Development and Validation of a Dynamic Model of the Gastrointestinal Tract

Development and Validation of a Dynamic Model of the Gastrointestinal Tract

Ontwikkeling en validatie van een dynamisch maagdarm model

(Met een samenvatting in het Nederlands)

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Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

This thesis describes the development and application of a new revolutionary *in vitro* model of the gastrointestinal tract (GIT). Models can be used to perform simplified experiments under uniform and well controlled conditions. However, simulating such a complex system as the GIT carries the risk of oversimplification. Models of the GIT to date do not successfully simulate the dynamic conditions in the lumen of the GIT (8,68,71), which is necessary to answer the variety of questions that are raised by specialists in various fields, for example in the food, feed and pharmaceutical industries. Increased knowledge of food processing, molecular biology and nutrition has resulted in the production of functional foods and novel foods. The health claims used for functional foods require the establishment of efficacy, while the safety of novel foods has to be assessed before they can be marketed (72). The feed industry aims at improving the bioavailability of their products, introducing alternative protein and fat sources and reducing the faecal output of environmental pollutants such as phosphates. Finally, the pharmaceutical industry has an interest in studying the dissolution of drugs, slow-release systems and drug-nutrient interactions. An *in vitro* model that would successfully simulate the relevant conditions of the GIT can therefore be used for a broad range of applications. The model introduced in this thesis is meant to have a high predictive value towards events occurring in the lumen of the gut of monogastric species including man. The development of such a model involves a multi disciplinary approach, combining microbiology, physiology, gastroenterology, process technology, and automation.

PARTS OF THE GIT AND THEIR FUNCTIONS

The main function of the GIT is to break down food and to transport nutrients into the body. This is performed in a system of cavities and tubes that starts with the stomach and ends with the rectum (Fig. 1), each of which with a specific task (Table 1). Although the GIT is situated inside the body, the interior (lumen) of the GIT can be regarded as the outside world. The wall of the GIT forms the barrier between lumen and body, similar in function to the skin. The walls of the separate sections of the GIT are highly differentiated with regard to their specific task in the digestive process. There are cells that secrete hydrochloric acid, mucus or enzymes, and absorb specific nutrients through active transport across the cell membrane, and cells with immunological functions. Furthermore, the GIT

harbours numerous microorganisms which interact with the host as well as with foods. In this section the human GIT will be discussed.

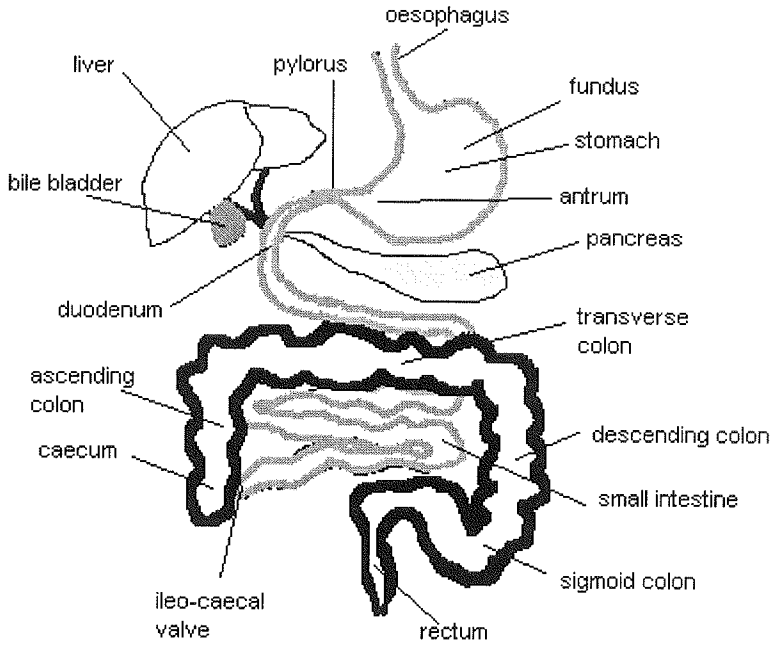

Fig. 1. Schematic presentation of the gastrointestinal tract

Stomach

The main parts of the stomach are the fundus for storage, the antrum for mixing, and the pyloric sphincter to separate the stomach from the small intestine (Fig. 1). The meal is ingested in a relatively short time and stored in the fundus to release the meal gradually into the small intestine to optimize digestion and absorption of nutrients (73). The antral motility consists of regular waves that squeeze the chyme towards the pylorus (29,46).

Table 1. Main functions of the separate parts of the digestive tract

Stomach	storage particle reduction digestion by pepsin and lipase, acidification barrier to microorganisms
Small intestine	emulsification of fat by bile digestion and absorption of protein, carbohydrates and fat absorption of nutrients and water
Large intestine	fermentation of undigested materials by the microflora absorption of water, fermentation products and sodium

This results in remarkably efficient grinding and mixing of the chyme with digestive fluids. Only small particles are emptied, and also the caloric density of the chyme entering the duodenum is carefully controlled (47). The delivery of chyme from the stomach into the duodenum is regulated by opening and closing the pyloric sphincter and through its coordination with antral waves and the motility of the duodenum (40). In the stomach extensive secretion of digestive fluids take place. Hydrochloric acid is secreted to solubilize and release components from the food, and to create a barrier to pathogenic microorganisms towards the lower gut. Just after ingestion of food with some buffer capacity, the pH can be sufficiently high to accommodate salivary amylase activity and gastric lipase activity. Especially in newborn infants this lipase activity is important and can hydrolyse 30% of the lipids (38). While the pH is decreasing due to hydrochloric acid secretion, proteolytic activity by the secreted pepsin increases to an optimum activity at pH 2.5. To protect the gastric wall, the pepsin is secreted as pepsinogen, an inactive zymogen, which is activated by acid and pepsin in the stomach. The gastric wall is protected against the secreted hydrochloric acid by a layer of mucus.

Due to the low pH not many resident microorganisms are present in the human stomach (Fig. 2). Recently, it has been shown that gastric ulcers may be correlated with the presence of *Helicobacter pylori* (64). Many microorganisms are able to pass the stomach with the food (transiting microorganisms). Their survival depends on the gastric pH (which is not always low, as pointed out above),

protective components in the food matrix such as fat, their physiological condition and their acid tolerance (52).

Small intestine

An important task of the small intestine is to absorb nutrients and water from foods and secreted digestive fluids. The small intestine is divided into three parts: duodenum, jejunum and ileum, which in the adult human have a length of about 25 cm, 190 cm and 285 cm, respectively, and together contain ca. 400 g of chyme. The wall of the small intestine is folded and covered with villi which increase the surface area tenfold (37). The villi are covered with microvilli which increase the surface area of the small intestine to ca. 200 m^2 (76). The zone of microvilli is also called the brush border. The large surface area – about the area of a tennis court – results in a very efficient absorption of water and nutrients. Bile is secreted in the duodenum and contains bile salts that emulsify the dietary fat into small droplets to increase the surface area for lipase activity. Most of the bile salts that are secreted in the duodenum are re-absorbed in the ileum for re-use in the bile (the 'enterohepatic circulation'). The acidic chyme entering from stomach is neutralized by bicarbonate to obtain the appropriate pH for small intestinal enzymes (27). The pancreatic juice secreted into the duodenum contains a complex mixture of pre-enzymes (zymogens) that are activated by trypsin (65). Trypsin is activated mainly by enterokinase in the lumen of the proximal small intestine (36). Secretion of zymogens is an efficient method to protect the pancreas from being attacked by its own enzymes. An overview of the main pancreatic enzymes is given in Table 2.

Table 2. Pancreatic enzymes and their substrates (40)

Enzyme		Substrates
proteolytic	trypsin	bonds adjacent to lysine and arginine
	chymotrpysin	bonds adjacent to aromatic amino acids
	elastase	neutral amino acids with aliphatic side chains and many others
	carboxypeptidase A	carboxy-terminal aromatic amino acids
lipolytic	lipase	1 and 3 ester bond of triglycerides
	phospholipase A	2 ester bond of lecithin
saccharolytic	α-amylase	starch

In the small intestine, the digestion of nutrients is determined by combined enzyme activities, secreted components such as bile salts, and residence time. When enzymes and substrate travel through the small intestine, their interaction changes in time. Substrates are changing due to degradation and solubilization, while also enzymes are gradually broken down. During the small intestinal transit, the chyme is concentrated due to water absorption. The products of digestion diffuse to the brush border, where they may be degraded further by brush border enzymes and absorbed.

The absorption of digestive products prevents build-up in the lumen of the small intestine, which could inhibit enzyme activities. *In vivo* absorption occurs very efficiently by different mechanisms depending on the nutrient. The most simple absorption mechanisms is by simple diffusion, with molecules passing directly through the membrane. The driving force is a concentration gradient. Passive absorption of hydrophilic molecules occurs through water pores in the cell membrane or through the tight junctions, depending on their size (14).

The products of fat digestion and compounds such as lipophilic vitamins are incorporated in mixed micelles, which diffuse to the brush border where they are absorbed through the lipid membrane of the enterocytes (74). Another mechanism of absorption is by facilitated diffusion, where molecules are transported across the membrane by carrier proteins. The driving force is the concentration gradient. Molecules can also be actively transported across the membrane by carrier proteins against a concentration gradient, using cell energy.

The motility of the small intestine prevents microorganisms from propagating in the lumen, as demonstrated by the overgrowth of microorganisms seen in patients with diminished motility (79). Under normal conditions only those microorganisms colonize which grow fast enough to resist the flow of chyme such as Enterobacteriaceae and enterococci. Others are associated with or grow in the mucus layer of the gut wall. The species that are resident in the small intestine are controlled by secreted bile salts, anaerobic conditions, the immune response of the host and the ability of the microorganisms to adhere to the cells of the gut wall (69,42,48). Also some transiting pathogenic microorganisms can adhere to the epithelium, which might be part of their invasive strategy (4). Recent research has revealed that indigenous microorganisms are able to modify the specific attachment receptor and thus prevent colonization of the pathogen (11,77). The body can react to invasive pathogens by increasing cell turn-over to dispose of the pathogens attached to the cells. In the lumen of the ileum significant numbers of

microorganisms are present (Fig. 2), mainly due to reflux from the caecum through the ileocaecal valve and reduced flow of chyme.

Large intestine

The function of the large intestine is break-down of undigested materials by microorganisms, and absorption of water and sodium. The large intestine is separated from the small intestine by the ileo-caecal valve (Fig. 1), and consists of the following sections: the caecum (7 cm long in the adult human), the ascending colon (18 cm), the transverse colon (50 cm), the descending colon (30 cm), the sigmoid colon (40 cm) and the rectum (15 cm). The large intestine contains ca. 350 g material, with a dry weight content from ca.14% in the caecum to ca. 23% in the rectum. The average time for ingested radiopaque markers to transit the colon is about 35 hours. Men have a shorter colonic transit time than women: 31 hours and 39 hours, respectively (54). Bacterial dry matter accounts for half of total dry matter, while bacterial counts range from 10^{10}/ml in the ceacum to more than 10^{11}/ml in the faeces, with over 400 different species being present (43). The large numbers of microorganisms ferment undigested materials delivered from the small intestine. The energy obtained through fermentation of dietary fibre, in particular resistant starch, accounts for 5–10% of the total energy requirements of people on western diets (17). The fermentation of dietary fibre can be an important energy source for human beings under close to starvation conditions. This saccharolytic fermentation is regarded as a healthy process (38).

It is assumed that fermentation decreases the pH which inhibits harmful enzyme activities such as 7α-dehydroxylase, an enzyme that converts primary bile salts into secondary deoxycholic and lithocholic acids which have carcinogenic properties (15). The production of butyrate is especially important because it is utilized as a source of energy by colonocytes; this nourishment is thought to prevent malignant proliferation (17).

Proteolytic activity of the colonic microflora might result in the production of toxic compounds and is hence generally regarded as unwholesome. Epidemiological studies have revealed that the consumption of red meat correlates with colon cancer (7). There is evidence that this is due to formation of N-nitroso compounds from red meat protein that has escaped digestion. The colonic microflora might possess several enzyme activities that generate toxic, genotoxic or carcinogenic products (Table 3; 67).

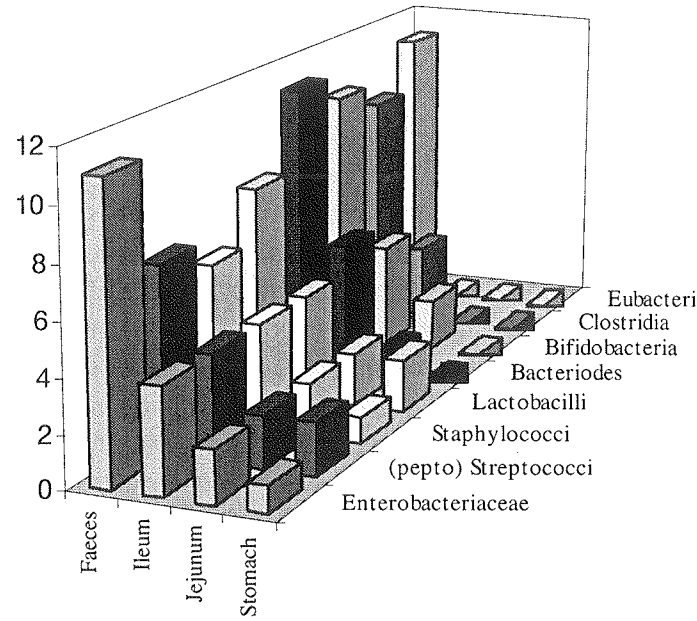

Fig. 2. Numbers of some groups of bacteria in different parts of the gastrointestinal tract (75)

Table 3. Conversion of substrates by gut flora enzymes into toxic products (66)

Substrate	Enzyme	Product
Azo compounds	azoreductase	amines
Aromatic and heterocyclic nitro compounds	nitroreductase	amines
Nitrate/nitrite	nitrate/nitrite reductase	N-nitroso compounds
Amino acids	decarboxylase	amines
Glucuronides	β-glucuronidase	induction of carcinogenic benzo(a)pyrene
Plant glycosides	β- glucosidase	aglycones, hydrocyanic acid

Water absorption is an important feature of the large intestine. The human large intestine may absorb 1.5 l/day, with a maximum absorptive capacity of about 6 l/day (21). Water is absorbed passively to maintain isotonic luminal conditions, coupled to absorption of sodium, which is transported actively against an electrochemical gradient. The flows of liquid in the GIT are presented in Figure 3.

IMPORTANT ASPECTS TO SIMULATE GASTROINTESTINAL FUNCTIONS

The digestive tract consists of separate compartments, dedicated to a specific step in digestion. To study the fate of ingested compounds, it is important to expose the meal to each step of digestion with a realistic transit time for each compartment. Because of secretion, transit of the meal and absorption of nutrients and water, the composition of the chyme is changing in time. In other words, different fractions of the meal are exposed to different conditions in each part of the GIT. The effect of gastric transit depends on the interaction between different parameters such as gastric emptying, secretion of enzymes, hydrochloric acid and water. The digestion of milk protein is a good example to illustrate the interaction of gastric parameters. Gastric pH after ingestion of the milk decreases slowly (Fig. 4). The meal is gradually delivered from the stomach and thus portions of the meal escape exposure to low pH and consequently to peptic activity. The gradual delivery of a meal can be described by mathematical equations that calculate the cumulative amount of meal delivered in time, expressed as a percentage of intake (22,25). Liquids and semi-liquids show an exponential gastric delivery pattern, while solids have a more linear pattern of gastric delivery (60). To obtain a realistic gastric delivery of the meal, specially when there are particles in the meal, the sieving activity of the pylorus should be mimicked.

To mimic the grinding of particles and to mix and transport the chyme in a physiological fashion, it is important to reproduce the characteristics of the motility of the gut. The peristaltic movements are a very efficient way to propagate the concentrated chyme through the gut.

Digestive processes such as gastric emptying, secretion of digestive fluids and mucus, and motility are highly controlled by hormonal and neural regulation mechanisms, influenced by the food and external factors (13). Often the absorption of nutrients is controlled to meet the body's requirements, or depending on the properties of the gut wall or on the presence of specific transport mechanisms. For study of the behaviour of microorganisms in the gut, also the interaction between microflora and the host is considerable (77). The immunological response of the host is a major determinant of the microflora's composition.

The aspects described above may lead to the following criteria to design a realistic GIT model, as have been proposed by Longland (49):

- sequential use of digestive enzymes,
- appropriate pH and co-factors, co-enzymes, bile salts, etc.,
- removal of end-products of enzyme activity to prevent product inhibition,
- mixing appropriately in each step of the digestion process,
- physiological residence times for each digestive step.

Not mentioned by Longland, but very important, is the influence of the body on digestion of the food.

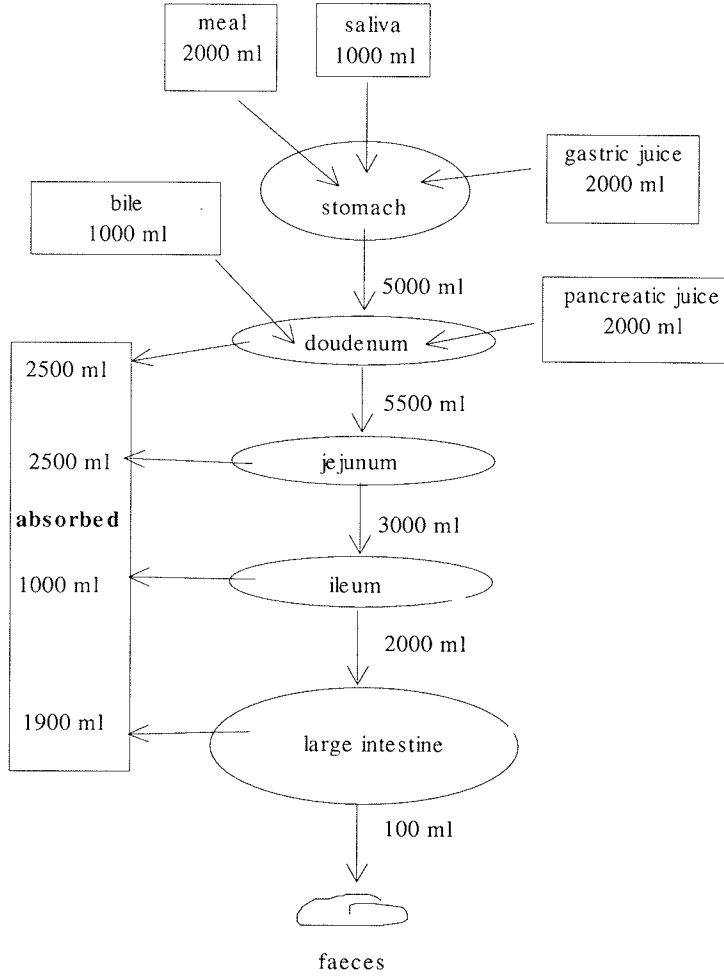

Fig. 3. Typical flows (ml/day) of water through the GIT. (5,6,18,61)

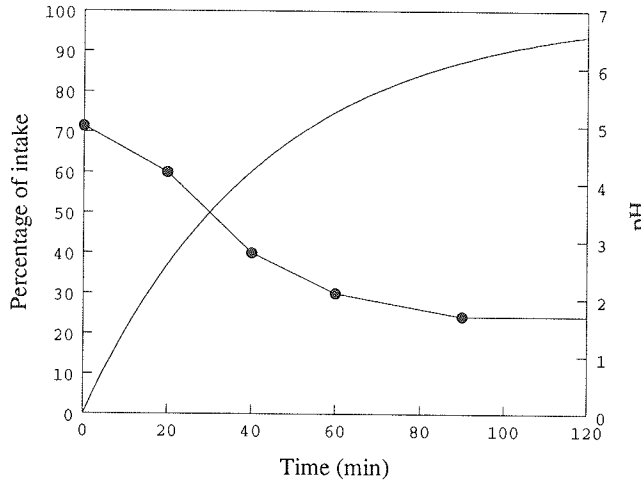

Fig. 4. Cumulative gastric meal delivery, expressed as a percentage of intake (solid line), and pH profile (●) after ingestion of milk.

MODELS OF GASTROINTESTINAL FUNCTIONS

This section will give an overview of GIT models developed to date and discuss them in relation to the criteria for an adequate simulation of gastrointestinal functions, as described in the previous section.

Models to study digestion

Several models for estimating digestibility have been described and evaluated by Boisen and Eggum (8) and Savoie (71). The methods described generally involve a single, two- or three-step digestion, simulating gastric digestion, gastric/small intestinal digestion and gastric/small intestinal/large intestinal digestion, respectively. The models are static models: the steps of digestion are simulated by exposing the test material to an enzyme solution at a fixed pH and temperature during a fixed period of time. In static gastric models, the test material is exposed to pepsin at a pH below 2.5 during a period that ranges from 0.5 h to 48 h (8). A

gastric model that incorporates a flow of secretion has been described by Vatier *et al.* (80) to assess the characteristics of an antacid. A similar model by Savalle *et al.* (70) was dedicated to the study of gastric digestion and evacuation of milk proteins. Gastric juice is pumped into a vessel, while the gastric contents are removed from the vessel to simulate gastric emptying. The gastric pH is not kept at a fixed value, but controlled by the addition of hydrochloric acid. These gastric models can be described as dynamic models since liquid flows and the pH are controlled and change in time.

After gastric digestion, the test material is generally exposed to digestion with pancreatic enzymes at around pH 7.5. Incubation times ranged from 1 h (2) to 18 h (9). Small intestinal digestion is most frequently simulated with pancreatin as a source of pancreatic enzymes (2,8,71), or with intestinal fluid (32). Some methods include an extraction step to avoid the interference of fat with the determination of protein digestibility (2). The digestive mixtures are generally mixed with a magnetic stirrer (71) or in a shaker. Englyst (26) used marbles to mimic small intestinal peristalsis in a system that determines the fraction of resistant starch.

The most advanced digestion system described to date is the dialysis cell method (33,34), which uses a dialysis tube to remove (enzyme-inhibiting) products of digestion. Another method that incorporates absorption is described by Diepenmaat-Wolters (23) who used a hollow-fibre system to evaluate mineral bioavailability.

To evaluate degradation several methods have been used such as measurement of acid production during proteolysis (62), colorimetric measurement of the products of starch digestion (45), and determination of undissolved nutrients by filtration or centrifugation after treatment with a precipitating agent such as trichloroacetic acid (12). The large intestinal digestion step is used to simulate microbial degradation of dietary fibre. This step is simulated with rumen fluid (81) or fibre-degrading enzymes (9,10).

Models to study microorganisms

The behaviour of microorganisms is generally studied in models of the large intestine. Only few models include the micro-ecology of the stomach and the mall intestine (16,59).

Models that simulate fermentation by colonic microflora are reviewed by Rumney and Rowland (68). Three types of models have been described to study different aspects of colonic microflora: batch culture systems, semi-continuous culture

systems and continuous culture systems. Batch cultures are mainly used for simple short-term carbohydrate fermentation experiments (1 to 2 days) and only involve incubation of test material with faeces or colonic contents (3,78,82). The experiments are generally performed in closed vessels under anaerobic conditions. Semi-continuous cultures need a more sophisticated set-up. The microflora is fed while some of the contents is removed from the fermentation vessel intermittently to mimic the entry of chyme from the ileum and a flow of chyme to the next part of the gut. Continuous cultures have the same set-up as the semi-continuous cultures, except that they have a regular feeding of microflora and removal of contents. This approach results in a steady-state situation in which the growth rate of the microorganisms is determined by the dilution rate. To simulate the consecutive parts of the colon, different systems have been designed with two, three or five vessels in series (35,56,59). These systems generally allow for growth of strictly anaerobic microorganisms by flushing with anaerobic gas. The pH is measured and controlled within the physiological range through addition of acid or alkali. Both continuous and semi-continuous cultures have been used to study the micro-ecology of the flora, degradation of undigested materials, enzyme activities and production of interesting metabolites such as short-chain fatty acids, gases and toxic compounds.

Limitations of the GIT models developed to date

This section will discuss the limitations of the GIT models to date with respect to a realistic simulation of the aspects that influence the fate of ingested compounds.

Composition of the chyme

The composition of the chyme is influenced by the transit of the meal, secretion of digestive fluids, and absorption of nutrients and water. None of the models described to date simulate these combined aspects and obtain a physiological chyme composition for the different digestive stages in time.

Enzyme, bile and electrolyte concentrations are not physiological (8). Especially the single enzyme methods are limited in their use since enzymes usually work together to digest a meal (71). Pancreatin or intestinal fluid are relatively cheap and contain a mixture of relevant enzymes. A disadvantage of these preparations is that their composition is not well defined, with a batch-to-batch variation of enzyme activities. Also, pancreatin contains a considerable amount of non-enzyme material. Generally, the gastric pH profile after ingestion of the meal is not

Small intestinal transit is also generally mimicked as a batch process, which is more physiological than simulating gastric passage as a batch process. The start of the experiment can be regarded as the beginning of the small intestine, where bile and pancreatic secretion are mixed with the gastric output. Incubation time can be regarded as the time needed for this mixture to travel through the small intestine. It has been suggested by McNeill Alexander (52) that the small intestine can be best described as a plug flow reactor (PFR, Fig. 5). A PFR is a tube along which the contents flows with only radial mixing. However, in the small intestine there is also extensive longitudinal mixing by segmentation (Fig. 7), especially when the chyme is liquid.

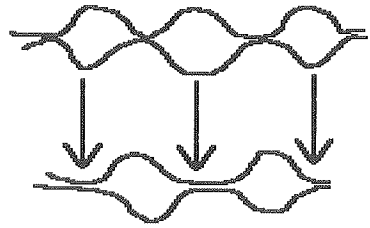

Fig. 7. Segmentation (18)

Probably the most realistic way to simulate the transit of the meal is to model the small intestine as a series of small continuously stirred tank reactors (CSTRs, Fig. 5). The size of the compartments determines the extent of longitudinal mixing. A large number of small compartments assumes little longitudinal axial mixing and act together as a PFR. Larger compartments assume homogeneous chyme along a section of the gut due to longitudinal mixing. The extent of mixing and propagation of the meal is determined by its bulk and viscosity. An ingested capsule travelled through the small intestine in 8 hours (27) which perfectly fits the PFR model with no longitudinal mixing. A typical gastric and ileal delivery of a solid meal is shown in Figure 8. The delay and pattern of ileal delivery shows that in this case the small intestine also approximates a PFR. Liquid meals, however, may have a much shorter transit time through the small intestine. Marteau *et al.* (52) demonstrated that 50% of a milk meal, and also of a yoghurt meal, was evacuated from the small intestine ca. 1.5 h after ingestion. The addition of indigestible components such as lactulose can decrease the small intestinal

transit time (44) because it retains fluid in the lumen of the small intestine through osmotic activity. The gastric and ileal delivery of lactulose is shown in Figure 9. The transit of lactulose can best be simulated in a model based on a cascade of CSTRs. The study with lactulose shows that water absorption is an important determinant for small intestinal transit.

Colon models are generally CSTRs connected together to mimic sequential regions of the colon. However, the colon can only act as a CSTR if the chyme is liquid enough to be mixed. Due to water absorption mixing of the chyme decreases when it travels downwards the colon, causing the colon to act increasingly like a PFR. Microorganisms cannot be maintained in a PFR because they wash out with the plug flow. In the large intestine resident microorganisms resist the flow of chyme because they are attached to the gut wall.

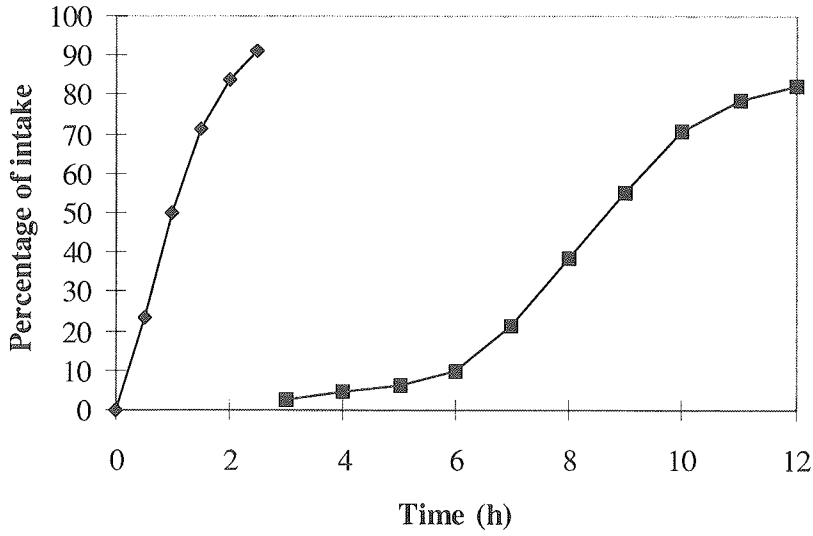

Fig. 8. Cumulative gastric (◆) and ileal (■) delivery of a solid meal (5).

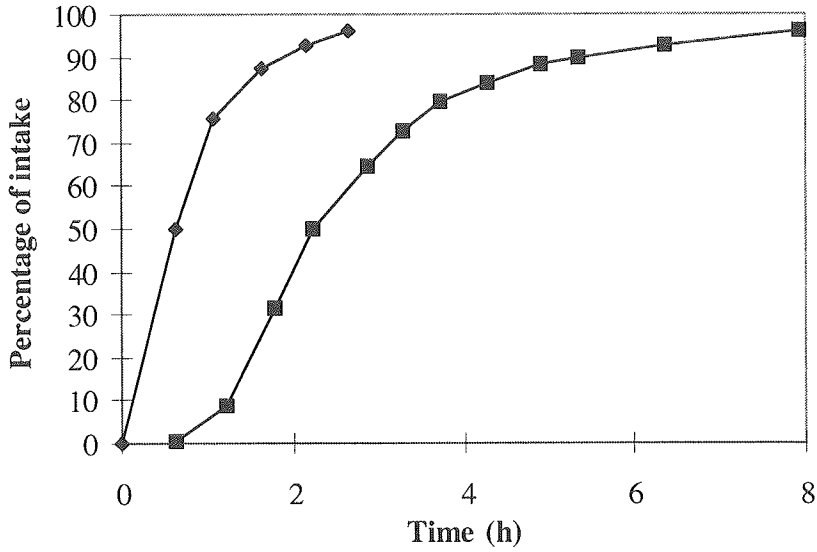

Fig. 9. Cumulative gastric (◆) and ileal (■) of a liquid meal with lactulose (5).

In fact, attachment to the gut wall is regarded as a prerequisite for invading microorganisms to overcome their lag phase in the new environment and to colonize the intestine (31). Experiments in continuous flow fermenters have shown that attachment to the glass wall of the vessel is also necessary for invading microorganisms to overcome the colonization barrier. Although the ecological principles might be similar, the mechanisms of adherence in a CSTR are very different from *in vivo* conditions. Therefore, an *in vitro* model may be useful to study microbial ecology, but not mechanistic aspects of gut colonization (31).

Response of the body

The fact that *in vitro* models cannot simulate the reaction of the body to compounds in the food can be regarded as an important limitation of *in vitro* models. Also, the rate of absorption of nutrients is difficult to predict *in vivo*, since removal of components in the chyme occurs in the model by a simple diffusion or filtration method. *In vivo* absorption occurs often by facilitated or active transport systems that involve carrier proteins to transport the molecules across the

enterocyte membrane. The complex interactions of microorganisms with the body cannot be simulated in any colon model to date.
It should be noted that the absence of feedback mechanisms can also be an advantage because it improves the reproducibility of the system and allows studies with the variation of only selected parameters.

SCOPE OF THE THESIS

The goal of the research presented in this thesis was to develop an *in vitro* model that mimics the dynamic conditions in separate parts of the gut. This model should meet the criteria for an adequate simulation of gastro-intestinal conditions, as mentioned in this chapter.
Several studies that show the development and application of the model are presented. These studies present validating evidence that the model adequately simulates the lumen of the GIT for the applications developed so far. However, it should be stressed that validation of the model for these and other applications will be an ongoing process depending on the availability of relevant *in vivo* data.
After this general introduction (Chapter 1), about the GIT and models of gastro-intestinal function, the features of the dynamic multi-compartmental model with respect to composition of the chyme, mixing and transit of the meal is explained for the gastric/small intestinal model in Chapter 2.
The applicability of the model to study the effects of gastric parameters such as the rate of meal delivery and pH on the efficacy of phytase, a feed enzyme, is demonstrated in Chapter 3.
In Chapter 4, the absorption of iron and phosphorus from cereals is compared to results obtained *in vivo*.
The use of the model to evaluate the survival of microorganisms, specifically lactic acid bacteria, is demonstrated in Chapter 5. Comparison of survival of lactic acid bacteria, with different responses to gastric acid and bile, in the model with *in vivo* data obtained from human volunteers offered a validation of the conditions prevailing in the model.
Chapter 6 and 7 deal with the digestion of protein contained in calf milk replacer and pig diets, respectively. Results in the model are compared with *in vivo* data obtained with cannulated animals.
In Chapters 3, 6 and 7, computer simulation techniques are introduced to evaluate the results.

In Chapter 8, the computer-controlled colonic model is introduced as a tool to study the effect of indigestible compounds on a high-density microflora.
The general discussion (Chapter 9) deals with the criteria for adequate simulation of the gastrointestinal conditions in the separate parts of the model. Furthermore, validation of the model, its applications, use and limitations are discussed. Finally, the discussion addresses some future developments.

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Chapter 2

A MULTICOMPARTMENTAL DYNAMIC COMPUTER-CONTROLLED MODEL SIMULATING THE STOMACH AND SMALL INTESTINE

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ABSTRACT

A multicompartmental *in vitro* model has been described, which simulates the dynamic events occurring within the lumen of the gastrointestinal tract of man and monogastric animals. The accuracy of this model for reproducing *in vivo* data on gastrointestinal transit, pH, bile salt concentrations and the absorption of glucose was tested. The *in vivo* conditions simulated in the model were based on studies in healthy human volunteers. Mathematical modelling of gastric and ileal delivery with power exponential equations was used for the computer control of meal transit. The model appeared to reproduce accurately the pre-set data on meal transit, pH and bile salt concentrations in the different gastrointestinal compartments. Glucose absorption from the small intestine was almost complete. This model reproduces very closely the dynamic conditions based on *in vivo* situations in monogastric animals and man. Therefore, the model can be an important tool in studying the fate of ingested components (for example, food, microorganisms, medicines) during gastrointestinal transit and, consequently, may contribute to the replacement of laboratory animals.

INTRODUCTION

When the fate of or the interactions between ingested compounds, such as food, microorganisms and medicines, in the lumen of the gastrointestinal tract have to be investigated, *in vitro* models may have several advantages over *in vivo* experiments. In general, *in vitro* experiments are less expensive, are easy to perform and are not limited by ethical constraints. They are not hampered by biological variations among subjects, they allow studies with toxic compounds or doses and they permit the manipulation of experimental conditions. However, most of the *in vitro* models developed to date have been dedicated to a single application and include a limited number of simulated parameters (2). As has been proposed by Longland (8), the following five aspects should be taken into account when devising new *in vitro* models of the gastrointestinal tract: (a) sequential use of enzymes in physiological amounts; (b) appropriate pH for the enzymes and addition of relevant co-factors such as bile salts and co-enzymes; (c) removal of the products of digestion; (d) appropriate mixing at each stage of digestion; (e) physiological transit times for each step of digestion. None of the models published so far (2,8) meet all of these five requirements and, most importantly, the dynamics of physiological transit of chyme have generally not been taken into account. Our aim was therefore to develop an *in vitro* model which would simulate, as closely as possible, the dynamic physiological

processes which occur within the lumen of the stomach and small intestine of man and monogastric animals, and which would be applicable to a broad range of studies. In this paper we have described such an *in vitro* model and its accuracy and reproducibility in simulating gastrointestinal transit, pH, bile salt concentrations and the absorption of glucose as an example of an end-product of digestion.

MATERIALS AND METHODS

The in vitro dynamic model of the gastrointestinal tract

The model consists of four successive compartments (Figure 1) simulating the stomach, duodenum, jejunum and ileum. Each compartment is formed by two connected basic units consisting of a glass jacket with a flexible wall inside. Water is pumped from a water bath around the flexible walls to control the temperature inside the units and the pressure on the flexible walls. Changes in water pressure are achieved by computer-activated rotary pumps. This enables mixing of the chyme by alternate compression and relaxation of the flexible walls. The compartments are connected by peristaltic valve-pumps consisting of three connected T-tubes, each with a separate tube-like flexible wall inside. If pressure is applied to the outside of the flexible wall, the valve is closed, leaving minimal dead space inside. In the open position the flexible walls facilitate unhindered passage of the chyme through the valves. Peristaltic pumping is achieved by regulating the sequence of opening and closing of the three parts of the valve-pump. During each peristaltic cycle, a constant volume of chyme is transferred. The frequency of peristaltic cycles is dictated by a computer, allowing the flow rate of the chyme to be controlled. The volume in each compartment is monitored with a level sensor connected to the computer.

A predetermined quantity of the meal is introduced into the gastric compartment within a pre-set period of time, with a peristaltic pump. The gastric and duodenal compartments are equipped with pH electrodes. The pH values are controlled via the computer by secreting either water or 1 M HCl into the stomach, or by secreting either water or 1 M $NaHCO_3$ into the duodenum, via syringe pumps. To avoid irregular pH values in the stomach due to incomplete mixing, HCl is secreted through a perforated tube. Secretions of gastric electrolytes and enzymes, bile and pancreatic juice are regulated by using computer-controlled syringe pumps. The jejunal and ileal compartments are connected with hollow-fibre devices to absorb

digestion products and water from the chyme and to modify electrolyte and bile salt concentrations in the chyme.

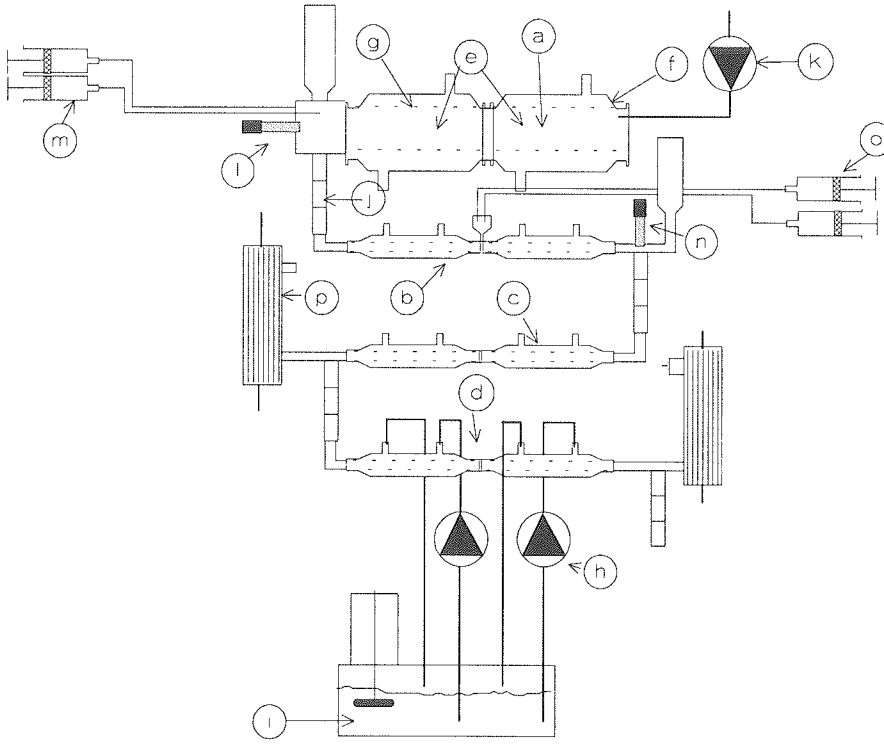

Fig. 1. Schematic diagram of the multicompartmental model of the stomach and small intestine. a, gastric compartment; b, duodenal compartment; c, jejunal compartment; d, ileal compartment; e, basic unit; f, glass jacket; g, flexible wall; h, rotary pump; i, water bath; j, peristaltic valve-pump; k, peristaltic pump; l,m, pH electrodes; n,o, syringe pumps; p, hollow-fibre device.

