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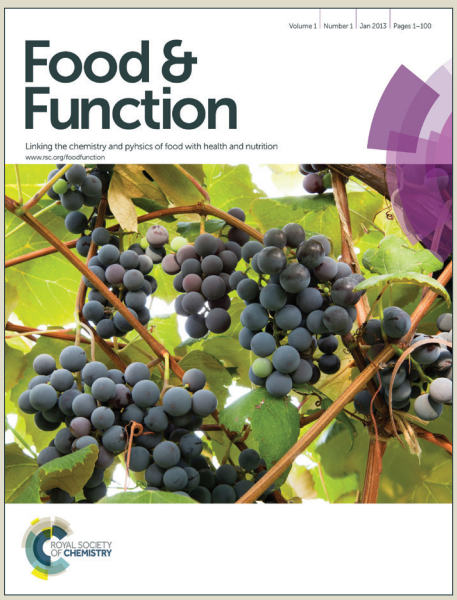

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Bi-compartmental elderly or adult dynamic digestion models applied to interrogate protein digestibility

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Abstract

The world's population is inevitably ageing thanks to modern progress; yet, the development of food and oral formulations tailored to the needs of the elderly is still in its infancy. *In vitro* digestion models offer high throughput, robust and practically ethics free evaluation of the digestive fate of ingested products. To date, no data has been made publicly available as to facilitate the development or application of an *in vitro* model mirroring the physicochemical conditions of the elderly gastro intestinal system. This study reports the development of a novel and highly bio-relevant *in vitro* model based on two serially connected bioreactors recreating the dynamic conditions of the adult or elderly alimentary canal. This report and its supplementary material describe in detail the set-up of the system, the physicochemical parameters applied and the development of the controlling software. These are intended to openly depict a versatile platform which could assist future efforts to develop age-tailored oral formulations. SDS-PAGE analyses of samples collected from *in vitro* digestion of beta-lactoglobulin, alpha-lactalbumin and lactoferrin suggest the bioaccessibility of "slow digesting" and "fast digesting" proteins identified in adult models do not necessarily maintain this trait under elderly gastro-intestinal conditions. Overall, this study brings forward a new generic yet advanced model that could help shed light into the underlying principles which could facilitate age-tailoring the digestive fate of liquid formulations.

Key words: Ageing, *In vitro* digestion, Proteolysis, Bioreactors

1. Introduction

Numerous agencies worldwide, including the WHO and the UN have identified that the world health population is tremendously ageing [1]. In light of the identified changes in the human gut physiology with age, it is important to help food manufacturers, scientists and health care professionals generate viable alimentary and pharmaceutical solutions that could help tackle the ill-symptoms and disorders of ageing. Such new edible alternatives should not only help extend and support human life but also improve its quality. Thus, rational design of food systems to meet the needs of consumers could be well advanced if generic, bio-relevant, robust and high throughput *in vitro* digestion models were made available, as advocated by various researchers[2-8].

A large collection of evidence shows that ageing is accompanied by a compromised quality of life, deteriorated physical fitness, inadequate food intake, reduced appetite, increased prevalence of chronic diseases as well as various changes in gut function, as recently reviewed[9]. Such studies specifically report that the geriatric population has marked changes in various gastrointestinal secretions and composition of various digestive components, starting from concentrated saliva, through reduced pepsin levels in the stomach, altered intestinal secretions (i.e. bile and pancreatic secretions) down to unique changes in the colon microbiome [10-15]. Due to the irreversible nature of these physiological changes, bio-processing and manufacturing could be re-thought to ensure adequate tailoring of foods and drugs to meet the geriatric needs.

In this respect, proteins and specifically milk proteins are macronutrients that have been identified as key nutrients affecting geriatric health and well-being [16]. Moreover, it is increasingly recognized that alimentary proteins can modulate various biological functions and consequently human health through the generation of bioactive peptides which may possess antihypertensive, opioid, immunomodulatory, antibacterial or even bifidogenic activities[17, 18]. Furthermore, recent

studies even suggest dairy processing, i.e. fermentation, may extend and enhance the ability of dairy products to affect human health through the bioactivity of peptides[19].

