both low solubility and permeability has limited or no IVIVC expected.[30,34] Further detailed discussion on establishment and limitations of IVIVCs in the pharmaceutical area are beyond the scope of this review. Readers are referred to existing reviews for more information.[25–29,32,33,35]

The fact that not all drug formulations can exhibit an IVIVC, as well as the presence of multiple classification systems of drug formulations, highlights the complexity in relating *in vitro* and *in vivo* pharmaceutical data. As a complex *in vivo* system cannot be entirely mimicked with an *in vitro* setup, the physicochemical properties of the drug, complexity of the delivery system, formulation composition, manufacturing method, dissolution method, and type of dissolution media used must be carefully considered during IVIVC development.[31] Similar principles also apply to studies on food digestion, but with more complexities due to the variations in food structure, mastication, and physiological responses related to food properties. Understanding available *in vitro* and *in vivo* approaches to study food digestion and how to relate the data generated in both types of studies are the first steps in interpreting findings from *in vitro* studies such that they can adequately mimic the *in vivo* food digestion process.

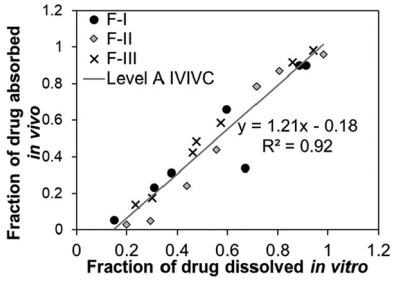
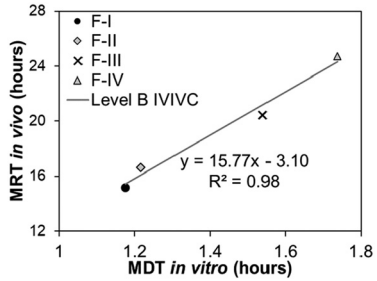
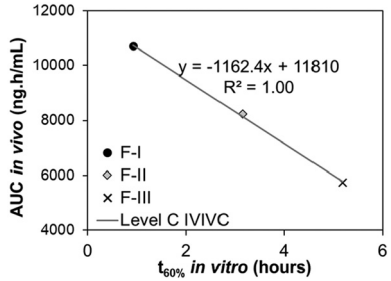
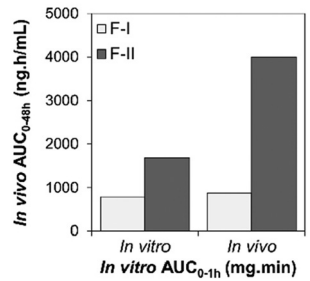
3. IVIVR development for food gastric digestion studies

3.1 General overview of available *in vivo* and *in vitro* gastric digestion approaches

Food digestion can be investigated through *in vivo* (human or animal studies), *in vitro* (laboratory experiments), or *in silico* (numerical/computational simulation) approaches.[37] As this review focuses on relating *in vitro* with *in vivo* gastric digestion data, only *in vivo* and *in vitro* gastric digestion approaches are discussed here.

For a comprehensive investigation of gastric digestion and the subsequent digestion processes, an ideal study design would be an *in vivo* study with the collection of the entire

Table 1 Levels of *in vitro–in vivo* correlation (IVIVC) in the pharmaceutical field and examples of the types of *in vitro* and *in vivo* data used to establish the correlations (adapted from Singh *et al.*[31]). In the example figure, different symbols in Level A, B, and C represent different formulations; different bar colors in Level D represent different formulations. Examples of types of *in vitro* models that might be utilized to generate the *in vitro* data include the USP I (basket) Apparatus, USP II (paddle) Apparatus, USP III (reciprocating cylinder) Apparatus, USP IV (flow through cell) Apparatus, Dialysis Bag.[29] Examples of *in vivo* studies that could be utilized to generate the *in vivo* data include dogs, domestic pigs, mini pigs, rabbits, rats, or human subjects[29,36]

Correlation	Level A	Level B	Level C	Level D
Example figure of correlation				
Relationship	Quantitative Point-to-point between the *in vitro* release time course and the *in vivo* response time course	Quantitative Correlation based on statistical moments/secondary parameters that characterize the overall rate of the process	Quantitative Point-to-point between an *in vitro* dissolution and a pharmacokinetic parameter	Qualitative Rank order (*e.g.*, comparison of trends)
Data points	Multiple formulations, multiple data points (time course) for each formulation	Multiple formulations, but only 1 data point (same *in vitro–in vivo* parameters) for each formulation	Multiple formulations, but only one data point (two different *in vitro–in vivo* parameters) for each formulation	Multiple formulations, one or multiple data points for each formulation
Examples of *in vitro* data	Dissolution curve (fraction of drug dissolved over time)	Mean dissolution time (MDT)	One of these parameters: • Disintegration time • Time for dissolution of *x*% of the drug (*e.g.*, $t_{60\%}$) • Dissolution rate • Dissolution efficiency	Any measurement, as long as similar variable/parameter *in vitro* and *in vivo* is measured
Examples of *in vivo* data	Absorption curve (fraction of drug absorbed in the blood over time; obtained by mathematical transformation of plasma concentration data)	One of these parameters: • Mean residence time (MRT) • Mean absorption time	One of these parameters: • C_{max} or T_{max} • Absorption constant • Area under the curve (AUC – total or cumulative) • Time for 20%, 60%, or 80% absorption	

content of the gastrointestinal tract or collection of gastrointestinal content from specific locations in the gut, which is invasive and difficult to ethically conduct in human subjects. With the analysis of food across the entire gastrointestinal content, physical, chemical, and microstructural changes at a particular digestion time point can be measured from the same subject, such as: pH and enzyme distribution in specific locations in the stomach, physical properties of the digesta, chemical content of the digesta, and microstructural changes in the digesta. Although non-invasive methods, which are commonly imaging-based methods (*e.g.*, magnetic resonance imaging and ultrasound), are preferred to study food digestion in the stomach,[38–40] these methods do not provide as complete of information as invasive methods. The development of non-invasive procedures to predict *in vivo* physicochemical changes in the stomach is an area for future research in food digestion.

The need for an invasive study design leads to the common use of non-primate, monogastric animals (*e.g.*, dogs, pigs, rodents) as physiologically-relevant models for the human stomach.[41–43] Among the available animal models, rodents and pigs are the most commonly used, with pigs having the closest physiological resemblance to the gastrointestinal tract of humans.[42,43] However, the use of animal models for research purposes is tightly regulated; it is only allowed with research ethics approval. Additionally, the planning and execution of an *in vivo* study is relatively complex and expensive, which is attributed to funding availability, the requirements for special expertise in preclinical studies or animal handling, longer time needed to conduct a study, and logistics of sampling. The lack of reference standards to compare the results between studies as well as inter-individual variations make data interpretation more complicated.[44–46]

The ethical constraints and complexities of *in vivo* studies have made *in vitro* digestion studies a more preferred and widely used approach in food digestion research, especially for rapid screening of digestibility and/or in-depth investigation of physical changes of foods in the gastrointestinal environment. Moreover, *in vitro* studies allow for more treatments or product formulations to be tested without significant cost and resource limitations, as well as the adjustment of digestion parameters to simulate certain physiological conditions for product development and testing purposes.[47,48] Available *in vitro* gastric digestion models can be generally classified into static, semi-dynamic, and dynamic models based on the approach used to simulate gastric digestion processes.[8] For the purpose of this review, gastric digestion models are defined and classified based on the presence of gastric wall contractions. Static models consist of a batch-type reaction (food mixed with limited or excess simulated gastric fluids) with no simulation of the mechanical breakdown and the removal of the entire digestion mixture after the assigned digestion duration.[46] Semi-dynamic models consist of a model without the simulation of gastric wall contractions (typically in the form of a jacketed vessel with slow stirring at the bottom) with the simulation of gradual acidification of the digestion mixture (by gradual addition of simulated gastric fluid to the food) and gastric emptying (by sample withdrawal at designated time points).[44,49] Dynamic models consist of mechanical models with a mechanism to simulate the contraction pattern of the *in vivo* stomach wall and the gradual acidification of the digestion mixture, as well as an outlet located at the pyloric part of the model to simulate gastric emptying.[8,41,49] Detailed discussion on *in vitro* digestion methods and models are outside the scope of this review, and readers are referred to recent review papers for further reading.[8,9,41,45,46,49–52]

An important aspect in an *in vitro* gastric digestion study is the appropriate selection of digestion parameters, such as digestive fluid composition and pH, simulated gastric fluid : food ratio or gastric secretion rate, and gastric emptying rate. There are various protocols used by different research groups to simulate physiologically relevant gastric digestion processes, although standardized protocols for static digestion and semi-dynamic digestion have been proposed by the COST-INFOGEST network.[44,53,54] Regardless of the digestion model and protocol used, it is critical to ensure that the approach is able to either mimic or provide an understanding of the food behavior *in vivo*, considering the lack of physiological aspects in many *in vitro* systems.[49] As such, we propose a framework to establish IVIVRs for food digestion studies that can be applied to any type of *in vitro* gastric digestion approach. The framework can be utilized by food researchers to quantitatively evaluate the usefulness and identify limitations of the *in vitro* digestion approach, and ultimately to improve *in vitro* digestion models and protocols such that they can be utilized to accurately mimic *in vivo* food digestion. While our focus here is on the gastric phase of digestion, this framework has the possibility to be utilized in other stages of digestion (*e.g.* oral or small intestinal phase) in the future.

3.2 Proposed IVIVR framework for food digestion research applications

In developing the proposed framework, the term IVIVR was selected to involve both linear and non-linear relationships between *in vitro* and *in vivo* data, instead of the term IVIVC from the pharmaceutical area that focuses mainly on linear correlations. This IVIVR framework can be applied to any type of food digestion data, but in the scope of this work, a focus on gastric digestion data is emphasized due to the importance of the gastric phase in the structural transformation of food and the large variations of the approaches reported in the literature to simulate *in vitro* gastric digestion. Moreover, with minimal or absence of nutrient absorption in the stomach, understanding food digestion and comparing *in vitro* with *in vivo* data in the gastric phase is more straightforward compared to other gastrointestinal regions. For example, in the small intestinal phase, there are numerous variables, such as variations in the rate of gastric emptying and size of emptied particles that are not well-understood (and as such, are difficult to mimic *in vitro*) and will play a critical role in the subsequent digestion processes. Once the framework for IVIVRs developed here has been adopted to gastric digestion data, it is recommended that additional studies work to refine

the framework to be utilized for later regions in the gastrointestinal tract (*e.g.* small or large intestine).

It is noteworthy that gastric digestion of food involves various changes in the food matrix, which may affect the breakdown and emptying mechanisms of the food in the dynamic gastric environment. As such, the structural changes of a food matrix during gastric digestion can be described by multiple parameters (or output variables), which may differ across food structures and composition, and may be limited by the type of *in vitro* or *in vivo* gastric digestion approach used. The need for having multiple different output variables for assessing IVIVRs in food digestion emphasizes the high level of complexity of such processes, compared to oral drug delivery that commonly focuses on the dissolution process of the drug. However, the use of multiple parameters also emphasizes the IVIVR framework proposed here provides enough flexibility to compare different parameters from various foods at different IVIVR levels, depending on the specific product(s) of interest and the specific outcome desired from the *in vitro* or *in vivo* study.

When developing an IVIVR, care should be taken in selection of the food products and the *in vitro* and *in vivo* methods utilized. It is recommended that a developed IVIVR may be applied to foods of similar composition and structure as those that were utilized in its development, and must note the specific *in vitro* (*e.g.* static, dynamic, *etc.*) method and *in vivo* species/population (*e.g.* healthy adult humans, growing pigs, *etc.*) that were utilized to develop the IVIVR. It should be noted that conclusions drawn from development of an IVIVR for a specific food product × *in vitro* method × *in vivo* study combination may not necessarily represent all food products (especially those foods with very different structure and/or composition to the foods utilized in the IVIVR development) or results from different *in vivo* species or populations. It is also important to select digestion parameters for development of the IVIVR that are relevant to the specific food products tested and will be applicable for future applications of the IVIVR.

3.2.1 Proposed IVIVR levels. Similar to the pharmaceutical IVIVC (Table 1), a food digestion IVIVR framework that consists of four levels is proposed here (Table 2). This IVIVR framework is proposed as a tool to evaluate the utility and limitations of an *in vitro* gastric digestion approach in mimicking an *in vivo* gastric digestion process for foods of similar composition and structure, instead of a tool to mathematically predict an *in vivo* output. Level A, B, and C IVIVRs describe quantitative relationships that are established by a statistical comparison of the *in vitro* and *in vivo* data, whereas a Level D IVIVR describes a qualitative or semi-quantitative relationship. The process of establishing an IVIVR at each level is summarized in Fig. 1.

A Level A IVIVR directly compares the kinetics of a gastric digestion process as a point-to-point relationship through pairing of *in vitro* and *in vivo* data collected at the same digestion time point in a scatter plot (Table 2). The digesta properties to be related must be carefully selected to represent a gastric digestion process and changes that may occur over time. For example, the pH of digesta remaining in the stomach can be selected as an indicator of gastric acidification; the fraction of hydrolyzed protein in the stomach relative to the initial total protein can be selected as an indicator of gastric proteolysis. For each food tested, at least three digestion time points are needed to establish a Level A IVIVR, which should cover the beginning, middle, and end of the digestion process. With the use of time-course data, evaluation of a Level A IVIVR also enables the evaluation of the physiological relevance of the *in vitro* approach (see section 3.2.2). Three different food products are suggested to obtain a Level A IVIVR. However, two types of foods may be acceptable if there is evidence of their contrasting digestive behavior obtained through *in vivo* studies (*e.g.* slow *vs.* fast gastric emptying). The behavior of each food should be monitored at early, middle, and late digestion times to capture the entire digestion kinetics. While a Level A IVIVR can also be explored on only one type of food, it is not recommended for extrapolation of the *in vitro* approach to other foods, because it is unclear whether the *in vitro* approach would produce similar accuracy to the *in vivo* system when applied to other types of food.