Computer program and mathematical modelling

The computer program has been designed to accept parameters and data obtained from *in vivo* studies in animals or human volunteers, such as the quantity and duration of a meal, the pH curves in the stomach and duodenum, secretion rates into

the different compartments, water absorption from the small intestine and gastric and ileal delivery into the duodenum and colon.
To control the transit of chyme, a power exponential formula for gastric and ileal delivery is used as described by Elashoff *et al.* (5):

$$f = 1 - 2^{-\left(\frac{t}{t_{1/2}}\right)^{\beta}}$$

Where f represents the fraction of meal marker delivered, t½ the half-time of delivery, t the time, and ß a parameter describing the shape of the curve.
This formula offers a method for controlling the gastric and ileal delivery in the model with only two parameters (t½, ß) to describe the curve.

Experimental design

Gastrointestinal transit and pH

The accuracy and reproducibility of the model for gastric delivery, intestinal transit and ileal delivery of chyme were assessed in two series of six experiments, simulating a slow and a fast transit time. The power exponential parameters describing the slow gastric and ileal delivery (Table 1) were obtained by calculating the curve fit of gastric and ileal delivery data from human volunteers who ingested yoghurt (9,10).

Table 1. Parameters for the power exponential equation used to describe the curves for slow and fast gastric and ileal delivery

	Fast		Slow	
	t ½ (minutes)	β	t ½ (minutes)	β
Gastric delivery	35	1.15	70	2
Ileal delivery	85	1.4	160	1.6

The parameters for the fast transit were based on *in vivo* data on gastric delivery of milk (9,10), while the parameters for ileal delivery were chosen arbitrarily to challenge the system under an extremely fast condition.

The volumes in the duodenal and ileal compartments were constant during the experiments (20 and 70 ml, respectively). The jejunal compartment was initially empty, and filled up during the experiments. A maximum volume of 70 ml, monitored by the pressure sensors, was maintained by water absorption.
Mixing in the gastric and small intestinal compartments was executed by 3 and 9 contractions of the flexible walls per minute, respectively. The test meal consisted of 100 ml of a 0.08% (w/v) solution of blue dextran (Sigma, St. Louis, USA) in water. Blue dextran was used as a marker because it is water-soluble, not absorbed and easy to quantify. The feeding time was 4 minutes. Gastric and duodenal secretion rates were set at 0.5 ml/minute and 1 ml/minute, respectively. The gastric pH values (Table 2) were set to mimic data observed *in vivo* after ingestion of milk (9). The duodenal pH value was set at 6.5 and recorded every minute.
From each compartment 2 ml samples were collected every 20 minutes for 5 hours. After measuring the blue dextran concentration, the samples were re-introduced into the compartments from which they originated.

Table 2. Predetermined pH values in the stomach

Time (minutes)	0	5	20	40	60	90	120
pH	4.8	4.5	4.2	2.8	2.1	1.8	1.7

Bile salt concentrations

Based on data from the literature for bile salt concentrations in the human small intestine (4,6,13), simulation of the following bile salt concentrations was attempted: in the duodenal compartment an initial concentration of 10-15 mmol/l, which progressively decreased during the first 2 hours after a meal to a constant level of ca. 5 mmol/l; in the jejunum, a constant concentration of about 10 mmol/l; and in the ileum, a concentration of 2-4 mmol/l. In order to simulate these conditions, 7 ml of a 4% (w/v) porcine bile (Sigma) solution was put into the duodenum at the beginning of the experiment, followed by the addition of a 4% bile solution at a flow rate of 0.5 ml/min during the first 30 minutes and a 2% bile solution (at a flow rate of 0.5 ml/minute) until the end of the experiment. The bile salt concentration in the jejunum was regulated by dialysing the chyme against a 1.55% bile solution. To obtain the lower bile salt concentrations in the ileum, the bile salts were dialysed

from the ileal chyme with a dialysis solution without bile. The bile salt concentrations were analysed in samples taken from each intestinal compartment at 1- hour intervals.

Absorption of digestion products

The efficacy of the model in absorbing products of digestion by dialysis was tested using glucose as an example of an end-product. To mimic a glucose load in the small bowel during starch digestion, a 10% (w/v) solution of glucose was delivered from the gastric into the duodenal compartment. Kinetics for transit of chyme and gastrointestinal secretions were similar to those used in the slow transit experiments. The glucose concentrations were analysed in serially collected ileal deliveries and in the jejunal and ileal dialysis fluids.

Analyses

Blue dextran concentrations were determined colorimetrically with a spectrophotometer (LKB Biochrom, Cambridge, UK) at a wavelength of 595 nm. Bile salts were analysed using the 3α-hydroxysteroid dehydrogenase method (Sigma), and glucose by using the glucose hexokinase method (Boehringer Mannheim, Germany).

Calculations

During each experiment the amount of marker (blue dextran) was determined in each intestinal compartment and in the ileal effluent at 20-minute intervals. The gastric delivery of blue dextran was calculated through the summation of the amounts of marker in all of the intestinal compartments and in the ileal effluent at each sampling time. Ileal delivery was determined by calculating the amount of marker in the ileal effluent at each sampling time. The cumulative gastric and ileal deliveries, expressed as a percentage of total intake, were fitted into the power exponential formula with the non-linear curve-fitting facilities of Slide Write 5.0 software (Advanced Graphics Software, Carlsbad, USA). The calculated curve fitting parameters were used to compare the variation among the six experiments and to compare these findings with the parameters of the pre-set delivery curves. Coefficients of variation (CV) were calculated.

RESULTS

Simulation of gastrointestinal transit

The distribution of blue dextran in each compartment, expressed as a percentage of total intake, during slow and fast gastrointestinal transit of chyme are shown in Figure 2 and Figure 3, respectively. The gastric and ileal deliveries of the model simulated accurately the pre-set curves for slow and fast deliveries of chyme calculated from *in vivo* data obtained from studies with human volunteers (Figures. 4 and 5, respectively). The cumulative blue dextran delivery data, expressed as a percentage of total intake, were fitted into the power exponential equation. For each experiment, the curve-fitting parameters (t½, ß) and the determination coefficient (r^2) are presented in Tables 3 and 4, to show the quality of the curve fit. The average values for these parameters, including their CV and standard deviations, show the accuracy and reproducibility of the model under conditions simulating a slow and a fast transit.

Reproduction of pH values

In all experiments the pre-set pH curve was closely simulated in the gastric compartment (Figure 6). In the duodenum the reproduction of the pre-set pH value of 6.5 was good, except during the first 30 min when the initial secretion of $NaHCO_3$ was not yet neutralized by the gastric contents.

Reproduction of bile salt concentrations

The evolution of the concentration of bile salts in the duodenal, jejunal and ileal compartments was in the required range (Figure 7).

Glucose dialysis

Of the glucose delivered into the duodenum, 96% was absorbed through dialysis from the small intestinal compartments. This absorption occurred predominantly from the jejunal dialysis device (79%) and was completed with that of the ileal compartment (17%).

Table 3. Parameters calculated from individual curves of gastric and ileal delivery in the model during the slow transit protocol

	Parameters of gastric delivery			Parameters of ileal delivery		
Experiment	t ½ (min)=70	β=2	r^2	t½(min)=160	β=1.6	r^2
a	70.3	2.13	0.999	154.8	1.66	0.998
b	69.9	2.04	0.995	158.2	1.55	0.996
c	70.4	2.00	0.997	156.8	1.61	0.995
d	70.3	2.22	0.991	161.3	1.58	0.995
e	73.9	2.03	0.998	163.3	1.60	0.996
f	71.3	2.02	0.995	160.6	1.70	0.997
Mean	71.0	2.07		159.2	1.62	
SD	1.35	0.08		2.86	0.05	
CV (%)	1.9	3.7		1.8	3.1	

t ½ =half time; ß= coefficient of the power exponential equation (3); r^2= coefficient of determination; SD= standard deviation; CV= coefficient of variation.

DISCUSSION

The proximal gastrointestinal tract of monogastric animals, including man, can be divided into separate sections such as the stomach, duodenum, jejunum and ileum. Each part has specific conditions related to its function in the digestion and absorption of food and its components. The mixing and transit time of food through the stomach and small intestine determine the period in which the food is exposed to the specific conditions in these compartments. The sum of these successive influences determines the fate of the ingested components.

The mixing of chyme in the model presented here simulates closely the natural contractions of the gastrointestinal tract. Both the frequency and strength of squeezing can be controlled to obtain the appropriate mixing and grinding actions. The *in vivo* gastric delivery can be described by a power exponential equation (5). In the *in vitro* model the transit of a meal is continuously regulated by gastric and ileal delivery curves based on this equation. The two variables in this equation allow the computer to regulate the pump-valves in such a way that the gastric and ileal deliveries in the model are simulated.

Table 4. Parameters calculated from individual curves of gastric and ileal delivery in the model during the fast transit protocol

	Parameters of gastric delivery			Parameters of ileal delivery		
Experiment	t ½ (min)=35	β=1.15	r^2	t½(min)=85	β=1.4	r^2
a	34.9	1.20	0.999	83.3	1.35	0.998
b	36.4	1.18	0.999	84.5	1.38	0.996
c	36.4	1.17	0.998	86.1	1.39	0.993
d	35.1	1.13	0.999	82.1	1.43	0.996
e	34.7	1.06	0.999	85.8	1.46	0.998
f	36.5	1.16	0.997	82.4	1.26	0.992
Mean	35.7	1.15		84.0	1.38	
SD	0.77	0.05		1.58	0.06	
CV (%)	2.2	4.0		1.9	4.6	

t ½ =half time; ß= coefficient of the power exponential equation (3); r^2= coefficient of determination; SD= standard deviation; CV= coefficient of variation.

In this study the accuracy and reproducibility of the model were tested, as well as the ability of the model to mimic the gastrointestinal transport of chyme under two different predetermined transit times. The slow and the fast transit were based on *in vivo* data for gastric and ileal delivery of yoghurt and milk, respectively, obtained in human volunteers (9,10). The results show that the *in vitro* data for gastric and ileal deliveries, under both slow (Figure 4) and fast (Figure 5) gastrointestinal transport conditions, simulated very closely the *in vivo* data. This demonstrates the potential of this model to mimic the dynamics of *in vivo* transit of chyme.

Since data in relation to *in vivo* transit through the duodenum and jejunum were not available, we could only use gastric and ileal delivery data to mimic the transit of a meal. To handle this problem, a method to control the transit through the duodenal and jejunal compartments had to be developed. The method tested in this study was chosen arbitrarily and assumed an initial empty state of the jejunum and the presence of a residue in the ileum before a meal.

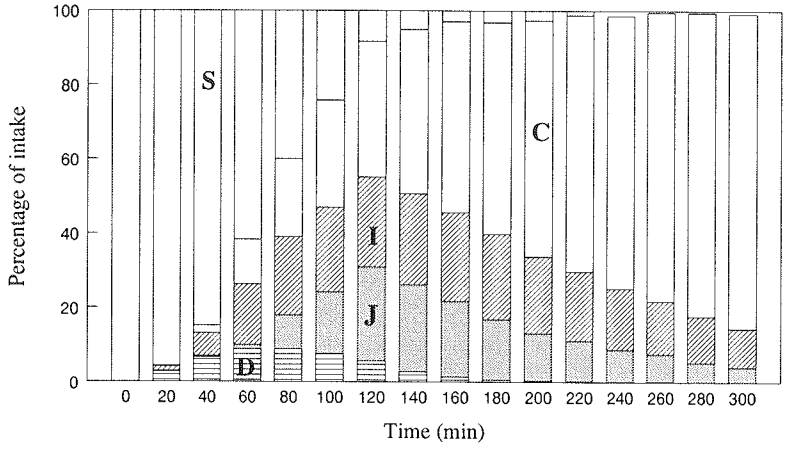

Fig. 2. The average distribution of blue dextran was measured, as a percentage of total intake, in the gastric (S), duodenal (D), jejunal (J), ileal (I) and the colon (=sampling beaker; C) compartments of the model, during a simulation of the slow transit of yoghurt (gastric emptying t½=70 minutes; ileal emptying t½=160 minutes).

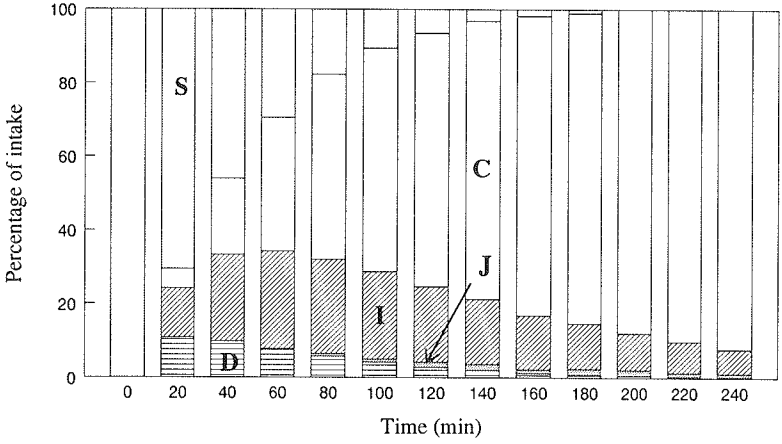

Fig. 3. The average distribution of blue dextran was measured, as a percentage of total intake, in the gastric (S), duodenal (D), jejunal (J), ileal (I) and colon (=sampling beaker; C) compartments of the model, during a simulation of the fast transit of milk (gastric emptying t½=30 minutes; ileal emptying t½=80 minutes).

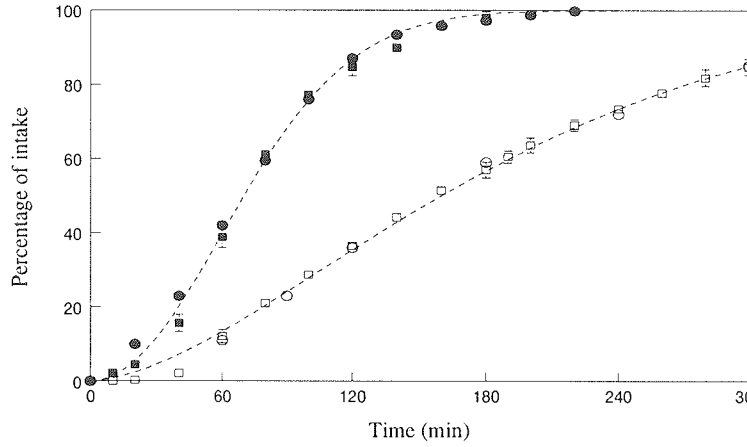

Fig. 4. Cumulative gastric and ileal delivery of a meal expressed as a percentage of total intake: *in vivo* gastric (●) and ileal (○) delivery of yoghurt (4,5); gastric (■) and ileal (□) delivery of blue dextran in the model simulating the slow transit of yoghurt (gastric delivery t½=70 minutes; ileal delivery t½=160 minutes). The lines present the pre-set curves. Values are means ± standard deviations of six experiments.

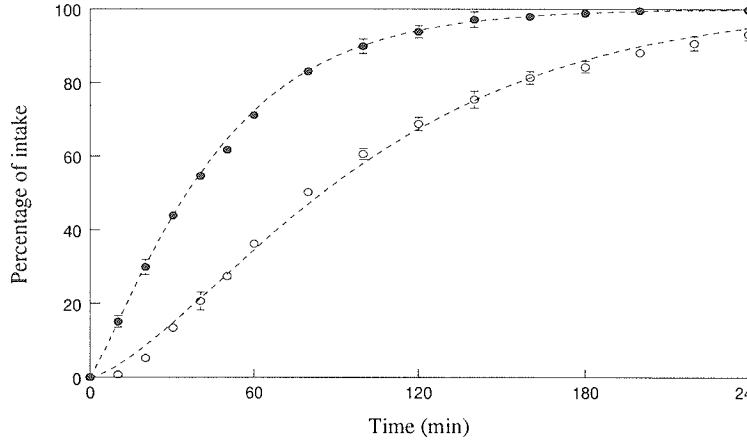

Fig. 5. Cumulative gastric and ileal delivery of a meal expressed as a percentage of the total intake: the gastric (●) and ileal (○) delivery of blue dextran in the model simulating the fast transit of milk (gastric emptying t½=35 minutes; ileal emptying t½=85 minutes). The lines represent the pre-set curves. Values are means ± standard deviations of six experiments.

The transit time increased if the jejunum was allowed to fill up. Once the jejunum was full, the transit time could be controlled by varying the rate at which water was absorbed. During the experiments with slow transit conditions, the jejunum filled up between the first and second hour after a meal, resulting in a significant increase in the amount of blue dextran in this compartment (Figure 2). In contrast, during the experiments with fast transit conditions, the jejunum did not fill up, due to fast intestinal transit which was necessary for a fast ileal delivery. This was reflected in a low amount of blue dextran in the jejunal compartment during these experiments (Figure 3).

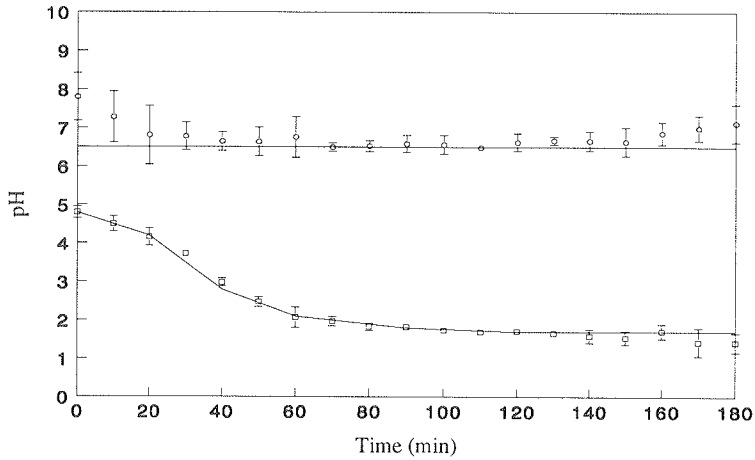

Fig. 6. PH values in the gastric and duodenal compartments. The points represent the measured pH values in the gastric (○) and duodenal (□) compartments. The lines represent the predetermined curves. Values are means ± standard deviations.

The activities of enzymes, the survival of ingested microorganisms and the physical state of molecules are strongly influenced by the pH values in the gastrointestinal tract. The gastric pH increases during the ingestion of food depending on the pH and the buffer capacity of the food and subsequently decreases due to acid secretion. In the presented dynamic *in vitro* model, the simulation of the *in vivo* gastric pH curve was very good. The diffuse secretion of HCl approved adequate to prevent irregular pH values due to incomplete peristaltic mixing of gastric contents.

The pH set in the duodenal compartment (pH 6.5) was also maintained adequately (Figure 6). Since the pH in the duodenum was controlled by the secretion of $NaHCO_3$, the accuracy improved if sufficient antagonising gastric contents were delivered (after ca. 20 minutes).

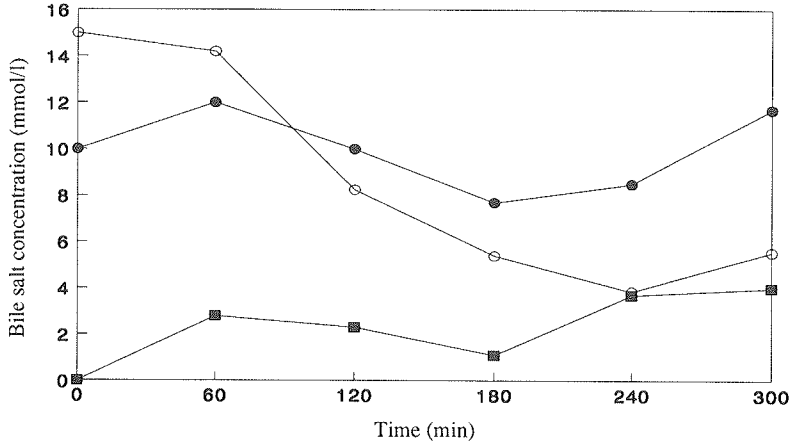

Fig. 7. Kinetics of bile salt concentrations analysed in the duodenal (○), jejunal (●) and ileal (■) compartments of the model during 300 min after ingestion of a meal, simulating physiological concentrations as found in human subjects (6,7).

Together with the gastric delivery curve, the pH curve determines the exposure time of food components to gastric acid. We simulated the gastric pH curve (Figure 6) and gastric delivery curve (Figure 4) after the ingestion of yoghurt. Combining these two curves shows that it takes approximately 1 hour before pH 2 is reached, while at that time, 40% of the consumed meal has already passed through the stomach. This clearly illustrates the power of this dynamic gastrointestinal model in comparison to static models in which gastric 'juice' exposure has a fixed pH during a fixed period of time for the whole meal (2,14).

Bile salts exert important physiological functions in the small intestine. For example, they act as co-factors for digestive enzymes, facilitate fat digestion, and influence the survival of ingested microorganisms. The methods used to control the bile salt concentrations in the model resulted in an accurate reproduction of the predetermined ranges of concentrations in the duodenal, jejunal and ileal compartments (Figure 7). Through the secretion of concentrated bile during the first hour, the high concentration observed *in vivo* in the duodenum just after a meal, which is due to gall bladder contraction, was mimicked. The dialysis devices proved

to be efficient in simulating the increased jejunal and decreased ileal concentration (Figure 7) as described for the *in vivo* situation (4,13).

The ability to absorb products of digestion is also an important feature of *in vitro* models. In particular when digestion is studied, absorption of digested food components should prevent product inhibition of the intestinal enzymes (1,2,8). Dialysis membranes have already been described for other models (14). The hollow-fibre devices, which have a large membrane area and consequently a fast dialysis capacity, appeared almost as efficient as the active process *in vivo* (3).

The strength of our *in vitro* model is that it combines all essential requirements, as proposed by Longland (8). The model mimics the gastrointestinal peristalsis which results in physiological mixing of the contents, in contrast to homogenisation with magnetic stirrers used in other models (7,12,15,16). The pH and the enzyme and bile salt concentrations simulate the dynamic physiological patterns found *in vivo*. These important features are lacking in static models (1,2,8,14). Peristalsis of the pump-valves, in combination with their construction between the different compartments, mimics a natural transport of gastrointestinal contents, even when they contain food particles. This is an essential improvement in comparison to the use of tubes and pumps described for other dynamic models (2,7,8,12,15,16). Using hollow-fibre devices, connected to the jejunal and ileal compartments, this model combines digestion and absorption, which is of utmost importance for reliable *in vitro* experiments.

CONCLUSIONS

We have shown that our multicompartmental, computer-controlled, dynamic model can accurately reproduce predetermined physiological parameters, such as meal size and duration, peristaltic movements, pH, gastric and intestinal secretions, gastrointestinal transit, and absorption of digested products and water. Individual parameters, such as those simulating non-physiological conditions, can be varied in the model. These predetermined parameters for the various compartments of the model can be used to simulate physiological as well as extreme or pathological conditions in the lumen of the stomach and small intestine of monogastric species. This provides an opportunity to study the fate of ingested components under complex dynamic conditions without the individual variation encountered in *in vivo* experiments. Therefore the model is very suitable for pre-screening studies and may be a good alternative to animal experiments. However, the limitations of this model should also be recognised: it does not allow physiological processes of the gut wall (enterocytes), such as active transport and feedback mechanisms, to be studied.

Applications of the model which are now being validated include the survival of ingested microorganisms (11; Marteau *et al.*, submitted), the digestion of starch and protein and the fate of ingested medicines.

ACKNOWLEDGEMENTS

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Chapter 3

EFFICACY OF FUNGAL PHYTASE DURING TRANSIT THROUGH A DYNAMIC MODEL OF THE PORCINE STOMACH

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ABSTRACT

Experiments were performed in the gastric compartment of the TNO gastro-Intestinal Model (TIM) to study the efficacy of fungal phytase in the stomach of the pig under various gastric conditions. Pure phytase and phytate were used to exclude interactions with feed components. Experiments with and without pepsin showed that the activity of phytase is not influenced by pepsin. The effect of gastric passage time was tested during a fast and a slow gastric meal delivery, with 50% delivery of the meal after 60 min and 120 min, respectively. A pH profile that mimicked the decreasing gastric pH after the ingestion of a meal was compared with a constant pH of 5, close to the optimum pH for phytase activity. After a collection period of 6 h with the pH profile and the fast gastric delivery, the cumulative recovery of free phosphorus in the gastric effluent was 23% of the total phytate phosphorus intake, while a constant pH of 5 yielded 32%. With the slow gastric delivery, the recovery of free phosphorus with the pH profile and the constant pH of 5 was 26% and 39% of total phytate phosphorus intake, respectively. Experiments with phytase concentrations of 70, 100, 200 and 280 FTU/L and 3 g/L phytate, resulted in cumulative recoveries of 14%, 23%, 42% and 51% free phosphorus as a percentage of phytate phosphorus intake, respectively. With a phytase concentration of 280 FTU/L and 6 g/L phytate, 31% of the phytate phosphorus was recovered as free phosphorus. The lower relative efficacy as compared to 280 FTU/L phytase and 3 g/L phytate demonstrated that when substrate limitation becomes a relevant effect, most of the meal already has been delivered from the stomach.

The present study demonstrates the potential of a dynamic computer-controlled model of the porcine stomach to study the influence of gastric conditions on the efficacy of phytase. The results show that gastric pH and gastric meal delivery have a large effect on the efficacy of phytase in the porcine stomach. Experiments with phytase that also contained acid phosphatase activity showed a low additional effect of acid phosphatase.

INTRODUCTION

A considerable amount of phosphorus (P) is present in feed as phytate phosphorus. This P is not or insufficiently accessible to meet the nutritional requirements of monogastric animals such as pigs. The P is liberated from phytate by the enzyme phytase (EC 3.1.3.8), a phosphatase contained in some seeds and produced by some fungal species. By adding fungal phytase to animal feed it is possible to increase the bioavailability of phytate P and thus to reduce the necessity to add

inorganic P to the feed. This may result in a decreased emission of P in manure. When ingested with the meal, phytase is predominantly active in the stomach. Phytase activity is low posterior to the duodenum, due the intestinal pH values and the degradation of the enzyme (4). The following gastric conditions are important factors for the efficacy of phytase: 1) rate of gastric delivery; 2) pH course in time; 3) peptic activity; 4) composition and structure of the meal; and 5) phytate and phytase concentrations and ratio. It is difficult to study the effect of these parameters on phytase activity *in vivo* because it is not possible to change a single parameter. With the TNO gastro-Intestinal Model (TIM) it is possible to mimic the dynamic conditions in the gastro-intestinal tract and to vary specific parameters under reproducible conditions. The effects of gastric delivery, pH profile, peptic degradation and various concentrations of pure phytate and pure phytase on the efficacy of fungal phytase were studied to demonstrate the potential of a computer-controlled model of the porcine stomach.

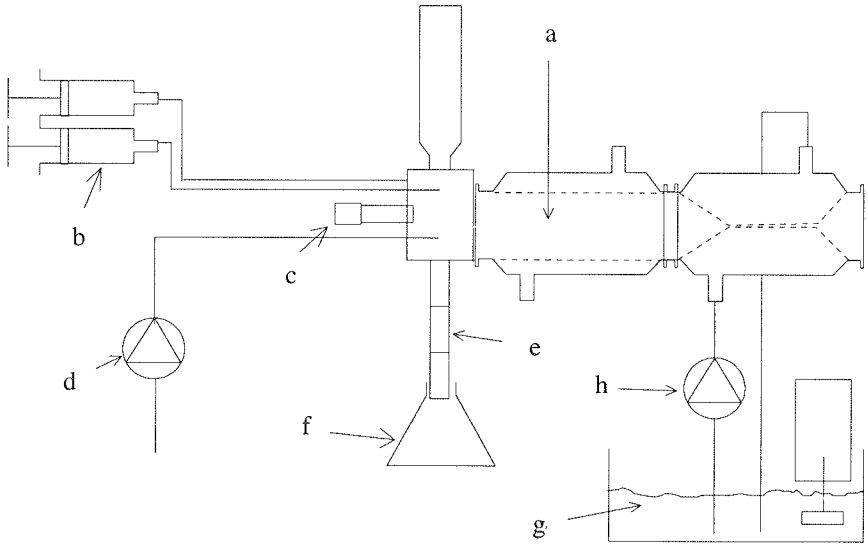

Fig. 1. Dynamic model of the porcine stomach

a, gastric compartment; b, secretion pumps; c, pH electrode; d, pump for HCl solution; e, peristaltic valves; f, sampling bottle; g, water bath; h, circulation pump

MATERIALS AND METHODS

Gastric model

The experiments were performed in the gastric compartment (Fig. 1) of the TNO gastro-Intestinal Model (TIM; 8). The model consist of two connected glass units (a) with flexible walls inside that are alternately squeezed, by increasing the pressure between the jacket and the flexible wall, to mimic the mixing motility of the stomach. Artificial saliva and gastric juice were secreted via a syringe pump (b). The pH of the gastric contents was measured with a pH electrode (c). Hydrochloric acid was introduced to adjust the pH in the stomach with a computer-controlled peristaltic pump (d). Gastric delivery was controlled by the computer by using a peristaltic valve pump system (e). The gastric delivery was collected in a vessel (f). The system was heated by circulating water from a water bath (g) with a pump (h).

Experimental design

Duplicate experiments were performed under various conditions in order to evaluate the influence of isolated parameters on phosphorus liberation from phytate by fungal phytase (Natuphos® 5000, containing 5500 FTU/g, Gist-brocades, Delft, the Netherlands). One FTU was defined as that amount of enzyme required to liberate 1 µmol inorganic P in 1 min from 1.5 mM sodium phytate solution at pH 5.5 and 37 ° (3).

Prior to the experiment 10 ml of electrolyte solution, containing per litre 0.22 g $CaCl_2$, 2.2 g KCl, 5 g NaCl, 1.5 g $NaHCO_3$ and 500 kU pepsin (Sigma, St. Louis, MO), was introduced into the gastric compartment to mimic a residue present in a sober stomach. Depending on the experiment (Table 1), 300 ml electrolyte solution with sodium phytate (MW 923.8, 20.13% P; Sigma) and phytase was introduced into the gastric compartment.

The secretion of saliva/gastric juice containing 500 kU/L pepsinogen (Sigma) was set on 0.5 ml per minute. To study the effect of peptic degradation of the phytase, experiments were performed with and without pepsin and pepsinogen (Table 1: Exp.1 and 2). Because pepsin has an optimum activity at pH 2.5, these experiments were performed with a phytase that also possesses a strong acid phosphatase activity (Sigma, 1150 FTU/g at pH 5.5, 1530 FTU/g at pH 3). This phytase was tested at an activity of 60 FTU/L at pH 5.5.

Table 1. Matrix of experiments

	Experiment										
Variable	1	2	3	4	5	6	7	8	9	10	11
pH profile	x	x	x	x			x	x	x	x	x
pH 5					x	x					
Pepsin		x	x	x	x	x	x	x	x	x	x
Slow delivery				x		x	x	x	x	x	x
Fast delivery	x	x	x		x						
Phytase (FTU/L)	60	60	140	140	140	140	70	100	200	280	280
Phytate (g/L)	3	3	3	3	3	3	3	3	3	3	6

To assess the effect of pH on the efficacy of phytase, experiments were performed with a pH profile (Table 1: Exp. 3 and 4) that mimicked the temporal decrease in pH in the porcine stomach after a meal (Fig. 2), or with a constant pH of 5 (Table 1: Exp. 5 and 6). The pH was controlled by secreting 0.5 M HCl or electrolyte solution. The model was programmed to control gastric delivery according to a predetermined curve using the following power exponential equation:

$$f = 1 - 2^{-\left(\frac{t}{t_{1/2}}\right)^{\beta}}$$

Where f is the fraction of meal marker delivered, t½ is half-time of gastric delivery, t is time after meal ingestion, and β is a parameter describing the shape of the curve. This equation has been used to fit the cumulative gastric delivery of a meal marker, expressed as a percentage of the intake (2). To assess the effect of the rate of gastric delivery on the efficacy of phytase, we used a slow and a fast gastric delivery curve (Fig. 2, Table 1). The fast delivery curve (t½ = 60 min, β = 1) and

the slow delivery curve ($t_{1/2}$ = 120 min, β = 1) described the gastric deliveries of a meal in two individual pigs (10). The accuracy of the model to control gastric delivery has been demonstrated in a previous study (8).

The experiments to test the effect of pH and gastric delivery were performed with 140 FTU/L phytase and 3 g/L phytate. To investigate the effect of enzyme and substrate concentration on the release of P, several phytase and phytate concentrations were tested (Table 1: Exp. 7 to 11) with the pH profile and slow gastric delivery. The gastric delivery was collected in bottles on dry ice (-40 °C), at 1 h intervals for 6 h. Each sample was weighed and stored at -40 °C. The free P concentration was analysed in each sample to determine the breakdown of phytate by phytase. Prior to analysis, the frozen samples were thawed and homogenized at 4 °C. Phytase activity was inactivated by incubating the samples at pH 1, at 100 °C for 10 min. Free P was determined in duplicate with the molybdate-vanadate method using an auto-analyser (11).

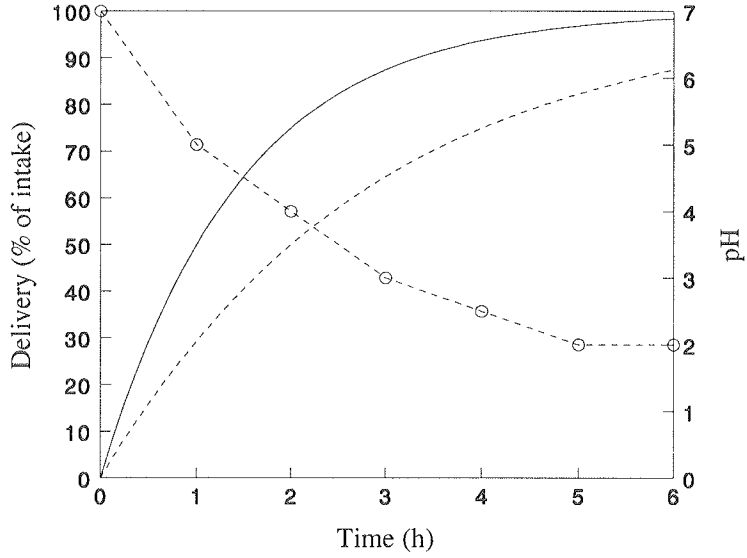

Fig. 2. Fast (solid line) and slow (dotted line) gastric meal delivery curves and pH profile (O) in the dynamic model of the porcine stomach.

Calculations and computer simulation

Digestibility coefficient

The P digestibility coefficient was calculated by expressing the measured amount of free P delivered from the gastric compartment as a percentage of the theoretically delivered total phytate P. The delivery of phytate was determined according to the gastric delivery curve used.

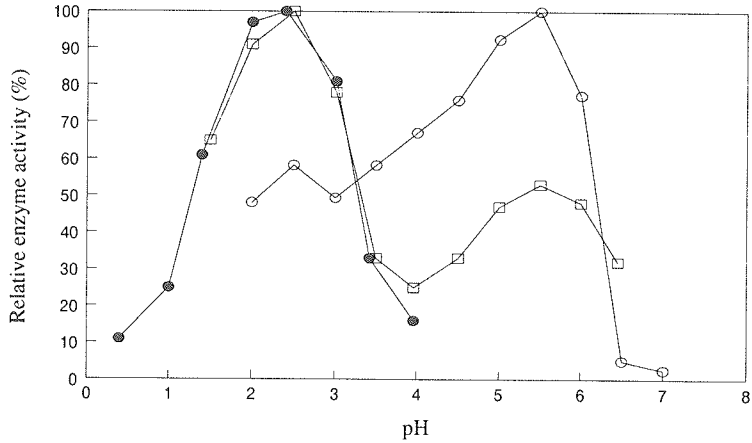

Fig. 3. pH versus activity profile of Natuphos® phytase (○), phytase with acid phosphatase activity (□) and pepsin (●).

Computer simulation

A computer simulation was used to interpret the results. The simulation program was based on the program that controlled secretion and gastric emptying in the model. Thus the dilution of the test solution and its gastric delivery could be predicted exactly. These known parameters were combined with the phytase activity based on the pH versus activity profile of phytase (Fig. 3) in relation to the pH profile in the model. The program calculated the relative phytase activity and the concentrations of liberated P during the experiments.

RESULTS

Pepsin

The experiments performed with phytase containing also acid phosphatase activity showed a significant release of P from phytase with an optimum between 2 and 3 h, irrespective of the presence of pepsin (Fig. 4).

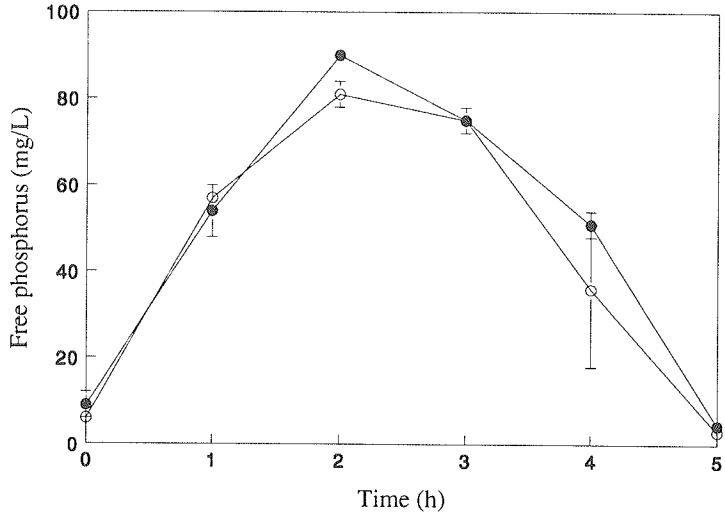

Fig. 4. Average concentrations of free phosphorus in time in gastric delivery, for phytase (Sigma, 60 FTU/L) with (●) and without (○) pepsin added. (n=2, error bars present the range)

Gastric pH and rate of meal delivery

A slow gastric delivery had a positive effect on phytase efficacy as compared to the fast gastric delivery (Fig. 5). The effect of gastric delivery was mainly apparent after 2 h and increased in time. An increasing positive effect of maintaining a fixed pH of 5 as compared to a physiological pH profile was measured until 3 h after the start of the experiment. After 3 h the concentrations decreased, while the difference diminished. The cumulative delivery of free P with the slow gastric delivery curve and a constant pH of 5 was 39% of intake after 6 h. With the pH profile during the slow gastric delivery curve CD of free P was 26% of intake (Fig. 6). The fast gastric delivery curve resulted in a faster delivery of free P as compared to the slow delivery with both the pH profile, as with a fixed pH of 5. The cumulative delivery of free P during the fast delivery experiments was completed after 5 h, because the stomach was empty as dictated by the fast delivery curve (Fig. 2).

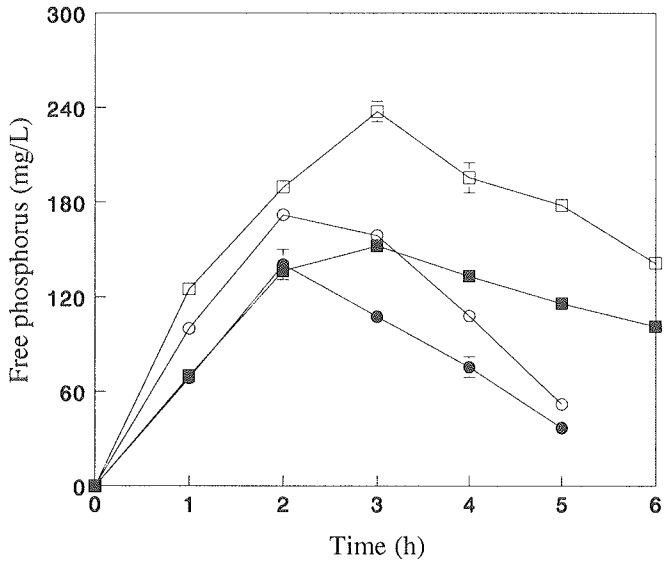

Fig. 5. Average concentrations of free phosphorus in time in gastric delivery during fast gastric delivery (t½ = 60 min) with a physiological pH profile (●) or a fixed pH of 5 (○) and slow gastric delivery (t½ = 120 min) with a physiological pH profile (■) or a fixed pH of 5 (□). (n = 2, error bars present the range)

The cumulative delivery during the fast delivery experiments at a fixed pH of 5 and with the pH profile were 32 % and 23 % of intake, respectively. Phytate digestibility coefficients, expressed as the amount of free P as a percentage of total P delivered from the stomach compartment, are presented in Table 2.

Table 2. Average digestibility coefficients (DC) from duplicate experiments with a fast (t½ = 60 min) and a slow (t½ = 120 min) gastric meal delivery, and a physiological pH profile or a fixed pH of 5. The experiments were performed with 140 FTU/L phytase and 3 g/L phytate.