Amongst the many obstacles towards age-tailored foods and personalized nutrition, understanding the digestive fate of foods and drug formulations is an inevitable yet elemental hurdle which could be tackled through *in vitro* digestion methods. This vivid field of research has already facilitated reconstruction of various aspects of the human alimentary canal. These models have guided the elucidation of the digestive fate of proteinaceous systems in healthy adults and even infants [4-6, 20-25]. Yet, no publicly-available data could be found on the development or application of an *in vitro* model mirroring the physicochemical conditions of the elderly gastro intestinal (GI) system. Thus, this study sought to develop a novel and highly bio-relevant *in vitro* model which would recreate the physicochemical conditions of the elderly gastro intestinal tract (GIT). The **main goal** of this research was to identify the physicochemical parameters unique to the elderly GI system, integrate them into a new bi-compartmental digestion model and apply it to investigate the digestive fate of a defined set of whey proteins. This was pursued under the hypothesis that the specific conditions of the aging GIT lead to modulated breakdown of proteins compared to their degradation in the healthy adult GIT, which is commonly used as a golden standard.

2. Methods and Materials

2.1. Development and application of the digestion models

The bi-compartmental digestion model developed in this study was constructed from two mini-bioreactor units, as outlined in **Figure 1**. These two bioreactors were serially connected through a silicon tube which passed through one of the peristaltic pumps of the first bioreactor controller unit. To enable bio-relevant mirroring of the dynamic characteristics of gastro-duodenal digestion, this model comprised of two continuous stirred tank reactors (CSTR) which were computer controlled

through a specialized program. The first bioreactor (V1) was defined as the gastric chamber and was controlled for its mixing, pH gradient and emptying into the second bioreactor. The second bioreactor (V2) was defined to mimic a duodenal compartment and was controlled for its mixing, pH and bile secretion gradient through the customized software program. Altogether, the model was designed and programmed to mimic either the gastro-duodenal digestion of a healthy adult or a healthy elderly person (defined as 75 years old).

Practically, two commercially available mini-bioreactor 250mL units (MiniBio, Applikon biotechnology, Netherlands) were serially connected through a silicon tube (115 cm in length, Medent, Israel cat. 054-010030, pre-calibrated according to the manual procedure of the bioreactors), filled with simulated digestive fluids and maintained at 37°C through "*my-Control*" software version 1.0X (Applikon, Netherlands). Experiment time was set to be a total of 2h from the initiation of the gastric phase in the adult model (or 3h for an elderly model) and samples could be aseptically collected from each bioreactor through a designated tubing system located in the vessel head plate. V1 was controlled through the controller panel of the bioreactor and the customized software program developed using the *"BioXpert*" V2 software Version 2.93 (Applikon, Netherland) which also controlled V2. Feeding of acid, alkali or bile secretions and drainage of digesta from V1 into V2 were performed through peristaltic pumps located on the controller units equipped with silicon tubes and commanded through the "*BioXpert*" software. Pancreatic secretions were injected into V2 by the operator in two doses, based-on physiological information[11]. This software not only regulated the experimental conditions but also recorded all measurements, i.e. volumes pumped through each peristaltic pump and all of the input from the temperature and pH sensors.

Post-prandial gastric pH gradient measured in healthy adults[12] was programmed to be generated through two peristaltic pumps (pump 1 and 2 included in the Applikon bioreactor controller 1) using HCl and NH_4HCO_3 to obtain a gradual pH drop between 4.5 to 1.5 (or 6.2 to 2.0 in an elderly model)

during the course of an experiment (demonstrated in **Figure 2A**). Gastric mixing and emptying are of great importance to chyme breakdown and transit, thus, both parameters were accounted for in V1. An average mixing profile of one to two mixing events per min, each pulse of 200 RPM (or 100 RPM for the elderly model) was defined, as to concur with the gastric contractions measured *in vivo*[26, 27]. An additional peristaltic pump was programmed to drain chyme from V1 into V2 according to the physiologically determined gastric emptying, also known as the Elashoff equation[28]:

[1] $f = 2^{-(t/t_{1/2})^{\beta}}$

Where f is the fraction of the meal remaining in the stomach at time t, t is the time from the beginning of the meal, $t_{1/2}$ is the time at which one-half of the meal has emptied and β is the coefficient describing the shape of the curve. This equation describes gastric volume remaining after initiation of emptying into the duodenum (demonstrated in **Figure 2B)**. This equation was derivatized into the following equation:

[2] $f' = \frac{\beta * log2}{t_{1/2}} * \frac{2^{(t/t_{1/2})^{\beta}}}{t^{1-\beta}}$

This equation was used to define the rate of gastric emptying through the pylorus and was applied to software programing of the peristaltic pump, taking into account a 5 min delay from the initiation of the experiment until initiation of gastric emptying, to concur with *in vivo* findings related to liquid formulations[12]. In V2, gastric chyme was neutralized to pH of 6.1 (or pH of 6.5 in the elderly model) using ammonium bicarbonate. Dynamic secretion of bile into duodenal compartment (demonstrated in **Figure 3**) was performed according to physiological data derived from a human study[13, 29]. Further details on the computer programming and application of the mathematical definitions can be found in the supplementary material.