A Level B IVIVR relates *in vitro* and *in vivo* descriptive parameters of food digestion. The digestion kinetics of each food are represented by a single descriptive parameter (*e.g.*, gastric emptying half-time, gastric breakdown half-time), thereby each food contributes to one data point in the scatter plot (Table 2). The descriptive digestion kinetic parameter for each food is obtained by collecting the kinetic data of a digestion process for each food and fitting the data from each food to an appropriate empirical or mathematical model. For example, a modified-exponential model can be used to describe the gastric emptying of solid foods with parameters of gastric emptying rate constant, lag phase, or gastric emptying half-time;[55,56] the Weibull equation can be used to describe the softening process (an indicator of breakdown process) of solid food in the stomach with parameters of softening half-time, the shape parameter, and the scale parameter.[10,57] At least four products with varying rate or degree of structural changes in the *in vivo* stomach are recommended to evaluate a Level B IVIVR.

Level C is a quantitative relationship between a value derived from *in vitro* gastric digestion time-course data and a value from *in vivo* small intestinal digestion/absorption time-course data. When a Level C IVIVR is present between an *in vitro* gastric digestion parameter with an *in vivo* absorption parameter, the structural transformation of the food during gastric digestion is likely to be the limiting factor to nutrient release and absorption in the small intestine, such that the trends in *in vivo* nutrient absorption can be predicted using *in vitro* gastric digestion. It is preferred that Level A and B IVIVRs have been evaluated prior to evaluating Level C IVIVR. However, in the case that Level A and B IVIVRs cannot be evaluated, a Level C IVIVR can be evaluated given the relationship between an *in vitro* gastric digestion parameter and a small intestinal digestion/absorption parameter has been reported

Table 2 Summary of proposed relationships between *in vitro* and *in vivo* gastric digestion data with the purpose of developing an *in vitro–in vivo* relationship (IVIVR) framework between an *in vitro* digestion method or model to *in vivo* gastric digestion or absorption data. Examples of the types of data shown here are limited to gastric digestion only, but the same concept could be adapted to *in vitro* and *in vivo* data from other stages of digestion. The following framework can be applied to data from multiple types of *in vitro* digestion methds or models (such as static, semi-dynamic, or dynamic methods) as well as both human studies and animal models utilized to study food digestion

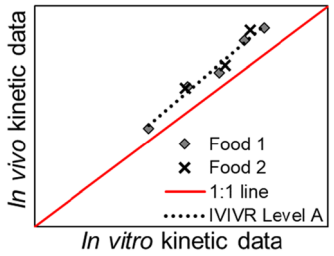
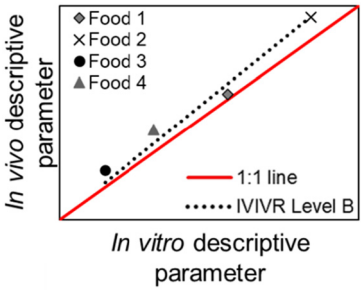
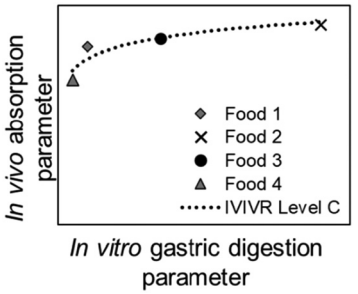
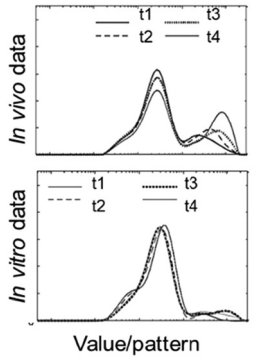
Relationship	Level A	Level B	Level C	Level D
Illustration of relationship				
Type of relationship	Quantitative *In vivo* kinetic data *vs. in vitro* kinetic data (paired at the same time point)	Quantitative An *in vivo vs.* an *in vitro* kinetic secondary parameter	Quantitative An *in vitro* gastric digestion parameter *vs.* an *in vivo* small intestinal digestion/blood nutrient appearance parameter	Qualitative/semi-quantitative Based on visual similarity between *in vitro* and *in vivo* data
Number of data points per food product involved	Multiple (time-course data, covering the beginning, middle, and end of digestion)	One (single descriptive value, typically derived from mathematical/empirical modeling of the data)	One *in vitro* gastric digestion and one *in vivo* small intestinal digestion/blood nutrient appearance (single descriptive value or derived from mathematical/empirical modeling of the data)	Can be a group of data that is observed as a whole, or a single qualitative observation
Minimum number of food products needed to develop relationship	Three (representing slow, medium, and fast breakdown rate) for each type or category of food	Four	Four	N/A
Examples of *in vitro* data	The same *in vivo–in vitro* data collected over time, such as: • Fraction emptied/retained • Fraction hydrolyzed • Moisture content • pH	The same *in vivo–in vitro* descriptive parameter obtained from data fitting to mathematical model, such as: • Breakdown rate (softening half-time, disintegration half-time) • Aggregation index half-time • Gastric emptying half-time • Gastric emptying rate constant • Mean retention time • Mean particle size (D_{50})	Gastric digestion parameter, such as: • Breakdown rate • Aggregation index ($t_{x\%}$) • Gastric emptying half-time • Area under the curve (AUC), incremental AUC (iAUC) • Nutrient degree of hydrolysis • Fraction released at a selected time point	The same *in vitro–in vivo* visually interpreted data, such as: • SDS-PAGE pattern • Particle size distribution • Microscopy observation • Intragastric pH change/ distribution pattern • Rank order/trend of measured values
Examples of *in vivo* data			Appearance of nutrient in blood parameter, such as: • AUC, iAUC • Maximum plasma nutrient concentration at a selected time point Small intestinal absorption parameter, such as: • Rate of absorption • % Bioaccessibility • % Digestibility	

Table 2 *(Contd.)*

Relationship	Level A	Level B	Level C	Level D
Significance to the evaluation of the *in vitro* approach	To evaluate the physiological relevance and limitations of the *in vitro* method or model	To determine if the *in vitro* approach has the same trend found *in vivo* and evaluate factors that cause differences between *in vitro–in vivo* trends	To identify if gastric digestion is a limiting factor in nutrient release and absorption	To complement quantitative observations
When to apply the approach	• When sampling time points are the same between *in vitro* and *in vivo* • When evaluating the performance of selected *in vitro* measurements to reproduce *in vivo* kinetics	• When sampling time points are not the same between *in vitro* and *in vivo* (due to different study designs) • When comparing kinetic parameters between *in vitro vs. in vivo* studies	• (Preferred, but not required) Level A and/or Level B IVIVR has been evaluated to show similarity between *in vitro* and *in vivo* gastric digestion processes • If there is *in vivo* evidence of the relationship between gastric digestion and nutrient absorption	• When data is qualitative • When comparing general trends between *in vivo* and *in vitro* processes
Cautions in interpretation	• Sufficient number of data points are needed to establish reliable correlation • Digestion time at which deviation from 1 : 1 correlation needs to be identified together with the possible factors causing the deviation • Relationship is applicable for foods within the same product category or type as those used to establish the relationship	• Different *in vitro* or *in vivo* profiles may produce similar secondary parameters • It may not indicate similarities in the kinetics, but in *in vitro* trend can reliably mimic the *in vivo* results • Relationship is applicable for foods within the same product category or type as those used to establish the relationship	• The presence of Level C correlation does not indicate similar kinetics between gastric and small intestinal digestion (it may occur "by chance") • Relationship is applicable for foods within the same product category or type as those used to establish the relationship	• Cannot be used as the sole basis to evaluate an *in vitro* approach • Qualitative comparison is very subjective and may be influenced by the observer's bias

in vivo. For example, gastric emptying rate has been reported to affect the maximum change of plasma glucose ($\Delta_{max,glucose}$) in humans and growing pigs for carbohydrate-based foods,[12,58] such that a Level C IVIVR can be evaluated for an *in vitro* gastric digestion emptying rate parameter and *in vivo* $\Delta_{max,glucose}$.

Level C IVIVRs should include at least four food products; the products should either have varying rates of structural changes *in vitro*, or varying rates of nutrient absorption properties (*e.g.*, area under the curve (AUC) of blood plasma concentration over time). Similar to a Level B IVIVR, each food tested contributes to one data point in the IVIVR plot. The relationship can be either linear or non-linear, and if needed, a non-linear relationship can be linearized through data transformation to evaluate the linear R^2 equivalent. A Level C IVIVR is not a point-to-point comparison between the same type of data, thus the scale (and units) between *x*- and *y*-axes can be different.

The last IVIVR level proposed is Level D, which implies certain similarities between *in vitro* and *in vivo* data, but with less certainty compared to a level A, B, or C IVIVR. In Level D, trends or overall profiles between *in vitro* and *in vivo* digestion data are compared based on visual inspection to determine the similarity between results, such as: (i) similarity in the overall appearance of protein bands in SDS-PAGE gels, (ii) similarity in the overall particle size distribution profile, (iii) similar appearance between *in vitro* and *in vivo* digesta, or (iv) visually similar intragastric pH profile. Level D relationships could also involve ranking of food products or trends, as in the Level D pharmaceutical correlation. Unless Level A and Level B IVIVRs have already been examined, the presence of Level D relationship does not necessarily indicate the accuracy of an *in vitro* gastric digestion approach in simulating an *in vivo* process. As such, the significance of Level D IVIVR in the evaluation of an *in vitro* gastric digestion approach is to provide complementary information that supports the similarities between *in vitro* and *in vivo* processes.

3.2.2 Quantitative parameters to evaluate level A, B, and C IVIVRs. Unlike the pharmaceutical IVIVC that has Level A as the highest importance, Level A and Level B IVIVR have the same importance in the proposed IVIVR framework for food digestion, as they provide complementary information. It is recommended that IVIVRs at both Level A and B to be examined when evaluating an *in vitro* gastric digestion approach. Level A and B IVIVRs can be examined on various gastric digestion variables that represent a certain gastric digestion process; these digestion parameters should be carefully selected, depending on the food materials being tested, and may vary based on food structure and composition.

The evaluation of Level A and B IVIVRs involves the statistical assessment of the agreement between *in vitro* and *in vivo* data using Pearson's correlation coefficient or ordinary linear regression (Fig. 1; section 3.2.2.1). For Level A and B IVIVRs, as the *in vitro* and *in vivo* data utilized should represent the same measurement (with the same units), an assessment of the accuracy of the *in vitro* approach to predict *in vivo* values can

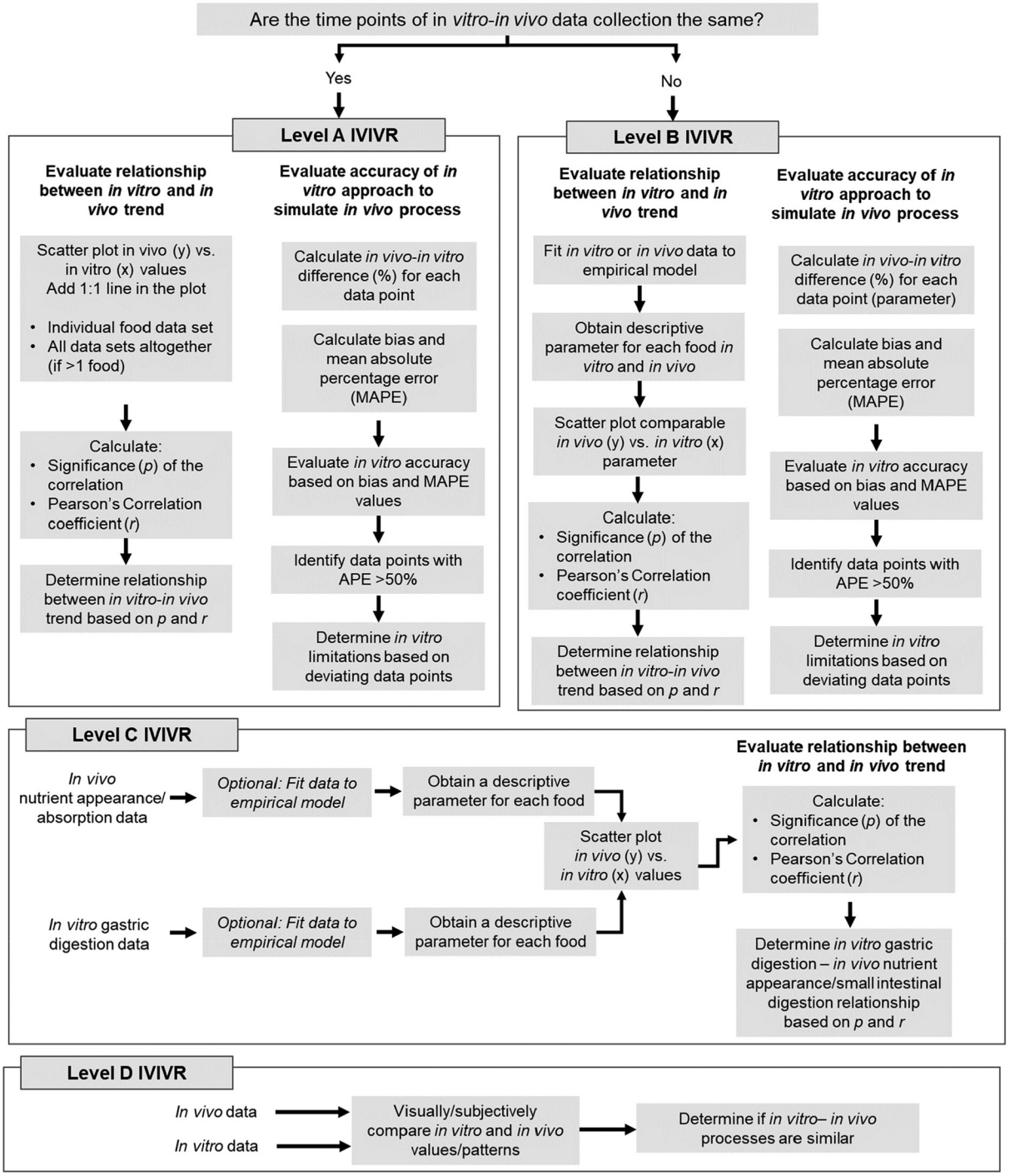


Fig. 1 Step-by-step diagram to establish IVIVR at the different levels according to the proposed IVIVR framework (Table 2). Thresholds for quantitative values are detailed in Table 3. A more detailed decision tree for establishing a Level A or B IVIVR is provided in Fig. 2. It should be noted that an IVIVR must also contain clear information on the *in vitro* and *in vivo* approach utilized for data generation, the food products tested, and any data excluded from the relationship to avoid unnecessary extrapolation.