Gastric delivery curve	Gastric pH	DC
fast	curve	23.7±0.2
fast	fixed	33.0±0.2
slow	curve	29.7±0.1
slow	fixed	44.6±0.8

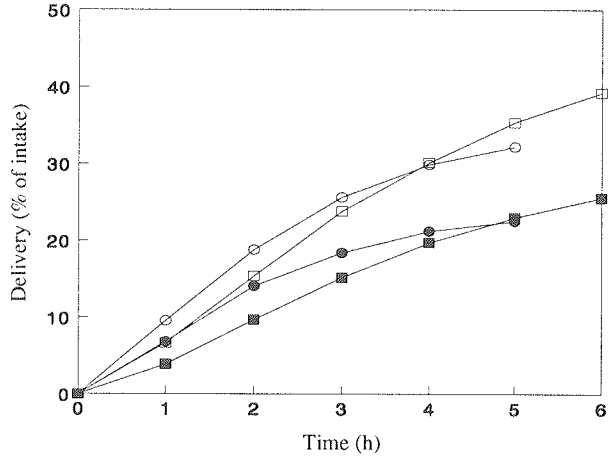

Fig. 6. Average cumulative gastric delivery of free phosphorus, expressed as a percentage of total intake of phytate phosphorus, during fast gastric delivery ($t_{1/2}$ = 60 min) with a physiological pH profile (●) or a fixed pH of 5 (○) and slow gastric delivery ($t_{1/2}$ = 120 min) with a physiological pH profile (■) or a fixed pH of 5 (□).

Enzyme and substrate concentrations

The results of the experiments with 70, 100, 200 and 280 FTU/L phytase and 3 g/L phytate showed a close-to-linear dose response (Figs. 7 and 8), with a correlation coefficient between phytase activity and P digestibility coefficient of 0.99. The P digestibility coefficient for each enzyme activity is presented in Table 3. The concentration of free P in time during the experiments with 280 FTU/L phytase and 3 g/L phytate showed only a more gradual decrease after 4 h as compared to the experiments with 280 FTU/L phytase and 6 g/L phytate (Fig. 9). After 4 h in the experiments with 280 FTU/L phytase and 3 g/L phytate, 72% of total phytate P in the sample was released from the phytate (Fig. 10). In the same time, in the experiments with 280 FTU/L phytase and 6 g/L phytate ca. 39% of total phytate P in the sample was released.

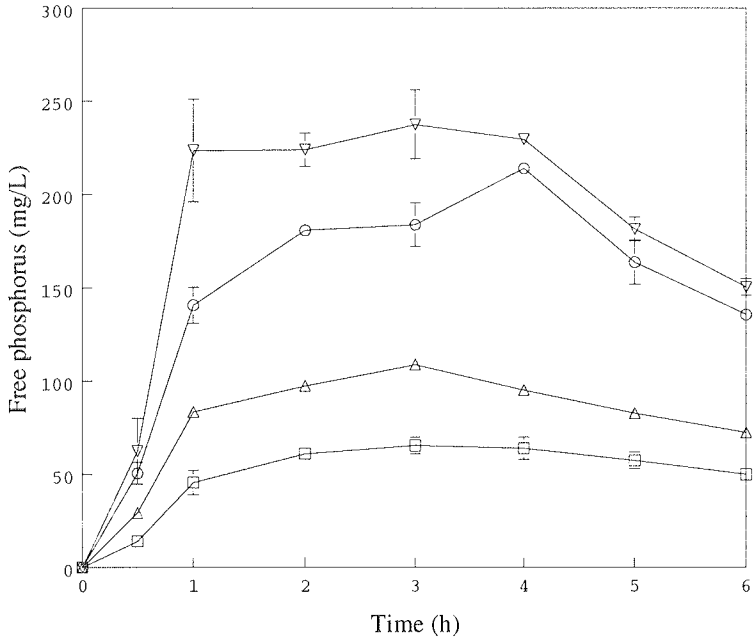

Fig. 7. Average concentration of free phosphorus in the gastric delivery released from phytate (3 g/L) in time by various phytase concentrations: 70 FTU/L (□); 100 FTU/L (Δ); 200 FTU/L (○) and 280 FTU/L (▽). (n = 2, error bars present the range)

Table 3. Average digestibility coefficients (DC) from duplicate experiments with various phytase concentrations. The experiments were performed with the slow gastric meal delivery ($t_{1/2}$ = 120 min) and a physiological pH profile.

Phytase (FTU/L)	Phytate (g/L)	DC
70	3	13.4±1.5
100	3	20.9±0.1
200	3	38.6±0.9
280	3	48.6±2.0
280	6	27.3±0.1

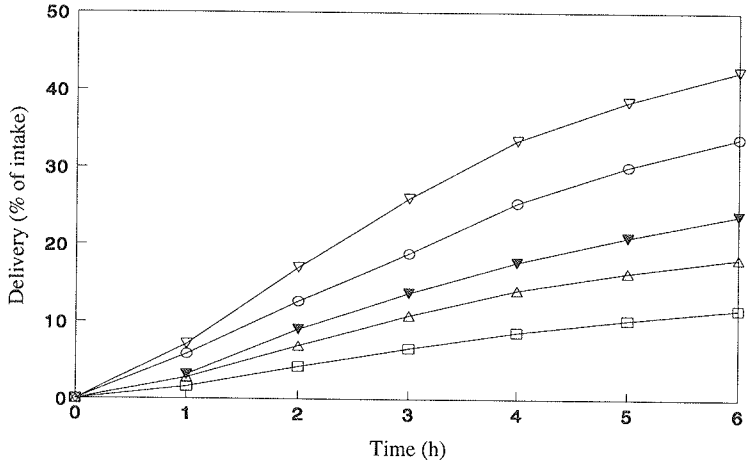

Fig. 8. Average cumulative gastric delivery of free phosphorus, expressed as a percentage of total intake of phytate phosphorus at various phytase concentrations: 70 FTU/L (□); 100 FTU/L (Δ) 200 FTU/L (○) and 280 FTU/L (3 g/L phytate:▽, 6 g/L phytate: ▼).

Computer simulation

Computer simulations of the theoretical phytase activity during the experiments with and without the pH profile are presented in Fig. 11. From this figure and the pH profile (Fig. 2) it can be derived that during the first hour of the experiments with the pH profile, the activity of phytase increased from almost zero at pH 7 to ca. 75% at pH 5, while the activity decreased again with a further decreasing pH. During the experiments at a fixed pH of 5, there was no loss of activity due to pH, but only due to dilution by secretion. The computer simulation also showed a similar phytase activity during the first hour of both the fast and the slow gastric delivery. After the first hour the difference in phytase activity increased. Based on the calculated relative activity of phytase, also the concentration of liberated P were estimated with the program (Fig. 12).

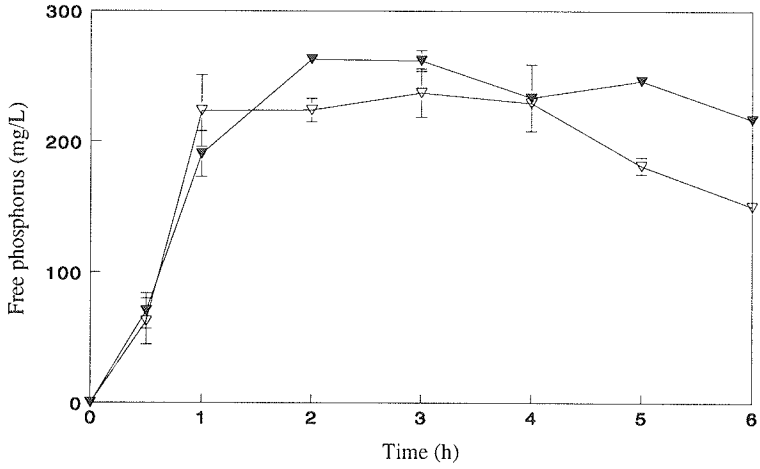

Fig. 9. Average concentration of free phosphorus in the gastric delivery with 280 FTU/L phytase and 3 g/L phytate (▽) or 6 g/L phytate (▼). (n = 2, error bars present the range)

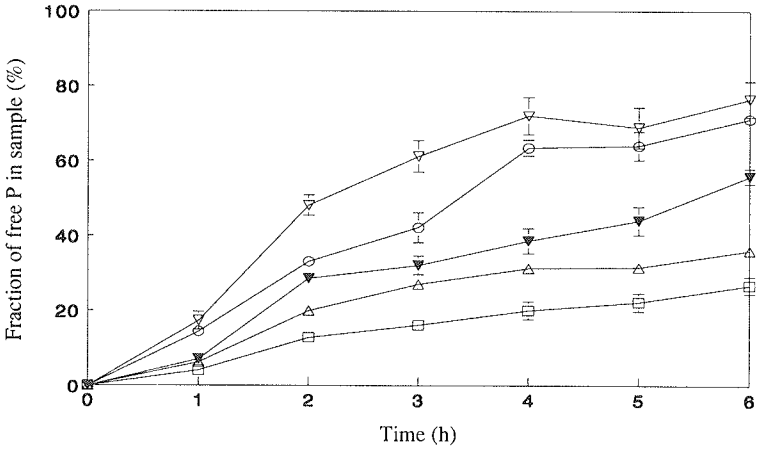

Fig. 10. Free phosphorus in each hourly sample of the gastric delivery with various phytase and phytate concentrations expressed as a percentage of the total P in each sample: 70 FTU/L phytase (□); 100 FTU/L phytase (Δ); 200 FTU/L phytase (○) and 280 FTU/L (3 g/L phytate:▽, 6 g/L phytate: ▼).

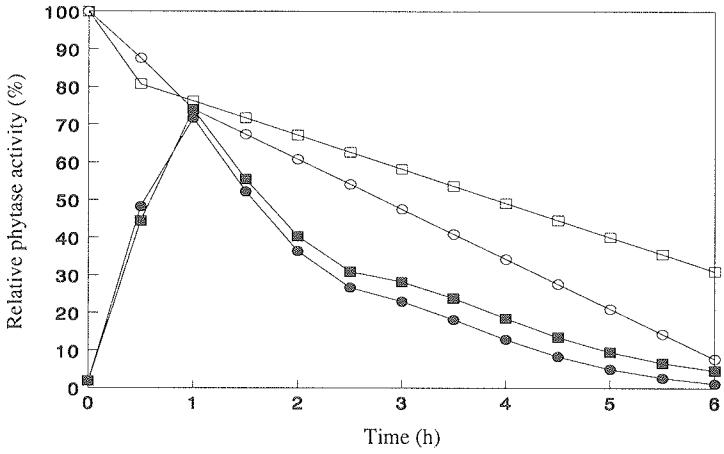

Fig. 11. Computer simulation of the influence of pH, gastric secretion and gastric meal delivery on relative activity of phytase in time, during fast gastric delivery (t½ = 60 min) with a physiological pH profile (●) or a fixed pH of 5 (○) and slow gastric delivery (t½ = 120 min.) with a physiological pH profile (■) or a fixed pH of 5 (□).

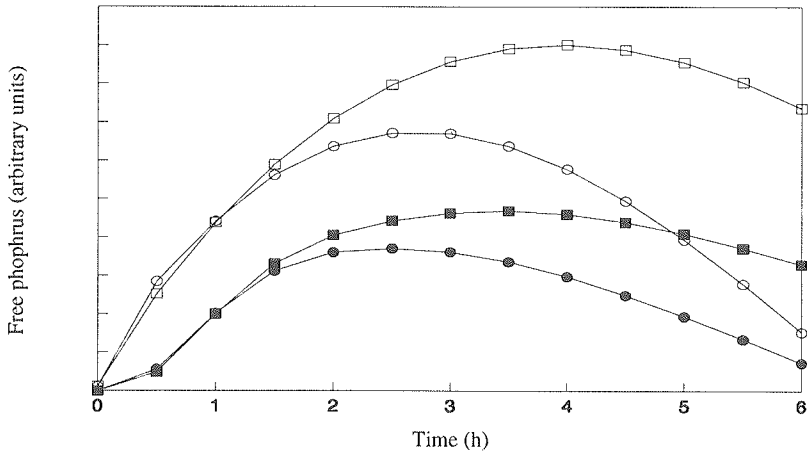

Fig. 12. Computer simulation of released phosphorus in the gastric delivery as influenced by pH, gastric secretion and gastric delivery in time, during fast gastric delivery (t½ = 60 min) with a physiological pH profile (●) or a fixed pH of 5 (○) and slow gastric delivery (t½ = 120 min.) with a physiological pH profile (■) or a fixed pH of 5 (□).

DISCUSSION

This paper describes the application of a dynamic gastric model as a tool to study the efficacy of feed enzymes in the stomach. A simple system with only phytase and phytate was used to exclude interaction with food components. This allowed to assess only the influence of physiological parameters, such as peptic activity, pH, secretion and rate of gastric emptying, on the efficacy of phytase during passage through the porcine stomach.

In vitro methods that have been used to study the interaction of phytate with other dietary components, such as minerals, are static models that mimic gastric digestion during a fixed time at a fixed pH (7,1,13). This is not a physiological condition, since gastric pH is not constantly low but increases after ingestion of the meal and decreases thereafter. Also enzyme, product and substrate are gradually diluted by salivary and gastric secretion and are gradually delivered from the stomach. We have developed a dynamic gastric model that combines all these continuously changing parameters during the passage of a meal.

The pH optimum for the fungal phytase used in this study is around 5.5 (Fig. 3). At pH 7 phytase activity is close to zero while at pH 3 it is about 50% of the maximum. The activity increases to 60% at pH 2.5 and decreases to 50% at pH 2. Theoretically, the pH effect can be reduced by combining the phytase with an acid phosphatase which is active below pH 4. We calculated that the decrease in P concentration after 3 h is almost completely due to dilution (Fig 5). Thus, there is very little enzyme activity left after 3 h, even under optimum pH conditions. This is in agreement with the degree of hydrolysis of the phytate (Fig. 10). Most of the meal (enzyme and substrate) is delivered from the stomach within 3 h (fast delivery: 87.5%; slow delivery: 65%). Therefore, it can be expected that the addition of acid phosphatase to phytate will have little benefit to the animal, which is confirmed in animal studies (9). The same rationale can also be used to explain the observation that the efficacy of phytase is not influenced by pepsin, given the fact that the pH activity profile of pepsin is similar to that of acid phosphatase (Fig. 3). These results emphasize the importance of residence time of the meal in the stomach for the efficacy of phytase.

Both the fast and the slow gastric delivery can be regarded as within a physiological range (10). The experiments with both delivery curves reveal the differences in phytase efficacy that can be expected due to individual variation within a population of pigs. This demonstrates the potential of a dynamic

computer-controlled model to study the variation of a single parameter or a combination of parameters under reproducible conditions.

The experiments with various phytase concentrations showed a close-to-linear response for the range tested (70-280 FTU/L). This is in agreement with other published data (14,5,6). Adding extra phytate did not increase the efficacy of phytase: with 6 g/L phytate approximately the same amount of P was released as with 3 g/L phytate. The K_m of phytase for myo-inositol hexakisphosphate is 27, 161, 1000 and 200 µM for IP-6, IP-5, IP-4 and IP-3, respectively (12), showing that the activity of phytase at the beginning of the experiment with 3 g/L was already close to V_{max}. During the experiments the enzyme activity decreased considerably due to the pH and dilution (Fig.11). This resulted in a limited release of P from the extra available substrate later in the experiments with 280 FTU/L phytase and 6 g/L phytate.

The parameters for meal transit, secretion and pH can be used in a computer simulation program to predict theoretical values. These exactly defined parameters can be combined with (less predictable) parameters such as pH-enzyme activity profile, enzyme kinetics, interactions with other food components and accessibility of substrates. To demonstrate the principle, we used mathematical modelling of the interaction between the pH-activity profile of phytase, the gastric pH profile, and gastric delivery and secretion. The profile of the measured data (Fig. 5) could be well reproduced by the calculated data (Fig. 12). Both the measured and the calculated results reveal that gastric pH and gastric delivery are major determinants for phytase efficacy in the stomach.

CONCLUSIONS

The study of the kinetics of phytase in the stomach proved to be an interesting case to demonstrate the potential of a gastric model that mimics the dynamic interaction between gastric pH, gastric emptying and gastric secretion. Controlled variation of specific parameters allowed to study their individual influence on the efficacy of phytase. Computer simulation proved to be a useful tool for obtaining more insight into the complex interaction between enzyme related parameters and physiological parameters. Using a phytase with acid phosphatase activity did not significantly increase the efficacy of the phytase under the conditions tested.

With knowledge of the behaviour of phytase in a simple test system with only a few compounds, as presented in this paper, phytase can be tested in more complex matrices such as feed ingredients or complete meals.

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Chapter 4

ESTIMATION OF THE BIOAVAILABILITY OF IRON AND PHOSPHORUS IN CEREALS USING A DYNAMIC *IN VITRO* GASTROINTESTINAL MODEL

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ABSTRACT

A recently developed *in vitro* gastrointestinal model was evaluated for the estimation of the bioavailability of Fe and phosphorus and its correlation with bioavailability *in vivo*. *In vitro* experiments were carried out without and with phytase supplementation (750 FTU/kg feed) using rapeseed, sunflowerseed, whole wheat and white wheat flour. Phytase addition during *in vitro* digestion of rapeseed and sunflowerseed resulted in markedly increased dialysability of iron (67% and 20%) and phosphorus (31% and 66%). The release of free phosphorus during digestion of whole wheat and white wheat flour in the *in vitro* gastrointestinal model was observed to be correlated with the endogenous phytase activity in wheat. Comparison with different *in vivo* studies revealed that the *in vitro* gastrointestinal model could be used for a relative estimation of the bioavailability of Fe and phosphorus.

INTRODUCTION

The bioavailability of minerals and trace elements from foods is defined as the proportion of the mineral that can be absorbed and utilised by the body. In the small intestine several components in the diet may form soluble or insoluble complexes with the minerals, thus increasing or decreasing their availability for absorption. Cereal-based products are important sources of essential nutrients, however, they also contain considerable amounts of phytate, a recognised inhibitor of zinc and iron absorption in man (5,9).

Human studies have indicated that activation of native phytase or the addition of microbial phytase to phytate-rich diets could counteract the antinutritive effect of phytate on the dietary bioavailability of minerals and trace elements (2,13,14). The solubility of minerals, the pH in the intestinal lumen determined by gastric, pancreatic and intestinal secretions as well as by dietary factors and the residence time at the absorption site may also exert a potent effect on the absorption efficiency. Knowledge of these various factors that affect the bioavailability of minerals and trace elements from different foods may help when designing diets for vulnerable groups and also be useful data when developing food processes.

Bioavailability studies of minerals should preferably be determined by measurements *in vivo*. Human studies, however, are time-consuming and expensive, rather complicated to perform and sometimes yield quite variable results. Animal studies are easier to perform but have the disadvantage of

uncertainties with regard to differences in digestion and absorption capacity, between animals and man. Therefore, a rapid and valid *in vitro* model would be a valuable tool in estimating bioavailability.

As the absorption of minerals and trace elements is taking place in the complex environment of the small intestine, simulation of the successive changing conditions in the gastrointestinal tract is probably the most critical step for the estimation of the bioavailability of minerals and trace elements. The *in vitro* model used in the present study has recently been described by Minekus *et al* (8). Predetermined physiological parameters, such as meal size and duration, peristaltic movements, pH, gastric and intestinal secretions, gastrointestinal transit and absorption of digested products and water based on the *in vivo* situation in man appeared to be reproduced accurately in the model. Therefore, this *in vitro* model resembles more closely the *in vivo* situation than other previously described *in vitro* methods.

The purpose of the study was to validate the use of the recently developed *in vitro* method for estimation of the bioavailability of iron in humans and animals, and to relate the absorption of iron to the phytate content in different cereals. The data obtained were compared to human and animal studies.

MATERIAL AND METHODS

Materials and reagents

Cereals; white wheat flour (Super Patent, Mount Everest, Ranks Meel, The Netherlands), whole wheat flour (Super Volkoren, Vesuvius, Ranks Meel, The Netherlands), rapeseed and sunflowerseed provided by Gist-brocades, The Netherlands.

The contents of Fe, phosphorus and phytate (sum of inositol tri-, tetra-, penta- and hexa- phosphates) in the cereals are presented in Table 1.

Pancreatin solution: 12.5 g pancreatin (Pancrex V powder, Pabyrn batch no: 93J28/50, Paines & Byrne, Ltd, West Byfleet; Surrey, UK), dissolved in 125 ml Milli Q water. After centrifugation for 20 minutes at 12500 r.p.m., 0.8 g $NaHCO_3$ was added.

Bile solution: 12 g porcine bile extract (B-8631, Sigma, St. Louis, MO., USA), was dissolved in 300 ml of Milli Q water.

Gastric electrolyte solution: prepared using; NaCl (4.8 g/L), KCl (2.2g/L), $CaCl_2$ (0.22 g/L) and $NaHCO_3$ (1.5 g/L).

Gastric enzyme solution: Pepsinogen from porcine stomach (P-4781, grade III-s Sigma, St. Louis, MO., USA), diluted with gastric electrolyte solution (end concentration 1100 kU/L).

Duodenal electrolyte solution: the electrolyte for the small intestine was prepared using; NaCl (50 g/L), KCl (6.0 g/L) and $CaCl_2$ (3.0 g/L), diluted ten times with Milli Q water.

Trypsin: Bovine pancreas (T-8253, Type III, Sigma, EC 3.4.21.4, St. Louis, MO., USA), 2.0 g/L dissolved in 0.1 M $NaHCO_3$.

Pepsin: Pepsin from porcine stomach (P-7012 , Sigma, St. Louis, MO., USA), dissolved in gastric electrolyte (end concentration 500 kU/L).

Microbial phytase (E.C. 3.1.3.8) prepared from *Aspergillus niger*; Natuphos®liquid, batch 4925, phytase activity 5000 FTU/g, was provided by Gist-brocades, BSD B.V., Delft, The Netherlands.

Table 1. Total Fe, P, phytate-P content and endogenous phytase activity in rapeseed, sunflowerseed, whole wheat flour and white wheat flour.

Cereal	Fe µg/g	Fe mg/experiment[a]	Total P g/kg	Total P mg/experiment	Phytate-P[b] mg/experiment	Phytase activity FTU[c]/g
Rapeseed	585	9.48	10.8	174.9	140.3	0.08
Sunflowerseed	300	3.87	13	167.7	155.7	0.26
Whole wheat	31	1.14	4	146.4	107	7.3
White wheat	11	0.6	1.3	71	31.1	3.5

[a] calculated on dry matter

[b] sum of inositol tri-, tetra-, penta- and hexaphosphates

[c] 1 FTU = the amount of the enzyme that liberates 1 µmol inorganic phosphate from 1.5 mM sodium phytate at pH 5.5 and 37°C in one minute.

All chemicals were of analytical grade and Milli Q water (Millipore, Bedford, Mass., USA) was used throughout the study. During the initial experiments all glassware was soaked in 2.5 M HCl and rinsed three times in Milli Q water before use. This procedure was later modified and soaking was made with 3% HNO_3

followed by rinsing six times with Milli Q water. Stock solutions for gastric and duodenal electrolytes were stored in plastic bottles.

Experimental design

The total volume of the test meals was 300 ml (20 % dry matter).

The phytate-P content in the different experiments was approximately 0.4 g/L test solution (or lower), corn starch was included to test meals containing cereals with a phytate-P content exceeding 2 g/kg.

Cereal		Corn starch
Wheat flour	60 g	----
Whole wheat flour	40 g	20 g
Rapeseed	17.4 g	42.6 g
Sunflowerseed	13.8 g	46.2 g

Gastric electrolyte solution (80 ml) and 10 ml pepsin solution (500 kU/L) was added to each sample. The mixture was made up to 300 ml with Milli Q water.

All experiments were performed in at least duplicate and with or without the addition of 45 FTU phytase (1 FTU = the amount of the enzyme that liberates 1 µmol inorganic phosphate from 1.5 mM sodium phytate at pH 5.5 and 37°C in one minute). Blank experiments (with only reagents and Milli Q water) were carried out to determine the amounts of dialysable Fe from the reagents.

Simulated gastrointestinal digestion procedures

The model consists of four successive compartments (Fig 1) simulating the stomach, duodenum, jejunum and ileum. The gastrointestinal *in* vitro model has been described in detail before (8). Each compartment is formed by two connected basic units consisting of a glass jacket with a flexible wall inside. Water is pumped from a water bath into the glass jackets around the flexible walls to control the temperature inside the units and the pressure on the flexible walls. Changes in the water pressure are achieved by computer-activated rotary pumps. This enables mixing of the chyme by alternate compression and relaxation of the flexible walls.

The compartments are connected by peristaltic valve-pumps consisting of three connected T-tubes, each with a separate tube-like flexible wall inside. In the open position, the flexible walls facilitate unhindered passage of the chyme, if pressure is applied to the outside of the flexible wall, the valve is closed.

Peristaltic pumping is achieved by regulating the sequence of opening and closing of the three parts of the valve-pump. During each peristaltic cycle, a constant volume of chyme is transferred. The frequency of peristaltic cycles is dictated by a computer, allowing the flow rate of the chyme to be controlled. The volume in each compartment is monitored with a pressure sensor connected to the computer.

The gastric and duodenal compartments are equipped with pH electrodes. The pH values are controlled via the computer by secreting either water or 1 M HCl into the stomach, or by secreting either water or 1 M $NaHCO_3$ into the duodenum, via syringe pumps. Secretions of gastric electrolytes and enzymes, bile and pancreatic juices are regulated using computer-controlled syringe pumps. The jejunal and ileal compartments are connected with hollow-fibre devices to absorb digestion products and water from the chyme and to modify electrolyte and bile salt concentrations in the chyme. The computer program has been designed to accept parameters and data obtained from *in vivo* studies in animals or human volunteers, such as the pH curves for the stomach and duodenum, secretion rates into the different compartments, water absorption from the small intestine and gastric and ileal delivery. To control the transit of the chyme, a power exponential formula for gastric and ileal delivery is used, as described by Elashoff *et al* (3).

At the start of the experiment the test material was introduced into the gastric compartment with or without phytase addition. The secretion of gastric and duodenal juices was set to 0.5 ml/min and 1 ml/min, respectively. The ileal deliveries were collected after 1, 2, 4 and 6 hours in bottles containing 8 ml concentrated HCl, in order to stop phytate hydrolysis. Jejunal and ileal dialysis fluids were collected after 2, 4 and 6 hours and the volumes were measured.

The proportion of the compounds diffusing across the hollow-fibre membranes during the intestinal passage was used as a measure of the bioavailability of minerals and trace elements for absorption in the gastrointestinal tract. After the simulated digestion, the residual amounts of contents in the gastric, duodenal, jejunal and ileal compartments were collected. The concentration of free phosphorus was determined in each sample.

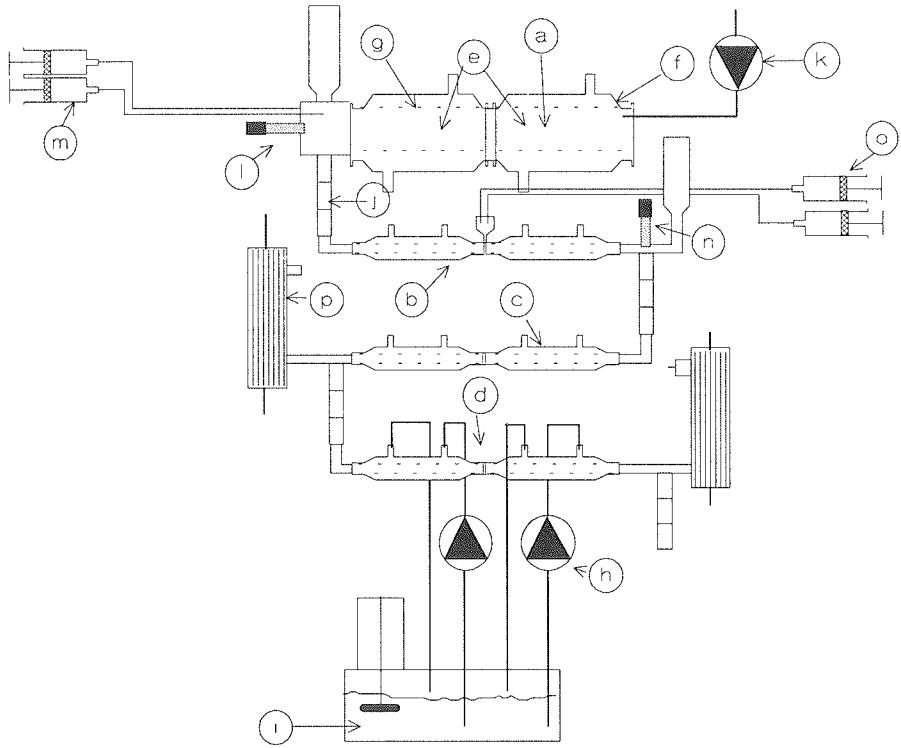

Fig. 1. Schematic diagram of a multi compartmental model of the stomach and the small intestine: (a) Gastric compartment; (b) duodenal compartment; (c) jejunal compartment; (d) ileal compartment; (e) basic unit; (f) glass jacket; (g) flexible wall; (h) rotary pump; (i) water beth; (j) valve pump; (k) feeding pump; (l,m) pH electrodes; (n,o) syringe pumps; (p) hollow-fibre device.

Endogenous phytase activity was analysed in each cereal and the total FTU/experiment was calculated (1 FTU = the amount of the enzyme that liberates 1 µmol inorganic phosphate from 1.5 mM sodium phytate at pH 5.5 and 37°C in one minute).

Analytical methods

The concentration of free phosphorus was determined in samples of ileal deliveries, jejunal and ileal dialysates, gastrointestinal residues and cereals with the molybdate-vanadate method using an auto-analyzer. Fe in dialysates and cereals

were determined by Atomic absorption spectrometry. Fe concentrations in dialysates were also deter-mined colorimetrically with a spectrophotometer at 565 nm (LKB, Biochrom, Cambridge, UK) using Spectroquant, Photometric Fe test 1.14761 (Merck, Darmstadt, Germany). Requisite precautions were employed for minimizing contamination of samples during handling, storage and analysis.

Inositol hexa-, penta-, tetra- and triphosphates were determined according to the method of Sandberg and Ahderinne (10) as modified by Sandberg *et al.* (11).

Calculation of bioavailability (dialysability)

The amount of dialysed mineral, which is supposed to be related to bioavailability, is expressed as percentage of the total amount present in the food sample:

Dialysability (%) = D/(W * A) * 100

were D is the total amount of dialysed mineral (µg), W is the dry weight of the cereal sample used in the digestion experiment (g) and A is the concentration of mineral in the dried cereal sample (µg/g).

RESULTS

Rapeseed

Addition of exogenous phytase during the gastrointestinal digestion of rapeseed was found to increase the amounts of dialysed iron by 67% (Table 2) or in total amounts from 382 µg to 636 µg Fe. The concentration of free phosphorus (% of total P intake) was 31% higher in dialysates from rapeseed with phytase addition as compared to rapeseed digested with only endogenous phytase (Table 2). The highest amounts of dialysable phosphorus were determined in the dialysates collected during the second and fourth hour of digestion (Fig 2). Free phosphorus concentrations in residues were almost equal in the stomach and duodenum (Table 3), with or without phytase addition. In jejunum and ileum the amounts of free phosphorus were higher in the residues with phytase addition (Table 3). The largest difference (77%) was found in the jejunum, where the recovery of free phosphorus was 3.9 and 2.2%, respectively.

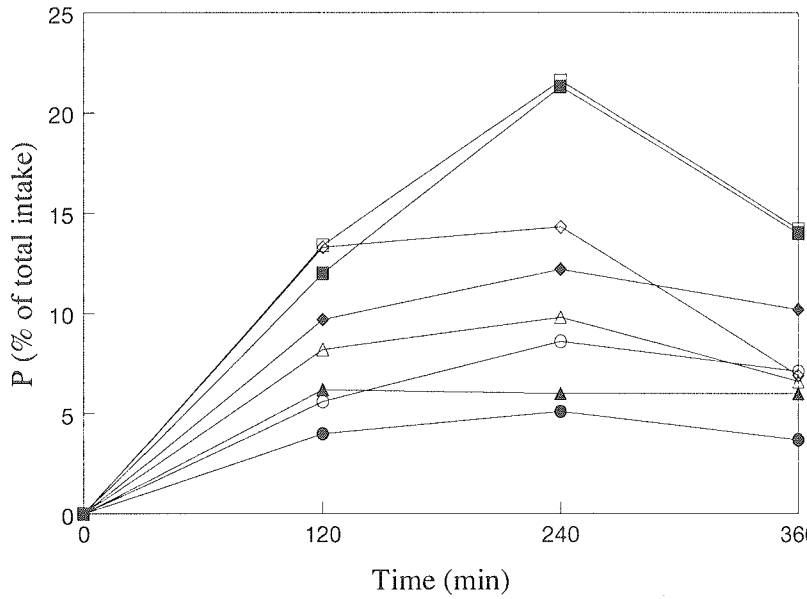

Fig. 2. Recovery of free phosphorus in jejunal and ileal dialysates during digestion of rapeseed (Δ,▲), sunflowerseed (○,●), whole wheat flour (□,■) and white wheat flour (◇,◆) with (open markers) and without (closed markers) addition of phytase (45 FTU).

Table 2. Dialysability (%) of Fe and P as percentage of total Fe and P intake during in vitro digestion of rapeseed, sunflowerseed, whole wheat flour and white wheat flour with and without addition of exogenous phytase.

	Fe dialysability			P dialysability		
Cereal	With phytase[a]	Without phytase	Difference (%)	With phytase[a]	Without phytase	Difference (%)
Rapeseed	6.7	4	6.7	24.6	18.8	30.8
Sunflower seed	18.8	15.7	20.1	21.3	12.8	66.4
Whole wheat flour	38.3	35.2	8.6	49.2	47.3	4
White wheat flour	144	95.1	51.4	34.5	32	7.8

[a] 45 FTU/experiment, 1 FTU = the amount of the enzyme that liberates 1 µmol inorganic phosphate from 1.5mM sodium phytate at pH 5.5 and 37°C in one minute.

White wheat flour

In dialysates from white wheat flour digestion, the Fe dialysability was 51% higher in the experiments with phytase supplemented wheat flour (Table 2). The dialysability of free phosphorus was increased by 8% after addition of phytase to the wheat flour. Analysis of free phosphorus in residues showed only a very small variation between experiments with and without exogenous phytase (Table 3). The recovery of free phosphorus in ileal delivery was similar in wheat flour digested with the addition of phytase, 5.8 % compared to 5.1% in samples from untreated wheat flour (Fig 3).

DISCUSSION

The key point for the development of a valid *in vitro* method is the close simulation of the *in vivo* situation. Compared to other *in vitro* gastrointestinal models, the model used in this paper has a number of specific advantages, such as peristaltic movements and absorption of nutrients. In addition it is a dynamic model with physiological emptying patterns and transit times for liquids and solids, which makes it more realistic than other *in vitro* methods.

In a previous study with this model (4), it was observed that a slow gastric emptying (half time of emptying = 2 h), comparable with gastric emptying in pigs on solid feed, had a positive effect on the enzymatic degradation of phytate as compared with a fast gastric emptying (half time of emptying = 1 h). This is in agreement with the findings described by Kemme and Jongbloed (6), who indicated that phytase of plant origin was able to dephosphorylate a part of the dietary phytic acid in the stomach of the pig, and that it was strongly dependent on the retention time in the stomach. Also the continuously changing pH after a meal has to be considered. In the present study the pH in the stomach was controlled to mimic the decrease of the pH in time after a meal. Figure 2 shows the release of free phosphorus in dialysates during digestion for six hours. During the first two hours of digestion we noticed a rapid increase in phosphorus dialysability, followed by two hours of rather slow phosphorus release (except for whole wheat, where the phosphorus continued to increase at the same rate). After four hours of digestion the amounts of dialysed phosphorus decreased or remained at almost the same level. This illustrate the importance of a dynamic model which simulates the successive physiological parameters in the gastrointestinal tract.

For the validation of the model, the results obtained *in vitro* should be compared with *in vivo* data. In a study by Lantzsch *et al* (7), the gastrointestinal hydrolysis of

phytate from different diets based on wheat, barley and soya was determined in growing pigs. In the stomach and the proximal half of the small intestine a mean breakdown of 51% of phytate from wheat was measured. In samples collected in the present study during the *in vitro* digestion of white wheat flour without additional phytase, we found that the total amounts of free phosphorus was 56%, which is in close agreement with the observation by Lantzsch *et al* (7).

The degradation of phytate in a rapeseed meal diet (without phytase activity) was measured in pigs by Sandberg *et al* (12), these authors found that 35-45% of the phytate was hydrolysed in the stomach and small intestine of ileal-fistulated pigs.

In the present study, the total amounts of free phosphorus in samples collected from rapeseed digested without phytase addition was determined to be 33%, thus indicating a dephosphorylation of the inositol-phosphates during the digestion in good agreement with the values obtained in the study by Sandberg *et al* (12). Furthermore, in the study by Sandberg *et al* (12), the absorption of phosphorus from a rapeseed meal diet with a phytate content of 3.79 mg/g feed was 44.5%, compared to the present study where the dialysability of phosphorus was 18.8%, from a rapeseed diet containing approximately twice as much phytate (8.06 mg /g), thus, indicating that our results are conclusive with respect to the relative relation in the composition of the diets on the bioavailability of phosphorus.

Barrier-Guillot *et al* (1) studied the phosphorus availability in broilers and pigs from four different wheat varieties with different phytate content (0.9 to 2.0 g/kg) and endogenous phytase activities ranging from 0.44-0.66 U/g. They found a mean apparent P digestibility of 40% and a linear relationship between P digestibility and endogenous phytase activity in wheat. In our *in vitro* digestion with whole wheat flour containing 2.7 g/kg phytate-P and a phytase activity of 0.73 FTU/g, 47% of the total P intake was recovered as free phosphorus in the dialysates which correlates well with the data from the study by Barrier-Guillot *et al* (1).

When we compare our results with the endogenous phytase activity in the different cereals (Table 1) we find that, in whole wheat flour, having the highest phytase activity of the tested cereals, the effect of exogenous phytase addition on the free phosphorus concentration in dialysates (Fig 2), was less pronounced than in the other cereals. The most marked effect of phytase supplementation was found in rapeseed and sunflowerseed, where also a lower endogenous phytase activity (Table 1) was determined.

The effect of intrinsic wheat bran phytase and a microbial *A. niger* phytase on iron absorption in man was investigated in a recent study by Sandberg *et al* (13). The

addition of *A. niger* phytase (200,000 PU) to a meal containing wheat bran (9 mg phytate-P) just before consumption was found to markedly increase the iron absorption from 14% to 26%. In our experiments with whole wheat flour the Fe dialysability increased by 9% after phytase addition (45 FTU to 107 mg phytate-P).

In conclusion, the gastrointestinal model appears to be a promising screening tool for the evaluation of mineral and trace element bioavailability from cereals. Although the experimental design does not allow for a direct comparison with *in vivo* values, the *in vitro* determinations of the bioavailability of iron and phosphorus represents well the effects of differences in the composition of the diets.

Furthermore, the results of *in vitro* methods cannot be used for a direct prediction of the bioavailability of minerals and trace elements, since not all physiological conditions that are important in determining the bioavailability of nutrients, such as active absorption, can be simulated *in vitro*. However, our data seems to be in qualitative agreement with the quoted studies, showing good correlations with respect to the relative relation between the *in vitro* availability and the *in vivo* availability of iron and phosphorus.

Further research with the *in vitro* model is needed to provide a means of screening foods and predicting mineral utilisation from complete meals.

ACKNOWLEDGEMENTS

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Chapter 5

SURVIVAL OF LACTIC ACID BACTERIA IN A DYNAMIC MODEL OF THE STOMACH AND SMALL INTESTINE: VALIDATION AND THE EFFECTS OF BILE

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ABSTRACT

This study was conducted to validate a dynamic model of the stomach and small intestine to quantify the survival of lactic acid bacteria and to assess the influence of gastrointestinal secretions. The survival of a single strain of the following species, *Bifidobacterium bifidum* and *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus,* was measured under the physiological conditions (e.g., peristalsis, changes in pH, changes in concentrations of enzymes and bile) and were compared with data obtained in humans. No significant differences were found between the *in vitro* and *in vivo* data, indicating that the model has a predictive value for the survival of these bacteria in humans.