also be conducted (section 3.2.2.2). Since Level C IVIVRs will not have equivalent data between *in vitro* (gastric digestion parameter) and *in vivo* (absorption or appearance parameter), a statistical evaluation of the relationship between the *in vitro* and *in vivo* data can be conducted, but the accuracy of the relationship cannot be evaluated (Fig. 1). Each of these steps are described in detail below. It should be noted that prior to starting an in-depth analysis of any *in vitro* or *in vivo* data, statistical outlier tests should be conducted to identify any data considered outliers, as the analyses described below will not serve to statistically detect outliers in the data sets or relationships.

3.2.2.1 Statistical evaluation of relationship between the in vitro and in vivo data (levels A, B, C)

a. Calculate the correlation (linear relationship) between *in vitro* and *in vivo* data

It is recommended that the correlation between the *in vitro* and *in vivo* data is determined using Pearson's correlation procedure[59] to determine the r value. An alternative approach is to use an ordinary linear regression equation that is fit to the data:

$$\textit{in vivo}\ \text{output} = \text{slope} \times (\textit{in vitro}\ \text{output}) + \text{intercept} \quad (1)$$

The significance of the correlation (determined at $p < 0.05$) can be obtained by calculating the t-score and p-value of the correlation. The t-score is calculated as follows:[60]

$$t = r \times \frac{\sqrt{(n-2)}}{\sqrt{(1-r^2)}} \quad (2)$$

where r: correlation coefficient, n: number of data points, and $(n-2)$: degrees of freedom. The p-value can be determined as the corresponding two-sided p-value for the t-distribution with $n-2$ degrees of freedom. It should be noted that while the p-value can be calculated using eqn (2), it may also be generated by a commercial statistics software, if such software is utilized to calculate the Pearson's correlation coefficient.

b. Interpret the *r* and *p* values

***p*-Value:** $p < 0.05$ is required to show that there is a significant relationship between the *in vitro* and *in vivo* gastric digestion processes. It is noteworthy that the probability of obtaining $p < 0.05$ is greater with increasing number of data points.[60] Therefore, caution is needed when interpreting the p value determined in datasets with small number of samples (<4 data points). As a result, at least 4 foods are recommended for Level B and C IVIVR (since each food contributes only one data point to the relationship).

Correlation coefficient (*r*): Strong correlation is defined by Pearson's correlation coefficient (r) ≥ 0.7 or the coefficient of determination of the linear regression (R^2) ≥ 0.5.[61] A high r or R^2 should not be mistakenly interpreted as physiological relevance (Table 3); it only indicates that the *in vitro* digestion process follows the same direction (Level A) or trend (Level B, C) as the corresponding *in vivo* process.

Regression line equation: If a linear regression approach is utilized, the regression line equation is useful to estimate *in vivo* values using *in vitro* data, given $r \geq 0.7$ and $p < 0.05$. However, it cannot be utilized to define the accuracy and limitations of the *in vitro* approach in simulating physiological reality, because the regression does not indicate which *in vitro* data points that deviate from 1 : 1 relationship with *in vivo* data. For example, if there are 10 pairs of *in vitro–in vivo* data points and the four last *in vitro* data points deviate further from the respective *in vivo* data points, but the trend of change between *in vitro* and *in vivo* data is consistent, the regression approach would still result in a high r or R^2 and a significant p-value. However, the regression fails to point out when the

Table 3 Interpretation of the parameters for Level A, B, and C IVIVRs obtained from statistical and quantitative comparisons. The parameters are listed in descending order of importance in the assessment of an IVIVR

IVIVR Level	IVIVR parameter	Value	Interpretation
A, B, C	Significance of correlation (p)	$p < 0.05$	Significant relationship exists between the examined *in vitro* and *in vivo* process
		$p \geq 0.05$	No significant relationship between the examined *in vitro* and *in vivo* process
A, B, C	Correlation coefficient (r) or coefficient of determination (R^2)	$r \geq 0.7$ (or $R^2 \geq 0.5$)	High similarity in the trend and direction of *in vivo* and *in vitro* process
		$r < 0.7$ (or $R^2 < 0.5$)	Limited relationship between *in vitro* and *in vivo* process. There are variations in the *in vivo* process that are not properly addressed in the *in vitro* approach, or the *in vitro* approach is not applicable for certain time point or food structure
A, B	Mean absolute percentage error (MAPE)	MAPE < 20%	Accurate simulation of an *in vivo* process by an *in vitro* approach; the *in vitro* approach can be used to accurately mimic *in vivo* output
		20% ≤ MAPE ≤ 50%	Reasonable simulation of an *in vivo* process by an *in vitro* approach. Interpretation of the *in vitro* approach to mimic *in vivo* output must be done with caution, and individual data points should be evaluated to identify limitations in the *in vitro* approach
		MAPE > 50%	*In vivo* process cannot be accurately simulated by an *in vitro* approach; *in vitro* approach needs modification
A, B	Bias	Bias < −20%	*In vitro* approach generally overpredicts *in vivo* trend
		−20% ≤ bias ≤ 20%	*In vitro* approach generally predicts the *in vivo* trend accurately
		Bias > 20%	*In vitro* approach generally underpredicts *in vivo* trend
A, B	Absolute percent error (APE)[a]	APE < 50%	Specific *in vitro* data point shows reasonable similarity to corresponding *in vivo* data point
		APE > 50%	Specific *in vitro* data point does not show reasonable similarity to corresponding *in vivo* data point; can be utilized to identify limitations in *in vitro* approach in accurately mimicking *in vivo* processes

[a] The APE is calculated on individual data points, while the other metrics in this table represent the entire dataset utilized to develop the IVIVR at each specific level.

in vitro data points start to deviate from the expected *in vivo* result. Alternatively, it could be that the *in vitro* data follows a linear trend, but this trend is not necessarily a 1 : 1 relationship. In this case, there may still be a high r or R^2 and significant p-value, while the *in vitro* and *in vivo* data may deviate significantly from a 1 : 1 relationship (*e.g.* the Level B correlation discussed in section 3.3.2.2 and Fig. 4). Therefore, it is not recommended to use the regression line equation to evaluate the accuracy of an *in vitro* approach in simulating *in vivo* process.

3.2.2.2 Evaluation of accuracy of an in vitro approach to simulate in vivo trends (levels A, B). Once the relationship between the *in vitro* and *in vivo* trends has been established, the accuracy of an *in vitro* digestion approach in simulating an *in vivo* digestion process also needs to be evaluated, as Level A and B IVIVRs facilitate the comparison of the same variable measured *in vitro* and *in vivo* (Fig. 2). For both Level A and B IVIVRs, the following steps should also be conducted after the statistical evaluation of the IVIVR has been completed:

a. Calculate the mean absolute percentage error (MAPE) and bias

MAPE and bias were selected to evaluate the accuracy of an *in vitro* approach, using the *in vivo* data as the "true" values, as they are commonly used in the quantitative analysis of the accuracy of modeling and forecasting data:[62–64]

$$\text{MAPE}\,(\%) = \frac{\sum_{i=1}^{n}\left|\frac{\textit{in vitro}\text{ value at time } t - \textit{in vivo}\text{ value at time } t}{\textit{in vivo}\text{ value at time } t}\right|}{n} \times 100\% \quad (3)$$

$$\text{Bias}\,(\%) = \frac{\sum_{i=1}^{n}\frac{\textit{in vitro}\text{ value at time } t - \textit{in vivo}\text{ value at time } t}{\textit{in vivo}\text{ value at time } t}}{n} \times 100\% \quad (4)$$

where n is the number of data pairs between *in vitro* and *in vivo* data evaluated in the IVIVR in both the MAPE and bias calculation.

b. Interpret the MAPE and bias values

MAPE: We define MAPE < 50% as the limit to consider that an *in vitro* approach reasonably simulates *in vivo* digestion process, and a MAPE < 20% is required to conclude that a specific *in vitro* approach accurately simulates the *in vivo* gastric digestion process.[63] MAPE > 50% indicates that the

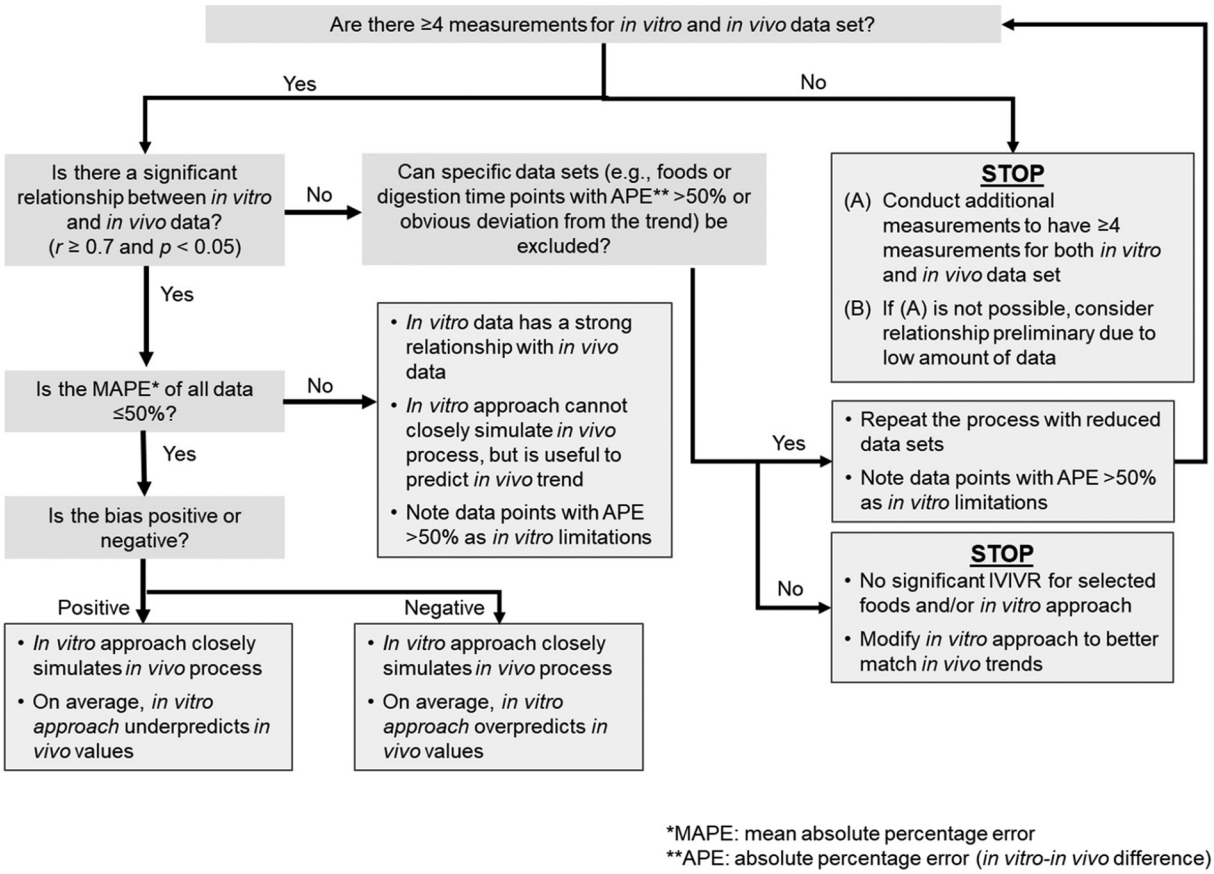


Fig. 2 Decision tree diagram to evaluate Level A and B IVIVRs, using the values determined through the framework in Fig. 1 and the thresholds for these values provided in Table 3. MAPE can be calculated following eqn (3), bias can be calculated following eqn (4), and APE can be calculated following eqn (5).

selected digestion parameters need adjustment to obtain a higher degree of similarity between the *in vitro* and *in vivo* digestion processes (Table 3).

If MAPE < 20% and the *r* and *p* values meet the suggested criteria (Table 3) to indicate agreement between *in vitro* and *in vivo* data, it can be concluded that the *in vitro* approach can be used as a substitute for *in vivo* data for the specific gastric digestion process examined (in specific *in vitro* and *in vivo* models) and for those foods utilized to establish the IVIVR.

However, if MAPE > 50% but the correlation values of *r* and *p* calculated above still meet the suggested values, the *in vitro* approach is still useful as a screening tool to predict *in vivo* trends in the measured digestion parameter, although there may be deviations between the specific values obtained *in vitro* compared to the *in vivo* data that need to be considered in the *in vitro* data interpretation (Fig. 2).

Bias: Bias provides more detailed information whether the *in vitro* approach underpredicts (bias > 20%), overpredicts (bias < −20%), or accurately predicts (−20% ≤ bias ≤ 20%) an *in vivo* digestion process (Table 3).

c. Calculate the absolute percent error (APE) of individual data points to determine limitations in the in vitro approach

Even when an *in vitro* approach meets the criteria for *r*, *p*, and MAPE (Table 3), it is possible that some *in vitro* data points exhibit large differences with *in vivo* data due to the complexity of *in vivo* gastric digestion processes. Such data points can be identified by calculating the absolute percent error (APE) between *in vitro* and *in vivo* data for each data point, with the *in vivo* study is considered as the "reference" method:[65]

$$\text{APE}\,(\%) = \frac{|\textit{in vitro}\,\text{value} - \textit{in vivo}\,\text{value}|}{\textit{in vivo}\,\text{value}} \times 100\% \qquad (5)$$

d. Interpret APE of individual values and repeat above steps (if needed)

We propose 50% as the maximum acceptable APE for any individual data point. Data points outside 50% difference from *in vivo* values can provide information pertaining to the limitations of the *in vitro* approach and may help to design future improvements in the current *in vitro* digestion models (examples will be discussed in section 3.3.1). If individual data points with APE > 50% can be removed from the data set, while still maintaining ≥4 total data points (of each *in vitro* and *in vivo* data), remove these points and consider them limitations of the *in vitro* approach and repeat the above steps with the reduced data set (Fig. 2). If data points are removed from any data set to develop the IVIVR, the limitations of the IVIVR should be noted clearly (*e.g.* if the relationship is only valid for certain digestion times or food products due to data points being removed when the relationship was developed).

3.3 Case studies: application of the proposed IVIVR framework to evaluate the performance of *in vitro* gastric digestion models

In this section, we provide three case studies to serve as examples of the implementation of the proposed IVIVR framework to solid and liquid foods from studies of the authors where original research data was available for use in the quantitative analysis. Through these case studies, examples of how the IVIVR framework provided here can help in the interpretation of a specific *in vitro* approach and its improvement for better physiological relevance are shown. While semi-dynamic digestion data were not available to compare with our previous *in vivo* studies, we hypothesize that the relationships would be similar to those observed with the static digestion model. In each case study, selected digesta properties were monitored both *in vivo* and *in vitro* to evaluate different aspects of gastric digestion. Not all data/observations in the previously published *in vivo* or *in vitro* studies was presented for each case study. This is because the goal of this review was to demonstrate how to assess an *in vitro* digestion approach using the proposed IVIVR framework, which will aid in future development of IVIVRs when both *in vitro* and *in vivo* data are available.

3.3.1 Case study 1 – relating starch-based solid food digestion data from dynamic *in vitro* digestion with *in vivo* data from a growing pig model

3.3.1.1 Description of the study. **Food products:** Six starch-rich foods of similar composition but varying food structure (durum wheat semolina porridge, white rice couscous, durum wheat couscous, white rice noodle, long grain white rice, durum wheat fettucine pasta) were used in the *in vivo* study. Two of the six foods (durum wheat semolina porridge (semolina) and durum wheat fettucine pasta (pasta)) were selected for the *in vitro* study, because they had the fastest (semolina) and slowest (pasta) gastric emptying rate in the *in vivo* study by Nadia *et al.*[10] These foods also had contrasting microstructure and buffering capacity.

***In vivo* study:** Growing pigs (~22 kg body weight) were used as an animal model of the adult human digestive system. Details on the animal handling and sampling protocols have been described elsewhere.[10,66,67] On sampling day, each pig was fed one of the six foods (250 g starch in the dry matter (DM) of the cooked product). The pigs were euthanized after 30, 60, 120, or 240 min of digestion to represent a food × digestion time combination. The stomach was removed and contents of the proximal (upper) and distal (lower) sections of the stomach were mixed carefully in separate containers before analysis.

The extension of this work to the absorption of glucose into the peripheral circulation was studied in an *in vivo* glycemic response study (growing pig model) that was previously described.[67,68] Briefly, in the glycemic response study, growing pigs were catheterized in the ear vein for blood sampling. On blood sampling day, the plasma glucose response of the pigs was measured before and up to 360 min after feeding the same test meals as described above when gastrointestinal content properties were measured.

***In vitro* study:** Dynamic gastric digestion was conducted in the second-generation human gastric simulator (HGS 2.0) built at the Riddet Institute, New Zealand, which has the

feature of a J-shaped simulated stomach. The details of this dynamic gastric model are described elsewhere.[69] The *in vitro* digestion started with an oral phase followed by a gastric phase with gastric secretions (pH = 0.8, 2000 U porcine pepsin per mL) introduced from the top of the HGS 2.0 at 4.1 mL min^{-1}. Every 30 min for up to 240 min gastric digestion, ~170 g digesta was withdrawn from the pyloric opening of the stomach bag to simulate a gastric emptying rate of 5.68 g min^{-1}. The experiment was terminated at 30, 60, 120, or 240 min of gastric digestion, then the remaining digesta in the stomach bag was divided to two approximately equal parts (proximal/upper and distal/lower) to mimic the proximal and distal samples collected in the *in vivo* study.

3.3.1.2 IVIVR evaluation (Fig. 3). **Level A:** Dry matter (DM) retention was selected to evaluate if the *in vitro* gastric emptying approach was able to mimic *in vivo* DM gastric emptying. Moisture content and pH were selected to evaluate the *in vitro* gastric secretion approach in mimicking *in vivo* gastric content acidification and mixing. Normalized hardness was selected to evaluate the *in vitro* gastric secretions and stomach loading phase approach in simulating food overall breakdown in the *in vivo* stomach. For each digestion variable, the *in vitro* and *in vivo* values at the same digestion time (average values across all replicates from each respective experiment) were used to build a scatter plot (Fig. 3A), and the *in vitro–in vivo* MAPE (eqn (3)), bias (eqn (4)), and APE (eqn (5)) were calculated (Table 4). The DM retention, normalized hardness (H_t/H_0), and moisture content had an $R^2 > 0.80$, $r \geq 0.90$, and $p < 0.05$ (Fig. 3A), indicating a high similarity in the direction and trends of the *in vivo* and *in vitro* DM emptying, gastric breakdown, and moisture uptake.

The DM retention data had a MAPE of 27% (Fig. 3A), which indicated that the selected gastric emptying rate and approach of emptying a constant amount of digesta simulated *in vivo* DM emptying with reasonable accuracy. Based on the *in vitro–in vivo* difference calculation (Table 4A), data points with >50% APE were pasta after 240 min and semolina after 120 min; both digestion times were the longest time that was tested for each food product *in vitro*. The differences in the dry matter emptying kinetics that occurred at longer digestion times can be attributed to the absence of physiological responses to slow gastric emptying at longer times in the *in vitro* approach.

Intragastric pH, moisture content, and normalized hardness had MAPE > 50% (Fig. 3A2–A4). In the intragastric pH and moisture content data, >50% APE was found mostly in the proximal stomach region and at the longest digestion time in both stomach regions (Table 4B and C), which may be associated with two reasons. First, the limitation in the gastric secretion introduction system of the HGS v2.0 (where secretions are introduced in the proximal stomach region), which is different from an *in vivo* stomach where gastric secretions are introduced from the gastric wall. Second, the use of generalized and constant secretion rate throughout the digestion time that caused high intragastric dilution *in vitro*, which also explained the poor overall correlation of intragastric pH data ($r = 0.25$, $p > 0.05$) and resulted in a higher moisture content *in vitro* compared to *in vivo* results. If the values with APE > 50% are removed from the moisture content and pH data, the MAPE and bias both fall within ranges suggesting a reasonable estimation of digestion processes (MAPE and bias of 11 and −1% or 17 and 17% for moisture content and intragastric pH, respectively), although with fewer data points ($n = 9$ for moisture content and $n = 5$ for intragastric pH).

In the normalized hardness data (Table 4D), all semolina data points had >50% APE, indicating much faster breakdown of semolina *in vitro*. These differences may be attributed to the prolonged contact with amylase in the *in vitro* experiment, a greater dilution of the semolina with gastric secretions, and the low level of mucins in the *in vitro* simulated gastric fluids, which may impact the overall digesta consistency.[70] However, for the pasta data, only two data points had >50% APE. If the remaining data set is considered, the MAPE and bias fall within reasonable ranges with >4 data points (MAPE = 26%, bias = −8%, $n = 6$), suggesting that the *in vitro* approach used here was able to mimic the breakdown of a solid meal (pasta) with better accuracy compared to a semi-solid meal (semolina).

Despite the identified limitations between *in vivo* and *in vitro* point-to-point data, it is worth noting that high correlation between *in vivo* and *in vitro* data was found ($r > 0.75$) for all selected parameters when the correlation was examined at individual food or food × stomach region level (Table 5), indicating changes in the selected digesta properties went in the same direction as what was found *in vivo* when examination was conducted only on one particular data set. The different slopes between the food or food × stomach region suggests that different foods responded differently to generalized gastric digestion parameters, thus the usefulness or validation of an *in vitro* approach should not be based on only a single type of food for future extrapolation to a wider variety of food materials.

Level B (*Preliminary*): DM gastric emptying half-time was obtained from fitting the DM retention data (Table 4A) to the modified-exponential equation, conducted separately for the *in vivo* and *in vitro* data sets.[55,56] Because there were only two data points (due to the two meals used in the *in vitro* study), the IVIVR is considered preliminary (Fig. 3B). However, an agreement in the *in vitro* and *in vivo* trend of DM emptying half-time for the two foods of contrasting structure likely suggests that the *in vitro* approach used in this case study can be used as a screening tool to predict the rank order of dry matter gastric emptying for starch-rich foods with similar structure to the six foods tested in the *in vivo* study. Overall, the bias and MAPE were both 45% (APE for pasta = 55%; APE for semolina = 35%). More food products need to be tested to determine the nature of the Level B IVIVR for DM emptying.

Level C (*Preliminary*): Previous *in vivo* studies have reported a relationship between gastric emptying rate with glycemic response,[12,58] hence a Level C IVIVR was assessed on the *in vitro* gastric digestion emptying rate parameter ($k_{in\ vitro}$) and the incremental area under the glycemic response *in vivo* ($iAUC_{in\ vivo}$). As there were <4 data points (due to the two meals used in the *in vitro* study), this relationship is considered preliminary. As in Level B, there was an agreement in the *in vitro*