The survival of these strains of lactic acid bacteria in the gastrointestinal model was investigated under two different conditions in the small intestine: simulation of physiological secretion of bile and a low bile secretion. Reductions in viability were significantly different between the bacterial species. The dose-response effect of bile on the survival of the tested bacteria was significant, demonstrating the bactericidal effect of bile salts. This study demonstrates the differences between bacterial species in their sensitivity to gastric and intestinal secretions.

INTRODUCTION

The survival of ingested microorganisms in the gastrointestinal tract (GIT) influences the risk of foodborne infections and the efficacy of probiotics and orally dosed live vaccines. Validated methods and models are required to study the mechanisms influencing the survival of microorganisms and to allow comparison and selection of probiotic or vaccinal strains. Ingested microorganisms are exposed during their transit through the GIT to successive stress factors that influence the survival of those microorganisms (20, 30). The roles of gastric pH and gastrointestinal peristalsis in preventing bacterial colonization of the small bowel are well established (12, 30); in contrast, the role of bile in this respect still is a matter of debate (30). Based on the results obtained in static *in vitro* models, some researchers (5, 30) have reported that the bactericidal effects of conjugated bile acids are weaker than those of free bile acids. However, this conclusion was questioned by others (31). In fact, the predictive value of results obtained in static *in vitro* models is limited for several reasons. First, the bile salt concentration in the gut is not static, but changes over time and in the different parts of the small intestine. After a meal, bile salt concentration sharply increases in the duodenum up to ca. 15 mmol/L, and then progressively decreases to 5 mmol/L. In the

jejunum, the bile salt concentration is ca. 10 mmol/L, and, in the ileum, the concentration falls below 4 mmol/L because of active ileal absorption (13, 24). Second, bile salts form micelles with phospholipids (as they are found in whole bile) and, therefore, have lower antibacterial activity than artificial solutions of pure bile salts (32). Finally, *in vivo*, the successive stresses by gastric acid and bile can be expected to exert a stronger antimicrobial effect than one of these parameters alone.

Recently, a dynamic, computer-controlled model has been developed that allows the simulation of successive *in vivo* conditions in the stomach and small intestine, such as the kinetics of pH, bile salt concentrations, and transit of the chyme (23). The objective of the present study is validate this model in relation to the survival of these bacteria. The species and strains used in this study were chosen because they are used in commercial fermented milks (6, 20) and because the survival of these bacteria has previously been quantified in humans (17, 19, 26, 27, 29), which allows validation of the model by comparison of the *in vitro* and *in vivo* results.

MATERIALS AND METHODS

Dynamic gastrointestinal model

Minekus *et al.* (23) have described the model: it comprises four serial compartments simulating the stomach, duodenum, jejunum and ileum, which are connected by computer-controlled valve pumps (Figure 1). Temperature is kept at 37°C. The chyme is gently mixed three or nine times per minute in the gastric or intestinal compartment, respectively, by alternate contractions of the flexible walls. Simulated salivary, gastric, biliary, and pancreatic secretions are introduced into the corresponding compartments by computer-controlled pumps. The jejunal and ileal compartments are equipped with hollow fibre devices that permit dialysis of the chyme. The pH conditions in the gastric and duodenal compartments are monitored with pH meters connected to the computer. Secretion of either 1 mol of HCl or a neutral electrolyte solution into the gastric compartment is dosed via the computer for pH control. The same procedure is applied for secretion of either 1 mol of $NaHCO_3$ or the neutral electrolyte solution into the duodenal compartment. Mathematical modelling to reproduce and control gastric and intestinal emptying is performed using a power-exponential equation with variables for half-time of gastric or intestinal emptying and the β-value as a parameter describing the shape of the curve.

Products and microorganisms

Two fermented milk products were used: Ofilus® (Yoplait, Paris, France) containing *Bifidobacterium bifidum* (ca. 10^6 cfu/g) and *Lactobacillus acidophilus* (ca. 10^7 cfu/g) and a yogurt containing *Lactobacillus delbrueckii* ssp. *bulgaricus* strain LB9 (ca. 10^7 cfu/g) and *Streptococcus thermophilus* strain ST20 (ca. 10^8 cfu/g). Ofilus® was studied 4 to 8 d after preparation as in the *in vivo* study described by Marteau *et al.* (19). Yogurt was prepared from one batch of milk powder (147 g/L). The reconstituted milk was sterilized at 110°C for 12 min, cooled at room temperature (± 20°C), and inoculated with *L. delbrueckii* ssp. *bulgaricus* strain LB9 and *S. thermophilus* strain ST20 (both kindly provided by Boll, Saint-Germain-les-Arpajon, France). The product was incubated aerobically at 37°C until the pH reached 4.6 (ca. 4 h) and subsequently stored overnight at 4°C.

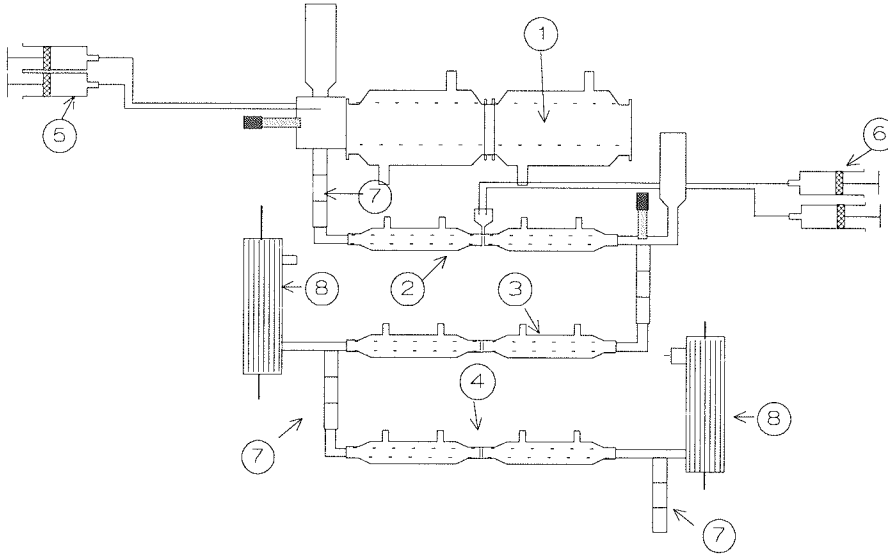

Fig. 1. The dynamic multicompartmental model of the gastrointestinal tract. 1, gastric compartment; 2, duodenal compartment; 3, jejunal compartment; 4, ileal compartment; 5, gastric secretions; 6, intestinal secretions (bicarbonate, bile, pancreas juice); 7, peristaltic valve pumps; and 8, dialysis devices connected to the jejunum and ileum.

Experimental design

The survival of the four bacterial species was assessed: 1) inside the gastric compartment, 2) in the chyme delivered from the gastric compartment (gastric delivery), 3) in the chyme delivered from the ileal compartment during experiments simulating physiological bile salt concentrations, and 4) in the chyme delivered from the ileal compartment during experiments with low bile concentrations in the small intestinal model. For each condition, six experiments were performed. Two species were tested simultaneously: the two species in the Ofilus® product and the two species in the yogurt product. Gastric and ileal delivery experiments lasted for 3 and 6 h, respectively.

Before each experiment, the model was decontaminated by steaming at 100°C for 45 min. The Ofilus® and yogurt products (50 ml each) were separately introduced via an inlet on the gastric compartment after dilution (1:1, vol/vol) in a sterile electrolyte solution containing 6.2 g/L of NaCl; 2.2 g/L of KCl; 0.22 g/L of $CaCl_2$ and 1.2 g/L of $NaHCO_3$ to simulate the *in vivo* dilution by saliva. The secretion into the gastric compartment comprised the electrolyte solution with 370 U/ml of pepsinogen (Sigma Chemical Co., St. Louis, MO) at a flow rate of 0.25 ml/min and 1 mol of HCl or the electrolyte solution at a flow rate of 0.25 ml/min. The pH curve in the stomach was computer–controlled (23) to reproduce the values found in humans after yogurt consumption (3): pH 5.0 at initiation, pH 4.1 at 20 min, pH 3.0 at 40 min, pH 2.1 at 60 min, and pH 1.8 at >80 min. In the small bowel compartments, pH was kept at 6.5 ± 0.5.

Gastric and ileal emptying in the model were regulated by computer via the pump valves to reproduce the gastric and ileal emptying of a nonabsorbable meal marker ingested with yogurt by human volunteers (21). For gastric emptying, the half-time and was 70 min, and the β coefficient of the power exponential equation was 2. For ileal emptying, the half-time was 160 min, and the β coefficient was 1.6 (23).

Duodenal secretion comprised 1 mol of $NaHCO_3$ or the electrolyte solution (0.25 ml/min); 7% Pancreatin® (Pancrex V, Paines & Birne, Greenford, England) in 0.3 mol of $NaHCO_3$, (0.25 ml/min) and bile (porcine bile extract, which is comparable with human bile; Sigma Chemical Co.) at a concentration differing among the experiments (flow rate 0.5 ml/min).

The dialysis fluid contained 5 g/L of NaCl, 0.6 g/L of KCl, 0.25 g/L of $CaCl_2$, and bile extract (concentration depending on the intestinal compartment and the experiment) and had a flow rate through the hollow fibres of 10 ml/min.

The concentrations of bile salts in the experiments simulating the physiological conditions were the same as described by Minekus *et al.* (23). Briefly; 12.5 ml of

4% bile solution was in the duodenal compartment initially, followed by secretion of 4% bile for the first 30 min and 2% bile for the remaining time; the jejunal dialysis fluid contained 1.55% bile, and ileal dialysis fluid was without bile. The concentrations of bile salts during the low bile condition were kept at 2 mmol/L over time in each intestinal compartment. This concentration was obtained by initially introducing 20 ml of a 0.8% bile solution into the duodenal compartment, followed by the secretion of 2% bile; the dialysis devices were not used.

Sampling and microbiological methods

To study the effect of gastric secretion on the survival of bacteria, samples from the gastric compartment were taken at 0, 20, 40, 70, 106, 127, and 180 min after feeding. To assess the delivery of viable bacteria from the gastric compartment into the duodenal compartment, the chyme was collected on ice immediately after the pyloric valve and fractionated in 30 min periods for 3 h (intestinal compartments were not used in these experiments). The delivery of viable bacteria from the ileal compartment was assessed in chyme collected on ice from after the ileo-cecal valve and fractionated in 60-min periods for 6 h. Previous experiments have shown that storage of the samples on ice for 2 h did not affect the colony counts.

The volume of each sample was measured. Serial decimal dilutions were plated onto validated selective media with a spiral plater (Spiral System Instruments, Bethesda, MD). The following agar media and culture conditions were used: Rogosa medium (Oxoid Ltd., Basingstoke, England) for *L. bulgaricus* and *L. acidophilus* with anaerobic incubation (BBL® GasPak™ ; Becton Dickinson, Cockeysville, MD) at 37°C for 48 h; M17 (Oxoid) for *S. thermophilus* with aerobic incubation at 37°C for 48 h; and Beerens medium (1) for *B. bifidum* with anaerobic incubation at 37°C for 72 h.

Calculations and statistics

The survival of bacteria were expressed as percentage of the ingested total number of bacteria (means ± SE), which allows the comparison of different species and different conditions regardless of differences in initially ingested numbers of bacteria. In the gastric compartment, the survival percentage was calculated in each sample, taking into account the volumes secreted and gastric emptying in time. Gastric and ileal deliveries of viable microorganisms were calculated from the bacterial counts in the samples and the corresponding outflow of chyme. The mean (± SE) initial viable numbers of lactic acid bacteria per millilitre in the

gastric compartment of the model were 2.2 (± 1.2)x10^7 and 3.6 (± 1.4)x10^6 for *B. bifidum* and *L. acidophilus* (Ofilus® product), respectively, and 7.8 (± 1.4)x10^7 and 2.1 (± 0.3)x10^8 for *L. bulgaricus* and *S. thermophilus* (yogurt product), respectively. Cumulative percentages of the live bacteria delivered from the gastric and ileal compartments for the total collection period were obtained by summing the results of successive sampling periods.

The cumulative delivery of *L. acidophilus* and *B. bifidum* was compared with results obtained previously in human volunteers with the same bacterial species in the same product under similar conditions (19) using ANOVA. The *in vitro* and *in vivo* data were compared for bacterial survival in ileal samples that were taken within a 1-h interval in six replicates.

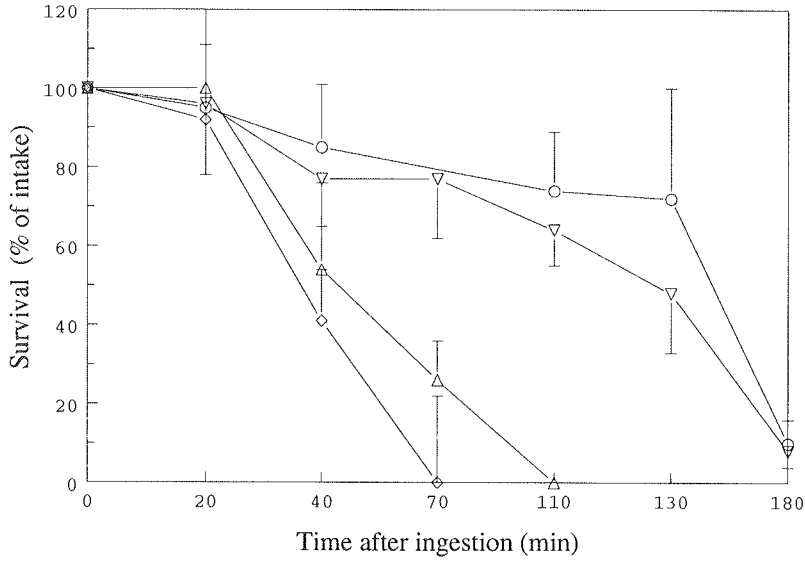

Fig. 2. The survival of ingested *Bifidobacterium bifidum* (○), *Lactobacillus acidophilus* (▽), *Lactobacillus bulgaricus* (△), and *Streptococcus thermophilus* (◇) in the gastric compartment of the model. Values are expressed as mean percentages (± SE) of live bacteria relative to the ingested numbers (n = 6 for each strain).

RESULTS

Streptococcus thermophilus and *L. bulgaricus* in the yogurt product survived only briefly in the gastric compartment (Figure 2). Viability after 40 min was significantly lower than that of *L. acidophilus* and *B. bifidum* in the Ofilus® product, and, within 70 and 110 min, the viable counts fell below 1% of the ingested numbers of bacteria. After 120 min, more than 40% of the ingested numbers of *L. acidophilus* and *B. bifidum* remained viable in the gastric compartment (Figure 2).

The deliveries of viable bacteria from the gastric compartment into the duodenal compartment (Figure 3) were significantly lower for *L. bulgaricus* (26%) and *S. thermophilus* (12%) than for *L. acidophilus* (64%) and *B. bifidum* (67%). The cumulative deliveries of viable *L. acidophilus* and *B. bifidum* cells from the gastric compartment increased continuously for more than 2 h (Figure 3), but those for *L. bulgaricus* and *S. thermophilus* reached a peak within 70 min.

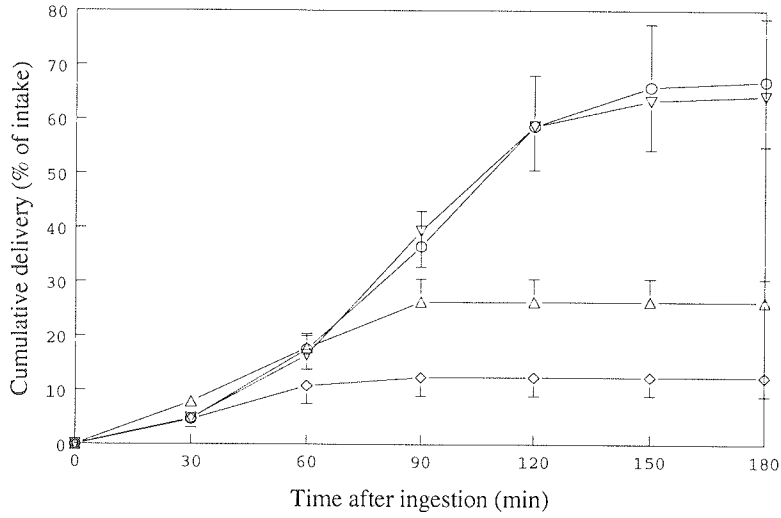

Fig. 3. Cumulative delivery of *Bifidobacterium bifidum* (○), *Lactobacillus acidophilus* (▽), *Lactobacillus bulgaricus* (△), and *Streptococcus thermophilus* (◇) from the gastric into the duodenal compartment of the model. Values are expressed as mean percentages (± SE) of live bacteria passing the simulated pyloric sphincter relative to the ingested numbers (n = 6 for each condition).

The cumulative deliveries of the viable bacteria from the ileum into the colon, using the physiological and the low (2 mmol/L) bile concentrations, are shown in Figure 4. Passage through the small bowel with physiological bile concentrations resulted in a decreased survival of all four species relative to gastric delivery. At the low bile concentration, deliveries of viable *L. acidophilus* and *B. bifidum* were significantly higher. For *L. bulgaricus* and *S. thermophilus*, no differences were significant between physiological concentrations and low bile salt concentrations, probably because of the low survival (close to the detection limit) under both conditions.

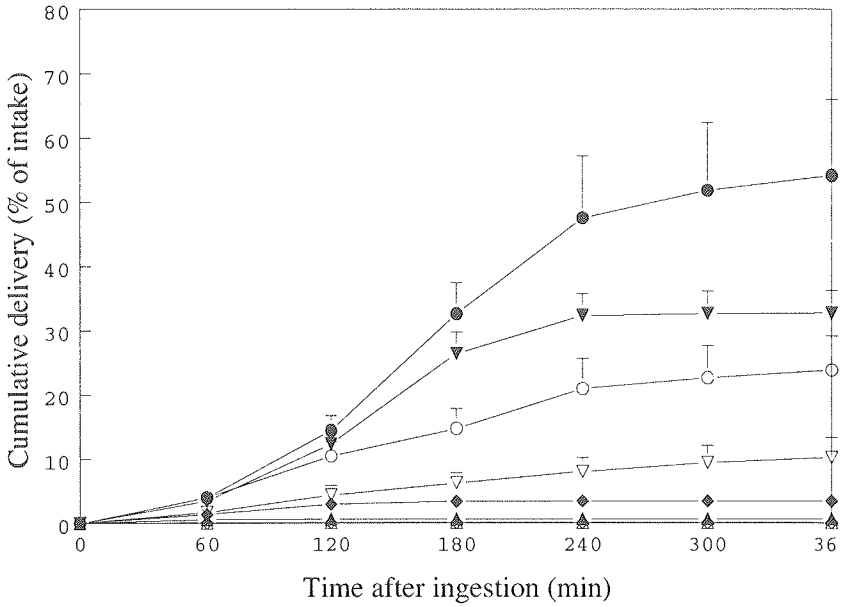

Fig. 4. Cumulative delivery of *Bifidobacterium bifidum* (○,●), *Lactobacillus acidophilus* (▽,▼), *Lactobacillus bulgaricus* (△,▲), and *Streptococcus thermophilus* (◇,◆) from the ileal compartment, simulating physiological bile salt concentrations (open markers) and low bile salt concentrations (solid markers) in the small intestinal compartments of the model. Values are expressed as mean percentages (± SE) of live bacteria passing the simulated ileo-cecal sphincter relative to the ingested numbers (n = 6 for each condition).

The cumulative survival of *B. bifidum* and *L. acidophilus* during passage through the gastric and the small intestinal compartments (until the simulated ileo-caecal

valve) did not differ significantly from those found previously in humans with the same bacterial strains in the same vehicle (19) (ANOVA: $P = 0.88$).

DISCUSSION

Research on the survival of ingested bacteria in the GIT is important for the selection and development of probiotics or oral vaccines as well as for a better understanding of possible mechanisms behind probiotic functions of beneficial microorganisms. Also, more knowledge is needed concerning the kinetics of survival of ingested pathogenic microorganisms in order to analyse the risk of foodborne pathogens (25). The survival of microorganisms has scarcely been quantified *in vivo*, except for some species of lactic acid bacteria (2, 17, 19, 26, 28, 29), because of difficulties in sampling from the human gut and because of ethical constraints. In some studies, attempts have been made to obtain information on the influence of acidic or bile salts on the survival of microorganisms using static single-compartmental models (3, 5, 8, 9, 14, 17, 28). However, the predictive value of such models is limited because they do not simulate the sequential stresses that are due to the continuously changing conditions to which ingested microorganisms are exposed during their passage *in vivo*. The model used in this study permits an accurate and dynamic simulation of the major factors influencing the survival of ingested microorganisms, such as pH, bile concentrations, and transit through the different parts of the GIT (23, 30).

Although the intakes of viable numbers of bacteria were standardized and were similar in each experiment, the expression of survival as a percentage of the total numbers of ingested bacteria facilitates comparisons among different species and different conditions, and estimates the absolute amount of passing microorganisms. The data on survival of *L. acidophilus* and *B. bifidum* in the model are not significantly different from those obtained with the same strains in the same vehicle in healthy volunteers using an intubation technique (ANOVA: $P = 0.88$) (19). in addition, the cumulative numbers of viable bacteria passing the end of the intestinal compartments of the model were similar with those passing the ileum in humans (Figure 5). The data on survival of *L. bulgaricus* in the duodenal and ileal compartments (Figure 4) are also consistent with other data (17, 26, 27). Although the comparison with *in vivo* data is limited to one strain of four different species, this study shows the validity of the dynamic model and the method for the prediction of the survival of ingested lactic acid bacteria in humans.

The model can be used to assess the successive influences of gastric secretion, gastric emptying, and bile concentrations on the survival of probiotic

microorganisms. Static experiments (3, 17) have shown that *Bifidobacterium* spp. and *L. acidophilus* are more acid-resistant than are *L. bulgaricus* and *S. thermophilus*. In this dynamic model, however, even for acid-sensitive species such as *L. bulgaricus* and *S. thermophilus,* a relatively large fraction of ingested bacteria reached the duodenum alive (Figure 3). This situation occurred mainly during the first 20 to 30 min after a meal when the pH in the stomach was still relatively high (above pH 3.8) (3, 27). This result emphasizes the importance of the initial period of gastric emptying for the delivery of live bacteria into the small intestine. The kinetics of gastric delivery simulating that of yogurt was relatively slow (halve-time of gastric emptying was 70 min). Therefore, the survival of ingested bacteria would have probably been even higher if the fast gastric emptying of a liquid would have been simulated (with a halve-time of gastric emptying of 30 min).

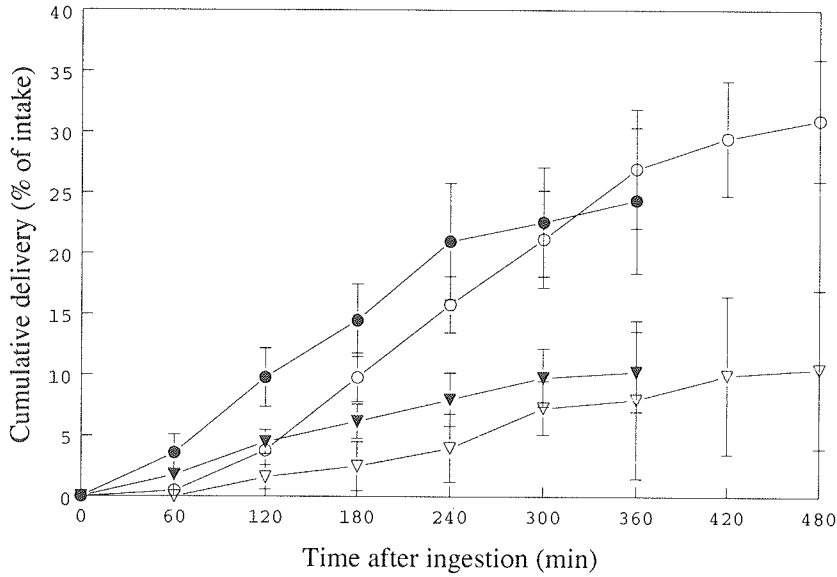

Fig. 5. Cumulative delivery of *Bifidobacterium bifidum* (○,●), and *Lactobacillus acidophilus* (▽,▼), from the ileum of human volunteers (19) (open markers) and from the ileal compartment of the model (solid markers). Values are expressed as mean percentages (± SE) of live bacteria passing the simulated ileo-cecal sphincter relative to the ingested numbers (n = 6 for each condition).

Until now, the lethality of bile on microorganisms in the human small bowel was thought to be low, even negligible (30) because of *in vitro* data that showed that conjugated bile salts, which constitute the majority of bile salts present in the small bowel, were less bactericidal than deconjugated bile salts (5, 13, 24, 30, 31). In the present study, bile exerted a strong influence on the survival of the bacterial species tested (Figure 4): the survival rate varied within a small range of bile concentrations. These findings support the importance of investigating the sensitivity of microorganisms to bile as a selection step for potential probiotics (6, 8, 11). The bile stress for ingested microorganisms in the GIT is complex, because bile concentrations and residence times vary in each compartment of the GIT. Furthermore, the bile stress occurs after the pH stress in the stomach. Sublethally injured microorganisms have a different and unpredictable resistance to new stress factors (16). For these reasons, a dynamic model is expected to be more appropriate for prediction of the *in vivo* effects of bile on microorganisms than a static model with a constant concentration of bile.

Although probiotic microorganisms are generally thought to survive the transit through the GIT for their functionality (6, 20), the damaging effect of bile salts on yogurt bacteria seems also to have positive consequences. Bile could liberate the lactase activity from yogurt bacteria in the small bowel, which could partially explain in part the better lactose digestion after digestion of yogurt by lactase-deficient subjects (7, 15, 18, 22). The bacterial lysis in the small intestine dependents on bile salt and could thus be considered as a way to deliver specific biologically active components to the duodenum using ingested microorganisms. Clinical applications can be investigated in this *in vitro* model, for example, lipase activity to treat pancreatic insufficiency for cases in which classical lipase delivery systems are not sufficiently active in the duodenum (4, 10)

CONCLUSIONS

The described dynamic *in vitro* model of the gastrointestinal tract offers new possibilities for quantitative studies of the survival of microorganisms in the gastrointestinal lumen. This model can be helpful in screening microorganisms for targeting in the gut, such as probiotics or oral vaccines, whether or not in combination with specific food components as selective substrate (prebiotics). Finally, the influence of certain microorganisms on the metabolic activity in the lumen can be studied.

ACKNOWLEDGMENTS

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Chapter 6

A DYNAMIC COMPUTER-CONTROLLED MODEL OF THE STOMACH AND SMALL INTESTINE TO STUDY COAGULATION AND PROTEIN DIGESTION OF CALF MILK REPLACERS

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ABSTRACT

TNO has developed an *in vitro* model that mimics accurately and reproducibly the kinetics of the gastrointestinal tract of calves. In this study the adequacy of the model was demonstrated as a tool to evaluate protein digestion and clotting of calf milk replacers (CMR). Three different calf milk replacers based on skim milk (SMP), skim milk / soluble wheat protein (SMP/SWP) and soya protein isolate (SPI) were tested while simulating normal conditions in the lumen of the calf gastrointestinal tract. The retention of protein nitrogen, which was used as a measure of coagulation of the CMR, was determined by comparing gastric protein nitrogen delivery of the CMR with theoretical delivery without coagulation. The ranking of gastric delivery of protein nitrogen for the three CMR parallelled *in vivo* delivery. Gastric delivery of nitrogen in time with SMP after clotting proved to correspond exactly with results obtained from cannulated calves. Digestion and absorption of protein in the model were compared with results obtained in experiments with cannulated calves. *In vitro* digestibility coefficients calculated after 360 min for SMP, SMP/SWP and SPI were 97.9%, 95.8% and 92.7%, respectively. The same products gave digestibility coefficients of 99.5%, 97% and 96.3%, respectively, in ileal-cannulated calves.
The absorption in each compartment and the ileal delivery of nitrogen in time for the diets in the model could be exactly reproduced with a computer simulation program. The results show that the TNO gastro- Intestinal Model (TIM) offers a valuable tool for evaluating both digestion and gastric delivery of calf milk replacers. These are important parameters for research on product improvement and quality control. The standardized and reproducible control of kinetic parameters allows for computer modelling and evaluation of the results even with a complex interaction of parameters.

INTRODUCTION

The most frequently used protein sources in calf milk replacers (CMR) are skim milk powder (SMP) and whey protein products. Soya protein is used as an alternative protein source for SMP. Coagulation and digestibility are important properties of proteins to be evaluated. Coagulation is regarded as an important property of CMR containing SMP (6,7), since it might improve the bioavailability of nutrients. The protein digestibility coefficient is an important parameter to describe the nutritional value of protein and can be used to evaluate the quality of protein sources, to improve production processes or diet composition, and to control product quality. The choice of an appropriate test system depends on the type of study, the available budget and ethical considerations. A live intact animal is the most complete test

system, especially if the test animal is of the same species as the target animal. However, due to considerable biological variation among animals, often a large number of animals will be needed to obtain reliable results. This makes animal studies relatively expensive and hence not very suitable for screening or routine experiments. From an ethical point of view, the use of invasive techniques on animals is debatable. *In vitro* models are cheap, relatively easy to perform and fast. However, their predictive value may be limited depending on the complexity of the model. TNO has developed a multi-compartmental, dynamic, computer-controlled model of the stomach and small intestine, which allows for a close simulation of the dynamic conditions that occur in the lumen of the gastrointestinal tract (GIT) of monogastric animals (1,4,9,11). These conditions include a gastric pH that follows a pre-set course, physiological concentrations of secreted molecules and active compounds such as electrolytes, bile salts and enzymes, transit of the meal through the successive parts of the GIT, and absorption of water and products of digestion. This paper describes the use of the model to evaluate coagulation and true protein digestibility of CMR under conditions that mimic the dynamic events in the gastric and small intestinal lumen of the preruminant calf.

MATERIALS AND METHODS

The model

The TNO gastro-Intestinal Model (TIM) has been described previously (9). Briefly, the model consists of four successive compartments, simulating the stomach (Fig. 1, A), duodenum (B), jejunum (C) and ileum (D). A meal can be fed to the gastric compartment during a pre-set time. In the gastric compartment gastric juice is added (E), while the pH is measured (F) and adjusted to follow a predetermined curve. The compartments consist of two connected glass units with flexible walls inside. The temperature is controlled by pumping water from a water bath (G) through the space between the jacket and the flexible wall. The walls can be squeezed by varying the pressure on the water. The chyme in each compartment is mixed by alternately squeezing the flexible walls. To control the transit of the meal, the compartments are separated by computer-regulated peristaltic valve pumps (H). Bile and pancreatic juice are secreted into the duodenal compartment (I). The duodenal pH is measured (J) and controlled through the addition of sodium hydrogen carbonate. Products of digestion and water are absorbed from the jejunal and ileal compartments by pumping dialysis liquid (K) through hollow fibre membrane units with a molecular

weight cut off of approximately 5000 (L). The ileal delivery is collected on ice in a vessel (M).

Transit of the meal

In vivo data on transit of the meal were obtained from earlier experiments with preruminant calves. The gastric meal delivery curve was calculated from the gastric lactose delivery curve of cannulated calves fed with CMR that contained soya protein concentrate (SPC) as the only protein source (G.H. Tolman, unpublished). Since this milk replacer does not coagulate, the lactose delivered from the stomach in time was assumed to be a representative liquid meal marker (9). The gastric meal delivery curve was expressed as the proportion of total intake delivered from the stomach in time (Fig. 2). Ileal meal delivery in the model was based on mean ileal delivery of the SMP, SMP/SWP and SPI diets in ileal-cannulated preruminant calves, using Cr_2O_3 as a meal marker (12).

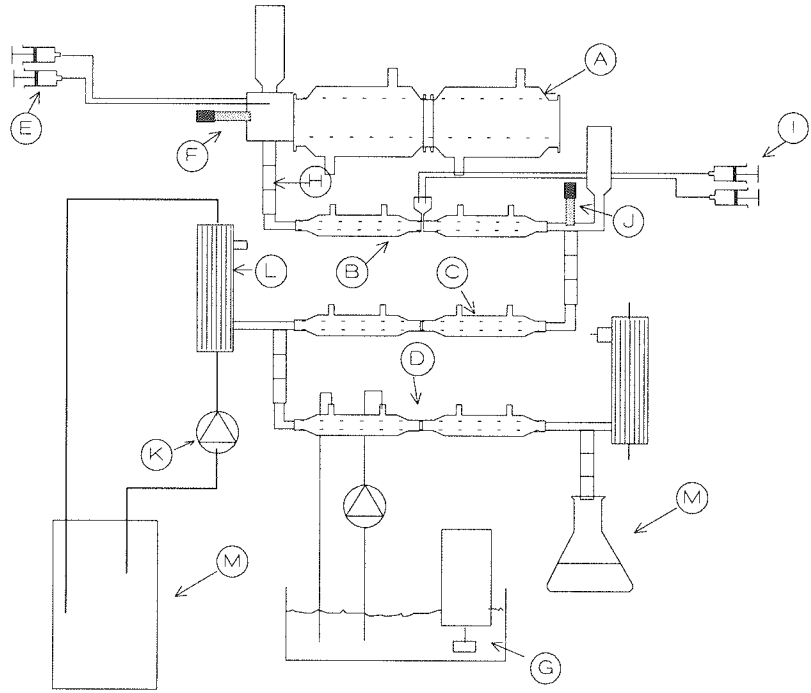

Fig. 1. Schematic diagram of the multi-compartmental model of the stomach and small intestine. A, gastric compartment; B, duodenal compartment; C, jejunal compartment; D, ileal compartment; E, gastric secretion pumps; F, pH electrode; G, water bath; H, peristaltic valve pump; I, duodenal secretion pumps; J, pH electrode; K, dialysis fluid; L, hollow-fibre device; M, collecting vessel for ileal delivery.

These *in vivo* data were curve-fitted using the formula proposed by Elashoff (2):

$$f = 1 - 2^{-\left(\frac{t}{t_{1/2}}\right)^{\beta}}$$

Where f presents the fraction of the meal delivered, $t_{1/2}$ the half-time (min) of delivery, t the time (min) and β a parameter describing the shape of the curve. The curve parameters $t_{1/2}$ and β were used in the protocol to dictate gastric and ileal meal delivery in the model. The values for $t_{1/2}$ and β were 87 and 1, respectively, for the gastric meal delivery curve and 406 and 1.9, respectively, for the ileal curve (Fig. 2).

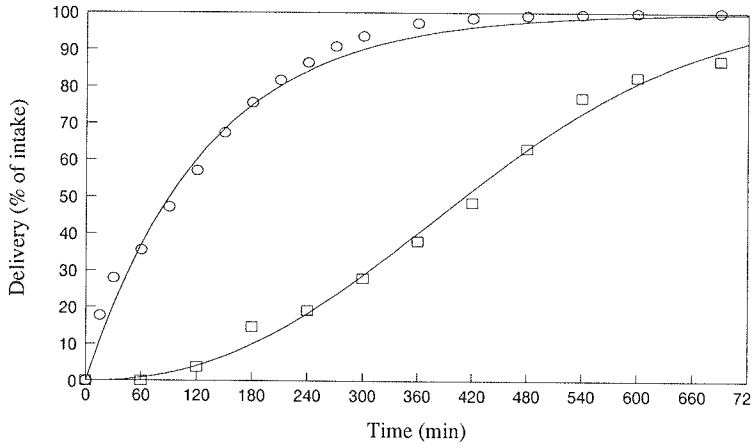

Fig. 2. Gastric meal delivery as a percentage of the intake of SPC (○) and mean ileal meal delivery as a percentage of the intake of SMP, SMP/SWP and SPI (□), measured in cannulated calves. The lines present the curve fit.

Test meals

Three different CMR were used, with SMP, a 70:30 mixture of SMP and SWP (SMP/SWP), and SPI as the only protein source (12). The composition of the CMR is presented in Table 1. The CMR were prepared by adding water to 37.5 g of the dry product until a volume of 300 ml. A predigested formula milk (Nutrilon Pepti, Nutricia, The Netherlands) was used as a reference to assess the absorption capacity of the model.

This predigested formula milk (PFM) was prepared by adding water to 60 g of the powder until a volume of 300 ml, to achieve a similar concentration of protein in the reconstituted milk as in the CMR.

Table 1. Composition of the milk replacers (% of dry matter)

Ingredient	SMP	SMP/SWP	SPI
Skim milk	60	30	-
Soluble wheat protein	-	12.625	-
Soya protein	-	-	24.5
Lysine	-	0.56	-
Fat	18	18	18
Starch	4	4	4
Lactose	17.25	30.915	47.65
Premix	0.5	0.5	0.5
$CaCl_2.2H_2O$	0.25	0.5	0.75
$CaCO_3$	-	0.475	0.8
$CaHPO_4.2H_2O$	-	0.5	1
NaCl	-	0.1	0.15
$NaHCO_3$	-	0.225	-
$KHCO_3$	-	0.65	1
KH_2PO_4	-	0.5	1
$NaH_2PO_4.2H_2O$	-	0.375	0.5
MgO	-	0.075	0.15

Experimental design

Gastric retention experiments.

Gastric retention of nitrogen was used as a measure of formation and successive breakdown of the coagulum of each CMR in time, during 360 min experiments in duplicate. To simulate dilution by saliva during ingestion of the meal, 100 ml of an electrolyte solution containing NaCl 6.2 g/L, KCl 2.2 g/L, $CaCl_2$ 0.22 g/L and

$NaCO_3$ 1.2 g/L was added to the reconstituted CMR. The meal was then introduced into the gastric compartment, which contained 5 ml of rennet (Rademaker, De Hoek, The Netherlands) to simulate a residue of gastric juice. Gastric secretion was mimicked by secreting electrolyte solution containing 2% (v/v) rennet at 0.25 ml/min (1). The pH was adjusted to follow a pre-set curve by switching between a 1 M HCl solution and water at a flow rate of 0.25 ml/min. The pH curve was set to the following course: $t = 0$ min, pH 6.7; $t = 90$ min, pH 3.5; and $t = 180$ (1). The pyloric peristaltic valve pump was controlled to empty the meal from the gastric compartment according to the pre-set gastric meal delivery curve (Fig. 2). Retention of protein in the gastric compartment was determined by calculating the difference between nitrogen measured in the gastric delivery and theoretical nitrogen delivery according to the gastric meal delivery curve.

Digestion experiments.

Preparation of the meal and gastric conditions were the same as for the gastric retention experiments mentioned above. Secretion of digestive fluids into the duodenum was mimicked by the addition of a 4% ox bile solution (Sigma, St. Louis, MO) at a flow rate of 0.5 ml/min and a 10% pancreatin (Pancrex V, Paines & Birne, Greenford, UK) solution at a flow rate of 0.25 ml/min. The pH in the duodenum was adjusted to 6.5 by switching between a 1 M sodium hydrogen carbonate solution and water at a flow rate of 0.25 ml/min. The dialysis fluid, containing NaCl 5 g/L, KCl 0.6 g/L and $CaCl_2$ 0.25 g/L, was pumped through the hollow fibre membrane units at a flow rate of 10 ml/min. To determine the contribution of secreted fluids to ileal delivery and absorption of nitrogen, blank experiments were performed with only electrolyte solution as the meal.

Sampling and analysis.

The ileal delivery was collected at hourly intervals for 6 h, a period similar to gastric delivery time. The dialysis liquid was collected every two hours. At the end of the experiment, the contents of each compartment were collected. The weight of each gastric and ileal delivery sample was measured to determine the gastric and ileal fresh matter delivery. Protein nitrogen in each sample was determined by the method of Kjeldahl.

Calculations.

In the experiments with cannulated preruminant calves, an unabsorbable meal marker (Cr_2O_3) was added to the meal. After collecting all digesta delivered via the ileal-caecal fistula, the recovered marker was used to calculate absorbed nitrogen as a percentage of intake. The *in vivo* true ileal protein digestibility coefficient was determined by correcting for endogenous nitrogen using the ^{15}N method (12).

The same method was used to calculate digestibility in the model. However, instead of a real meal marker we used a calculated virtual meal marker (VMM) representing the calculated amount of meal in each compartment and in the ileal delivery, which is used by the computer to control the transit of the meal (9). The *in vitro* true ileal digestibility coefficients were calculated as a percentage of delivered VMM. The ileal delivery of VMM was calculated taking into account the gastric retention of nitrogen due to coagulation, as measured in separate gastric retention experiments. Blank experiments without a meal were performed to correct for the contribution of secreted ('endogenous') nitrogen.

Statistical analysis.

The results of the experiments in the model are presented as the mean ± SD for duplicate experiments. The digestibility coefficients of the experiments in cannulated calves are presented as the mean ± SD of four experiments. The ability of the *in vitro* and *in vivo* methods to discriminate among diets was evaluated by determining the least significant difference (LSD) with an unpaired Student's *t* test at a 5% level of significance, based on the pooled standard error.

Computer modelling

The results were further analysed with a computer simulation program of the kinetic processes in the model. The calculations in the simulation program were based on the algorithms used in the program that controlled the physical model. In addition to the kinetics of liquid flow, also gastric retention, enzymatic digestion and absorption of the products was taken into account. Nitrogen absorption was calculated by assuming a decrease in concentration in time proportional to the concentration. The rate of absorption of nitrogen from the jejunal and ileal compartments was described by a constant (K_{jej}, K_{il}). Although the kinetics of liquid flow through the model were

based on the gastric and ileal delivery curves, the kinetics of nitrogen flow were largely determined by gastric retention. Therefore, the measured gastric emptying curve for nitrogen was also incorporated in the simulation program. The results in the model were fitted by iteration of K_{jej} and K_{il}. A detailed mathematical description of the computer modelling will be presented elsewhere (Minekus and Krüse, in preparation).

RESULTS

Reproduction of in vivo kinetics

The average cumulative gastric fresh matter delivery in all the gastric delivery experiments in the model was close to the gastric fresh matter delivery of the SPC diet in calves (Fig. 3). The *in vivo* gastric fresh matter delivery showed a slight decrease in flow between 30 and 120 min which was not reproduced in the model. For all the experiments, average cumulative ileal fresh matter delivery was slightly higher in the model than in calves (Fig. 4). Cumulative gastric delivery of nitrogen after the intake of the SPI diet in the model was similar to the pre-set gastric delivery curve based on *in vivo* gastric emptying of the SPC diet (Fig. 5).

Coagulation

Cumulative gastric nitrogen delivery of each diet in the model is presented together with the pre-set emptying curve (Fig. 5) and the emptying curve of nitrogen from the SMP diet *in vivo* (G.H. Tolman, unpublished). The SPI diet in the model emptied in conformity with the pre-set curve, indicating that no retention of protein nitrogen took place. The SMP/SWP diet showed a medium retention of nitrogen while the SMP showed a strong retention. *In vitro* gastric nitrogen delivery with the SMP diet appeared to be similar to that *in vivo*.

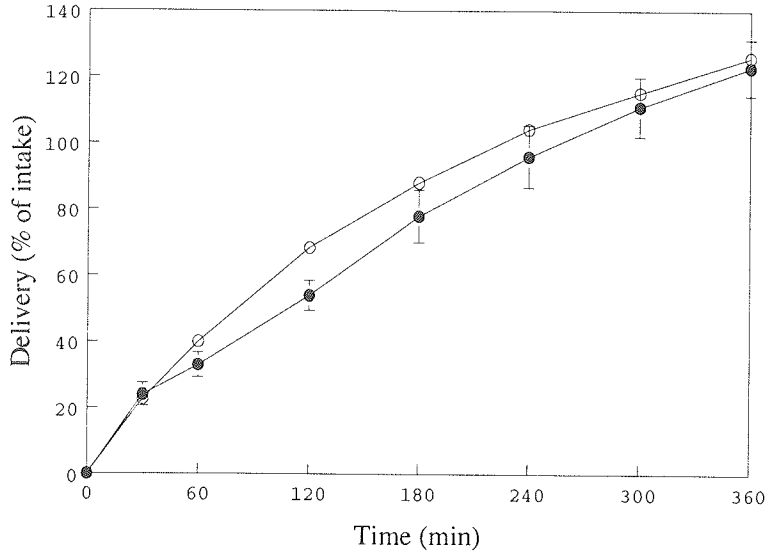

Fig. 3. Average cumulative gastric fresh matter delivery as a percentage of the intake of SPC *in vivo* (● ± SD, $n = 5$) and average fresh matter delivery of SMP, SMP/SWP and SPI in the model (○ ± SD, n = 6).

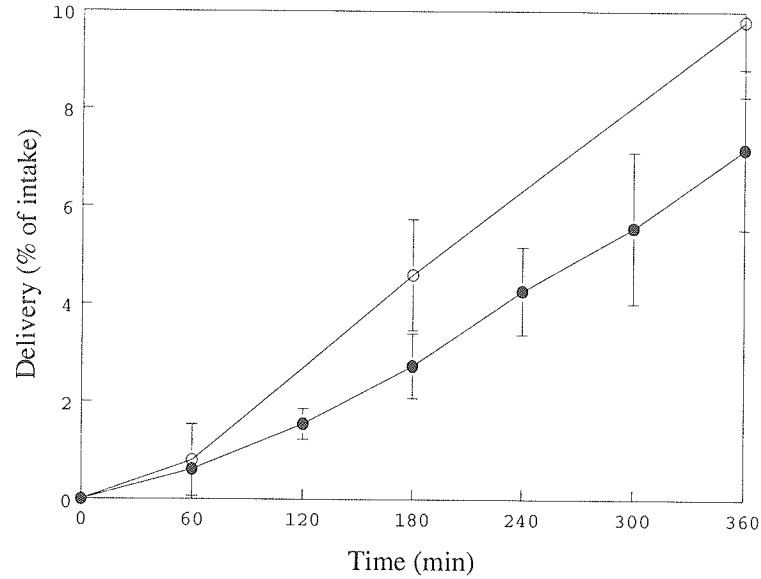

Fig. 4. Average cumulative ileal fresh matter emptying as a percentage of the intake of SMP, SMP/SWP and SPI *in vivo* (● ± SD, $n = 11$) and *in vitro* (○ ± SD, $n = 6$).

Protein digestibility

Cumulative ileal delivery of protein nitrogen, corrected for secreted nitrogen, for the three CMR as a percentage of total intake is shown in Fig. 6. A significant difference between the diets was found after 360 min (P=0.05, LSD = 0.77). Because this difference in ileal delivery of protein nitrogen can also be explained by gastric retention, the ileal true digestibility coefficients in the model were corrected for the gastric retention of nitrogen. It was calculated that, due to coagulation, 20% and 33% of the VMM was delivered from the ileal compartment for the SMP and SMP/SWP diets, respectively. Without coagulation, as with the SPI diet, 42% of the VMM was delivered from the ileal compartment.

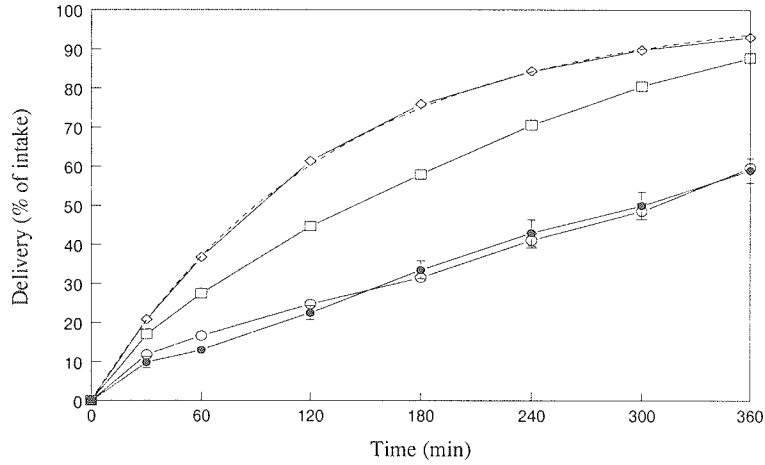

Fig. 5. Gastric delivery of nitrogen during experiments with SMP (○), SMP/SWP (□)) and SPI (◇) in the model, and SMP *in vivo* (● ± SD, n = 6) as compared to the gastric delivery curve (dotted line). (n = 2, error bars present range)

The ileal true digestibility coefficients corrected with these VMM deliveries were in good correspondence with *in vivo* true digestibility data (Table 2). However, no significant difference was found between the SMP and the SMP/SWP diets. The digestibility coefficients measured in cannulated calves showed only a significant difference between the SMP and the SPI diets.

Distribution of protein nitrogen after 360 min

The distribution of nitrogen in the model at the end of the experiment, after 360 min, for the three CMR and the predigested formula milk (PFM) is shown in Fig. 7. The experiments with the PFM diet showed that 90% of the nitrogen delivered from the gastric compartment was absorbed from the jejunal compartment, while 6% was absorbed from the ileal compartment .

Table 2. True digestibility coefficients expressed as percentage units for three calf milk replacers *in vivo* (± SD, $n = 4$) and *in vitro* (± SD, $n = 2$).

	in vivo (% units)	*in vitro* (% units)
SMP	99.5±1.1	97.9±1.1
SMP/SWP	97.5±0.7	95.8±0.6
SPI	96.4±1.1	92.7±0.7
LSD (P=0.05)	2.23	2.66

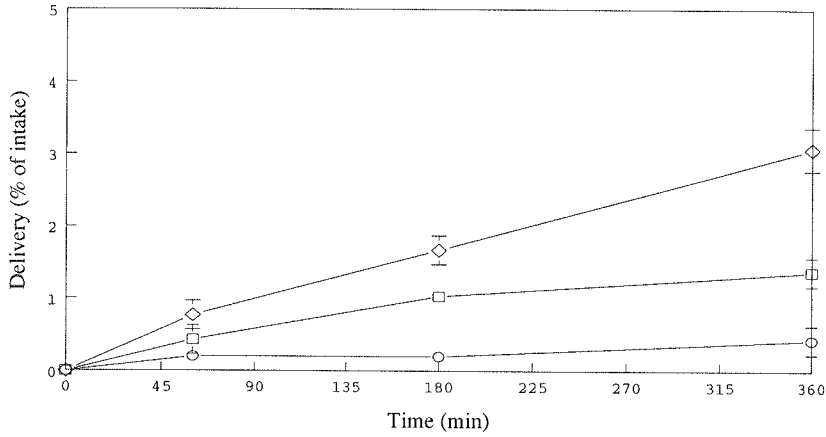

Fig. 6. Cumulative ileal delivery of nitrogen as a percentage of intake for SMP (○), SMP/SWP (□) and SPI (◇). ($n = 2$, error bars present range)

During the experiments with the SPI diet the absorption of nitrogen from the jejunal and ileal compartment was 78% and 11% of the gastric delivery, respectively. With the SMP diet 90% of the nitrogen delivered from the gastric compartment was absorbed from the jejunal compartment, and 4% from the ileal compartment. With the SMP/SWP diet the absorption was 88% and 5% from the jejunal and ileal compartment, respectively. The fraction of the meal remaining in the gastric compartment was 6% for the PFM and SPI diets, and 13% and 41% for the SMP/SWP and SMP diets, respectively.

Absorption of nitrogen in time and computer modelling

The measured and simulated absorption of nitrogen from the jejunal and ileal compartments in time is presented in Fig. 8 a,b,c,d for the three CMR meals and the PFM reference meal.

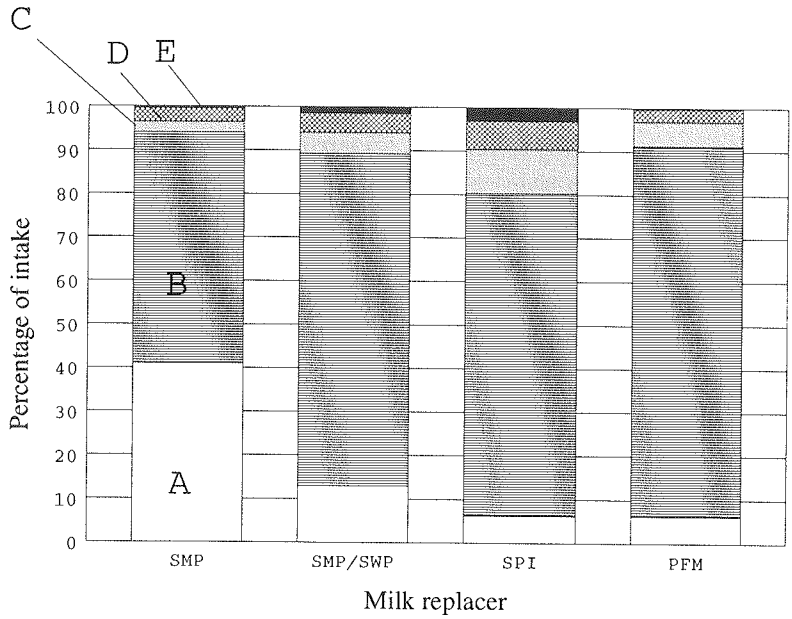

Fig. 7. Distribution of protein nitrogen after 360 min, for SMP, SMW, SPI and PFM. A, gastric compartment; B, jejunal dialysate: C, ileal dialysate: D, small intestinal compartments; E, ileal delivery.

Table 3. Rate of nitrogen absorption from the jejunal compartment (K_{jej}) and the ileal compartment (K_{il}) of the model for each test meal.

	K_{jej} (h^{-1})	K_{il} (h^{-1})
SMP	7.2	0.9
SMP/SWP	4.1	0.9
SPI	2.0	0.7
PFM	6.3	1.8

Absorption rates in the jejunal and ileal compartments for each test meal are presented in Table 3. The results show that dialysis of nitrogen occurs mainly in the jejunal compartment. The K_{jej} shows the same ranking as the digestibility coefficients. The K_{il} value for the PFM diet shows a relatively high rate of nitrogen absorption in the ileal compartment as compared to the CMR's.

DISCUSSION

Reproduction of the in vivo situation

Gastric emptying was controlled to reproduce gastric delivery of the SPC diet, taking into account the dilution by salivary and gastric secretion. The similarity between the model and the *in vivo* situation indicates that the magnitude of gastric secretion closely simulated *in vivo* gastric secretion (Fig. 3). Ileal fresh matter delivery is the result of gastric fresh matter delivery, small intestinal secretion and water absorption. Ileal fresh matter delivery from the model showed that the net fluxes of water in the small intestinal compartments were similar to mean ileal delivery of the three CMR in cannulated calves.

Coagulation

Coagulation occurs when casein is enzymatically denaturated by pepsin and chymosin present in the stomach of the calf. *In vitro* coagulation of milk is normally tested with commercially available rennet used in the cheese industry. Gastric emptying of the coagulum during digestion is hard to test in a simple *in vitro* system (10). An *in vitro* model capable of simulating gastric emptying of a coagulum in time should have a physiological gastric pH profile and allow for secretion of acid and

enzymes. The model should have a realistic pyloric system that is able to retain particles and provide unhindered passage for liquids.

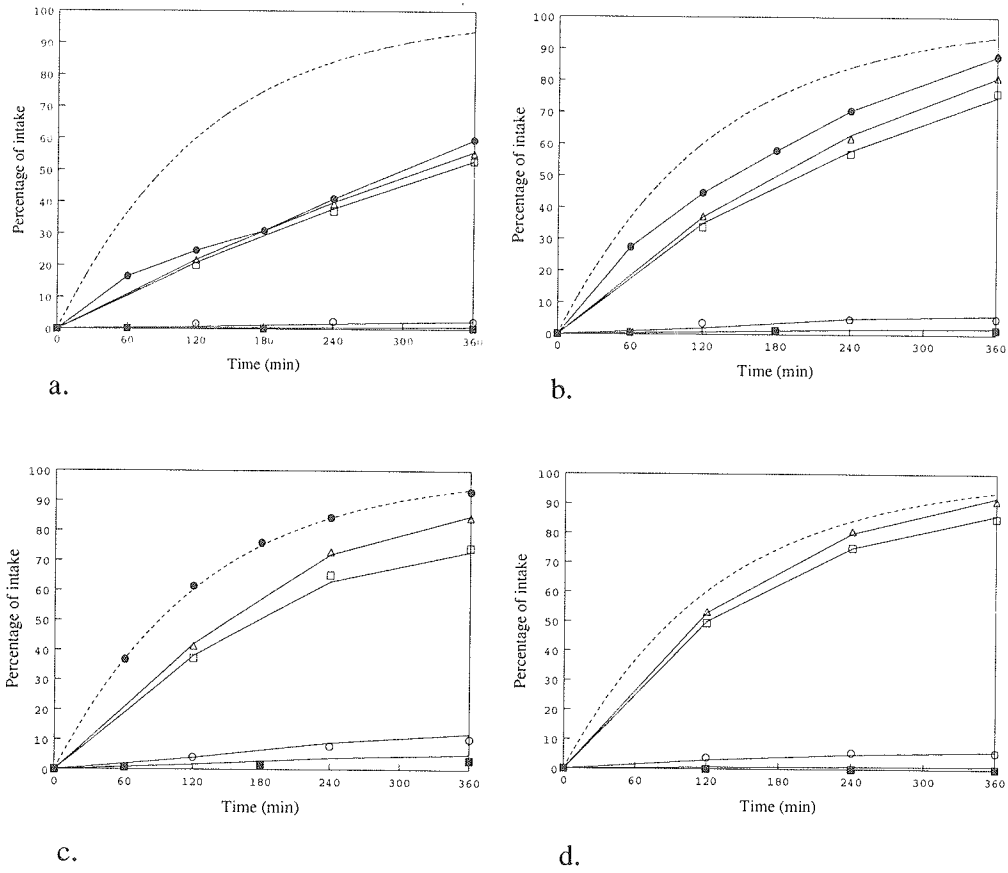

Fig. 8. Computer simulation of absorption and ileal delivery of protein nitrogen for SMP (a), SMP/SWP (b), SPI (c) and PFM (d), expressed as a percentage of intake. The markers present measured delivered nitrogen from the ileal compartment (■), measured gastric delivery of nitrogen (●), and measured nitrogen absorption from the jejunal compartment (□), from the ileal compartment (○) and from both compartments (Δ). The bold lines present the computer simulation, the dotted line presents the gastric meal delivery curve.

Furthermore, the model should have a mixing and grinding motility that resembles the motility of the stomach. The model presented in this paper incorporates all these features and provides distinction between the three different calf milk replacers. The gastric delivery of the SMP diet in time was similar to results obtained in cannulated calves on the same diet (Fig. 5). Gastric delivery of the SMP/SWP diet in time was not tested *in vivo* and could hence not be compared with results in the model. However, it may be assumed that this diet gives a moderate coagulation *in vivo*, which was also found in the model. The SPI diet did not coagulate in the model and was delivered from the gastric compartment in correspondence with the gastric delivery curve, thus showing the ability of the model to deliver a meal according to a pre-set profile.

Protein digestibility

In vivo protein digestibility is generally determined with the use of a meal marker. The ideal meal marker should have the same behaviour as the nutrient to be examined, without being absorbed. Some meal markers follow the liquid phase of the meal, whereas other ones follow the solid phase. In the case of gastric retention of nitrogen due to coagulation of casein, it would be preferable to use a marker that is trapped and consecutively released from the clot similar to the casein protein. During *in vivo* experiments the digesta are generally collected over a period including several successive meals. The digestibility is determined by the ratio between recovered meal marker and nutrient. With this method, differences in transit time between nutrient and marker do not have a large effect on the calculated digestibility. However, if the delivery of nutrient is studied in time after a single administration of marker, differences in transit time between nutrient and marker lead to misrepresentation of reality. During our *in vitro* experiments we could calculate continuously the distribution of a virtual meal marker in the model. For a non-coagulating CMR the distribution of the virtual meal marker could exactly be calculated and controlled by the computer. For a coagulating CMR gastric retention of nitrogen is a physical process that cannot be regulated by the control computer. To overcome this problem we measured gastric delivery of nitrogen in time for each CMR. Gastric fresh matter delivery was similar for each CMR, since this was controlled by the computer as dictated by the protocol. Using this curve combined with measured gastric nitrogen delivery, the flow of nitrogen – when not absorbed – (virtual nitrogen marker) could exactly be calculated. We determined the protein digestibility coefficient as a percentage of delivered virtual nitrogen marker after 360 min. The results show a good correlation between the results obtained in the model

and those obtained in cannulated calves. However, the digestibilities are too similar to achieve significant differentiation of all CMR tested, for both the *in vivo* and the *in vitro* method.

The fact that gastric retention and the ileal digestibility coefficient have the same ranking suggests that digestion is related to gastric emptying. However, this is not the only factor since soya protein is less digestible than casein (4,5). For SMP/SWP 50% of the protein originates from wheat. It is not clear whether only the lower intrinsic digestibility of the wheat protein contributed to the slightly lower digestibility or if it is also due to a smaller amount of coagulating protein. A positive effect of coagulation on digestibility of the protein was not conclusively demonstrated. A study of Petit et al. (9) revealed only a small, non-significant, difference in digestion between coagulating and non-coagulating calf milk.

Distribution of nitrogen after 360 minutes

The distribution of nitrogen after 360 min was measured to balance the nitrogen fluxes in the system. The efficacy of the system to absorb digested protein was demonstrated with predigested formula milk. This resulted in almost complete absorption of nitrogen. The SPI diet appeared to be less digestible than SMP and SMP/SWP. Although more protein was delivered from the gastric compartment into the small intestinal compartments, a lower percentage was absorbed. This is in agreement with both *in vivo* digestibility coefficients and literature data (4,5). The difference in absorbed nitrogen between SMP and SMP/SWP is small, in agreement with the digestibility coefficient.

Computer modelling

A computer simulation of the events that occur in the model was used to obtain more insight into complex interactions among parameters involved in the kinetics of meal transit, digestion and absorption. The amount of protein nitrogen in time could be well simulated with only the kinetics of transit and a constant factor for dialysis (Fig. 8a, b, c, d). Measured gastric emptying of nitrogen was included in the system and proved to be the main determinant for protein absorption in time. This is in good agreement with the literature (3). The absorption constants give extra insight in the digestion and absorption of protein in time, from the jejunal and ileal compartment of the model. For example, although the digestibility coefficients are similar, a different absorption pattern for SMP and PFM can be demonstrated. The relatively rapid absorption of the SMP protein in the jejunal compartment is compensated by a faster absorption of the PFM nitrogen in the ileal compartment. This difference can be due to intrinsic factors of the complete milk proteins and hydrolysed wey proteins

in the SMP and PFM diets, respectively. However, the lower absorption rate of the PFM nitrogen can also indicate a limitation of the absorptive capacity of the jejunal hollow fibre membrane unit. This limitation does not occur with the SMP, because the nitrogen is much more gradually delivered from the gastric compartment (Fig. 5).

An *in vitro* model which mimics physiological processes that occur in the lumen of the gastrointestinal tract offers the opportunity to perform studies under strictly controlled and reproducible conditions. This makes the physical model a perfect intermediate between the *in vivo* situation and mathematical modelling of gastrointestinal processes. The physical model presents a complex (but simplified) system that can be validated with *in vivo* results. The mathematical model can be validated and tested with the physical model.

CONCLUDING REMARKS

It was demonstrated that the delivery of fresh matter and meal marker from the stomach and small intestine in calves could be well reproduced in a dynamic multi-compartmental model. Besides, the results on protein digestion obtained with the model had the same level and ranking as *in vivo*. These results indicate that the performance of the model was in accordance with selected *in vivo* conditions. This study shows that the model is a valuable tool to study coagulation and true protein digestion in liquid meals such as calf milk replacers. Further validation of the model awaits the availability of experiments in animals that can be reproduced in the model, preferably over a wider range of digestibilities.

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Chapter 7

A DYNAMIC COMPUTER-CONTROLLED MODEL SIMULATING THE PORCINE STOMACH AND SMALL INTESTINE TO STUDY THE PROTEIN DIGESTION OF PIG FEED

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submitted for publication

ABSTRACT

The applicability of the TNO gastro-Intestinal Model (TIM; 8) was tested to study the digestion of protein and dry matter with different pig feed diets of various ileal digestibilities. Digestibility data of diets with barley, rape-seed, wheat gluten, faba beans and meat-and-bone meal obtained from experiments in TIM were compared with results from experiments with ileal-cannulated pigs with the same diets. All diets were tested in the model with a protocol that described a normal physiological situation in the stomach and small intestine of healthy pigs. The protocol included data on meal mixing and transit, gastric and duodenal secretion, pH in the stomach and small intestine, and water absorption. Hollow-fibre membrane systems connected to the small intestinal compartments were used to remove digestive products. The ileal delivery of fresh matter, dry matter and nitrogen, expressed as a percentage of intake, of the rape seed diet in the model was similar to that found in cannulated pigs. This demonstrates the ability of the model to mimic the pre-set dynamic physiological conditions. Various methods were evaluated to calculate protein digestibility in TIM. Comparison of the ileal delivery during 6-hour experiments in TIM with that from cannulated pigs resulted in correlation coefficients of 0.84 and 0.95 for dry matter and protein digestibility coefficients, respectively. The digestibility coefficients of the diets calculated from the total amount of absorbed nitrogen gave a correlation coefficient of 0.95 with the results obtained *in vivo*. The high correlation coefficients indicate that the model is a good alternative to study the true ileal protein digestibility of pig feed containing protein from a broad variety of sources. The high correlation between true digestibility in TIM with a constant amount of secreted protein and the apparent digestibility in pigs indicate a small variation in endogenous protein secretion between the diets tested in the pigs.

The kinetics of nitrogen absorption from the small intestinal compartments of the model were studied with a computer program that simulated the events in the model. Absorption constants were determined that describe the rate of nitrogen absorption in time. This method proved to be a useful additional tool to study protein degradation in the model in time.

INTRODUCTION

Animal diets are blended from feedstuffs of diverse origin. Differences in nutritional value between feedstuffs and variation among batches of the same feedstuff require regular evaluation of the diet's composition to meet the

nutritional demands of pigs. *In vivo* methods, especially those based on the use of surgically modified animals, require special facilities. In addition, they are expensive and time-consuming and are increasingly subject to ethical objections. *In vitro* models could offer an interesting tool to evaluate the digestibility and nutritional value of feedstuffs and animal diets. They are relatively inexpensive, rapid, reproducible and easy to perform and they avoid the use of animals. *In vitro* models to date are generally static, which means that the gradually changing conditions during each step of digestion - related to secretion of digestive fluids, transport of the meal and absorption of water and nutrients - are not simulated (1,2,15).

Recently, a dynamic multi-compartmental computer-controlled model was developed that simulates the stomach and the small intestine of monogastric animals. This TNO gastro-Intestinal Model (TIM) allows for a close simulation of the dynamic conditions that prevail *in vivo* in the lumen of the gastrointestinal tract (8,9,10).The aim of this study was to demonstrate the applicability of this dynamic model to study the digestion of different protein sources. The model was programmed to mimic important physiological conditions in the lumen of the porcine gut, based on experiments with a rape-seed diet in ileal-cannulated pigs, combined with parameters obtained from the literature (3,13,14). The experiments were performed with five pig diets and different protein sources covering a wide range of digestibilities. The digestibility of the protein for each diet in the model was compared to the protein digestibility coefficients for the same products obtained in ileal-cannulated pigs (16). A computer program that simulated the complex interactions of dynamic events in the model was used as a tool to study the absorption characteristics of nitrogen in time for each diet.

MATERIALS AND METHODS

The dynamic gastrointestinal model

The TNO gastro-Intestinal Model (TIM) has been described previously (8). Briefly, the model consist of four successive compartments (Fig. 1), simulating the stomach (A), duodenum (B), jejunum (C) and ileum (D). A meal can be fed to the gastric compartment during a pre-set time. In the gastric compartment gastric juice is added (E) while the pH is measured (F) and controlled according to a predetermined curve. The compartments consist of two connected glass units with flexible walls inside. Water to control the temperature is pumped from a water bath (G) through the space

between the jacket and the flexible wall. The walls can be squeezed by varying the pressure on the water. The chyme in each compartment is mixed by alternate squeezing of the flexible walls. To control the transit of the meal, the compartments are separated by computer-regulated peristaltic valve pumps (H). Bile and pancreatic juice are secreted into the duodenal compartment (I). The duodenal pH is measured (J) and controlled by the addition of sodium hydrogen carbonate. Products of digestion and water are absorbed from the jejunal and ileal compartments by pumping dialysis liquid (K) through hollow-fibre devices with a molecular weight cut off of approximately 5000 (L). Retention of larger particles in the hollow-fibre devices is prevented by a filter (M). Ileal delivery is collected on ice in a vessel (N).

Test diets

Five test diets were selected with proteins sources from different products groups (Table 1; 16). The composition of the test diets is given in Table 2. The diets were ground to a particle size smaller then 0.5 mm to prevent retention of larger particles in the gastric compartment. This allowed for comparison of diets with identical gastric delivery rates and minimized the effect of particle size on digestibility (17). The test meals were prepared by mixing 60 g of diet with 90 ml of saliva/gastric electrolyte solution containing $CaCl_2$ 0.22 g/L, KCl 2.2 g/L, NaCl 5 g/L and $NaHCO_3$ 1.5 g/L and adjusted to 300 ml with water.

Gastric conditions

Prior to the introduction of the meal into the gastric compartment, 10 ml gastric juice consisting of the saliva/gastric electrolyte solution with 500 kU/L pepsinogen (Sigma, St. Louis, MO), was introduced into the gastric compartment and adjusted to pH 1.5 with a 1 M HCl solution to mimic a residue of gastric juice.

The pH in the gastric compartment was controlled according to a pre-set profile (Fig. 2) by secreting a 1 M HCl solution or water. Gastric juice was secreted at 0.5 ml/min.

Small intestinal conditions

The pH in the small intestine was kept at 6.5 by means of secretion of $NaHCO_3$ or water at 0.25 ml/min. Pancreatic output was simulated by secreting 10% pancreatin (Pancrex V, Paines and Birne, Greenford, UK) in small intestinal electrolyte solution (containing NaCl 5 g/L, KCl 0.6 g/L, $CaCl_2$ 0.22 g/L) at 0.25 ml/min.

Biliary output was simulated by secreting a 4% bile (Porcine Bile Extract, Sigma) solution at 0.5 ml/min. Prior to the experiment the duodenal compartment was filled with 10 ml trypsin solution (2 mg/ml) and 10 ml 4% bile solution. The jejunal and ileal compartments were filled with 100 ml of small intestinal electrolyte solution, which was also used as the dialysis fluid which was pumped through the hollow fibres at 10 ml/min.

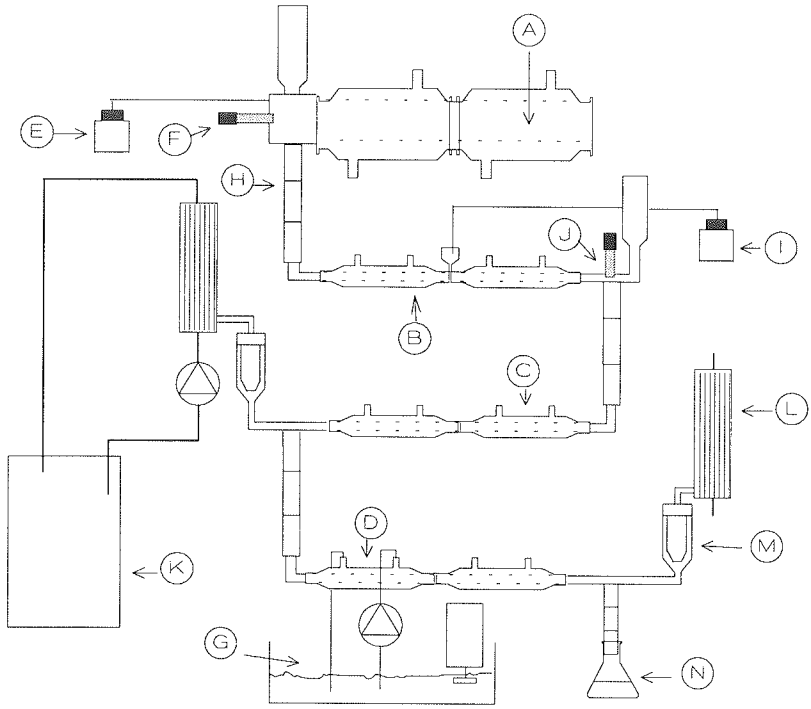

Fig. 1. Schematic diagram of the TNO gastro-Intestinal Model (TIM).

A, gastric compartment; B, duodenal compartment; C, jejunal compartment; D, ileal compartment; E, gastric secretion pumps; F, pH electrode; G, water bath; H, peristaltic valve pump; I, duodenal secretion pumps; J, pH electrode; K, dialysis fluid; L, hollow-fibre device; M, pre-filter; N, collecting vessel for ileal delivery.

Table 1. Product groups and representative protein sources in the test diets.

	Group	Feedstuff
1	Cereals	Barley
2	By-products of cereals	Wheat gluten
3	Legume seeds	Faba beans (high-tannin)
4	Expellers	Rape-seed
5	Products of animal origin	Meat-and-bone meal

Table 2. Composition of the test meals (%).

Diet	Barley	Wheat gluten	Faba beans (high-tannin)	Rape-seed	Meat-and-bone meal
Barley	84				
Wheat gluten	7	17.9			
Faba beans			58.2		
Rape-seed				51	
Meat-and-bone meal					27
Starch		51.9	19.53	26.25	48.05
Dextrose		15	15	15	15
Soya oil	2	1.5	1	3	1
Cellulose		5			5
$CaCO_3$	0.93	0.8	0.9	0.3	
$CaHPO_4$	1.75	2.25	1.85	1.4	
NaCl	0.5	0.5	0.5	0.5	0.5
MgO		0.2			0.1
$KHCO_3$	0.9	1.8	0.2		1.25
$NaHCO_3$	0.3	0.4	0.3	0.3	
L-lysin HCl	0.37	0.5			
DL-methionin			0.25		0.13
L-tryptophane			0.02		0.02
Premix	1	1	1	1	1
Cr_2O_3	0.25	0.25	0.25	0.25	0.25
Diamol	1	1	1	1	1

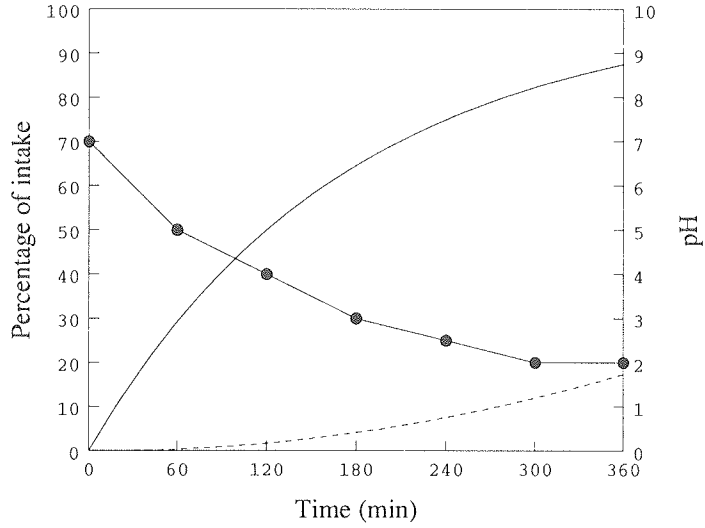

Fig. 2. Gastric (solid line) and ileal (dotted line) delivery curves of a non absorbable meal marker expressed as a percentage of intake, and the gastric pH profile (●).

Control of meal transit

Transit of the meal through the model was controlled according to gastric and ileal delivery data of an indigestible meal marker obtained from experiments in pigs. These data were transferred into a gastric and ileal delivery curve by curve-fitting the cumulative gastric and ileal deliveries of the meal marker, expressed as a percentage of intake, using a power exponential formula as proposed by Elashoff *et al.* (5) and modified by Decuypere *et al.* (4).

$$f = 1 - 2^{-\left(\frac{t}{t_{1/2}}\right)^{\beta}}$$

Where f represents the fraction of meal marker delivered, $t_{½}$ the half-time of delivery, t the time, and β a parameter describing the shape of the curve. This method allows for computer control of gastric and ileal delivery of the meal in the model by using curves with only 2 parameters. The transit of the meal through the model was determined by calculating the amount of a virtual meal marker (VMM) in each compartment at regular intervals, taking into account the flow of chyme and the secretion and absorption of water. The accuracy and reproducibility of this

method has been determined previously (8). This study was performed with a gastric delivery curve described by a $t_{½}$ of 120 min and a β of 1 (14). The ileal delivery curve was based on data obtained from the *in vivo* ileal delivery of the rape seed diet in cannulated pigs, using titanium oxide as a meal marker (Fig. 2). Curve fitting of the cumulative ileal delivery of titanium oxide, expressed as a percentage of intake, resulted in a $t_{½}$ of 652 min and a β of 2.17.

Sampling and analysis

The diets were tested in the model during duplicate 6 h experiments. Blank experiments with only electrolyte solution as a meal were performed to determine the 'endogenous' protein in the secreted fluids. During the experiments the ileal delivery was collected in 2 h fractions. The weight of the samples was measured to determine fresh matter emptying. Dry matter was determined by drying the samples at 80 °C until no further loss of weight was detected. At the end of the experiment the contents of each compartment were collected and dried. The dried samples were stored at -40 °C for nitrogen analysis using the Kjeldahl method. The bottles with dialysis fluid were replaced every two hours. An aliquot of each contents was stored at -40 °C for analysis of dialysed nitrogen.

Digestibility coefficients

Apparent *in vivo* dry matter and protein digestibility coefficients were expressed as the amount of dry matter or nitrogen absorbed in the small intestine as a percentage of total intake. Chromic oxide and HCl-insoluble ash was used as a meal digestibility marker that was added to the meal to calculate ileal digestibilities according to the following formula (16) :

$$DC\ (\%) = 100 - ((N_{digesta} \times M_{feed}) / (N_{feed} \times M_{digesta}) \times 100)$$

where DC is the ileal digestibility coefficient of the nutrient expressed as a percentage of intake, $N_{digesta}$ is the content of nutrient in the collected digesta, N_{feed} is the content of nutrient in the feed, $M_{digesta}$ is the content of marker in the collected digesta and M_{feed} is the content of marker in the feed. The protein and dry matter digestibility coefficients obtained from experiments in the model were calculated according to the same formula as described for the *in vivo* experiments, with the

amount of VMM delivered from the ileal compartment instead of M_{feed}. Protein digestibility was also determined in the separate ileal delivery samples collected between 120 and 240 min, and between 240 and 360 min. Since these digestibility data were obtained with a relatively low percentage of VMM delivery (6, 10 and 17.4% of intake for the 240 min, 360 min and total delivery, respectively), the digestibility coefficients were also calculated from the amount of absorbed nitrogen as a percentage of duodenal delivery after 6 h.

Statistical analysis

The digestibilities of the diets in the model were compared to the apparent ileal digestibilities of the same diets in cannulated pigs by calculating the correlation coefficient (R) with the residual standard deviation (RSD), using the statistic facilities of Slide Write 5.0 software (Advanced Graphic Software, Carlsbad, NM). The ability of the *in vitro* and *in vivo* methods to discriminate among diets was evaluated by determining the least significant difference (LSD) with an unpaired Student's t test at a 5% level of significance, based on the pooled standard error.

Computer modelling

A computer simulation program was used to analyse the absorption data of nitrogen in the model. This simulation program was based on the computer program used to control the model. Thus the digestion protocol was exactly simulated, including transit of the meal through each compartment, gastric and duodenal secretions and water absorption. These kinetic parameters were combined with constants for absorption of nitrogen in the jejunal and ileal compartment (K_{jej}, K_{il}) through the dialysis membranes. The constants represent the rate of absorption of nitrogen. Absorption is the result of digestion and the capacity of the membrane. Earlier studies have shown that the capacity of the membranes is sufficiently high to remove all low-molecular-weight digestive products (8,11), hence the rate of absorption is mainly determined by the degradation of protein. Analysis of the absorption data offers insight into the degradation of protein in time. This method was successfully used to analyse the absorption of nitrogen during the digestion of calf milk replacers (11). As compared to the calf milk replacers, the diets in this study contained less digestible and less defined proteins. Therefore, in addition to the absorption constant, a parameter was used to determine the change of this absorption constant in time. Each absorption constant and the parameter that determined its change were determined by fitting them iteratively on the measured

nitrogen absorption and ileal delivery data. A more detailed description of the simulation method will be reported elsewhere (Minekus and Krüse, in preparation).