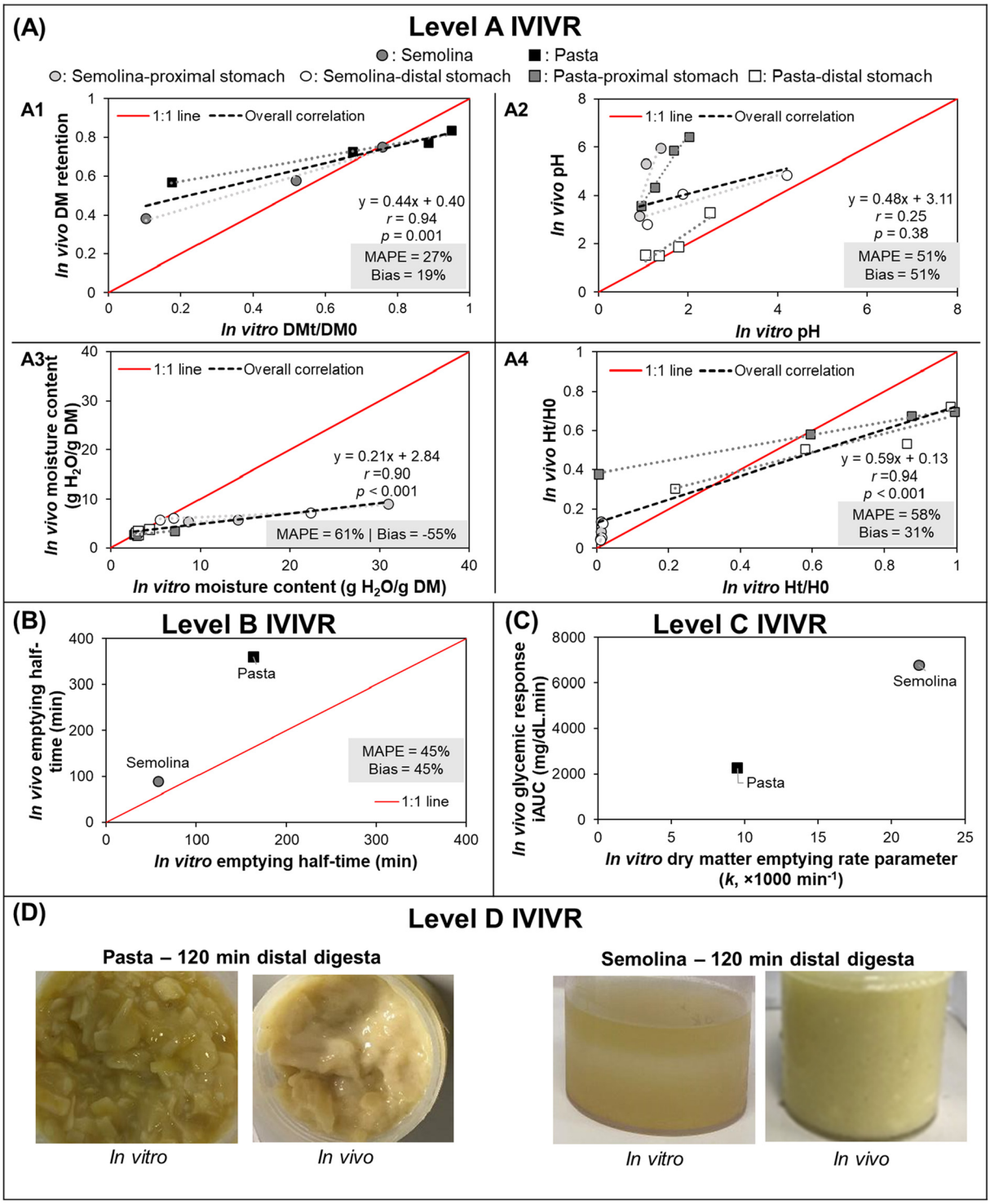


Fig. 3 IVIVRs developed for Case Study 1 (*in vitro* dynamic gastric digestion using the HGS 2.0 *vs. in vivo* digestion in growing pigs). *In vitro* data was taken from Nadia *et al.*;[69] *in vivo* study data was taken from Nadia *et al.*[10,66,67] For plots in (A), black dashed lines represent overall correlation line, displayed together with the regression equation; dotted lines with matching color to the symbol represent ordinary linear regression lines for each type of food; MAPE and bias were calculated using eqn (3) and (4), respectively. For plots in (A) and (B) the solid red lines represent a 1 : 1 correlation between *in vitro* and *in vivo* values. The coefficients for the regression lines for individual type of food can be found in Table 5. Values shown are means (n = 3–6), error bars are not shown for ease of viewing.

Table 4 Data used to establish the IVIVR in the Level A IVIVR plots of Case Study 1 (Fig. 3) and to identify points that deviated from 1 : 1 line. Data points with *in vitro–in vivo* difference (APE) greater than 50% are preceded with a hash (#) superscript. *In vivo* data were obtained from Nadia *et al.*,[10] *in vitro* data were obtained from Nadia *et al.*[69] Bias and MAPE were calculated using eqn (3) and (4), respectively

Food	Time (min)	Stomach region	Average values: *In vivo* (y)	Average values: *In vitro* (x)	$(y-x)/y \times 100$	$\lvert(y-x)/y\rvert \times 100$
A. Dry matter (DM) retention						
Pasta	30	Not	0.84	0.95	−13%	13%
	60	applicable	0.77	0.89	−15%	15%
	120		0.73	0.68	7%	7%
	240		0.57	0.18	#69%	#69%
Semolina	30	Not	0.75	0.76	−1%	1%
	60	applicable	0.58	0.52	10%	10%
	120		0.38	0.10	#73%	#73%
Average difference					Bias = 19%	MAPE = 27%
B. Intragastric pH						
Pasta	30	Proximal	6.42	2.03	#68%	#68%
	60		5.86	1.68	#71%	#71%
	120		4.33	1.25	#71%	#71%
	240		3.58	0.95	#73%	#73%
	30	Distal	3.30	2.49	25%	25%
	60		1.87	1.78	5%	5%
	120		1.52	1.34	12%	12%
	240		1.53	1.05	32%	32%
Semolina	30	Proximal	5.96	1.39	#77%	#77%
	60		5.31	1.06	#80%	#80%
	120		3.17	0.91	#71%	#71%
	30	Distal	4.84	4.20	13%	13%
	60		4.07	1.87	#54%	#54%
	120		2.82	1.08	#62%	#62%
Average difference					Bias = −55%	MAPE = 61%
C. Moisture content, dry basis (g H_2O per g DM)						
Pasta	30	Proximal	2.54	2.96	−16%	16%
	60		2.72	2.60	4%	4%
	120		3.07	2.72	11%	11%
	240		3.51	7.18	#−104%	#104%
	30	Distal	2.77	3.09	−11%	11%
	60		3.12	2.84	9%	9%
	120		3.44	3.03	12%	12%
	240		3.83	4.29	−12%	12%
Semolina	30	Proximal	5.39	8.69	#−61%	#61%
	60		5.80	14.18	#−145%	#145%
	120		9.10	30.95	#−240%	#240%
	30	Distal	5.81	5.53	5%	5%
	60		6.19	7.03	−14%	14%
	120		7.28	22.30	#−206%	#206%
Average difference					Bias = 51%	MAPE = 51%
D. Normalized hardness (H_t/H_0)						
Pasta	30	Proximal	0.68	0.88	−29%	29%
	60		0.69	0.99	−43%	43%
	120		0.58	0.59	−2%	2%
	240		0.38	0.01	98%	98%
	30	Distal	0.72	0.98	−36%	36%
	60		0.53	0.86	#−62%	#62%
	120		0.51	0.58	−14%	14%
	240		0.31	0.22	29%	29%
Semolina	30	Proximal	0.14	0.01	#91%	#91%
	60		0.09	0.01	#87%	#87%
	120		0.05	0.01	#78%	#78%
	30	Distal	0.13	0.02	#87%	#87%
	60		0.05	0.01	#74%	#74%
	120		0.04	0.01	#76%	#76%
Average difference					Bias = 31%	MAPE = 58%

Table 5 *In vivo–in vitro* linear regression coefficients (slope, intercept), correlation coefficient (r), and the significance of the correlation (p) for the digestion parameters examined in case study 1 (section 3.3.1), examined at specific food (dry matter data) or food × stomach region level (other than dry matter retention data). Significant correlation is present when $p < 0.05$

Food	Pasta		Semolina	
Region	Proximal	Distal	Proximal	Distal
Dry matter (DM) retention				
Slope	0.32		0.55	
Intercept	0.51		0.32	
r/R^2	0.99/0.97		0.99/0.99	
p	0.014		0.073	
Moisture content (dry basis)				
Slope	0.16	0.52	0.17	0.08
Intercept	2.33	1.57	3.64	5.48
r/R^2	0.84/0.71	0.76/0.58	0.99/0.98	0.99/0.97
p	0.159	0.240	0.088	0.108
pH				
Slope	2.75	1.27	5.20	0.58
Intercept	0.98	−0.06	−1.02	2.54
r/R^2	0.99/0.98	0.94/0.89	0.87/0.76	0.92/0.84
p	0.009	0.057	0.330	0.261
Normalized hardness (H_t/H_0)				
Slope	0.33	0.47	60.14	11.57
Intercept	0.38	0.21	−0.60	−0.09
r/R^2	1/1	0.94/0.88	0.96/0.92	0.86/0.74
p	0.002	0.060	0.186	0.340

and *in vivo* parameters (Fig. 3C), indicating that the *in vitro* approach may be utilized to predict the trend in the resulting glycemic response of starch-rich foods with similar structure to the six foods used in the *in vivo* study based on the *in vitro* gastric emptying rate parameter, however additional data is required to establish a Level C IVIVR with at least 4 food products.

Level D: The overall consistency of the digesta can give an approximation of the dilution of the sample with gastric secretions, the particle breakdown, and the mixing of gastric secretions with the solid meal. The digesta consistency (assessed visually) was similar between the *in vitro* and *in vivo* studies, particularly for pasta. Similar differences can also be

observed between the proximal and distal region both *in vivo* and *in vitro*. Images suggest that the semolina diet had a greater dilution with gastric secretions *in vitro*, which is supported by the moisture uptake data (described in Level A, above) and greater phase separation between the semolina particles and gastric secretions, likely due to the absence of mucin in the *in vitro* digestion approach. This comparison provides complementary information to support the quantitative relationships described in the Level A IVIVR.

3.3.1.3 Summary. A Level A IVIVR could be established for the *in vitro* approach in Case Study 1 (using the HGS with generalized digestion parameters) for the DM gastric emptying, and examination of the individual APE values of the DM emptying data indicated that there is a limitation in the *in vitro* approach in mimicking the gastric emptying process at longer digestion times, due to the lack of physiological regulation. A Level A IVIVR could only be established for moisture update, gastric acidification (pH), and breakdown (hardness change) with removal of data points with APE > 50%, suggesting that the *in vitro* approach still can be improved to adequately mimic these *in vivo* gastric processes. A preliminary Level B and C correlation could be established, although additional data points are needed to fully assess these relationships.

Use of the IVIVR parameters allowed for identification of areas in which the *in vitro* approach could be improved to better mimic *in vivo* processes. Physiological responses that caused reductions in dry matter emptying rate and gastric secretion rate over time were absent, which was hypothesized to result in an overestimation in the gastric acidification and breakdown *in vitro*, especially in semolina (fast-breakdown food). Adjustment is needed in the stomach loading phase, gastric secretion introduction, and gastric secretion rate variation to improve the physiological relevance of the *in vitro* approach. However, despite its limitations, the *in vitro* approach used in this case study can be used to mimic the *in vivo* trends in DM emptying rate, food breakdown rate, and resulting glycemic response of foods with similar structure to semolina and pasta.

3.3.2 Case study 2 – relating liquid milk digestion data from a dynamic *in vitro* model with *in vivo* data from a piglet model

3.3.2.1 Brief description of the study. **Food products:** Raw whole milk from cow, goat, and sheep were studied as examples of complex liquid foods. The three milks varied in composition and physicochemical properties. Sheep milk had higher protein, fat, and mineral content than the other two milks. Goat milk had a larger number of small fat globules, but bigger casein micelles than cow and sheep milk.[71,72] The milk composition of the three species varied slightly between the *in vitro* and *in vivo* studies due to seasonal differences in the fresh milk used, but the overall differences between the milks remained consistent.

***In vivo* study:** Piglets (3 weeks old on sampling day) were used as a model of gastric digestion in infants. Details on the animal handling and sampling protocols have been described elsewhere.[72] On the sampling day, each piglet was fed fresh raw whole milk (milk volume was determined to provide an equal amount of protein per kg body weight) and was euthanized after an assigned digestion duration (30, 90, 150, or 210 min), such that each piglet represented a replicate for a milk type × digestion time combination. The stomach content of each piglet was separated into curd and liquid fractions for analysis.

***In vitro* study:** Dynamic digestion experiments were conducted using the first generation human gastric simulator (HGS),[73] which has a simulated stomach with an inverted cone shape. Detailed descriptions of the experiments are given elsewhere.[71] Briefly, 200 mL pre-warmed milk (37 °C) was added to the stomach bag. Gastric secretions were introduced into the stomach bag as two separate mixtures (combined pH of 1.0–1.3): 176 mL electrolyte solution + 6 M HCl solution at 0.73 mL min^{-1} and 24 mL electrolyte solution (pH 7) containing pepsin (96 U porcine pepsin per mg milk protein) at 0.1 mL min^{-1}. The total volume of simulated gastric secretions introduced to the HGS was 1 : 1 to the weight of the milk. No gastric lipase was used, as the alternative to human gastric lipase with similar activity and specificity (*e.g.*, rabbit gastric lipase) was not commercially available when the study was conducted. Gastric emptying (~1.66 mL min^{-1}; theoretically calculated as the rate needed to empty 200 mL milk + 200 mL simulated gastric secretions within 240 min) was simulated by withdrawing ~50 mL sample every 30 min. The digestion experiments were terminated at 30, 90, 120, 150, 180, 210, or 240 min to simulate different total digestion time, then the remaining digesta in the stomach bag was separated into the curd and liquid fractions for analysis.

3.3.2.2 IVIVR evaluation (Fig. 4). **Level A:** The pH of the liquid fraction of digesta was selected to assess the simulation of gastric secretion pattern and acidification kinetics in the stomach. Curd DM retention was selected to evaluate the simulation of curd disintegration behavior and its gastric emptying, whereas protein and fat retention in the curd were selected to evaluate the release of protein and fat globules from the curd using the *in vitro* approach as compared to the *in vivo* system. For each of the selected variables, the average *in vitro* and *in vivo* values (Table S1†) were paired at each digestion time and used to build a scatter plot (Fig. 4A) and calculate *in vitro–in vivo* MAPE, bias, and APE (Table S1†).

In the overall comparison across the three different types of milk, a Level A relationship could be established based on the trend in the *in vivo* and *in vitro* pH kinetics ($r = 0.97$, $p < 0.05$, bias and MAPE = 15%; Fig. 4A1 and Table S1A†). This indicates that the *in vitro* gastric acid secretion approach was physiologically relevant and could mimic the gastric acidification kinetics of all three milks. However, it should be noted that while the MAPE was 15% between the *in vitro* and *in vivo* approaches, as pH is on a logarithmic scale to represent the H^+ ion concentration, such differences in pH may ultimately impact the enzyme activity or subsequent breakdown of the meals.