RESULTS

Reproduction of in vivo data

The dry matter and fresh matter deliveries from the ileal compartment of the model were similar to the data obtained with cannulated pigs after ingestion of the rapeseed diet (Fig. 3). At the end of the *in vitro* experiments, after 6 h, the ileal fresh matter and dry matter deliveries were 28% and 12%, respectively, compared to 23% and 11%, respectively, for cannulated pigs. Fat is not absorbed in the model; when corrected for this fraction, dry matter delivery was 11.3%.

Dry matter digestibility

Ileal dry matter digestibilities of the five diets resulted in a correlation coefficient of 0.84 with the apparent ileal digestibility coefficients obtained in cannulated pigs (Fig. 4). The linear regression equation was: $y = -18.4 + 0.86x$, were y and x are the *in vitro* and *in vivo* digestibility coefficient values, respectively, with a RSD of 3.0. The faba bean HT diet deviated most from the regression line.

Protein digestibility

To assess the kinetics of nitrogen absorption, the absorption and ileal delivery of nitrogen in time for each diet were measured and calculated as a percentage of intake. The data were corrected with a blank experiment, to assess only the kinetics of nitrogen from the diet.

Cumulative absorption of dietary nitrogen in time and ileal delivery of nitrogen for the five diets were compared to the results obtained with the computer simulation (Fig. 5a, b, c, d and e). The absorption and ileal delivery data fitted for all the diets, with absorption constants for the jejunal and ileal compartments (K_{jej}, K_{il}) that decrease in a linear pattern from the start to the end of the experiment (Table 3). The total amount of nitrogen absorbed from the diets after 360 min, expressed as a percentage of duodenal delivery, resulted in a correlation coefficient of 0.95 with the apparent digestibilities of the same diets in cannulated pigs (Fig. 6). The LSD calculated with the absorbed nitrogen data obtained in the model was 6.5 as compared to 8.1 for the *in vivo* apparent digestibility data (Table 4). The cumulative

ileal delivery of nitrogen for each diet as compared to the ileal delivery curve showed an almost linear increase for the wheat gluten, barley and faba bean diets (Fig. 7). The patterns for the meat-and-bone meal diet and the rape-seed diet were close to the ileal delivery curve until 240 min and increased in a linear pattern after 120 min. The protein digestibilities of the diets in the model gave correlation coefficients of 0.96, 0.90 and 0.95 as compared to the *in vivo* digestibility data for the 240 min, 360 min and total ileal delivery samples, respectively (Fig. 8a, b, c). An overview of the linear regression data is presented in Table 5.

Table 3. Calculated constants for absorption of nitrogen from the jejunal compartment (K_{jej}) and ileal compartment (K_{il}) at the start (t=0) and after 360 min during the digestion of the diets in the model.

Diet	K_{jej} (h^{-1}) t=0	K_{jej} (h^{-1}) t=360	K_{il} (h^{-1}) t=0	K_{il} (h^{-1}) t=360
Barley	0.45	0.26	0.41	0.06
Meat-and-bone meal	0.43	0.24	0.25	0.06
Rape-seed	0.36	0.17	0.23	0.04
Wheat gluten	1.55	0.09	1.26	0.29
Faba beans	0.31	0.31	0.45	0.06

DISCUSSION

Reproduction of in vivo conditions.

A dynamic multi-compartmental model has been developed that simulates the changing conditions in the lumen of the gastrointestinal tract of the pig in time. Simulation of the transit of the meal was achieved by using mathematical curves based on *in vivo* data. The ability of the model to reproduce the specified *in vivo*

situation was tested by comparing the ileal dry matter and fresh matter delivery of the rape-seed diet in the model with the corresponding delivery of the same diet in cannulated pigs. Ileal fresh matter delivery is influenced by the amount of liquid in the meal, the amount of secreted fluids and absorption of water. Ileal fresh matter delivery in time from the model indicates that the result of secreted and absorbed water in the model was in good agreement with that in pigs after ingestion of the rape-seed diet (Fig. 3).

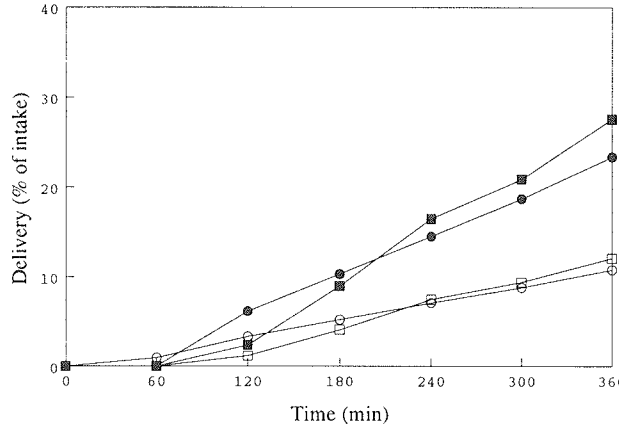

Fig. 3. Cumulative fresh matter delivery from the small intestine after the intake of the rape-seed diet, expressed as a percentage of intake in the model (●) and *in vivo* (■), and dry matter delivery from the small intestine in the model (○) and *in vivo* (□). The *in vivo* data were obtained from experiments with cannulated pigs.

The realistic dry matter delivery indicated that the digestion and absorption of all components in the diet including nutrients such as starch was also close to the *in vivo* situation. Fat was not absorbed in the system but contributed little to the delivered dry matter.

Dry matter digestibility

There was a good correlation between results obtained in the model and results obtained in cannulated pigs, with the slope of the linear regression line being close to the line of optimal correlation. However, the exact values were lower in the model than *in vivo* (Fig. 4).

Protein digestion

The kinetics of protein digestion and nitrogen absorption were assessed by the absorption and subsequent ileal delivery of nitrogen for each diet in time. Gastric delivery regulates the load of protein in the small intestine and is therefore a main determinant of the absorption rate of nitrogen (6). Under normal conditions *in vivo* absorption of digestive products is very efficient and not the rate-limiting step for breakdown and absorption of protein. The high absorptive capacity of the dialysis units connected to the jejunal and ileal compartments of TIM also warrants a fast removal of digestive products with a molecular weight less then 5000 (8,11). Peptides absorbed through the membrane are assumed to be sufficiently soluble *in vivo* to diffuse to the brush border where they are further degraded and absorbed. In the model, the luminal availability of nutrients for absorption is determined, which is dependent on release from the matrix, digestive degradation, solubilization and interaction with other components in the chyme. The luminal availability for absorption includes sufficient parameters to assess the digestion and absorption of proteins when it can be assumed that the rate of absorption *in vivo* and in the model is not the limiting step. By applying the same gastric delivery curve for each diet, it was possible to evaluate luminal availability for absorption of the protein by comparing absorption of nitrogen and ileal nitrogen delivery.

The linear increase in cumulative ileal nitrogen delivery indicates a regular outflow of nitrogen from the small intestine. This is in agreement with results obtained *in vivo* (16). The VMM is delivered in an exponential pattern, as dictated by the ileal delivery curve. From this it can be derived that the extent of digestion increased during the first hours of passage through the model. This is in agreement with the absorption constants obtained with the computer simulations and with the digestibility coefficients in the separate and total ileal delivery samples. In the 240 min sample (Fig. 8a), the digestibility coefficients were lower than in the 360 min ileal delivery sample and those calculated from the absorbed fraction of nitrogen (Fig. 6). Possibly, the digestion of this first fraction of the meal is limited by relatively short gastric and small intestinal residence times. Diets with larger less digestible particles might have irregular ileal delivery, as indicated by the larger error bars (Fig. 8a, b, c). The slope of the linear regression line for absorbed nitrogen (Fig. 6) was close to the slope of maximum correlation. Reproduction in the model of exact apparent digestibility coefficients *in vivo* is only possible when the secreted nitrogen in the model is the same as the endogenous nitrogen *in vivo*. The LSD ($P = 0.05$) for the nitrogen absorption data obtained in the model as well

as from experiments in pigs shows that for both methods it is not possible to discriminate between all the diets to a significant extent (Table 4). The low LSD obtained from duplicate experiments in the model as compared to experiments in six animals per diet demonstrates the good reproducibility of the model and the needlessness of larger-scale experiments.

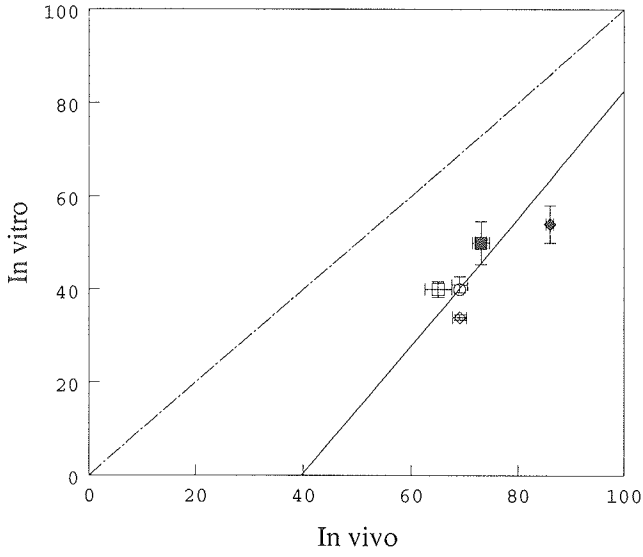

Fig. 4. Dry matter digestibility coefficient *in vitro* compared to *in vivo* for pig feed with barley (○), meat-and-bone meal (■), rape-seed (□), faba beans (◇) and wheat gluten (◆). The vertical and horizontal error bars present the SD for the *in vitro* and *in vivo* data, respectively.

Our model does not include a response to anti-nutritional factors which might increase endogenous protein secretion. The good correlation with *in vivo* data suggests that the contribution of variation in endogenous protein to the digestibility coefficient *in vivo* is negligible for the diets tested. This is likely since the diets contain low levels of anti-nutritional factors, except for the faba bean diet which contained 5.5 mg/g tannin (16) .

All the diets were tested with the same protocol which contained transit data based on results obtained with the rape-seed diet in ileal-cannulated pigs. Gastric

delivery and other physiological parameters show variation among diets as well as among animals. The good correlation between results obtained in the model and those in pigs suggests that digestibility in pigs is not strongly influenced by physiological variation among the diets tested.

The concept of the model allows to study the effect of gastric delivery on digestibility by varying only the gastric delivery curve. This method has been used to study the effect of bile concentration on survival of ingested lactic acid bacteria (7) and in a study of the efficacy of phytase in the gastric compartment (12).

Computer modelling

The absorption of nitrogen and subsequent ileal delivery were analysed with the use of a computer program that simulated all kinetic events that occurred in the model. The measured absorption and ileal delivery of nitrogen for each diet could exactly be fitted with linearly decreasing absorption constants (K_{jej}, K_{il}) in time.

Table 4. True digestibility coefficients of the diets obtained the model (mean ± SD, n = 2) and the apparent digestibility coefficients obtained in cannulated pigs (mean ± SD, n = 6), with the least significant difference (LSD, P = 0.05).

Diet	*in vivo* (% units)	*in vitro* (%units)
Barley	79±2.1	56±1.2
Meat-and-bone meal	62±4.2	48±4.3
Rape-seed	58±7.0	43±2.8
Wheat gluten	92±1.2	75±1.2
Faba beans	74±2.0	52±1.5
LSD	8.1	6.5

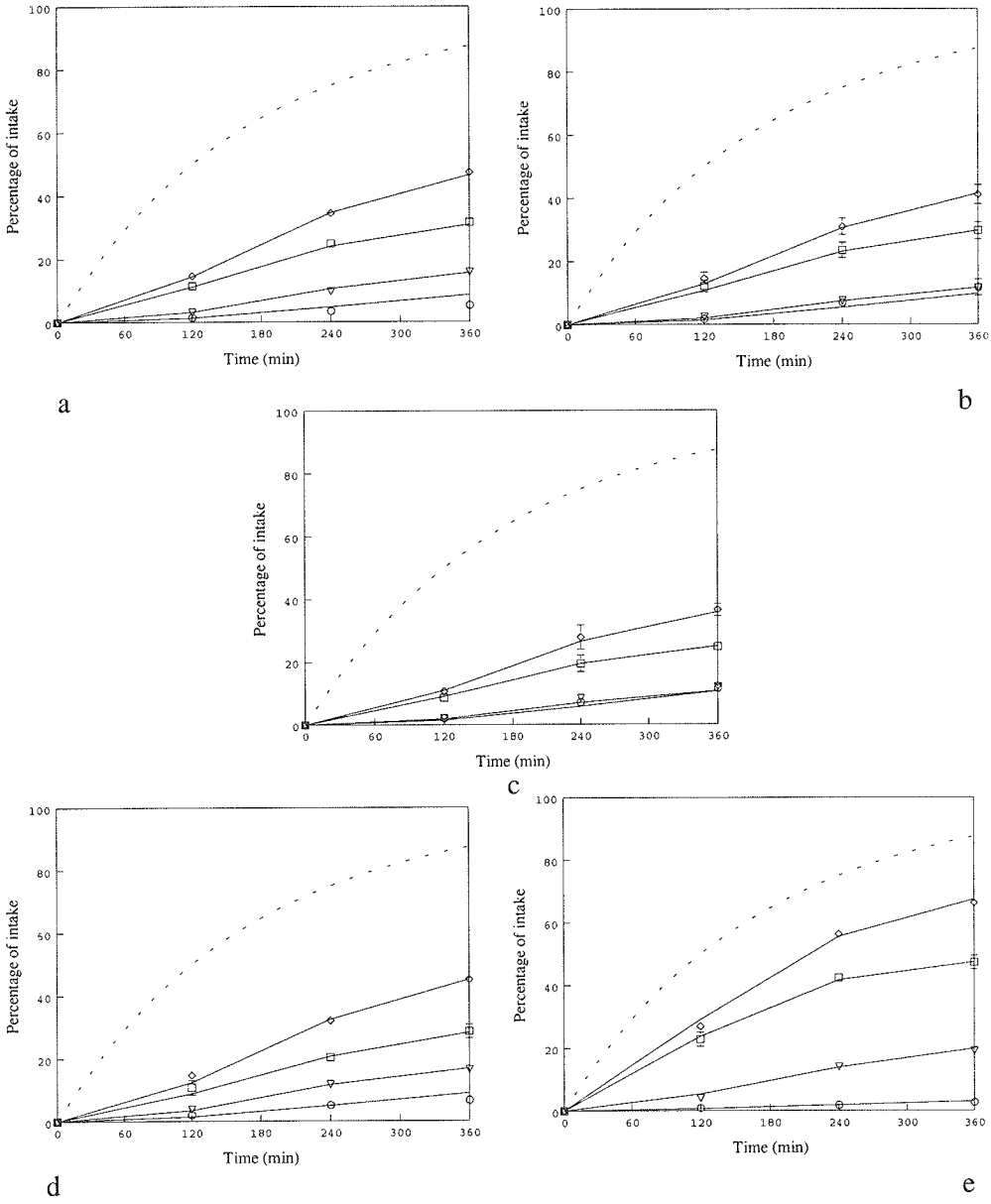

Fig. 5. Computer simulation (lines) and measured absorption (markers with range, $n = 2$) of dietary nitrogen from the jejunal compartment (□), ileal compartment (▽) and both compartments together (◇), and ileal delivery of nitrogen (○) expressed as a percentage of intake for barley (a), meat-and-bone meal (b), rape-seed (c), faba beans (d) and wheat gluten (e).

Table 5. Linear regression data of the correlation between the protein digestibility in different samples from the model and the protein digestibility coefficients obtained from experiments in pigs. y and x present *in vitro* and *in vivo* digestibility, respectively. RSD is the residual standard deviation and R is the correlation coefficient.

Fraction	Equation	R	RSD
Ileal delivery 120-240 min	$y = -119 + 2.2\,x$	0.96	8.1
Ileal delivery 240-360 min	$y = -21 + 1.11\,x$	0.90	6.5
Total ileal delivery	$y = -68 + 1.55\,x$	0.95	6.4
Absorbed nitrogen	$y = -7.41 + 0.85\,x$	0.95	3.3

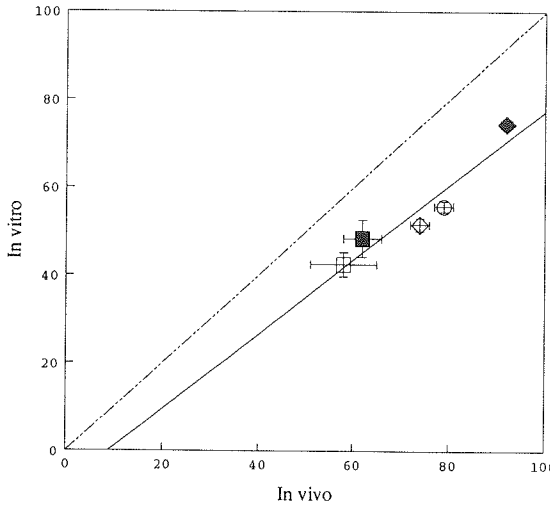

Fig. 6. Digestibility coefficients based on absorbed nitrogen in the model compared to the protein digestibility coefficients obtained in cannulated pigs for diets with barley (○), meat-and-bone meal (■), rape-seed (□), faba beans (◇) and wheat gluten (◆), with the linear regression line. The vertical and horizontal error bars present the SD for the *in vitro* and *in vivo* data, respectively.

The calculated absorption constant and its behaviour in time can be used to anticipate the digestion of protein during passage through the small intestinal compartments of the model. Previous experiments with excellently digestible

protein sources such as skim milk and soya isolate have shown absorption constants that do not change in time (11). In the present study it was not possible to obtain good fitting of the nitrogen absorption data without changing the absorption constants in time. This indicates a changing rate of digestibility of the protein, which is plausible since in this study we used complex protein sources, with different types of protein having different rates of digestibility and present in different matrices.

Digestibility might decrease due to a relative accumulation of less digestible protein or decreasing enzyme activities. Improvement of digestibility might be expected, for example, after a longer period of exposure to low gastric pH and peptic activity. The data obtained with the test diets showed different patterns of absorption.

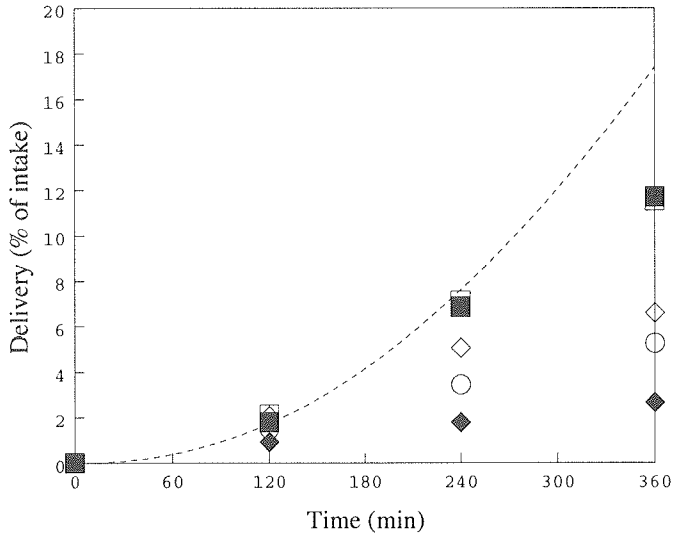

Fig. 7. Ileal delivery of nitrogen expressed as a percentage of intake and corrected for secreted nitrogen for pig diets with barley (○), meat-and-bone meal (■), rape-seed (□), faba beans (◇) and wheat gluten (◆). The line presents the ileal delivery of virtual meal marker (VMM).

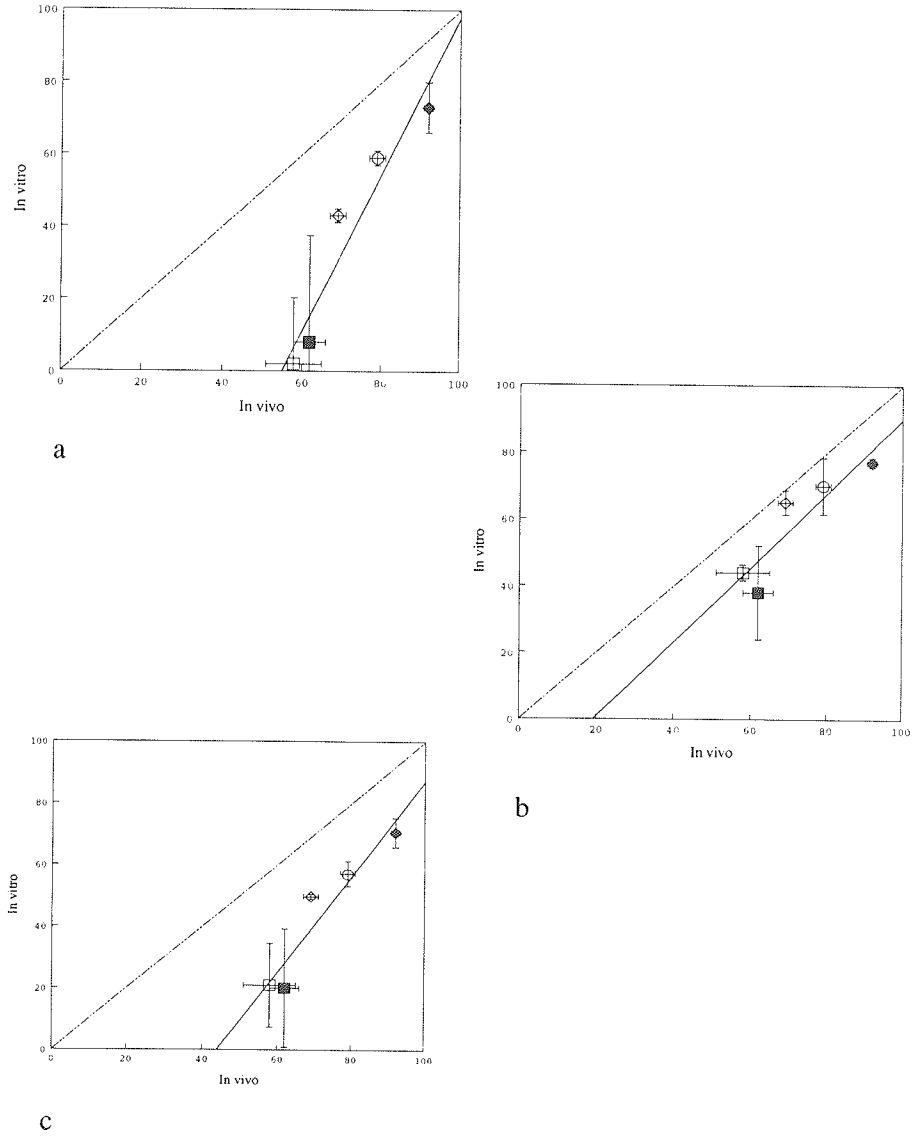

Fig. 8. Protein digestibility coefficients obtained in the model in ileal delivery between 120 and 240 min (a), between 240 and 360 min (b) and in total ileal delivery after 360 min (c) compared to the protein digestibility coefficients obtained in cannulated pigs for diets with barley (○), meat-and-bone meal (■), rape-seed (□), faba beans (◇) and wheat gluten (◆), with the linear regression line. The vertical and horizontal error bars present the SD for the *in vitro* and *in vivo* data, respectively.

The rape-seed and meat-and-bone meal diets have similar patterns. K_{jej} decreases to half the initial value, while K_{il} starts at a lower value than K_{jej}. The rape-seed diet has lower values than the meat-and-bone meal diet, which is in agreement with their digestibility coefficient. The barley and faba bean diets have different patterns for K_{jej}, but similar patterns for K_{il}. The barley diet had a decreasing K_{jej} similar to the meat-and-bone meal diet, and an initial K_{il} similar to K_{jej}. The faba bean diet had a constant K_{jej}, while K_{il} starts at a higher value than K_{jej}. The digestibility coefficient was higher for the barley diet than for the faba bean diet because the initial K_{jej} was higher when most of the nitrogen was absorbed. The higher K_{il} of faba beans accounted for a better digestibility as compared to that of the meat-and-bone meal. The low value in the ileal compartment after 360 min indicates almost complete digestion for these four diets.

The pattern of the wheat gluten diet differs from all other diets. The high initial values for K_{jej} and K_{il} support the superior digestibility as compared to the other diets, although both K_{jej} and K_{il} show a more rapid decrease as compared to the K values for the other diets. K_{jej} at 360 min is lower than for all other diets, while K_{il} shows that a relatively high digestive activity is present after 360 min. An explanation could be that the enzyme activity in the jejunal compartment exhausted the readily digestible protein until a residue of very slowly digestible protein remains after 360 min. The more slowly deceasing K_{il} as compared to K_{jej} might be explained by a lower enzyme activity in the ileal compartment, due to degradation of enzymes, and less digestible protein as compared to the jejunal compartment. This resulted in a slower degradation of protein, not reaching the point of exhaustion after 360 min. Another explanation could be that a fraction of the protein becomes more digestible with increasing residence time in the ileal compartment. The calculated extra time for K_{il} of wheat gluten to reach the same value as the other diets is 180 min. Further research will be necessary to add insight into the complex interaction of factors that determine digestibility, including additional animal studies to confirm the results obtained in the model.

CONCLUSIONS

The presented model offers the opportunity to evaluate the true digestibility of dry matter and protein in pig feed under strictly controlled conditions close to dynamic physiological conditions. Digestibility can be evaluated not only in ileal delivery but also during the digestion period by analysing the nitrogen absorption in time. These data can provide valuable support to research on improvements of ingredient

processing or diet composition. Furthermore, this model can be a useful tool to study the fate and efficacy of feed additives such as enzymes and slow-release additives.

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Chapter 8

A COMPUTER- CONTROLLED MODEL OF THE LARGE INTESTINE WITH PERISTALTIC MIXING, ABSORPTION OF FERMENTATION PRODUCTS AND A HIGH-DENSITY MICROFLORA

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in preparation

ABSTRACT

Laboratory models of the large intestine are widely used to study the metabolic and ecological behaviour of colonic microflora because experiments in human volunteers and animals are often hard to perform and have drawbacks with respect to ethics and costs. However, colonic models to date do not include removal of metabolites and water, which is essential to achieve physiological concentrations of microorganisms, dry matter and microbial metabolites. This paper introduces a new type of large intestinal model that combines these features and has been designed to be complementary to the dynamic multi-compartmental model of the stomach and small intestine described by Minekus *et al.* (18). High densities of microorganisms, comparable to those found in the colon *in vivo*, were achieved through absorption of water and dialysis of metabolites through hollow-fibre membranes inside the compartments. The dense chyme was mixed and transported by peristaltic movements. The potential of the model as a tool to study fermentation was demonstrated in experiments with pectin, fructo-oligosaccharide (FOS), lactulose and lactitol as substrates. Parameters such as total acid production and the short-chain fatty acids (SCFA) pattern were determined in time to characterize the fermentation. The stability of the microflora in the model was tested after inoculation with fresh faecal samples and after inoculation with a microflora that was maintained in a fermenter. Both approaches resulted in total anaerobic bacterial counts higher than 10^{10} CFU/ml with physiological levels of *Bifidobacterium*, *Lactobacillus*, Enterobacteriaceae and *Clostridium*. The dry matter content was ca. 10%, while total SCFA concentration was maintained at physiological concentrations with similar molar ratios for acetic acid, propionic acid and butyric acid as measured *in vivo*.

INTRODUCTION

To evaluate the health claims of functional foods and safety of novel foods, it is important to have models available that mimic the conditions in the gastro-intestinal tract (GIT). Models of the stomach and small intestine have been designed to study the digestion of nutrients (3,23). Recently, a multi-compartmental model of the stomach and small intestine has been developed that closely simulates the dynamic conditions in the lumen of the GIT (18). To study the effect of undigested compounds on the microflora, several *in vitro* models of the large intestine have been described (23). Microbial cell density in these models is generally limited by the fact that bacterial metabolites, such as short-chain fatty

acids (SCFA), are not removed from the fermentation process separately from the microorganisms. In order to absorb water and metabolites, a tubular model has been designed with hollow-fibre membranes inside. Furthermore, specific peristaltic mixing was achieved in order to handle physiological cell densities. The colonic model can be connected to the gastric/small intestinal model (18), which makes it possible to evaluate the effect of products or microorganisms on the large intestinal flora after simulated passage through the stomach and small intestine.
The aim of this paper is to introduce this new type of large intestinal model and to evaluate its use to study ecological aspects and metabolic activity of the microflora under close to colonic conditions. Composition, stability and enzymatic and metabolic activity of the microflora were measured and compared to *in vivo* values during feeding of a complex standard medium and several types of carbohydrates.

MATERIALS AND METHODS

The large intestinal model

The large intestinal model consists of units based on the same concept as that developed for the gastric/small intestinal model (18,19,20). Four glass units (Fig. 1, A), each with a flexible wall inside, are connected to each other. Peristaltic movements are achieved by pumping water into the space between the glass jacket and the flexible wall. The computer controls the sequential squeezing of these walls, thus causing a peristaltic wave which forces the chyme to circulate through the loop-shaped model. The tubular shape of the lumen of the model prevents 'obstipation'. The pH is measured with a pH electrode (B) and controlled by the addition of a 5 M NaOH solution (C). The dialysis liquid is pumped (D) from a bottle (E) through hollow-fibre membranes (molecular weight cut-off 50,000) positioned in the lumen of the model (F). Used dialysis liquid is collected in a waste bag inside the bottle, to obtain a closed system in order to prevent unintended flow of water across the hollow-fibre membrane. The amount of chyme in the model is monitored with a pressure sensor (G) and kept on a pre-set level through the absorption of water with a pump (H) in the dialysis circuit. The ileal effluent medium is mixed and kept anaerobic with nitrogen, and is introduced into the large intestinal model with the peristaltic valve system (I) as described previously (18). A peristaltic valve pump is used to remove chyme from the model. Gas is allowed to leave the model through a water lock (J).

Experiments

Experiments were performed to assess the efficacy of the model in removing SCFA, and to study the activity and stability of the microflora with two different types of inocula: 1) a standard microflora that was maintained in a fermenter, and 2) freshly collected faeces.

Efficacy of SCFA dialysis

To study the efficacy of the model in removing fermentation products such as SCFA, a test solution containing 20 mmol/L acetic acid, propionic acid and butyric acid was dialysed against water. During dialysis the concentrations of SCFA in the model were measured at 1 h intervals. Samples for SCFA analysis were prepared by mixing 500 µl of a 10-fold diluted sample with 100 µl internal standard (diethylbutyric acid) and 100 µl formic acid and centrifuged at 9000×*g* for 5 min. A 0.2 µl aliquot of the supernatant was analysed on a gas chromatograph equipped with a Stabilwax-DA column (Restek, The Netherlands). The temperature of the injector and of the FID detector was set on 200 °C. The oven temperature was 140 °C.

Experiments with a standard fermenter microflora

The model was inoculated with a standard microflora that was maintained under steady-state conditions in a 1600 ml fed-batch fermenter. The fermenter was inoculated with 80 g of fresh faeces, obtained from 5 healthy volunteers. The fermenter was flushed with nitrogen to maintain anaerobiosis, the pH was set on 5.8 and the incubation temperature was 37 °C. The composition of the microflora was monitored on a weekly basis. Each day 800 ml of culture was taken from the fermenter and refilled during the following 24 h with a modified medium as described by Gibson et al. (10). This medium contained per litre: 4.5 g NaCl, 2.5 g K_2HPO_4, 0.45 g $CaCl_2.2H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.005 g $FeSO_4.7H_2O$, 0.05 g ox bile, 0.01 g haemin, 0.4 g cystein, 0.6 g pectin, 0.6 g xylan, 0.6 g arabinogalactan, 0.6 g amylopectin, 5 g starch, 2 ml Tween 80, 3 g bactopeptone and 3 g casein, plus 1 ml of a vitamin mixture containing per litre: 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B-12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg *para*-aminobenzoic acid, and 4 mg thiamine. An inoculum for the colon model was prepared by concentrating the effluent from the fermenter about 5 times with a hollow-fibre micro-filtration cartridge (Microgon, USA). All handlings were performed under a flow of carbon dioxide. The colonic model was flushed with N_2

overnight prior to the introduction of 160 ml concentrated inoculum from the fermenter. The culture was fed with the same medium as described above for the fermenter, except that 10-fold concentrated carbohydrates, peptone, casein and Tween 80 were used. The feeding was set at 4 ml/h, while the chyme was removed at a flow rate of 2 ml/h and the flow of dialysis fluid was set on 6 ml/min. The dialysis fluid was the same medium as described above, without the carbohydrates, peptone, casein and Tween 80. The model was incubated at pH 5.8 and a temperature of 37 °C.

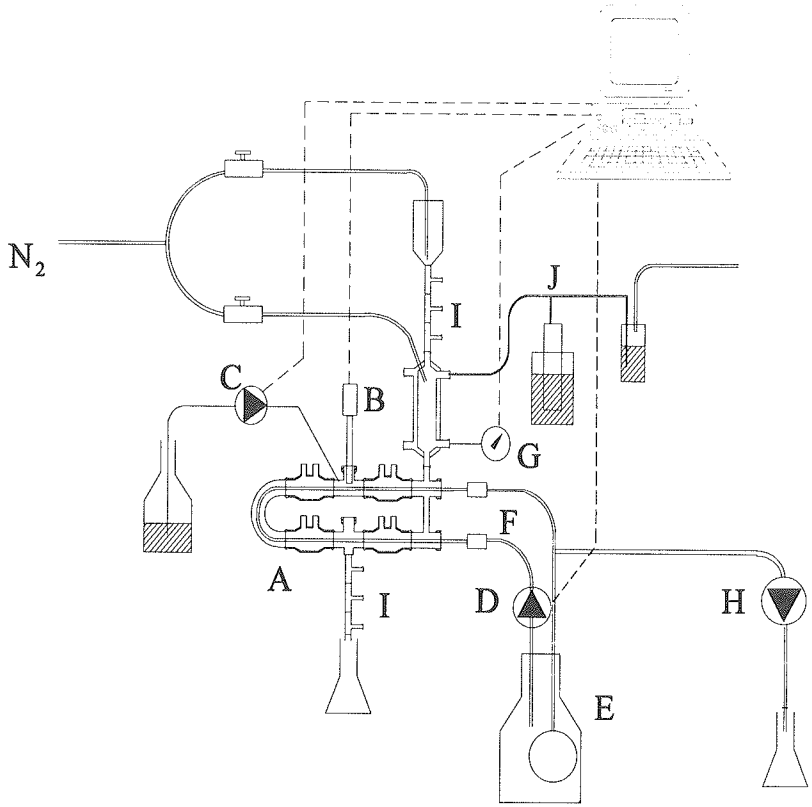

Fig. 1. Schematic presentation of the colonic model.
A, mixing units; B, pH electrode; C, alkali pump; D, dialysis pump; E, dialysis liquid; F, dialysis circuit with hollow fibres; G, level sensor; H, water absorption pump; I, peristaltic valve pump; J, gas outlet with water lock

Fermentation of carbohydrates

The fermentation experiments with carbohydrates in the model were performed with pectin (Sigma, St. Louis, MA), lactulose (Sigma), lactitol (Purac, The Netherlands) and fructo-oligosaccharide (FOS, Orafti, Belgium). After inoculation, the microflora was adapted to the conditions in the model for 12 h. Then the feeding was stopped to allow the microflora to ferment all available substrates. Dialysis was continued until all substrates were fermented, as monitored by acid production. Thereafter, dialysis was stopped to prevent small molecules from escaping during fermentation of the test substrates. Samples were taken from the chyme for SCFA analysis to determine initial values. The fibre test solution was prepared by diluting 0.25 g of dietary fibre in 10 ml of pre-reduced dialysis liquid in an anaerobic glove box. The dietary fibre was introduced into the model in such amounts that the SCFA concentration did not exceed physiological values. The pH was adjusted to 5.8 before introducing the test solution into the model. After fermentation of the product, as monitored by acid production, samples were taken from the chyme for SCFA analysis. SCFA production was determined by calculating the difference between levels before and after fermentation of the substrate. This method is schematically presented in Figure 2.

Composition and stability of microflora

The composition and stability of the microflora and its metabolic activity were evaluated during duplicate experiments with an incubation period of 5 days. Bacterial groups were enumerated on pre-reduced media in an anaerobic glove box. Total anaerobe bacteria s were counted on Reinforced Clostridium Agar (Oxoid, UK), *Lactobacillus* on De Man Rogosa Sharp Agar (Oxoid), *Bifidobacterium* on a selective medium according to Beerens (2), Enterobacteriaceae on Violet Red Bile Glucose Agar (Oxoid), and *Clostridium* on Perfringens Agar Base (Oxoid) with polymixin B as selective supplement. All plates were incubated at 37 °C under anaerobic conditions. Enzyme activities were determined with the API-zym (API, France) test according to the instructions of the manufacturer. The dry matter content was determined by drying 100 µl of sample on a pre-weighed filter in a microwave oven until no loss of weight could be detected.

Experiments with a faecal inoculum

An inoculum was prepared by mixing 200 g of faeces from a volunteer with 200 ml 0.1 M phosphate buffer pH 6.5. After filtration through a gauze, the filtrate was centrifuged. The pellet from the filtrate was combined with the retained material on the filter and suspended in 200 ml buffer. All handlings were performed under a flow of nitrogen. The model was inoculated with 200 ml of this faecal inoculum. Three experiments were performed successively, using faecal samples from three different volunteers. The pH was set on 6.5, the feeding was supplemented with 2% mucin (Sigma). The other conditions were similar to those described for the experiments with the standard fermenter flora.

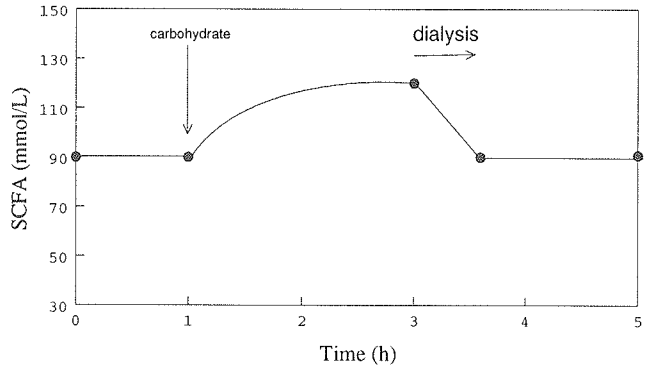

Fig. 2. Schematic representation of SCFA concentration in the model after introduction of a carbohydrate and dialysis.

Samples for SCFA analysis were mixed with 10% phosphoric acid and kept at -20 °C for gas chromatographic analysis according to the method of Jouany (13).

Dialysis and nitrogen flow were stopped for 2 h. The gas produced was collected and analysed on a gas chromatograph for H_2, CO_2 and CH_4 (12). Bacterial counts were carried out in the faecal inocula and in the model after 3, 6 and 9 days of incubation.

Total anaerobes and methanogens were determined with the 'most probable number' method according to Clarke and Owens (4). Total anaerobes were incubated on a medium according to Leedle and Hespell (14). Methanogens were cultivated in the medium described by Balch *et al.* (1) supplemented with 20%

(v/v) rumen fluid. This medium was selective for methanogen by the addition of clindamycin and cephalotin, and a mixture of 80% H_2/20% CO_2 as the only energy source. The other groups were enumerated on solid media. *Bacteriodes* was enumerated on Brain Heart Infusion agar (BHI, Biokar Diagnostics, France), *Bifidobacterium* on MRS agar at pH 7 (Biokar Diagnostics), *Lactobacillus* on MRS agar at pH 5 (Biokar Diagnostics), Enterobacteriaceae on Deoxycholate agar (DCA, Biokar Diagnostics), and facultative anaerobes were counted on G20 (22). All media were incubated at 37 °C for 48 or 72 h. Facultative anaerobes, *Lactobacillus* and Enterobacteriaceae were counted after aerobic incubation, whereas *Bacteroides* and *Bifidobacterium* were enumerated after anaerobic incubation.

RESULTS

Efficacy of SCFA dialysis

The concentrations of SCFA during dialysis in the model are shown in Table 1. The data could be well fitted with an exponential equation (Equation 1) to describe the efficacy of the dialysis process.

equation 1.

$$f = 100 \times 2^{-\left(\frac{t}{t_{1/2}}\right)}$$

Where f presents the percentage of SCFA remaining in the lumen of the model, t the time (h) and $t_{1/2}$ (h) the time needed to dialyse 50% of the SCFA.

The results show that the individual SCFA were removed from the lumen of the model at rates proportional to the molecular weight of the product, as is demonstrated by a $t_{1/2}$ of 1.35, 1.46 and 1.52 for acetic acid, propionic acid and butyric acid respectively. The efficacy of dialysis during fermentation experiments was demonstrated by the increasing concentration of SCFA after stopping the dialysis (Fig. 3).

Studies with standard fermenter flora

The composition of the microflora in the fermenter, which was monitored weekly, showed little variation during an 8-week period (Fig. 4).

During the experiments in the colonic model, the numbers of total anaerobes, bifodobacteria, clostridia, lactobacilli and Enterobacteriaceae were similar to those in the inoculum (Table 2). The concentration of SCFA was kept at 130 ± 50

mmol/L during the experiments, with an average molar ratio of 72%, 14% and 14% for acetic acid, propionic acid and butyric acid, respectively (Fig. 5). Enzyme activities showed little variation throughout the experiment (Table 3), while the dry matter content increased from 4.8±0.7% to 9.9±1.5% during the 5-day experiments.

Table 1. Percentage of SCFA in the model during dialysis. Mean of triplicate experiments (± SEM).

Time (h)	Acetic acid (%)	Propionic acid	Butyric acid
0	100	100	100
1	60.0 ±3.8	61.6 ±3.5	61.4 ±6.6
2	36.6 ±5.8	40.3 ±5.4	42.7 ±4.9
3	22.3 ±2.2	24.8 ±2.8	26.3 ±3.0
4	10.3 ±3.0	13.1 ±3.4	13.8 ±3.3

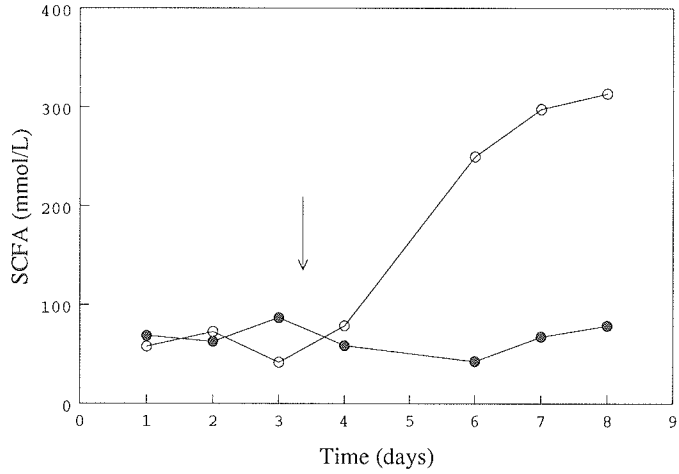

Fig. 3. Total concentration SCFA during an experiment with continuous dialysis (●) and during an experiment with dialysis until the time indicated by the arrow (○).

Pectin, lactulose and FOS showed a rapid fermentation pattern (Fig. 6). The amount of acid produced in 2 h was 1.55, 1.70 and 1.75 mmol respectively. Lactitol showed a slower pattern of fermentation, with 0.6 mmol acid produced in 2 h. The molar distributions of acetic acid, propionic acid and butyric acid after the fermentation of pectin, lactulose, FOS and lactitol are shown in Table 4.

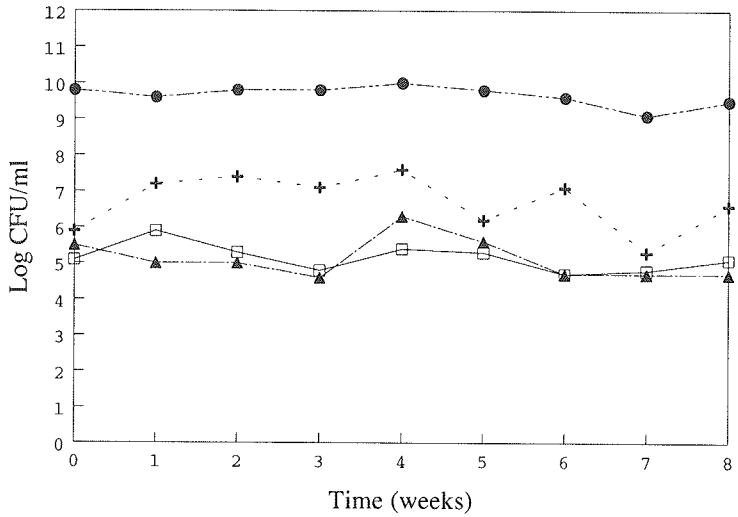

Fig. 4. Numbers of colony-forming units (log_{10}/ml) of total anaerobes (●), Enterobacteriaceae (□), *Lactobacillus* (▲) and *Clostridium* (+) in the standard fermenter flora, monitored for 8 weeks.

Table 2. Average numbers of some groups of bacteria from duplicate experiments (± SEM) in the fermenter inoculum and during incubation in the model. The data presented are expressed as log_{10} CFU/ml.

	Fermenter inoculum	Day 1	2	3	4	5
Total anaerobes	10 ±0.2	10.1 ±0.1	9.8 ±0.5	10.5 ±0.1	9.9 ±0.6	10.3 ±0.3
Facultative anaerobes	ND	8.5 ±0.2	8.0 ±0.5	8.0 ±0.6	ND	ND
Clostridia	7.1 ±0.0	7.7 ±0.3	6.1 ±1.3	5.8 ±1.5	7.9 ±0.6	7.4 ±0.2
Enterobacteriaceae	6.0 ±1.2	6.2 ±0.3	6.9 ±0.2	6.8 ±0.3	6.7 ±0.1	6.0 ±0.6
Bacteriodes	ND	9.0 ±0.1	8.6 ±0.1	8.6 ±0.1	ND	ND
Bifidobacterium	9.6 ±0.1	9.7 ±0.1	9.8 ±0.2	9.5 ±0.5	9.4 ±0.1	9.6 ±0.4
Lactobacillus	4.6 ±0.1	4.5 ±1.1	5.4 ±0.7	5.9 ±0.1	4.7 ±0.0	6.2 ±0.2

ND = not determined

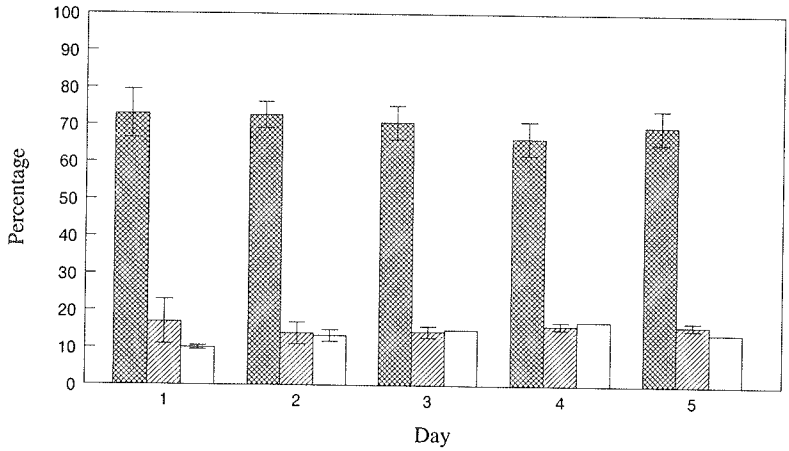

Fig. 5. Average molar ratios (± SEM, $n = 2$) of acetic acid (double-hatched), propionic acid (single-hatched) and butyric acid (blank) during experiments in the model after inoculation with standard fermenter flora.

Studies with faecal inoculum

The results show that the numbers of viable microorganisms in the model during incubation were similar to the initial numbers in the faecal inoculum (Table 5). The mean molar ratios of acetic acid, propionic acid and butyric acid were 55%, 22% and 23%, respectively. The ratios did not change significantly in time, with little variation between the experiments (Fig. 7).

The mean concentration of total SCFA, calculated from all three experiments, was 144 ± 40 mmol/L. The ratio between H_2, CO_2 and CH_4 was stable and similar for each experiment (Fig. 8).

DISCUSSION

There is a growing interest in models to study the efficacy of functional foods and to evaluate the safety of novel foods. Because experiments in animals or human volunteers have drawbacks with respect to cost, ethics and complexity, studies are often performed in *in vitro* models. *In vitro* models of the large intestine have been used successfully (23), but they generally do not combine physiological concentrations of metabolites with physiological numbers of microorganisms. If possible, this would increase the predictive value towards the *in vivo* situation, especially with respect to fermentation rates of substrates and also when the interaction between microorganisms and cell-to-cell contacts are of importance (10).

Table 3. Enzyme activity pattern over a period of 5 days in the model.

Enzyme activity	Day				
	1	2	3	4	5
Alkaline phosphatase	5	5	5	4	5
Esterase (C4)	3	2	2	1	2
Esterase Lipase (C8)	1	2	2	1	2
Lipase (C14)	0	0	0	0	0
Leucine arylamidase	4	3	3	3	5
Valine arylamidase	1	0	0	0	1
Cystine arylamidase	5	0	0	1	1
Trypsin	2	1	0	1	1
α-chymotrypsin	2	0	0	0	1
Acid phosphatase	4	5	5	5	5
Naphtol-AS-BI-phosphohydrolase	5	5	5	5	5
α-galactosidase	2	3	3	3	3
β-galactosidase	4	5	5	5	5
β-glucuronidase	5	5	5	5	5
α-glucosidase	5	5	5	5	5
β-glucosidase	3	4	3	2	4
n-acetyl-β-glucosaminidase	4	5	5	5	5
α-mannosidase	0	0	0	0	0
α-fucosidase	3	1	1	1	1

Table 4. Molar ratio between acetic acid, propionic acid and butyric acid after fermentation of several types of dietary fibre. Mean of duplicate experiments (± SEM).

Dietary fibre	Acetic acid (%)	Propionic acid (%)	Butyric acid (%)
FOS	82±6	12± 5	6 ± 0
Lactulose	89±0	3 ± 0	8 ± 1
Pectin	74±1	19± 0	8 ± 0
Lactitol	66±3	13± 1	21± 2

Simple static models accumulate metabolites which eventually might influence the metabolic activity of the microflora. A drawback of more complex continuous culture systems is that they operate under a steady-state condition and that the concentrations of metabolites are kept within the physiological range by the dilution rate and a limited amount of substrates in the influent (8).

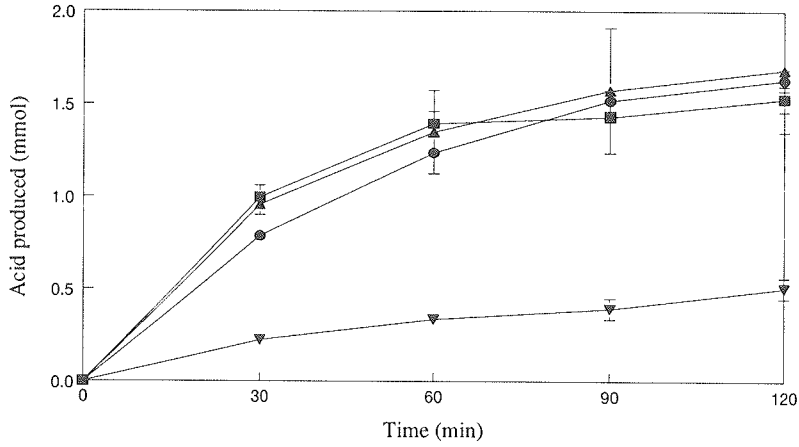

Fig. 6. Average acid production (± SEM, n = 2) during fermentation of lactulose (●), pectin (■), FOS (▲) and lactitol (▼) in time as monitored by the amount of secreted alkali.

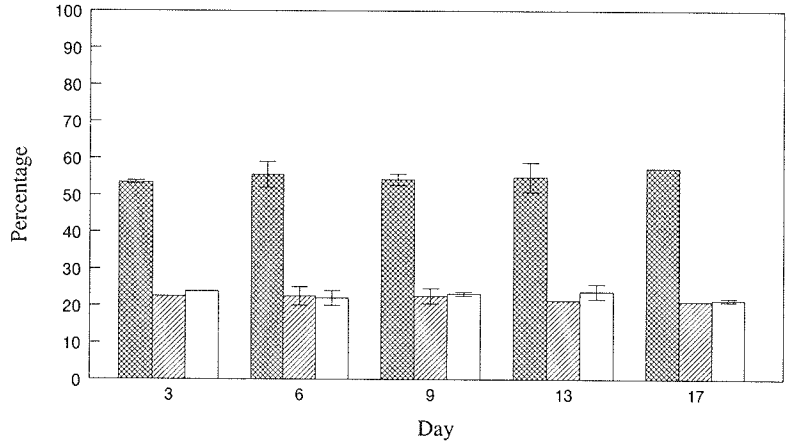

Fig.7. Average molar ratios (± SEM, n = 3) of acetic acid (double-hatched), propionic acid (single-hatched) and butyric acid (blank) during experiments in the model after inoculation with faecal flora.

Table 5. Average number (± SEM) of some groups of bacteria from 3 experiments and during incubation in the model after inoculation with a faecal inoculum. The data presented are expressed as $\log_{10}$ CFU/g for the faeces and $\log_{10}$ CFU/ml for the samples for the model.

	Faecal inoculum	Day 1	Day 3	Day 9
Total anaerobes	11.0 ±0.4	10.5 ±0.4	9.9 ±0.0	10.2 ±0.3
Facultative anaerobes	8.7 ±0.7	8.6 ±0.2	8.9 ±0.8	8.7 ±0.2
Enterobacteriaceae	8.6 ±0.8	7.8 ±0.7	7.8 ±0.4	7.6 ±0.5
Bacteroides	8.5 ±0.4	8.5 ±0.2	8.7 ±0.1	8.6 ±0.2
Bifidobacterium	8.1 ±0.9	8.1 ±0.5	8.3 ±0.2	8.3 ±0.1
Lactobacillus	7.9 ±1.3	7.7 ±1.0	7.6 ±0.3	8.0 ±0.7
Methanogens	6.9 ±1.9	8.0 ±1.8	8.5 ±1.2	8.5 ±0.7

Dilution, substrate limitation and product inhibition are the main causes that limit the number of microorganisms in these models. Therefore, removal of the metabolites from the chyme separately from the microorganisms, and absorption of water and concentrated feeding to allow an increased input of nutrients without increasing the dilution rate, are prerequisites to maintain the number of microorganisms as well as their metabolites at physiological levels. However, the feeding and mixing of dense fibrous and viscous materials is a common problem in large intestinal models (8). In our model, mixing with nitrogen offered an adequate method to keep the influent homogeneous and anaerobic. Besides, the peristaltic valve pumps proved adequate in pumping viscous and fibrous materials into the model and pumping chyme out of the model.

Water and metabolites were absorbed adequately through hollow-fibre membranes inside the compartments. By feeding concentrated ileal delivery medium, water absorption could be minimized in order to decrease fouling of the membranes. During the experiments up to 750 ml water could be absorbed. Experiments were performed to demonstrate that the SCFA could be dialysed efficiently and that their concentration could be maintained within physiological limits (Fig. 3). The applicability of the model to study the fermentation of carbohydrates has been demonstrated with several substrates (Table 4).

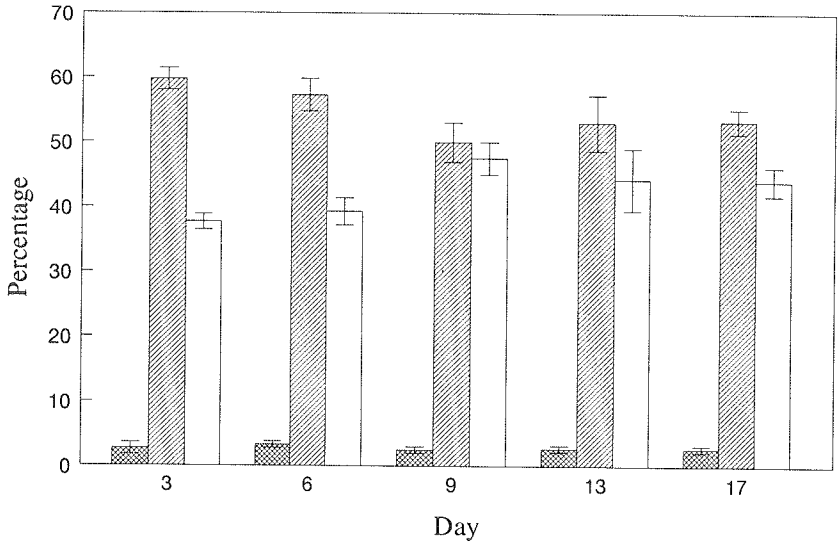

Fig. 8. Average percentage (± SEM) of H_2 (double-hatched), CO_2 (single-hatched) and CH_4 (blank) during experiments in the model after inoculation with faecal flora ($n = 3$).

The kinetics of fermentation could easily be monitored by the added alkali to neutralize the acid produced. We calculated a yield of ca. 40 g SCFA per 100 g of FOS, pectin and lactulose. This is in agreement with data presented by Cummings (7). The yield of 15 g SCFA per 100 g lactitol in 2 h of fermentation is low and in agreement with the slow fermentation pattern based on total acid production (Fig. 6). The molar ratios of SCFA after fermentation of FOS and pectin were similar to those obtained by Wang and Gibson (27). For lactulose, the relative amount of acetic acid was higher than those reported by others (25,27), whereas lactitol resulted in a relatively low production of acetic acid. The molar ratio of SCFA after inoculation with faeces was similar to values obtained *in vivo* (5), while the fermenter flora showed higher percentages of acetic acid, similar to Miller and Wolin (17) and Manning (16).

The number of bacteria in human colonic contents usually ranges between 10^{10} and 10^{11} CFU/ml, while the dry matter content increases from ca. 14% in the caecal material to 23% in the sigmoid rectum. It has been shown that about half of the dry matter originates from microorganisms (6), the other half being composed of bulking components such as plant cell material. The dry matter content of ca. 10% was reached with a low level of bulking components in the medium and hence consisted mainly of microorganisms. With the addition of extra cellulose to

the feeding medium, a dry matter content of up to 20 % was obtained (data not shown). The enzyme activities measured in the model were close to the activities found in faeces (21). The stability and activity of the microflora were tested during experiments with a faecal inoculum and with an inoculum from a fermenter, both of which have specific advantages. A faecal inoculum is easily to obtain, is considered representative of the colonic microflora and is therefore widely used as inoculum (23). However, standardization of faecal inocula is problematic. The fermenter microflora offered the possibility to obtain a standardized culture that was adapted to the ileal delivery medium and was directly available. The counts of the different microbial groups in the model for both studies were stable and within physiological ranges (15). The presence of physiological quantities of methanogens indicated a redox potential sufficiently low to maintain strictly anaerobic microorganisms.

CONCLUSIONS

This study presents a new, advanced model that simulates accurately the conditions in the lumen of the large intestine. The circular shape of the model, resembling a loop reactor, proved adequate to circulate a high-density chyme with peristaltic movements. The shape allowed the internal positioning of hollow fibres for the removal of microbial metabolites and water. The ability of the model to simulate closely the physiological conditions in the lumen of the large intestine was demonstrated for the levels of several groups of bacteria, microbial enzyme activities, production of SCFA and dry matter content of the chyme. As in all *in vitro* models of the gastrointestinal tract, the use of this model is limited by the fact that it does not include interactions with the host. Also, the mechanism of absorption used in the model is not the same as *in vivo*. However, taking these limitations into account, this model is a useful tool to study the fate of undigested components and their effect on microbial metabolism and ecology in the lumen of the large intestine. Such studies may include safety evaluation of foods that contain genetically modified material or antibiotic residues, bio-transformation of food components into toxic compounds, and the study of prebiotics and probiotics.

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Chapter 9

GENERAL DISCUSSION

INTRODUCTION

Models of the gastrointestinal tract (GIT) developed to date do not simulate the dynamic conditions in the gut adequately to meet the increasing need to study the efficacy or safety of foods, feeds, ingredients and pharmaceuticals (11; Chapter 1 of this thesis). The aim of the research presented in this thesis was to develop a model that simulates the dynamic conditions in the lumen of the GIT of monogastric animals with a high predictive value towards *in vivo* conditions. The studies resulted in a model that can be described as a computer-controlled multi-compartmental dynamic model that simulates the luminal conditions of the GIT, probably the most advanced GIT model to date (Chapters 2 and 8). The model can be regarded as a concept according to which a gastric, a small intestinal and a large intestinal model has been developed, that can be adapted for specific applications. So far, experiments have been performed with the gastric model alone (Chapter 3), with the gastric model connected to a three-compartmental small intestinal model (Chapters 2, 4, 5, 6 and 7), and with the large intestinal model (Chapter 8). This discussion will first evaluate the aspects of the model that reproduce a predetermined *in vivo* situation. Next, the discussion will focus on: the possibilities and limitations to use the model for specific applications; presentation of results; computer modelling; and some future developments.

REPRODUCTION OF LUMINAL CONDITIONS

A model should be a simplified but adequate representation of a specific reality. For the introduction and acceptance of a model it is important to validate it by presenting evidence that it is adequate for its purpose. Because the model can potentially be used for a wide variety of applications, it is better to evaluate the adequacy of the model to reproduce accurately the conditions in the lumen of the GIT, and the use of the model for specific applications. First, the aspects in relation to luminal conditions will be discussed, and how the model meets the pre-determined criteria as laid down in the General introduction (Chapter 1) to this thesis. To obtain realistic conditions in each compartment in time after the ingestion of food, the model should simulate:

- Physiological pH values in each compartment during the passage of food.
- Appropriate mixing in each compartment.
- Realistic transit of the meal through the compartments.
- Secretion of digestive fluids with physiological amounts of electrolytes, bile salts and enzyme activities.
- Absorption of water, nutrients and microbial metabolites from the lumen.

Regulation of pH

The reproduction of a gastric pH profile in the gastric compartment and maintenance of a pre-set value in the duodenum was demonstrated in every study presented in this thesis. The use of the predetermined gastric pH profile allows meals with different buffer capacities to be compared under the same gastric conditions. It is also possible to programme a secretion curve of hydrochloric acid to include the buffer capacity of the meal as a determinant for the gastric pH profile. Recently, also a pH control was introduced in the jejunal and ileal compartments to reproduce different pH values in these parts of the small intestine. The pH control in the colonic model proved a convenient way to assess the production of acid. Secreted alkali, which neutralized the produced acid , was used to present the rate of fermentation of several carbohydrates (Chapter 8).

Mixing and transit of the meal.

To mimic the peristaltic type of mixing in the GIT, a modular system has been designed with flexible walls that allow for contractions with a computer-controlled frequency, amplitude and strength. The design of the mixing units and the peristaltic valve pumps (PVP, Fig. 1),which are used to transport the chyme, form a tubular system with connecting compartments, which has been patented in the USA (12) and in Europe (13). To control quantitatively the transport of the meal, only the PVPs that simulate the pyloric- and ileo-caecal sphincter need to be calibrated. The latter is normally the outlet of the system, which allows easy monitoring of the flow rate from the ileal compartment. The flow rate of the pyloric valve is hard to monitor and is more variable due to the changing composition and viscosity of the gastric output. Gastric and ileal delivery are controlled to follow predetermined mathematical curves that describe the cumulative delivery of a non absorbable meal marker as a percentage of intake (Chapter 2). The control of meal transit with pre-set gastric and ileal delivery curves proved to allow for accurate and reproducible experiments. The curves also allow for easy translation of *in vivo* data into curve fitting parameters that can be used in the digestive protocol for the model. The parameters that describe a pre-set condition can be derived from *in vivo* data and are combined in a digestion protocol for the model.

An algorithm had to be used to control the transit from the duodenal compartment through the jejunal compartment to the ileal compartment, because there are little *in vivo* data available on meal transit through these parts of the small intestine.

Generally, a simple flow-through system is used, similar to a cascade of continuous-flow stirred-tank reactors (CSTR).

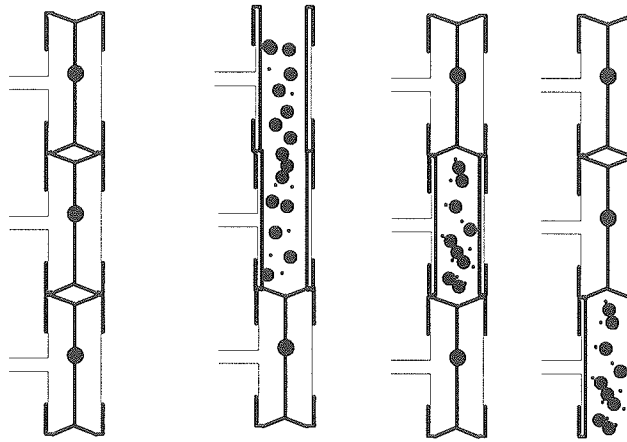

Fig. 1. Four stages in the cycle of a Peristaltic Valve Pump (PVP), showing the transport of a portion of chyme.

The higher flow of liquid into the system as compared to the ileal delivery of liquid is compensated by water absorption in the jejunal and ileal compartments. In other words, water absorption in time is calculated as the amount of water that needs to be removed from the small intestine, to balance the difference between the liquid entering the duodenal compartment and the liquid leaving the ileal compartment (Fig. 2).

The number of small intestinal compartments is based on a compromise between a realistic simulation of meal transit and the complexity of the model. For the studies presented in this thesis, a system with three compartments was used, since a division of the small intestine in duodenum, jejunum and ileum is generally accepted from a physiological point of view.

This configuration allowed for physiological bile salt concentrations in three sections of the small intestine (Chapter 5), and for a study of absorption

characteristics of nutrients by the separate absorption systems in the jejunal and ileal compartments (Chapters 4,6 and 7).

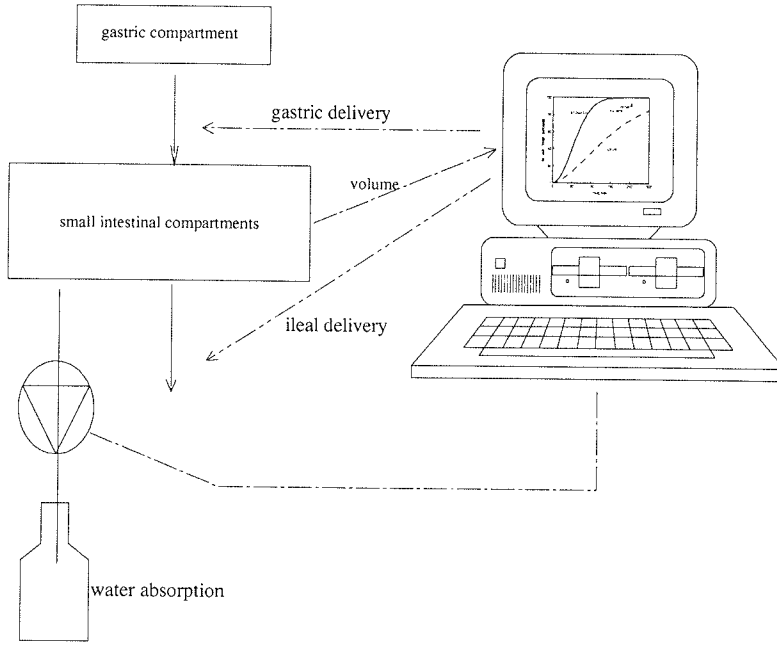

Fig. 2. Control of volume in the small intestinal compartments. The water absorption pump is activated when the volume in the small intestinal compartments exceeds the preset value and the ileal delivery curve does not dictate ileal delivery of chyme.

The handling of solid foods in the model introduces specific difficulties due to particles that might block the flow through the compartments or accumulate between the hollow fibres. To deal with foods that contain particles, specific adaptations have to be made to the model. Accumulation of particles between the hollow fibres during the pig feed experiments was successfully prevented by a pre-filter. The pendular movement of the chyme prevented fouling of the filter.

The pressure needed to squeeze the flexible walls in the gastric and the small intestinal model was supplied by the water circulation pumps. This was not

sufficient to obtain a good mixing of the high-density chyme in the colonic compartment. Therefore, a system was designed that uses nitrogen gas to supply pressure on the circulated water to contract the flexible walls.

The colonic model was designed to follow two strategies to simulate the transit of the chyme. As with other CSTR-type colon models, several compartments can be connected in a cascade (18). This configuration can be used to perform studies with a microflora that is maintained in a steady-state condition. The model can also be set to mimic a plug of chyme that travels through the large intestine in a single compartment. At the start of the experiment an amount of ileal delivery containing the test compound is introduced into the model, to simulate the caecum. Thereafter, pH, nutrients, electrolytes and rate of water absorption are changed in time to mimic the conditions in the successive parts of the large intestine. A simplified version of this system was used to study the fermentation of dietary fibre (Chapter 8).

Secretion of digestive fluids

The design of the model is based on the secretion of physiological amounts of liquid, enzymes and electrolytes into the gastric and duodenal compartments. Data on physiological values were obtained from the literature, which appeared to be not a simple task, especially for enzyme activities. Enzyme activities are measured under very different conditions, by a large variety of methods, and with a large inter-individual variation. Fortunately, under normal conditions enzymes are secreted in excess which makes the secretion of enzymes in the model less critical for most applications. The same arguments justify the use of pancreatin, which is a crude mixture of pancreatic enzymes with undefined activities, except for amylase, total protease and lipase activities.

In the gastric compartment a combined artificial salivary and gastric juice mixture is used that contains pepsinogen and a fungal lipase as an alternative to gastric lipase. The fungal lipase showed an activity similar to that of gastric lipase, although the specificity in respect to the chain length of fatty acids was not similar. The use of real gastric lipase would be an important improvement, especially to simulate neonate conditions (6). However, to our knowledge gastric or lingual lipase is not available in sufficient amounts and purity.

The fixed flow of gastric and salivary secretion is a simplification of the variable secretion of liquids *in vivo*, caused by reflex and feed-back mechanisms. Although metering pumps have recently been introduced that allow variable liquid secretion in time, generally a fixed flow of secretion was used. As explained in Chapter 6, gastric fresh matter delivery with a fixed flow of gastric secretion in the model hardly deviated from that found in calves. In the small intestine and in the large intestine the secretion of liquid is not very critical, because the flux of liquid in the lumen is the result of secreted and absorbed liquid.

Absorption of nutrients

Absorption of nutrients is an important aspect of realistic simulation of intestinal conditions. However, the mechanisms of absorption *in vivo* cannot be reproduced in an *in vitro* model that lacks the complexity of the gut wall. *In vivo* absorption of nutrients occurs very efficiently through diffusion or carrier-mediated transport of molecules across the enterocyte membrane. In the small intestinal model, hollow-fibre membrane devices are used with a molecular weight cut-off of approximately 5000 to absorb products of digestion and other small molecules, depending on their molecular mass. To deal with this discrepancy, the assumption has to be made that molecules small enough to diffuse through the membrane in the model are sufficiently accessible *in vivo* to pancreatic and brush border enzymes to be further degraded and absorbed. This implies false results in the model when there are molecules smaller than 5000 Da, which are not degraded and absorbed *in vivo*. If such molecules are important, a membrane with a lower molecular-weight cut-off could be selected. For the study of mineral absorption, the molecular weight cut off of 5000 implies that all soluble minerals are absorbed rapidly through the membrane. *In vivo,* absorption is also dependent on the condition and requirements of the body. This brings up the determination of the bioavailability of nutrients in the model. Bioavailability can be defined as the availability of a nutrient for utilization in the body (19), which is determined by a series of events as presented in Fig. 3. The first events, such as release from the food matrix, interaction with other compounds in the lumen and solubilization, determine the luminal availability for absorption. The digestibility, or the amount of nutrient that is absorbed, is determined by the luminal availability and the digestion and absorption processes in the in gut wall. Bioavailability is determined by transport of the nutrient to the target site after absorption through the gut wall.

Because absorption of nutrients in the model is determined by the nutrients' size, matrix, solubility, binding to other compounds and digestibility, only luminal availability can be assessed. The model is not suitable to determine the bioavailability of a nutrient if the mechanism of absorption and the transport to the target site are important.

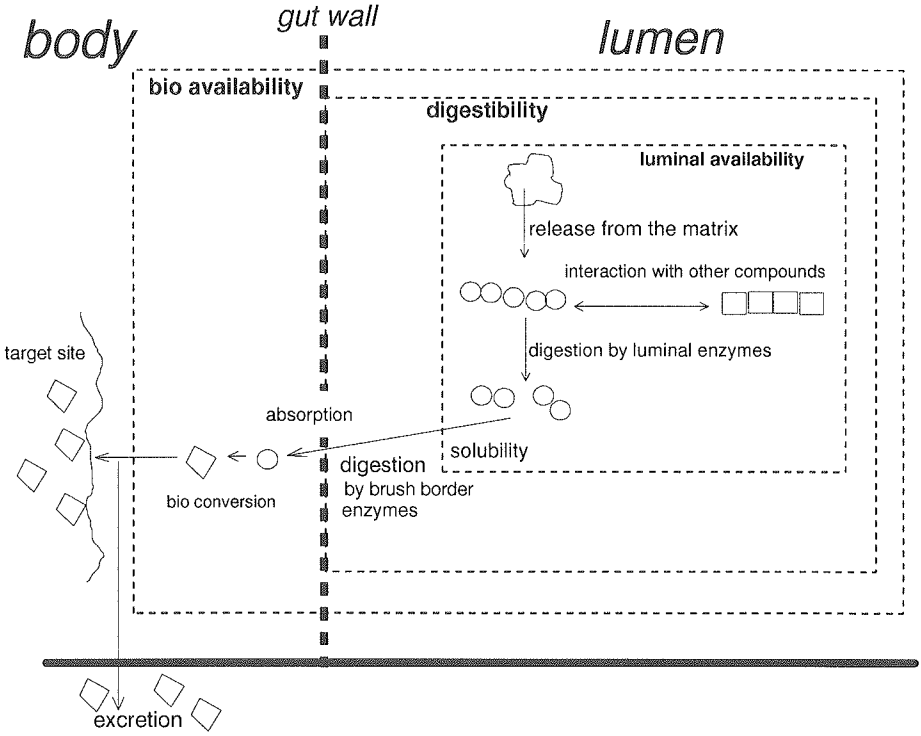

Fig. 3. Fate of ingested compounds, relation between luminal availability, digestibility and bioavailability.

Also, the model is not suitable to determine the exact rate of absorption of a nutrient if this is limited by processes following luminal availability. Another application that is limited by the type of absorption in the model is the study of undigested compounds in the large intestinal model. Some molecules are hardly digested and absorbed in the small intestine while their size is small enough to be absorbed through the membrane (2). Thus, a membrane has to be use with a cut-off

that is sufficiently small to retain these molecules but at the same time large enough to absorb, for example, digestible disaccharides, a prerequisite virtually impossible to meet. A study of undigested compounds is only possible if the composition of the relevant undigested fraction in the model is similar to that *in vivo*. This is only valid when the undigested fraction *in vivo* is larger than the cut-off of the membrane, while all compounds below the cut-off are digested and absorbed *in vivo*. Sometimes microorganisms such as yeasts are used to ferment digestible carbohydrates before introducing the test solution into the colonic model (26).

The absorption system with the hollow-fibre membranes only allows for the absorption of water-soluble compounds such as products of digestion of protein and carbohydrates. A special system is in development to absorb the products of fat digestion and other lipophilic compounds. *In vivo*, the products of lipid digestion and lipophilic compounds are generally incorporated into mixed micelles. These micelles diffuse to the brush border, where they become unstable, after which their constituents are absorbed through the lipophilic membrane of the enterocytes (20). Because processes occurring in the gut wall cannot be simulated in the model, a system was designed that removed the intact micelles but not the fat. Figure 4 shows a graphic comparison between lipid absorption *in vivo* and *in vitro*. A schematic presentation of the lipid absorption system is shown in Fig. 5. A microfiltration membrane was used that retains fat globules but not the micelles. A solid-phase column with C18 as the active compound is used to collect the lipophilic compounds in the filtrate, while the hydrophilic compounds are pumped back into the lumen. Removal of bile salts from the lumen was prevented by saturating the columns with bile salts before use in the system.

It must be realised that not all types of nutritional experiments can be performed in *in vitro* models. Availability of appropriate and not over-expensive materials, such as specific membranes, or (bio)chemicals are often limiting factors. Furthermore, it must be accepted that experiments which rely on interactions between food components, microorganisms and the host cannot be performed in the model. The expansion of the model with enterocyte cultures and mucosal tissue is an exciting development which will be discussed in the next section.

Response of the host

The host responds to characteristics of food in order to optimize digestion and uptake of nutrients. These interactions are very complex and involve neural and

hormonal mechanisms that regulate, for example, the secretion of digestive fluids, as well as gastrointestial motility (1).

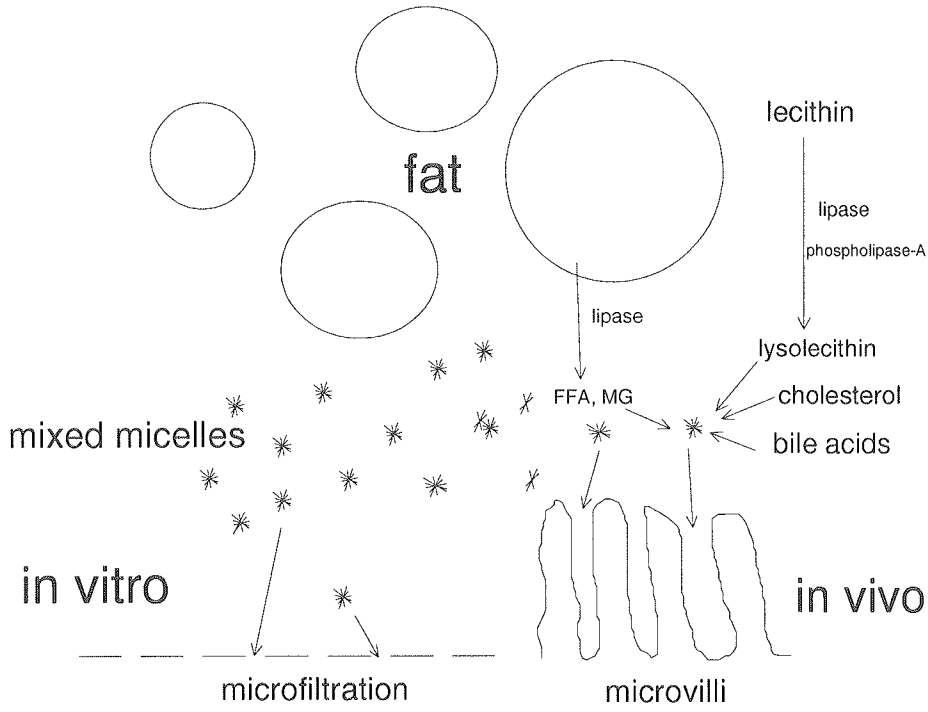

Fig. 4. Comparison between *in vivo* and *in vitro* absorption of lipids.

Although the interactions between the food and the host result in efficient utilization of nutrients, they are associated with day-to-day variation in physiological conditions within one person, and inter-individual variation within a population. This biological variation implies that, when the fate of ingested compounds is studied *in vivo,* the tests should be performed in a population sufficiently large to detect relevant treatment effects with sufficient statistical power. The model does not respond to the food: it is programmed to simulate a specific physiological condition, which results in reproducible experiments

irrespective of characteristics of the food. This lack of response might limit the predictive power of the model.

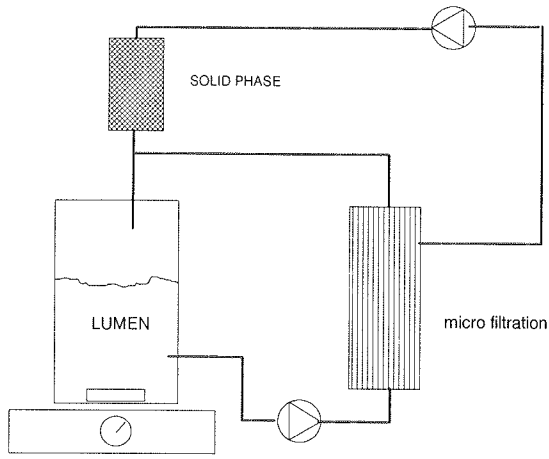

Fig. 5. Simplified, schematic presentation of the lipid absorption system.

However, because in the model isolated parameters can be varied, the effect of variation can be determined for specific parameters. Thus, not the mean result obtained in a population can be predicted, but rather the fate of a compound accessed under conditions that are normal or extreme for the population studied. This principle is demonstrated in Chapter 3, in which the effect of gastric meal delivery on the fate of phytase is described with two different gastric delivery curves which are both assumed to be normal for pigs (16). The principle is also applied in chapter 5 describing a study in which the survival of ingested lactic acid bacteria was tested with low and high bile concentrations in the small intestinal compartments.