The *in vivo vs. in vitro* data linear regression for of curd DM and fat retention (Fig. 4A2 and A4) indicated an acceptable

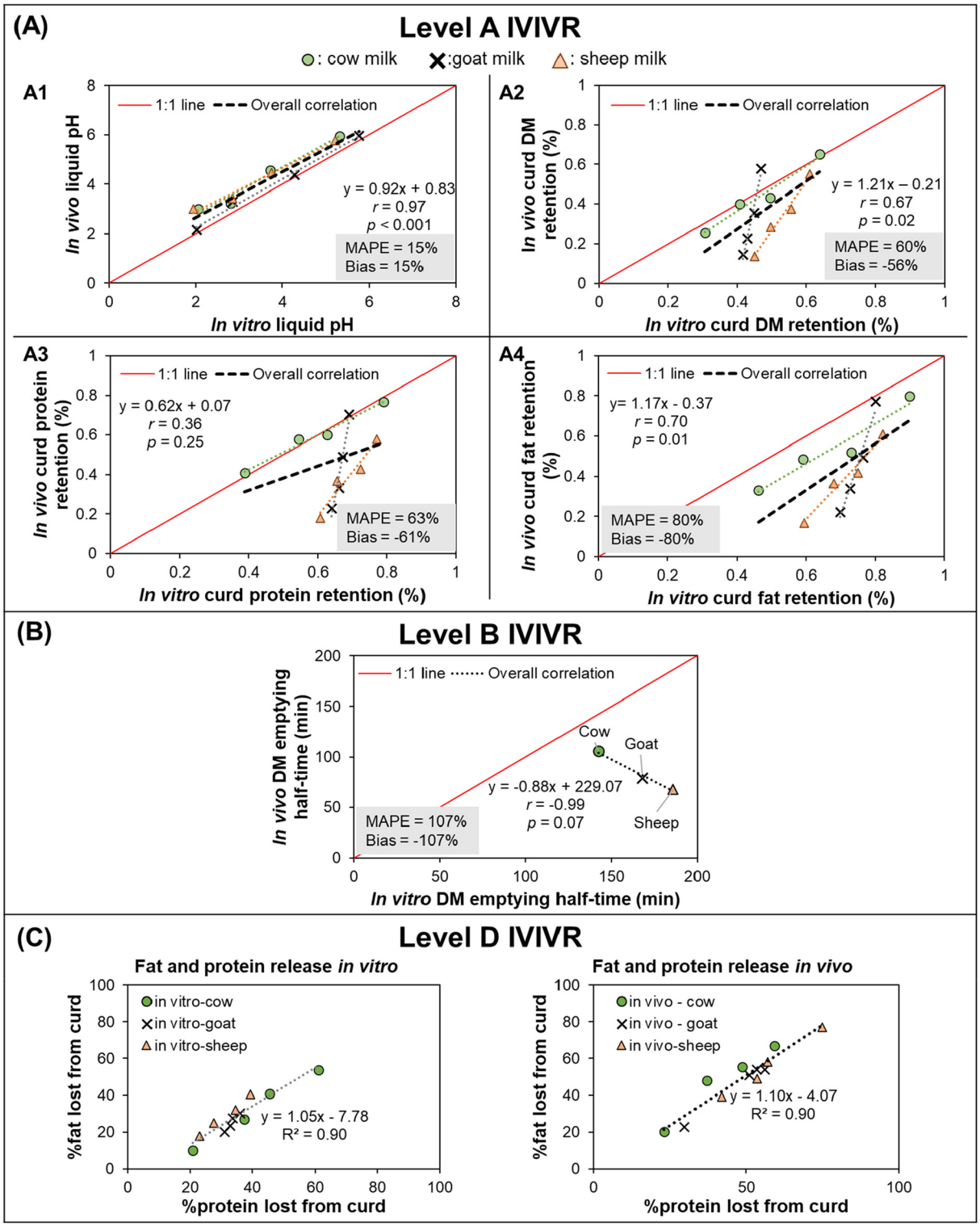


Fig. 4 IVIVR illustration for Case Study 2 (*in vitro* dynamic digestion using the human gastric simulator v1.0 *vs. in vivo* digestion in piglets). *In vitro* study data were obtained from Roy *et al.*,[71] *in vivo* study data were obtained from Roy *et al.*[72] For plots in (A), black dashed lines represent the overall correlation line, displayed together with the regression equation; dotted lines with matching colors to the symbol represent ordinary regression lines for each type of milk; MAPE and Bias were calculated using eqn (3) and (4), respectively. For plots in (A) and (B) the solid red lines represent a 1 : 1 correlation between *in vitro* and *in vivo* values. The coefficients for the regression lines for individual type of milk can be found in Table S2.† Values shown are means ($3 \leq n \leq 4$), error bars are not shown for ease of viewing.

strength of relationship (r = ~0.7, $p < 0.05$). However, these digesta properties had MAPE > 50% and bias ≤−56%, indicating that large differences were observed between the *in vitro* and *in vivo* data and improvement in the *in vitro* approach is required to establish a Level A IVIVR for these measurements. Calculation of the APE for the curd DM retention and curd protein retention (Table S1B and C†) revealed that significant *in vitro–in vivo* differences were found mostly in the goat and sheep milk (at times longer than 30–60 min). Meanwhile, in the curd fat retention data (Table S1D†), APE > 50% was found for almost all data points for goat and sheep milk. In the deviating data points, *in vivo* values were always lower than their *in vitro* counterparts, which indicated that *in vivo* curd disintegration occurred faster than *in vitro.* Such faster curd disintegration *in vivo* might be attributed to differences in the milk amounts fed on equal protein basis to piglets, differences in the gastric enzyme concentrations or secretion rates, or that the peristaltic contractions in the HGS were not as strong as those in *in vivo*, which ultimately underpredicted the disintegration of milk curds in the dynamic gastric model utilized in this study (HGS).

Interestingly, cow's milk had a significant ($p < 0.05$, $r \geq 0.97$) *in vitro–in vivo* relationship for the pH, and the curd DM, fat, and protein retention. When only the data from cow's milk is considered for these parameters, the MAPE and bias are within acceptable ranges for all data points except for dry matter retention after 90 min digestion. This suggests that the *in vitro* approach used may be able to adequately mimic the *in vivo* digestion of cow's milk but needs modification to adequately mimic the *in vivo* digestion of milk from other species in future studies. The discrepancy between the IVIVRs for the three milk types when analyzed altogether or separately within each type of milk may indicate a missing critical physiological factor in the *in vitro* approach that is needed for it to be applied across complex liquid food systems of varying composition.

Level B (*Preliminary*): As there were only three types of milk used in this study (resulting in <4 data points for the IVIVR), this IVIVR is considered preliminary. DM emptying half-time for each milk *in vitro* and *in vivo* was obtained from fitting the DM retention data (*in vitro* and *in vivo* separately) to the power-exponential equation.[74] There was a negative, linear relationship between *in vivo* and *in vitro* DM emptying half-time (Fig. 4B), indicating a contradictory *in vivo–in vitro* trend. Although in the Level A IVIVR, the DM retention was significant between the *in vitro* and *in vivo* data, the correlation coefficient was r = 0.67, and the MAPE > 60%, which agreed with this contradictory IVIVR Level B trend. This suggests that IVIVR evaluation needs to consider food-specific variation to gain holistic information relating to food-specific variations in digesta properties. In addition, this supports the recommendation of evaluating both Level A and B IVIVRs, as they provide complementary information and may help identify limitations of the *in vitro* method.

The contradictory trend between *in vivo* and *in vitro* DM emptying half-time could indicate a lack of simulation of critical physiological aspects in the *in vitro* approach (*e.g.*, gastric fluid composition and secretion rates including optimum levels of enzyme concentrations) or a mismatch in the design of *in vitro* and *in vivo* study that interfered with the factors that might affect gastric emptying of liquids *in vivo* (*e.g.*, food amount, food composition, calorie content).[75,76] It is also possible that the gastric emptying approach of withdrawing a constant volume at a regular interval may not be suitable for milk digesta, which might affect the DM emptying; such possibility is a topic for future investigation.

Level C: Ideally, *in vivo* plasma amino acid concentration as an indicator of protein concentration should be related with either the *in vitro* protein gastric emptying half-time or protein hydrolysis. However, as no published information on the absorption properties of the milk diets was available from the *in vivo* study, it was not possible to develop a Level C correlation with these previously published data.

Level D: In both the *in vitro* and *in vivo* stomach models, a high correlation (R^2 = 0.90; Fig. 4C) was found between the percent of protein and fat lost from the curd. These trends indicate that the release of fat globules from the curd was dependent and proportional to the rate of breakdown of the curd protein network in both the *in vitro* and *in vivo* approaches.

3.3.2.3 Summary. *In vivo* gastric acidification kinetics of milk were accurately mimicked using the *in vitro* approach by varying the amount of HCl depending on the type of milk. However, due to the differences in meal size (and composition), lack of physiologically relevant enzyme concentration (or secretion rates), and gastric emptying in the *in vitro* study, the *in vitro* approach utilized was not suitable to mimic the *in vivo* trends in the retention of milk curd components (DM, protein, fat). Regardless of the lack of agreement in curd disintegration and emptying kinetics, the *in vitro* approach was still useful for investigating the release mechanisms of fat globules from the milk curd into the liquid phase of digesta for an individual type of milk.

3.3.3 Case study 3 – relating data from starch-based solid foods from a static *in vitro* digestion model with *in vivo* data from a growing pig model

3.3.3.1 Description of the study. **Food products:** Six starch-rich products of different food structures, as described in Case Study 1, were used in the *in vivo* study. Five of them were selected for the *in vitro* study (semolina was excluded because of its semi-solid form).

***In vivo* approach:** The same *in vivo* studies (growing pig model) as described in Case Study 1 were utilized. Gastric digesta properties data from the distal stomach, as well as overall gastric dry matter retention (from proximal and distal stomach), was used for IVIVR evaluation. For the glycemic response study, the maximum change plasma glucose concentration within 360 min period after feeding the test meals relative to the baseline plasma glucose concentration was measured and expressed as Δmax.

***In vitro* approach:** Batch-type, static *in vitro* gastric digestion was conducted, as previously described.[77] In each digestion

experiment, 60 g food was incubated with simulated saliva (1 mL g^{-1} DM of food) for 30 s in a shaking water bath (37 °C) to simulate the oral phase. Subsequently, simulated gastric secretions (pH 1.8, containing 2000 U porcine pepsin per mL; 3 mL g^{-1} cooked food) was added to the food–saliva mixture, then incubated in the shaking water bath for an additional 15, 30, 60, 120, or 180 min to simulate the distal gastric phase. The pH of the digestion mixture was maintained at pH 2.0 ± 0.1. After each distal phase time, the entire digestion mixture was separated into large solid fraction (>2 mm particles) and liquid fraction (containing suspended solid particles ≤2 mm) for analysis.

3.3.3.2 IVIVR evaluation (Fig. 5). **Level A:** The simulation of moisture uptake into solid food particles was evaluated by comparing the moisture content of the *in vitro* solid digesta fraction with the moisture content of the *in vivo* distal stomach digesta (a mixture of solids and free liquids). The simulation of the softening process due to the action of gastric secretions in the *in vitro* approach was evaluated by comparing normalized hardness of the solid digesta fraction *in vitro* with the normalized hardness of the *in vivo* distal stomach digesta. The simulation of solid loss due to the dissolution and/or erosion into gastric secretions and its relationship with solid gastric emptying *in vivo* was evaluated by comparing the dry matter retention (DM) of *in vitro* solid digesta fraction (*e.g.* the amount of dry matter remaining in the solid particles after digestion) with the *in vivo* overall stomach digesta DM retention. The average *in vitro* and *in vivo* values for each of the selected variables (Table S3†) were paired at each digestion time to build a scatter plot (Fig. 5A) and calculate MAPE, bias, and APE.

Normalized hardness and moisture uptake met the correlation coefficient criteria listed in Table 3 (r = 0.90, p < 0.001 for normalized hardness and r = 0.75, p = 0.002 for moisture uptake). The high and significant correlation indicates that the static *in vitro* approach can be used in mimicking the direction and trend of *in vivo* softening and moisture uptake processes. However, in the normalized hardness data, the MAPE was 470%, and 13 of the 15 data points had *in vitro–in vivo* difference >50% with higher *in vivo* values compared to *in vivo* values (Table S3†). This suggests that although the trend and general direction of the softening kinetics was the same between the *in vitro* and the *in vivo* studies, the *in vitro* approach did not accurately simulate all aspects of the *in vivo* softening process. This is likely due to the absence of mechanical breakdown in the static digestion approach. It should be noted for data points with very small values *in vivo* (*e.g.* rice couscous at all digestion times), deviations in the trend resulted in very large APE for these data points (>500%).

A high, significant correlation (r = 0.75, p < 0.05), but low MAPE (8%) and bias (−3%), was obtained between the moisture content of *in vitro* solid digesta fraction and *in vivo* distal stomach digesta, indicating that the *in vitro* approach of incubation in excess gastric secretions was useful in predicting the direction and trend in the moisture uptake kinetics of solid starch-rich foods *in vivo* with reasonable accuracy. None of the individual data points had an APE > 30%, further supporting the utility of the static *in vitro* approach to mimic the moisture uptake *in vivo*.

A low general correlation (r = 0.35, p = 0.20) between *in vitro* solid digesta DM fraction and the *in vivo* DM gastric retention was obtained. Although the MAPE of DM retention was <19%, it cannot be concluded that the *in vitro* and *in vivo* solid loss process was similar because the general correlation was poor. When examined within individual food structure, high, positive correlation was found for couscous and rice couscous ($r \geq$ 0.97; Table S4†). The high correlation only for couscous and rice couscous, which were reported as fast-breakdown foods in the *in vivo* approach, suggests that the static *in vitro* approach was only useful in mimicking the trend and direction in the solid loss kinetics of food structure that undergoes fast breakdown in the gastric environment, and may not require as much breakdown due to the mechanical forces (that were not present in the static digestion approach used here).