In the future, it might be possible to use sensors or on-line analysis to control gastric meal delivery or other functions in the model. However, application of such techniques requires more knowledge on the control mechanisms *in vivo*.

An important development is the combination of the model with cultured monolayers of intestinal mucosa cells or segments of gut wall mucosa (5), for nutritional, toxicological and pharmaceutical studies. The model allows to study the effect of luminal conditions on an ingested compound, while biological models can be used to study the uptake of the compound or its breakdown products. Biological models can also be used as indicators of (non-specific) toxicological or pharmacological effects of ingested compounds. A selected panel of cell cultures, tissues or micro-organisms could be exposed to the luminal conditions in special containers or to the dialysate of the model. Such a system might be very effective in toxicological and pharmacological evaluation of ingested compounds and their breakdown products in the small intestinal model as well as in the colonic model. The colonic flora has been shown to exhibit enzyme activities that might give rise to toxic products (14,17). These activities are generally measured with specific assays. However, the conditions in an enzymatic assay might not be the same as those in the large intestine *in vivo* or in the model, and therefore might produce different results. Also, unknown enzyme activities resulting in the formation of toxic products might be overlooked. Non-specific determination of toxic compounds in the colonic model would be an interesting additional method. As for the gastric/small intestinal model, a panel of indicators for toxic compounds could be connected to the colonic model to determine if those compounds are produced *in situ* under close to colonic conditions.

APPLICATIONS

The use of the model for a specific application requires a defined protocol that includes the physiological parameters that describe the luminal conditions. Such a protocol is used by the computer program to reproduce the *in vivo* conditions in the model (Fig. 6). Besides the protocol, an application might require a specific configuration of the model and composition of secreted fluids. The use of protocols allows reproducible experiments which makes the model especially suitable for efficacy and safety testing during product development. The effect of different production processes or product compositions on luminal availability can be studied in comparative experiments under identical conditions with the same protocol in the model. A product can also be tested while varying the protocol, to test the behaviour of the product under normal, extreme or pathological gastrointestinal conditions. The model can be used for quality control by regularly testing a product with a standard protocol to monitor changes in gastrointestinal

behaviour due to variation in the production process or base materials. In conjunction with *in vivo* studies, the model might prove a powerful combination for mechanistic studies. *In vivo* studies often show large variation among individuals and are limited in measurable parameters and measuring points (black-box concept). In others words, animals and human beings might be too complex to study mechanisms, while the model might be too simple to give the complete picture.

To validate an application, experiments have to be performed to collect scientific evidence that results obtained in the model correlate well with results from similar experiments *in vivo*. Ideally, validation needs *in vivo* experiments that give sufficient physiological data to allow reproduction in the model with products that are also available for testing in the model. The number of products available for testing should be sufficiently high to generate results that can be (statistically) compared. Because there are almost no *in vivo* experiments available that meet all of these prerequisites, *in vivo* data have to be combined from different sources to generate specific protocols. The studies on survival of lactic acid bacteria (Chapter 5), protein digestion of calf milk replacers (Chapter 6) and pig diets (Chapter 7) could be performed with the same products as used in *in vivo* experiments.

In addition, these *in vivo* studies presented ileal delivery and digestibility data, which are necessary to compare the results with the gastric/small intestinal model. Although the studies presented in this thesis indeed provide validating evidence, more studies are necessary to corroborate the statistical significance and to establish that the model is adequate for a specific application. Furthermore, the validating evidence for the model increases when more different types of studies have been evaluated. For example, both the pig and the calf studies in the model on protein digestion give a good correlation with *in vivo* results.

The main differences in the standard protocols for pigs, calves and man are the gastric and ileal meal delivery curves, due to different types of food, eating patterns and relative dimensions of the gastrointestinal sections (22). Results with the recently developed dog protocol show a similar trend (21). Performing studies that have validating value for several species of monogastric mammals would enhance the possibilities to validate the model. Studies with volunteers to validate the model are often expensive and difficult to perform. Therefore, it would be interesting to be able to extrapolate results obtained from experiments in animals with a physiology almost similar to human physiology, such as the pig.

If validation is not possible, it can be decided that the conditions in the model are appropriate for the study based on academic considerations.

The colonic model has been used to study the fermentation of indigestible carbohydrates (Chapter 8), gene transfer (25) and effects of ingested compounds such as prebiotics, probiotics and antibiotics on microflora. Validation of these applications is hampered by the fact that it is difficult to measure relevant parameters, such as acid production and concentrations of test compounds, in different parts of the colon.

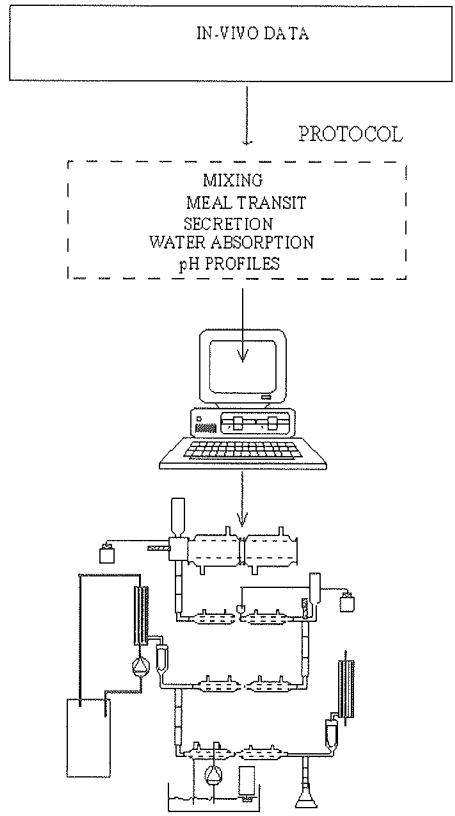

Fig. 6. Reproduction of a predetermined *in vivo* situation in the model

Results from faecal samples are often not representative of the colon due to the high bacterial metabolic activity and absorption of compounds by the host. The use of animals as models to study the events in the human large intestine is restricted by considerable differences in composition of microflora. Germ-free animals that are associated with specific microorganisms of human origin exhibit physiological abnormalities as compared to animals with a conventional large intestine (8). Simplified ecological large intestinal models, such as the continuous flow (CF) model described in the Introduction to this thesis, have been found to maintain the (major) microbial species in physiological ratios, which indicates ecological control mechanisms similar to those *in vivo* (8). Freter (8) has also presented some validating evidence for the study of colonization of microorganisms in CF models. The following similarities between CF models and the large intestine have been described:

- A microorganism needs to adhere to the wall to colonize.
- The amounts of microorganisms are regulated by substrate competition.
- Anaerobiosis.
- Continuous flow.

Other factors, such as the mechanisms of adherence and interactions between micro-organisms and the host, limit the application of any *in vitro* model of the large intestine including the one described in this thesis.

SAMPLING, ANALYSIS AND INTERPRETATION OF RESULTS

The results obtained with the gastric/small intestinal model are normally digestion data of proteins and carbohydrates or comparative absorption data for compounds such as drugs, minerals and trace elements. Prior to the experiment, a sample is taken from the meal to determine the intake of the test compound. Generally, jejunal and ileal dialysates and ileal effluent are collected during the experiment. The residual contents of each compartment are collected at the end of the experiment. This allows to determine the recovery of the test compound after the experiment, expressed as a percentage of intake. A high recovery is an important quality feature for the experiment. The recovery depends on the accuracy of the analysis and the behaviour of the test compound, and should generally be higher than 90% of intake. The digestibility or absorption coefficient can be calculated on the basis of the amount of unabsorbed test compound, which is consequently

present in residues and in ileal delivery (Chapters 4, 6 and 7). Another possibility is to calculate the digestibility from the amount of absorbed nutrient in the dialysate liquids. In *in vivo* experiments digestibility coefficients are generally expressed as a percentage of intake calculated with the use of a meal marker during a period of consecutive feedings (23,24). In the model, absorption of the nutrient is generally evaluated 6 hours after ingestion of the meal. During this period not all of the meal has travelled through the gastric and small intestinal compartments. Therefore, it is better to express the results for the model as a percentage of the delivery of a marker. For homogeneous (semi-)liquid meals the virtual meal marker (VMM) calculated by the control program can be used (Chapters 6 and 7). For example, the absorbed nutrient in the dialysate can be expressed as a percentage of duodenal delivery of the VMM, or the ileal delivery of the nutrient can be expressed as a percentage of delivered VMM. For meals that show gastric retention, the VMM cannot be used because this parameter does not anticipate retention of the nutrient. In such cases a physical meal marker can be applied with a similar transit as the nutrient in the meal as is used during *in vivo* experiments. The ileal delivery of the VMM can also be calculated with the gastric delivery curve of the nutrient, measured in separate experiments with only the gastric compartment (Chapter 6). Another approach to assess the absorbability or digestibility of a nutrient is to express the amount of absorbed nutrient as a percentage of an optimally absorbable marker. This method has the advantage that variations in the absorbing system are eliminated.

With *in vivo* experiments generally the apparent digestibility of a nutrient is determined because the contribution of secreted nutrient is variable and can only be measured with special techniques such as radioactive labelling of the nutrient in the meal.

In the model the true digestibility of a nutrient is determined, because there is no variation of secreted nutrient in response to the meal. To determine the contribution of secreted nutrient in the samples, blank experiments are performed without the meal. True digestibility is calculated from the difference between results from the blank experiments and those with the nutrient. These results can be compared with true digestibility data measured *in vivo*. True digestibility is not necessarily the same as digestibility of the dietary nutrient. Experiments with labelled protein have revealed that, due to interaction with other meal components, the absorption of secreted protein nitrogen is influenced by the food, which might lead to underestimation of the digestibility of dietary protein (4).

Sometimes additional blank experiments can be avoided by measuring the amount of secreted test compound during the experiment. This fraction, together with the amount of test compound in the meal, is described as total input. Thus, the amount of compound in samples from the model can be expressed as a percentage of total input. Generally, dialysis- and ileal delivery samples are taken at regular intervals to assess the kinetics of digestion and absorption in the model. The results can be further analysed with computer modelling.

Analysis in the colonic model is normally related to the microflora and its activity. The composition of the microflora is traditionally determined by enumeration on elective and selective media. However, although the colon contains numerous microbial species, only the main species can be determined by enumeration. Modern techniques have been introduced that allow for a closer look, and therefore contribute to our insight into the effect of ingested compounds on the microflora (7). Such techniques include DNA probes (10), fluorescence *in situ* hybridization (9), polymerase chain reaction (27), and denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis (15).

Fermentation products generally analysed are short-chain fatty acids, lactic acid, ammonia and gases (Chapter 8). Although these are main products, there is also a need to detect more obscure products that might affect the health of the host. Analytical methods such as Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry allow to compare fermentation patterns and to detect small differences that might be relevant.

COMPUTER MODELLING

The dynamic model combines some of the complexity of the *in vivo* situation with the reproducibility and accuracy of a computer-controlled machine. Results obtained with the model are the result of 'hard' parameters controlled by the computer, such as meal transit, and 'soft' parameters intrinsic to the test compound, such as digestibility, coagulation and interactions among food compounds. Computer simulation with a program that exactly reproduces the algorithms used to control the hard parameters can be an interesting tool to study the influence of soft parameters on the measured results. This is demonstrated by the study with calf milk replacers (Chapter 6). The coagulation of casein in the gastric compartment could be assessed by comparing the controlled theoretical gastric delivery with the measured gastric delivery of nitrogen.

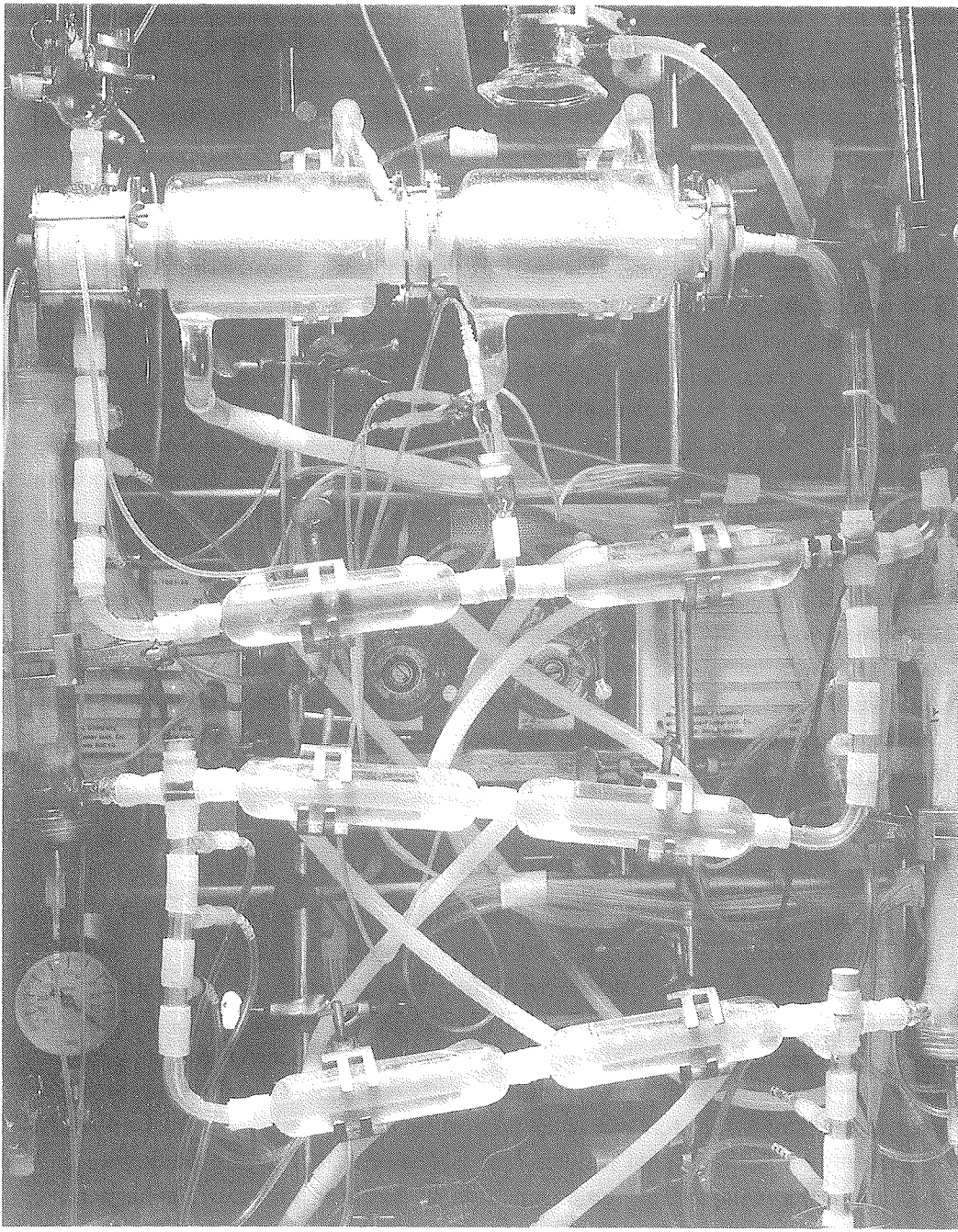

Fig. 7. Desktop prototype of the gastric / small intestinal model.

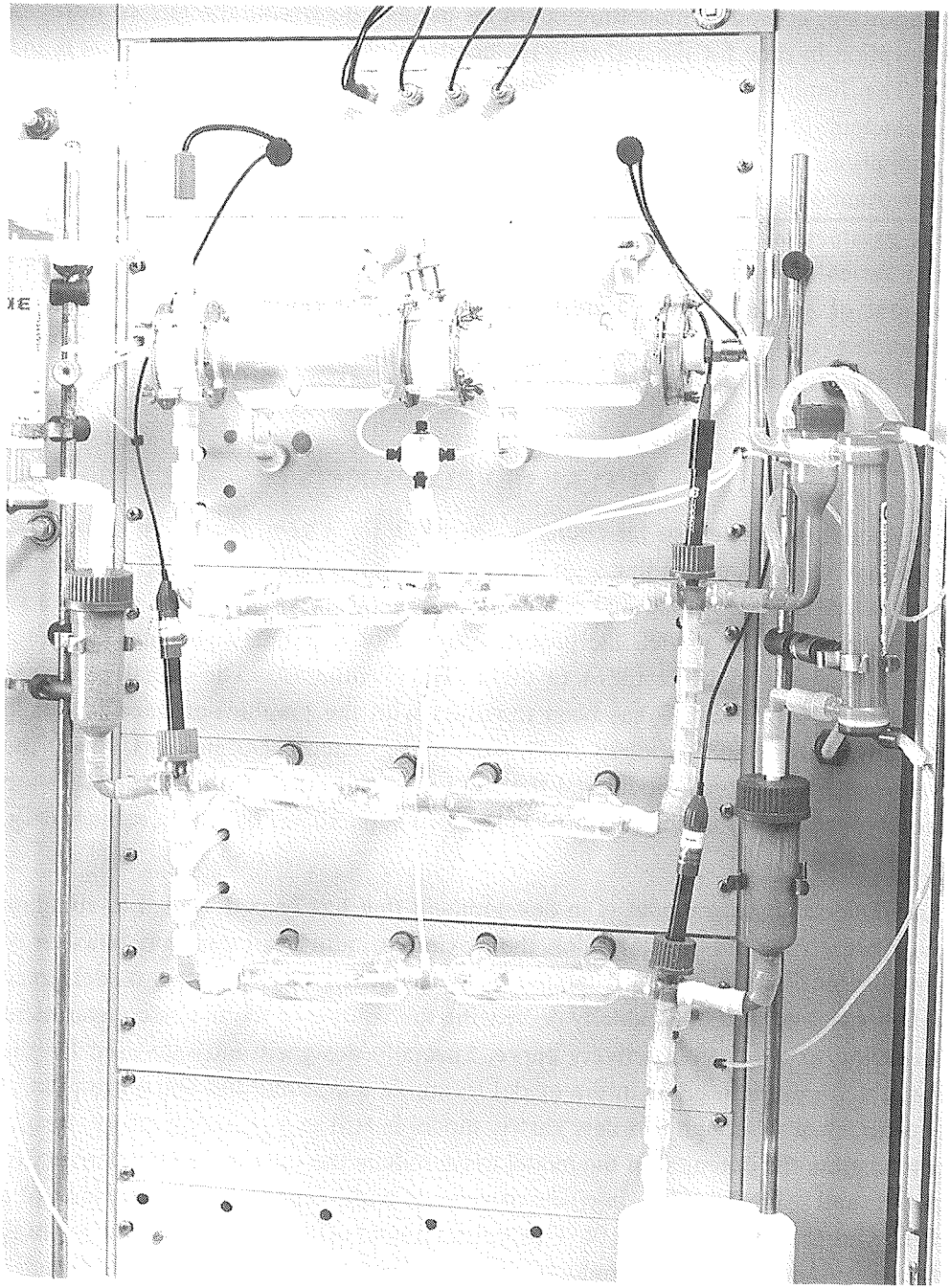

Fig. 8. Cabinet version of gastric / small intestinal model.

In addition, computer modelling could be used to determine the rate of nitrogen absorption in time for the calf milk replacers as well as pig feeds (Chapters 6 and 7). The limitation to modelling lies in the fact that the weight of individual parameters involved is often unknown. This results in 'black boxes' where assumptions are made for a combination of parameters. The complexity of the *in vivo* situation implies that many parameters have to be combined in black boxes. The parameters in the model are limited to luminal availability, which includes the first series of events involved in the uptake of compounds (Fig. 3). The limited amount of soft and hard parameters and their accessibility makes the model a promising tool to improve computer models that predict, for example, the bioavailability of specific compounds *in vivo*.

FUTURE DEVELOPMENTS

The model developed so far could be the basis for a new generation of *in vitro* models of the GIT, expanding with new technologies and ideas, to keep up with the demands of the feed, food and pharmaceutical industries. During the six years of development of the model, the processor speed of a standard personal computer increased from 25 MHz (386) to 200 MHz (Pentium®). The initial computer program in GWBASIC® has been rewritten with the graphics-oriented language DELPHI® for the Windows 95® operating system. During this time, the gastric/small intestinal model and the colonic model have evolved from table-top prototypes (Fig. 7) to sophisticated laboratory apparatuses built in a cabinet (Fig. 8).

An advanced gastric model is in development that has been designed to simulate the storage function of the fundus, the mixing, grinding and transport function of the antrum, and the sieving function of the pylorus (Fig. 9). In this model, meal delivery is regulated by gradually decreasing the volume of the fundic compartment according to the gastric delivery curve. Specially designed units are used for the antral and pyloric regions in the model to mimic antral motility and antro-pyloric coordination. Although this new gastric model is still in a prototype phase, results demonstrate the potential of the model to reproduce the separate gastric delivery of liquids and solids based on *in vivo* experiments with volunteers after ingestion of a meal with ground beef (Fig. 10). This gastric model offers the opportunity to study the behaviour of ingested products, such as tablets and capsules, in the stomach while mimicking a specific controlled situation.

Plans are being drawn up to develop new models that mimic the GIT of the chicken and ruminants based on the newly developed technology. In the future, smaller models will be designed to allow experiments with less reagents and test material, and to improve the efficiency of laboratory space.

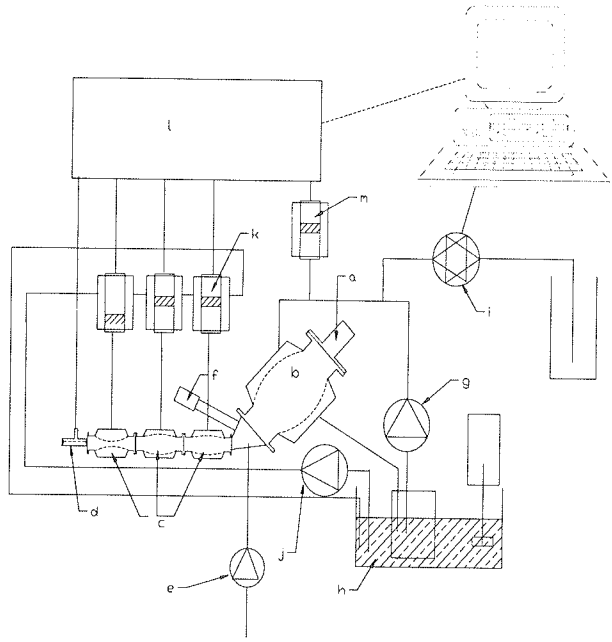

Fig. 9. Schematic drawing of the advanced gastric model.

a: inlet; b: fundic part; c: antral part; d: pyloric part; e: secretion pump; f: pH electrode; g: water circulation pump; h: waterbath; i: contraction pump; j: control unit; k, m: contraction controllers; l: pneumatic controller

The models will be further developed to include automated cleaning, filling and sampling. Also, a system is in development to monitor and control the model from a distance by a modem. A 'watch dog' system is in development that will monitor the processes and initiate a warning procedure when something goes wrong. In the near future, specific modules for absorption of lipophilic compounds, gut wall

simulation and liver function will be connected to the model to increase its applicability.

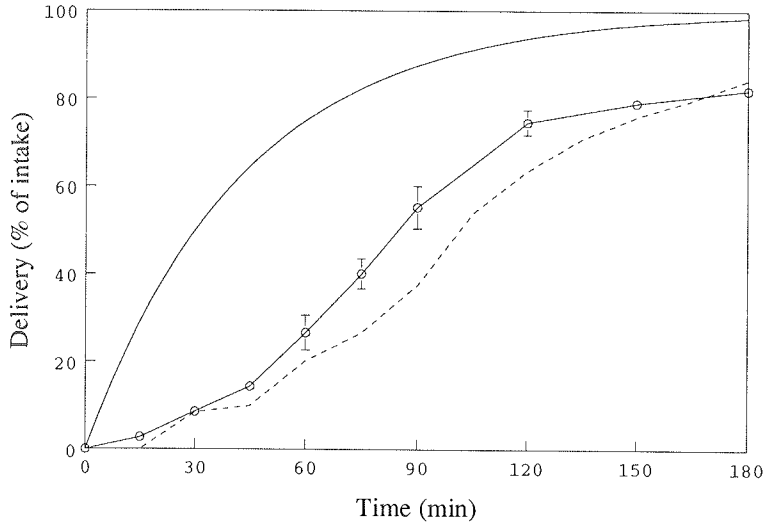

Fig. 10. Liquid (solid line) and solid delivery of a meal with ground beef from the advanced gastric compartment (O) and the solid delivery from the same meal in vivo (dotted line; 3).

CONCLUDING REMARKS

Tools such as the model presented in this thesis can improve our insight into the fate of ingested compounds in order to improve foods, feeds and pharmaceuticals and to evaluate their effects on health. In addition, the model will help reduce the use of test animals, especially in the pre-screening phase during product development.

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Summary

This thesis describes the development and testing of a gastrointestinal model that simulates as realistically as possible the conditions prevailing in the gastrointestinal tract (GIT) of man and monogastric animals. Such a model can be used to evaluate accurately the nutritional value of foods and the efficacy of drugs and, in addition, their mechanisms of action and safety.

The introduction (Chapter 1) presents a picture of the GIT and relevant aspects to be simulated with the model. Further, Chapter 1 presents an overview of models currently being used for studying the digestion of foods in the small intestine and models used to study the behaviour of bacteria in the large intestine. These models are tested in this chapter against conditions for realistic simulation of conditions in the GIT. It is concluded that existing models have the important drawback of being static, i.e. they are not able to mimic changes with time in the conditions foods are exposed to during their passage through the GIT. In practice, foods are transported gradually through the GIT. During their passage, conditions change as does the composition of gastric and intestinal contents under the influence of the action of digestive juices, digestion and absorption of nutrients and absorption of water. This thesis describes a model that mimics as realistically as possible these changing conditions across the GIT. In this dynamic model, the gastric and intestinal contents are mixed and driven forward by kneading movements.

Chapter 2 describes this dynamic model and demonstrates the accuracy and reproducibility of the model in simulating the conditions of someone eating yoghurt. The model for the stomach and the small intestine consists of a gastric compartment and three compartments for the small intestine. A computer program governs mixing of gastric and intestinal contents, the addition of digestive juices and the absorption of water. Because these functions vary both by animal species and by type of meal, for each application a specific protocol is drafter which can be read by the computer. In implementing the protocol all settings are combined, thus resulting in a dynamic process. For a proper functioning of digestive enzymes and to simulate the intestinal contents realistically, it is essential to have (digested) food components absorbed. This absorption is mimicked by means of hollow-fibre membranes coupled to some intestinal compartments.

The advantages of a dynamic gastric model are described in Chapter 3. The effects of such parameters as the gastric juice profile and gastric emptying on the effectiveness of the enzyme phytase were studied. Phytase is used as a feed additive to break down phytate and thus to make the phytate-bound phosphorus

available to animals such as pigs. Gastric conditions can be simulated accurately and reproducibly. Moreover, one single parameter can be changed in the model without altering other variables. This offers many opportunities for studying specific conditions in the stomach that may influence the action of the added phytase. In this study computer simulation techniques were used to further examine the measured effects.

Chapter 4 describes the use of the model for predicting the absorption of iron and phosphorus derived from cereals. Experiments were conducted with and without phytase added to several cereals. In grain types that did not naturally contain phytase, the bioavailability of iron and phosphorus was markedly improved by the addition of phytase. Phytase metabolizes phytate in cereals and thus counteracts the phosphorus-binding action of phytate. Results of experiments with the model appeared to correspond with results of *in vivo* experiments.

Chapter 5 describes a comparative study of survival of lactic acid bacteria strains in the model of the stomach and the small intestine and their survival in volunteers. The conditions in the model as laid down in the protocol mimicked as closely as possible average conditions in the volunteers. The survival rate of lactic acid bacteria in the model was quite similar to survival *in vivo*. As these bacterial strains are differentially sensitive to gastric juice and bile, the results indicate that the conditions the bacteria are exposed to in the model are comparable to those *in vivo*. The results of this study, like those in the previous study, demonstrate that one specific condition, i.e. bile concentration, can be changed in order to evaluate the effect of bile concentration on survival of lactic acid bacteria.

The settings of the model were chosen such as to represent the stomach and small intestine of the calf (Chapter 6) and the pig (Chapter 7) to compare digestion protein from calf milk replacers and pig feed, respectively, with results obtained with cannulated calves and pigs. Both gastrointestinal passage and the protein digestibility coefficients of the feeds tested were quite similar for the model and the cannulated animals. The decomposition of the various feeds with time could be closely examined through computer-aided calculations of events in the model and to compare the outcome with the measured values.

In Chapter 8 the model for the colon is introduced which can be used to study the effects on undigested compounds on intestinal flora. In this model water and bacterial excretion products are absorbed via hollow-fibre membranes across the model. This approach allows of high bacterial densities approximating physiological conditions. The concentrated contents of the large intestine are mixed and moved forward through kneading movements. The computer-controlled model allows of feeding, water absorption and mixing of intestinal contents

according to a standardized protocol. Because acid production is monitored by the computer the course of carbohydrate fermentation can be followed. The stability, composition and metabolic activity of intestinal microflora were studied in this model after inoculation with a standard microflora from a fermentor or with human faeces. Microbial composition and activity appeared to be stable for both inoculation methods, whereas counts of relevant groups of bacteria corresponded with those found in man.

The studies described in this thesis demonstrated that the newly developed dynamic model offers interesting possibilities for standardized studies of substances in the GIT under conditions strongly resembling those prevailing in man and in animals. The model's limitations are mainly in the fact that only processes in the intestinal lumen can be simulated. The complex processes in the intestinal wall such as specific absorption mechanisms and interactions with micro-organisms cannot be mimicked in the model. However, because the availability of food components is determined to a large extent by conditions in the gastrointestinal lumen, the model is still a very useful research tool. Future developments of the model will allow of coupling pieces of intestinal wall tissue or cell cultures with the model, thus enabling additional studies. The use of this model may effect a reduction in use of animals in experiments, in particular in the pre-screening phase on product development.

Samenvatting

Dit proefschrift beschrijft de ontwikkeling en het uittesten van een maag-darmmodel dat de omstandigheden in het maag-darmkanaal van de mens en van éénmagige dieren zoveel mogelijk nabootst. Zo'n model kan worden gebruikt om nauwkeurig te onderzoeken hoe voedingsmiddelen en medicijnen t.a.v. hun voedingswaarde of effectiviteit kunnen worden verbeterd. Tevens kunnen deze producten worden onderzocht op werkingsmechanismen en veiligheid.
In de inleiding van het proefschrift (hoofdstuk 1) wordt een beeld gegeven van het maag-darmkanaal en welke aspecten belangrijk zijn om na te bootsen met een model. Vervolgens wordt een overzicht gegeven van bestaande modellen die gebruikt worden voor het bestuderen van de vertering van voedingsstoffen in de maag en dunne darm en van modellen die gebruikt worden voor het bestuderen van het gedrag van bacteriën in de dikke darm. Deze modellen worden in deze bespreking getoetst aan voorwaarden die zijn gesteld voor het goed nabootsen van de omstandigheden in het maag-darmkanaal. Hierbij komt naar voren dat een belangrijk nadeel van de huidige modellen is dat ze statisch zijn. Dat wil zeggen dat deze modellen de in de tijd veranderende condities, die de voeding ondergaat tijdens de passage door het maag-darmkanaal, niet goed nabootsen. In werkelijkheid wordt de voeding geleidelijk door het maag-darmkanaal getransporteerd. Hierbij veranderen de omstandigheden en de samenstelling van de maag-darminhoud door de secretie van spijsverteringssappen, de vertering en de absorptie van nutriënten, en de absorptie van water. Dit proefschrift beschrijft de ontwikkeling en toetsing van een model dat deze veranderende omstandigheden in de verschillende delen van het maag-darmkanaal zoveel mogelijk nabootst. Ook wordt de maag-darminhoud in het model met knedende bewegingen gemengd en voortbewogen.
In hoofdstuk 2 wordt het dynamisch model beschreven en wordt getoond hoe nauwkeurig en reproduceerbaar dit model onder andere de condities nabootst van iemand die een beker yoghurt eet. Het maag-dunne darmmodel bestaat uit een maag compartiment en drie compartimenten van de dunne darm. De menging van de maag- en de darminhoud, de toevoeging van spijsverteringssappen, de zuurtegraad in de verschillende compartimenten, het transport van de voeding en de absorptie van water wordt door een computerprogramma geregeld. Deze functies zijn per diersoort en per type maaltijd verschillend en worden daarom voor elke toepassing vastgelegd in een protocol dat kan worden ingelezen door de computer. Bij uitvoering van het protocol worden alle instellingen gecombineerd,

waardoor een dynamisch proces omstaat. Voor een goede werking van de spijsverteringsenzymen en om de samenstelling van de darminhoud zo goed mogelijk te simuleren is het belangrijk om (verteerde) voedingsbestanddelen te absorberen. Dit wordt nagebootst met behulp van systemen met holle vezel membranen die aan enkele dunne darm compartimenten zijn gekoppeld.
De voordelen van een dynamisch maagmodel worden beschreven in hoofdstuk 3, waarin het effect van parameters zoals het maagzuurprofiel en de maaglediging op de effectiviteit van het enzym fytase wordt bestudeerd. Fytase wordt gebruikt als toevoeging aan onder andere varkensvoeders om fytaat af te breken waardoor het hieraan gebonden fosfor beschikbaar komt voor het dier. De omstandigheden in de maag kunnen nauwkeurig en reproduceerbaar worden nagebootst. Bovendien kan in dit model één enkele parameter veranderd worden, terwijl de overige parameters gehandhaafd blijven. Dit biedt vele mogelijkheden om te kijken naar specifieke omstandigheden in de maag die invloed hebben op de werking van het toegevoegde fytase. In dit hoofdstuk worden ook computersimulatie-technieken gebruikt om de gemeten effecten nader te kunnen bestuderen.
Hoofdstuk 4 beschrijft het gebruik van het model om de absorptie van ijzer en fosfor uit granen te voorspellen. Experimenten werden uitgevoerd met en zonder toevoeging van fytase aan verschillende granen. Het bleek dat de beschikbaarheid van ijzer en fosfor uit granen die niet van nature fytase bevatten aanzienlijk verbetert na toevoeging van fytase. Het fytase breekt het fytaat in de granen af, waardoor de ijzer en fosfor bindende werking van fytaat wordt opgeheven. De resultaten van proeven met het model bleken goed overeen te komen met resultaten uit *in vivo* experimenten.
In hoofdstuk 5 wordt een studie beschreven waarin de overleving van enkele verschillende soorten melkzuurbacteriën in het maag-dunne darmmodel worden vergeleken met de overleving van dezelfde bacteriën in vrijwilligers. De condities in het model, vastgelegd in het protocol, waren zoveel mogelijk identiek aan de gemiddelde condities in de vrijwilligers. De mate van overleving van de melkzuurbacteriën in het model vertoonde grote overeenkomst met die *in vivo*. Omdat deze bacteriën in verschillende mate gevoelig zijn voor maagzuur en gal, toont het resultaat aan dat de blootstelling aan de condities in het model vergelijkbaar is met die *in vivo*. Evenals de vorige studie toont ook deze studie aan dat in het model een specifieke conditie, in dit geval de galconcentratie, kan worden gewijzigd, waardoor het effect van de galconcentratie op de overleving van de melkzuurbacteriën kan worden onderzocht.
In de hoofdstukken 6 en 7 wordt de eiwitvertering van kalvermelkvervangers en varkensvoeders in het model, ingesteld als maag-dunne darm van respectievelijk

kalf en varken, vergeleken met de resultaten verkregen uit studies in gecannuleerde kalveren en varkens. Zowel de maag-darmpassage als de eiwitverterings-coëfficiënten van de geteste voeders, bepaald met het model, vertoonden grote overeenkomst met die in de dieren. De afbraak van de verschillende voedingen in de tijd kon nauwkeurig worden bestudeerd door de gebeurtenissen in het model met een computer na te rekenen en dit te vergelijken met de gemeten resultaten.
In hoofdstuk 8 wordt het colonmodel geïntroduceerd, waarmee het effect van onverteerde stoffen op de darmflora kan worden bestudeerd. In dit model vindt absorptie van water en bacteriële uitscheidingsproducten plaats via, door het model lopende, holle-vezelmembranen. Hierdoor kunnen hoge bacterieconcentraties worden bereikt die de werkelijkheid dicht benaderen. De geconcentreerde dikke darminhoud wordt gemengd en voortbewogen door kneedbewegingen. De computersturing van het model maakt het mogelijk om de voeding, de absorptie van water en de menging van de darminhoud volgens een gestandaardiseerd protocol uit te voeren. De zuurproductie wordt door het computer programma bijgehouden, waardoor het verloop van de fermentatie van koolhydraten kan worden gevolgd. De stabiliteit, samenstelling en metabole activiteit van de microflora werden in dit model bestudeerd na het beënten met een standaard microflora uit een fermentor of met faeces van mensen. De samenstelling en activiteit van de microflora bleken voor beide beëntingsmethoden stabiel, terwijl tellingen van belangrijke groepen bacteriën goed overeenkwamen met die in mensen.
Het onderzoek beschreven in dit proefschrift heeft aangetoond dat het ontwikkelde dynamische model interessante mogelijkheden biedt voor gestandaardiseerd onderzoek aan stoffen in het maag-darmkanaal onder omstandigheden die sterk overeenkomen met die in mens en dier. De beperkingen van het model liggen voornamelijk in het feit dat met het model alleen processen in het lumen van de darm kunnen worden nagebootst. De ingewikkelde processen in de darmwand zoals specifieke absorptiemechanismen en de interacties met micro-organismen kunnen nog niet worden nagebootst. Echter, omdat het beschikbaar komen van voedings-componenten in belangrijke mate wordt bepaald door de omstandigheden in lumen van het maag-darmkanaal kan het model toch een belangrijk hulpmiddel zijn voor onderzoek. In de toekomst kunnen stukjes darmwandweefsel of celcultures aan het model worden gekoppeld waardoor nog meer onderzoek mogelijk is. Door toepassing van het model kan, speciaal in de pre-screeningsfase bij productontwikkeling, het aantal proeven met dieren worden verminderd.

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Curriculum Vitae

De auteur van dit proefschrift werd op 1 januari 1957 geboren in Deventer. Na het behalen van het HAVO diploma in 1974 aan de Rijksscholengemeenschap "Schoonoord" te Zeist, volgde hij tot 1977 een opleiding tot medisch microbiologisch analist aan het Dr. Ir. W.L.Ghijsen instituut te Utrecht. Na de militaire dienst, doorgebracht als analist in het Militair Hospitaal te Utrecht en een korte carrière als uitkerings-deskundige bij de Bedrijsvereniging voor de Gezondheid in Zeist, kwam hij in 1980 bij TNO-voeding te Zeist in dienst als microbiologisch analist. Al snel gingen zijn werkzaamheden richting het besturen van fermentatie processen en het produceren van nuttige producten met microörganismen. In 1988 behaalde hij het deelcertificaat Biochemie aan de Hoge School Utrecht en volgde in 1991 de cursus "Advanced Course on Microbial Physiology and Fermentation Technology" aan de Technische Universiteit van Delft. Vanaf 1992 werkt de auteur aan de ontwikkeling van het, in dit proefschrift bescheven, maagdarm model. Begin 1996 ontving hij hiervoor de "TNO-erkenning", een prijs die wordt uitgereikt voor een uitzonderlijke bijdrage aan de TNO doelstellingen.

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Stellingen

1. Een maagdarm kanaal nabootsen met een statisch model is een rivier nabootsen met een vijver.

2. A lot of large intestinal models are just a piece of shit.

3. Helaas betekent CLAIM voor functional foods nog vaak: Cannot Locate Any Interesting Mechanism.

4. TIM schnabbelt reeds aardig bij als foto-model.

5. De blues is nader dan de rock.

6. Witte blues wordt vaak zwart gespeeld.

7. Op een slappe snaar valt niet te spelen, maar een te strakke snaar breekt snel. (Boedhistische wijsheid)

8. Wie af en toe niet stil staat en omkijkt, komt niet vooruit.

9. Testosteron maakt meer kapot dan je lief is.

10. Met een Landrover hoef je niet uit te wijken voor een Eland.

11. Een poep-machine is nergens voor nodig, je kunt toch zelf wel poepen! (Dyko Minekus, 20-1-92)

Stellingen behorende bij het proefschrift "Development and Validation of a Dynamic Model of the Gastrointestinal Tract" van Mans Minekus, 28 mei 1998.