Level B: The breakdown rate of the foods in the gastric environment, measured as softening half-time, was obtained from fitting the normalized hardness data to the Weibull equation.[10,57] A linear, but not 1 : 1 relationship (r = 1, slope = 0.57) between *in vitro* and *in vivo* softening half-time was identified in 4 out of the 5 foods tested (Fig. 5B). This suggests that the static *in vitro* approach can be utilized to predict the rank order in the breakdown rate of varying structures of solid starch-rich foods, despite the absence of mechanical breakdown in the *in vitro* approach. The deviation of one food type (rice noodle) from this strong trend possibly indicates that the breakdown of certain food structures in the stomach was not just controlled by diffusion of gastric fluid, but also other factors that were not mimicked in the static *in vitro* approach.

Level C: As a previous *in vivo* study reported a relationship between hydrolyzed starch content in the liquid fraction of gastric digesta with glycemic response *in vivo*,[58] the relationship between hydrolyzed starch content in the liquid fraction of *in vitro* digesta with *in vivo* maximum change in plasma glucose concentration was examined. A high linear relationship ($r \geq$ 0.94) between *in vivo* maximum change of plasma glucose and *in vitro* hydrolyzed starch content in the liquid digesta fraction measured at both early and late digestion time points (15 and 180 min distal phase). This high correlation suggests that the general trend in the maximum change of glycemic response *in vivo* of a wide range of starch-rich foods may be predicted using the specific static *in vitro* approach with gastric fluid added in excess, either by evaluation of the liquid fraction at an early or late time point of digestion.

Level D: Couscous, which showed a good correlation and acceptable *in vitro–in vivo* differences in the Level A IVIVR assessment, had a similar solid breakdown profile *in vitro* and *in vivo* based on the particle size distribution graphs after 30 min digestion (Fig. 5D). In both *in vivo* and *in vitro* graphs, the particle size distribution curve shifted to the left and flattened after 30 min digestion, compared to the undigested food. The similarity in how the particle size distribution changed *in vitro and in vivo* may suggest that the breakdown

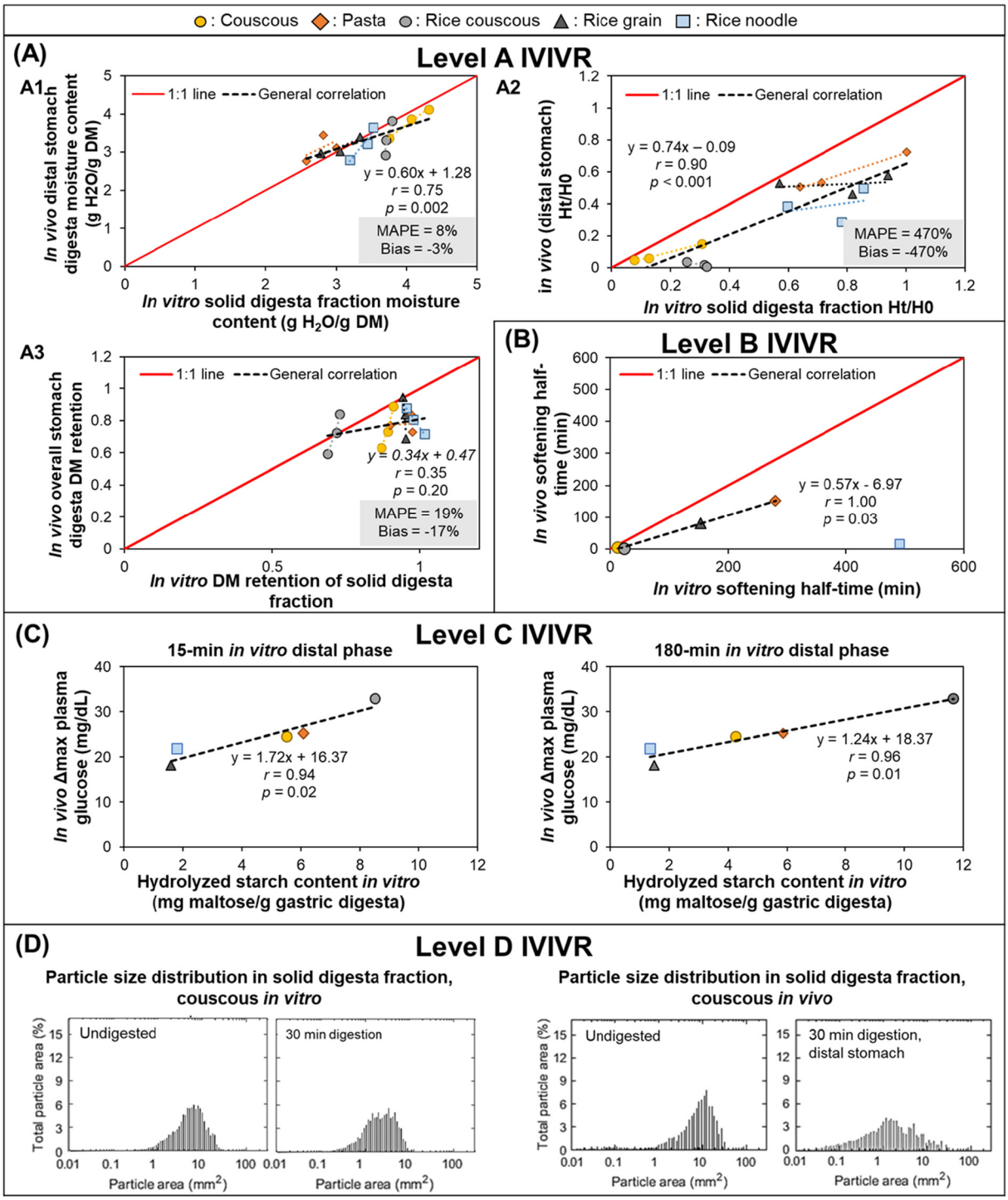


Fig. 5 IVIVR illustration for Case Study 3 (*in vitro* static digestion in excess gastric fluid *vs. in vivo* digestion in growing pigs). *In vitro* data were taken after 0 min proximal phase followed by up to 180 min distal phase data from Nadia *et al.*,[77] *in vivo* data were taken from Nadia *et al.*[10,66] For plots in the Level A IVIVR panel, black dashed lines represent the overall correlation line, displayed together with the regression equation; dotted lines with matching colors to the symbol represent ordinary regression lines for each type of food; MAPE and Bias were calculated using eqn (3) and (4), respectively. For plots in (A) and (B) the solid red lines represent a 1 : 1 correlation between *in vitro* and *in vivo* values. The coefficients for the regression lines for individual type of food can be found in Table S4.† Values shown are means (n = 3–6), error bars are not shown for ease of viewing.

process of couscous was driven by dissolution by gastric secretions in both *in vitro* and *in vivo* gastric digestion scenarios.

3.3.3.3 Summary. The *in vitro* static digestion approach can be utilized to predict the *in vivo* trend and direction of moisture uptake of solid starch-rich foods. It also has been demonstrated to accurately predict the *in vivo* gastric emptying of solids and breakdown process of food structures that do not require significant mechanical breakdown (*e.g.*, couscous, rice couscous). Despite the absence of mechanical breakdown, the static *in vitro* approach is still useful in predicting the rank order of food breakdown rate due to softening by gastric secretions. However, some food structures (*e.g.*, rice noodle) deviated from the rank order, and food-related aspects that may cause such deviations need to be examined to improve the static *in vitro* approach in future studies.

4. Challenges and methodological considerations in evaluating IVIVR for gastric digestion of foods

In addition to the case studies discussed above, the proposed IVIVR framework was also applied to previously published studies that relate *in vitro* and *in vivo* gastric digestion data (Table 6). An example of the extension of the proposed framework to the small intestinal phase is included as ESI Table S5.† It should be noted that in some of the examples from previous studies, the number of foods may not meet the criteria proposed in this IVIVR framework, but they are listed with the corresponding IVIVR level to exemplify the type of comparisons that can be made. The application of this IVIVR framework to the three detailed case studies and previous studies demonstrated that relationships between *in vivo* and *in vitro* data can be observed, regardless of the *in vitro* approach used. Based on this evaluation of *in vitro* and *in vivo* data, several considerations that should be taken into account to assist in future development of IVIVRs are presented here to aid future studies in development of physiologically relevant *in vitro* approaches and determination of quantitative IVIVRs with *in vivo* data.

4.1 Selection of relevant digestion variables for comparison

Depending on the purpose and type of the *in vitro* approach, digesta properties or the digestion variables to be compared within the proposed IVIVR framework may differ between studies and should be strategically selected. As a result, the three case studies and studies listed in Table 6 were selected as they examined different aspects of gastric digestion (*e.g.*, dry matter retention, pH, nutrient hydrolysis, mean breaking time) that are relevant to the food being tested. For example, the Level A IVIVR evaluation of the static *in vitro* digestion presented in Case Study 3 was conducted to relate the *in vitro* and *in vivo* softening behavior of solid foods due to the diffusion of gastric secretions, therefore the relevant digestion parameters to be evaluated were those related to secretion uptake (measured as moisture content) and softening (measured as normalized hardness) of the solid particles. On the other hand, there is more flexibility in the selection of digestion parameters that could be measured when using a dynamic *in vitro* approach, such as the pH, material retention (*e.g.*, dry matter, curd, fat, protein), moisture uptake, and physicochemical breakdown (see Table 6 and case studies 1–2). It is recommended that to minimize external factors that may affect the relationships between *in vitro* and *in vivo* data, the same measurement methods to characterize digesta properties should be utilized for samples from both the *in vitro* and *in vivo* studies.

4.2 Selection of food structures for IVIVR development

In the proposed IVIVR framework, it is strongly suggested to select a wide range of food structures to be studied *in vivo* and *in vitro*, which is particularly useful for the evaluation of Level B and Level C IVIVRs. However, many of the previous studies that focused on Level A IVIVRs typically investigated only one type of food consumed as a single-component meal (Table 6) to validate the physiological relevance of the *in vitro* approach; few studies listed in Table 6 reported Level B or C IVIVRs for more than one food.[57,80] Although good agreement with *in vivo* data was reported in the studies that reported the equivalent of a Level A IVIVR (Table 6), the use of only a single product in these studies may result in uncertainty whether the *in vitro* approach would be applicable for other types of foods to produce a high agreement with *in vivo* data.[8] For example, in the Level B IVIVR of Case Study 3, the static *in vitro* approach used to mimic the softening half-time worked only for four out of five foods tested (Fig. 5B). Similarly, the work of Drechsler and Bornhorst[57] indicated that only five out of the six foods studied showed a good agreement with *in vivo* data. This suggests that an *in vitro* approach may be applicable to only a certain range of food structures, and that drawing a conclusion of the usefulness or physiological relevance of an *in vitro* approach based on a single food structure may not result in similar physiological relevance for other food structures.

The limited number of studies that reported Level B and Level C IVIVRs, combined with the tendency of reporting Level A IVIVR for one type of food only in the literature (Table 6), suggests that future evaluation of *in vitro* gastric digestion approaches should involve more types of food products and more quantitative comparisons to examine IVIVRs across the three quantitative levels. Additionally, similar to the IVIVC in the pharmaceutical industry where not all drug formulations exhibit an IVIVC (depending on the solubility and permeability), aspects of food structure and composition that may affect the likelihood of establishing an IVIVR for food digestion need to be investigated. For example, in the food breakdown classification system (FBCS), it was proposed that the likelihood of an IVIVR for solid food digestion may be predicted by the initial texture (hardness), the rate of softening of the food in the gastric environment, and the rate-limiting mechanism of the breakdown process.[17] In the field of food

Table 6 Selected examples from previously published works of *in vitro* gastric digestion studies that were conducted to evaluate relationships with *in vivo* data and how they would fit in the proposed IVIVR framework. Unless otherwise stated, the data used for IVIVR establishment for each example is the same between the *in vitro* and *in vivo* study. For the definition of each IVIVR level, see Table 2. Detailed descriptions of the *in vitro* and *in vivo* methods for each row of the table can be found in the reference given in the table. Extended application of the IVIVR framework to small intestinal digestion data is provided in Table S5†

Food	*In vitro* approach	*In vivo* method	Data used for IVIVR evaluation	Findings	IVIVR within proposed framework	Ref.
Pretzel, white rice, brown rice, couscous, quinoa, orzo pasta	Static model: shaking water bath (excess gastric fluid)	Published data (international tables of glycemic index)	• *In vitro* softening half-time (time required to reduce the food hardness by 50%) • *In vivo* glycemic index	• The trend in the breakdown half-time (measured as softening half-time) *in vitro* aligned with the trends in *in vivo* glycemic index for five out of six of the foods studied	Level C	57
Reconstituted infant formula	Semi-dynamic model: glass jacket reactor	Piglet model (slaughter method)	• Remaining volume of gastric digesta	• Similar kinetics of protein hydrolysis *in vitro* and *in vivo*, based on no significant differences in the proportion of immunoreactive caseins and β-lactoglobulin *in vitro* and *in vivo*	Level A	78
			• Percentage of caseins and β-lactoglobulin in the stomach over time	• Good correlation between *in vitro* and *in vivo* proteolysis (correlation coefficient = 0.987) • No significant difference between *in vitro* and *in vivo* volumes of digesta remaining in the stomach ($p < 0.05$)	Level D	
α-Lactalbumin solution	Dynamic model: artificial gastric dynamic system (AGDS)	Human study (capsule endoscopy and nasogastric intubation in healthy adults)	• Gastric force, pepsin and gastric juice secretion	• Similar contractile force between the force exerted by the AGDS stomach and the mechanical force in the human stomach	Level D	20
	Static model: water jacketed reactor		• Gastric emptying	• Similar pH reduction profile in the gastric antrum of the AGDS and semi-dynamic system to that of human stomach		
	Semi-dynamic model: water-jacketed reactor		• pH at various intragastric locations	• Order of similarity to *in vivo* protein hydrolysis pattern and mechanisms: AGDS > semi-dynamic > static		
Red delicious apples (cored and skinned) consumed with water	Dynamic model: artificial stomach response kit (ARK®)	Human study (magnetic resonance imaging [MRI] in healthy adults)	• Meal retention in the stomach over time	• Linear correlation between *in vitro* and *in vivo* gastric content retention ($r = 0.99$)	Level A	79
			• Gastric emptying parameters (lag phase, gastric emptying rate constant (k), y-intercept (β))	• Similar gastric emptying patterns between *in vitro* and *in vivo* data • No significant difference between *in vitro* and *in vivo* emptying half-time, lag phase, k, and β	Level D	
Locust bean gum test meals (low and high viscosity) containing agar beads of varying fracture strengths	Dynamic model: dynamic gastric model (DGM)	Published data (MRI in healthy adults using multiple agar beads)	• Mean breaking time (MBT) of the agar beads	• Linear correlation between *in vivo* and *in vitro* MBTs (regression line $R^2 = 0.824$ for low viscosity meal, $R^2 = 0.685$ for high viscosity meal)	Level B	80

Table 6 *(Contd.)*

Food	*In vitro* approach	*In vivo* method	Data used for IVIVR evaluation	Findings	IVIVR within proposed framework	Ref.
Beef stew and orange juice	Dynamic model: new version of the near-real dynamic *in vitro* human stomach (new DIVHS)	Published data (dual phase isotopic study; gastric aspiration study in healthy adults)	• Gastric retention ratio of solid and liquid	• <12% average relative *in vivo–in vitro* difference in solid and liquid gastric content fraction	Level A	81
			• pH profile	• Similar solid and liquid emptying half-time, and average gastric emptying rate between *in vitro* and *in vivo* system • Consistent trend in the pH change during digestion with *in vivo* data	Level D	
Unheated-homogenized, pasteurized-homogenized, or UHT-homogenized cow's milk	Dynamic model: human gastric simulator (HGS)	Rat model (Sprague Dawley rats, slaughter method)	• Appearance of the milk curds formed • Protein hydrolysis (assessed from SDS-PAGE patterns)	• Similar trends between *in vitro* and *in vivo* data in curd mass retention and protein hydrolysis	Level D	82
Medium grain white rice	Dynamic model: human gastric simulator (HGS) v2.0	Growing pig model (slaughter method)	• Dry matter retention in the stomach	• Linear correlation between *in vivo* and *in vitro* dry matter retention (almost 1 : 1 correlation)	Level A	8
			• pH in the pylorus and fundus regions of the stomach	• Similar values between *in vitro* and *in vivo* pH in the pylorus or fundus region for up to 120 min digestion	Level D	
			• Textural changes (hardness) in the proximal and distal region of the stomach	• Similar trends between *in vitro* and *in vivo* hardness of digesta from the proximal and distal regions at all digestion time points		
Nutrilon® liquid meal, beef stew meal mixed with orange juice	Dynamic model: TIM advanced gastric compartment (TIMagc)	Published data (nasogastric intubation and scintigraphy studies in healthy adults)	• Gastric motility pattern, gastric wall pressure and shear forces	• Comparable gastric secretion profile, motility pattern, pressure height, pressure peak, and shear forces duration between *in vitro* and *in vivo* data, but sharp pressure peaks *in vivo* could not be fully simulated	Level D	83
			• Gastric emptying over time	• Similar solid fraction emptying curve to the *in vivo* study, but faster liquid emptying *in vitro* dan *in vivo*		
			• Gastric secretions component (overall gastric juice, pepsin, HCl) flow rate over time	• Close trend between the *in vitro* and *in vivo* gastric juice rate, pepsin, and HCl flowrate profile over time		

digestion, such concept has not been established in complex liquid foods and is an area for future investigation. In the process of evaluating IVIVRs across various food structures, it is also suggested that each food is first studied as a single-component meal, then followed up by the evaluation of IVIVRs using mixed meals. It should also be noted in any IVIVR the types of food composition and structures utilized for its development, as other foods with very dissimilar properties may not necessarily follow the same trends *in vitro* or *in vivo*.

4.3 *In vivo* study design

The usefulness of an *in vitro* digestion approach, either as a tool to mimic *in vivo* behavior or as a tool to investigate mechanisms that occur *in vivo*, is determined by the design of the *in vitro* and *in vivo* studies.[49] The first aspect of the study design is the selection of the *in vivo* model. For human digestion applications, the most preferred *in vivo* approach is a human study. Previous studies listed in Table 6 and the case

studies, however, have utilized animal models for more specific comparison of digesta properties that are difficult to measure in human subjects, such as averaged physical properties of digesta, nutrient hydrolysis in the stomach, and gastric emptying of a specific nutrient. As such, future studies are recommended to evaluate the utility of the proposed IVIVR framework using *in vivo* data from human studies. In the case of limited access to a pig model, smaller animal models such as rats may also be used, such as in the study of Ye *et al.*[82] However, caution in the data interpretation is needed due to the anatomical and physiological differences between smaller animal models (*e.g.*, rats) and large monogastric animals.[9,43] In the case of *in vivo* digestion approach evaluation using animal model, once Level A and B IVIVRs are established for the *in vitro* approach (*i.e.*, the breakdown processes have been confirmed to be similar to *in vivo* system), the next step should be validating the *in vitro* approach with measurable properties in human studies. If an animal model is utilized in developing an IVIVR, this should be stated clearly to avoid misinterpretation of the data in future studies to other species or populations that may not be relevant. In the planning of the *in vivo* study, it is also important to ensure that the variability of *in vivo* results is anticipated by carefully planning the study and having a sufficient number of experimental replicates to achieve a statistical power of at least 80%.

4.4 *In vitro* study design

In addition to methodological considerations of the *in vivo* study, the design of the *in vitro* study plays a crucial role in future IVIVR development. One aspect of the *in vitro* study design that should be carefully considered is the amount of food to be studied, especially if using a dynamic digestion model. Whenever possible, the scale of the *in vitro* approach also needs to be matched to the scale of the *in vivo* reference study, because it is unclear if gastric digestion parameters scale linearly. In certain cases, *in vitro* studies with different food amount, slightly different composition, and different digestion time scale from *in vivo* studies may still be useful as a predictive tool of general *in vivo* behavior (Table 3).

For example, in the work of Vardakou *et al.*,[80] the mean breaking time of agar beads of varying fracture strength *in vitro* (using the dynamic gastric model) and *in vivo* (published human study data) followed a similar trend, although in the *in vitro* study a single agar bead was used, whereas multiple agar beads were used in the *in vivo* study. Similarly, in the study of Drechsler and Bornhorst,[57] the trend of *in vitro* softening rate of solid carbohydrate-rich foods generally aligned with the trend in published glycemic index of similar types of foods (human study data), although the specific foods were not exactly the same in the *in vitro* and *in vivo* studies that were compared. In contrast, in Case Study 2, the dry matter emptying half-time *in vitro* and *in vivo* across three different types of milk was inversely related, which may have been caused by the different amounts of milk volume used in the studies (equal protein amounts fed *in vivo vs.* equal volume of milk utilized *in vitro*).

In addition to meal amount, the specific *in vitro* digestion parameters utilized also play an important role in whether an IVIVR can be established. For example, in Case Study 2, the differences in the simulated gastric fluid enzyme composition and secretion rates may have resulted in inaccurate simulation of curd disintegration kinetics *in vitro* for raw whole milks. Also, in Case Study 1, due to the differences in gastric secretion rate and location utilized in the *in vitro* study, the kinetics of gastric acidification and mixing of gastric secretions *in vitro* did not mimic what was observed *in vivo*.

Due to the importance of the specific digestion parameters in IVIVR development, it is critical to select digestion parameters (*e.g.* gastric secretion rate, gastric emptying rate, ratio of meal : secretions, enzyme and mucin content, *etc.*) that are derived from *in vivo* findings. It is recommended that future studies examine such parameters within this IVIVR framework for wide variety of food structures, as it is not practical (nor resource efficient) to conduct an *in vivo* study for each food product to determine specific digestion parameters to be utilized for that food. In addition, future studies are encouraged to utilize the quantitative approaches developed as part of this IVIVR framework to identify limitations in the current *in vitro* approaches, such that they can be improved to become more physiologically-relevant for a wide variety of foods. As the specific digestion method and parameters have been shown to be critical in the ability to develop an IVIVR, such details should be carefully noted in any future IVIVR, and the applicability of the IVIVR should be limited to use of the same *in vitro* digestion method and parameters.

4.5 Identification of physiological factors that contribute to lack of an IVIVR

One of the keys to define the limitations of an *in vitro* gastric digestion approach is identifying physiological factors that may contribute to the lack of relationships between *in vitro* and *in vivo* data, some of which have been highlighted in the case studies. These factors include: mastication, gastric secretory response, gastric sieving mechanisms, and hormonal feedback that affects gastric emptying and digestion processes.

For example, a common attribute in the *in vitro* approaches listed in Table 6 is the use of varying gastric secretion rate over time to closely mimic the pH profile in the *in vivo* stomach, because gastric secretions are important to biochemical changes in the gastric environment due to the presence of multiple digestive enzymes, each with a different range of active pH.[23,84,85] Such approach of varying the gastric secretion rate is ideal to achieve the closest resemblance to physiological reality, as also highlighted in the studies that involved the comparison between static, semi-dynamic, and dynamic gastric digestion models.[20,86] However, gastric secretion data may not be available for every food type or for specific food combinations, and a previous *in vivo* study reported that gastric secretions vary not only with digestion time, but also across food types.[10]

Another example is the use of a constant gastric emptying rate over time in dynamic *in vitro* digestion models. In Case

Study 1, while the *in vivo* dry matter gastric emptying was mimicked well *in vitro*, larger APE values were observed at later digestion times. This suggests that that incorporation of a variable gastric emptying rate (where the gastric emptying rate changes with digestion time) during the digestion period may help to incorporate the physiological regulation that occurs *in vivo*. Understanding mechanisms of how to control the gastric emptying rate (and if this process varies with different food types or structures) is an area for future study.

5. Conclusions & future recommendations

Use of *in vitro* gastric digestion studies to mimic food structural changes and nutrient release *in vivo* are expected to keep growing in the future. With the various advantages of *in vitro* studies over *in vivo* studies, it is anticipated that *in vitro* studies will eventually be utilized in the food industry to redefine the healthiness and nutrition information panels of food products and support certain health claims of functional food products. Prior to drawing certain conclusions based on *in vitro* gastric digestion study results, it is important that researchers understand the utility and limitations of their *in vitro* gastric digestion approach. To facilitate this evaluation, we proposed an IVIVR framework to relate *in vitro* gastric digestion data with *in vivo* gastric digestion and nutrient blood appearance/small intestinal absorption. The current version of the IVIVR framework serves as a tool to assess the utility of a specific *in vitro* approach in mimicking *in vivo* gastric digestion process. The IVIVR framework is also useful to evaluate the selection of *in vitro* digestion parameters and improve their accuracy, and to help understand key aspects of gastric digestion. If an *in vitro* approach has been evaluated with this IVIVR framework and its usefulness to mimic *in vivo* process of certain food groups has been defined, one can be confident that the *in vitro* method can be applied for foods with similar composition and structure and result in similar findings to an *in vivo* study with the species or population used in the IVIVR development.

In the future, the implementation of this IVIVR framework in food digestion research will be valuable for:

- Identification of food structures/compositions that may not exhibit an IVIVR
- Reviewing existing *in vitro* gastric digestion approaches (especially dynamic *in vitro* approaches that are likely to achieve a 1 : 1 relationship with *in vivo* data) to develop physical models, process parameters and protocols that accurately simulate the kinetics of digestion and nutrient release in the human stomach
- Coupling the existing experimental work *in vitro* with *in silico* approaches to improve prediction of food digestion variables
- Development of a generalized regression model to predict *in vivo* gastric digestion and/or nutrient absorption output based on *in vitro* data, taking into account variations of the structure and composition of foods
- Application of the framework to investigate the IVIVRs of mixed meals, which is a more realistic scenario of *in vivo* food consumption

While the methodology and assessment criteria of this IVIVR framework have been carefully formulated to be applicable for any type of gastric digestion method and food structure, it is expected that the robustness of the methodology and assessment criteria will be refined in the future when it is widely implemented in more experimental data, such as the inclusion of case studies that utilize *in vivo* data from human studies. It is also recommended that after application of this IVIVR to a wider variety of food products, and types of *in vitro–in vivo* studies, refinements should be made, including extension of the framework to other sections of the gastrointestinal tract, such as the small intestine.

Author contributions

J. Nadia: conceptualization, data curation, methodology, formal analysis, visualization, writing – original draft preparation; D. Roy: methodology, writing – review & editing; C. A. Montoya: methodology, writing – review & editing; A. Acevedo-Fani: methodology, writing – review & editing; H. Singh: writing – review & editing; G. M. Bornhorst: conceptualization, methodology, supervision, writing – review & editing.

Data availability

No primary research results have been included and no new data were generated as part of this review.

Conflicts of interest

There are no conflicts of interest to declare.

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