Remodeling of the system to reflect an elderly person. The developed bi-compartmental model was adjusted to mirror the physiological conditions of the elderly population (defined as 75 years old) through the conversion of the software parameters to meet the physiological parameters of the aged GI system. In order to identify the physicochemical parameters of the elderly GIT and breach gaps in current pH-stat methods, a literature survey was performed on two major databases: PUBMED and ISI Web of Science. This survey was specifically targeted to realistic physiological data gathered through adequate human studies and followed an initial screening of search results which scoured through 44 papers that were identified. Exclusion criteria were then defined to be subject characteristics (age, number and type of background medications and cohort size). Specifically, mean subject age was set to be 75 and no less than 70, number and type of background medications was defined as two: medication for hypertension and for hypercholesterolemia (which are vastly prescribed in western countries) and cohort size was sought to exceed 20 subjects. Following the application of these exclusion criteria, only 8 studies were found suitable, with cohort sizes of up to 206 subjects[10-14, 29-31]. These articles were used to further refine the adult model and to develop the elderly gastro-duodenal model, as detailed and justified in **Table 1**. In practice, the elderly model was adjusted to account for the distinct elderly gastric mixing, gastric pH gradient (**Figure 2A**), gastric emptying (**Figure 2B**), duodenal pH and mixing, pancreatic secretion, bile secretion (**Figure 3**) as well as divergence in biochemical composition of the luminal content. In this respect, simulated gastric (SGF), duodenal (SDF) and bile (SBF) fluids and enzymatic levels were adjusted to relevant physiological levels which are also described in **Table 1** and **Table 2**. Moreover, saliva ionic composition, gastric lipase levels as well as amylase activities (in saliva and pancreatic secretions) occurring in the elderly were identified[30, 31] but unaccounted for in this model due to its scientific focus on proteolysis.

Implementation of the digestion models to probe protein digestibility. Samples of 2.5% (w/v) protein solutions at pH 7.0 were prepared using double distilled water (DDW). A simulated bolus of 40mL along with 9 μL of $CaCl_2$ (4 M) were carefully injected through a designated opening in the head plate of bioreactor V1 which was pre-filled with 60 mL of SGF containing pepsin (1000 or 750 u/mL for adult or elderly, respectively) warmed up to 37°C. At this time, bioreactor V2 was filled with 10 mL of pre-heated SDF and kept at 37°C. Simultaneously, the pH of V1 was adjusted to 4.5 or 6.2 for adult or elderly model, respectively, and the *"Bioxeprt"* software was initialized. Once gastric empting into V2 was initiated, system operator introduced a burst of pancreatic enzymes (as detailed in **Table 2**) into V2 which was followed by a second dose of enzymes after 40 min, to ultimately obtain physiological enzymatic levels in V2. The first burst into V2 also contained 4 M $CaCl_2$ (3 or 6 μL for adult or elderly model, respectively) and was performed at the beginning of gastric emptying from V1 into V2. Throughout these digestion experiments, sample aliquots were aspirated after 6, 10, 30, 60, 120 minutes from V1 (representing gastric contents), and also after 180 minutes at the end of the elderly program. From V2 (representing the duodenum), samples were collected after 15, 30, 60, 120 minutes during the adult program and in addition after 180 minutes at the end of the elderly program. All gastric digesta samples were rapidly neutralized to pH 7 using freshly prepared 1M NH_4HCO_3, while duodenal digesta samples were inactivated using the irreversible serine-protease inhibitor PMSF (final concentration of 0.5mM PMSF). All samples were placed on ice and stored at -20°C until further analysis.

Evaluation of protein breakdown through SDS-PAGE. Qualitative evaluation of protein breakdown and peptide profiles in digesta samples was performed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Dilution of digesta samples for analysis was normalized to contain a fixed protein concentration which enabled adequate comparison between samples. Electrophoresis was performed using a 15% gel at 180V for 50 min in a Tris/Glycine/SDS

running buffer. Gels were then fixed in 30% (v/v) ethanol, 10% (v/v) acetic acid and 60% (v/v) DW, rinsed in DW and stained with Comassie Brilliant Blue R-250 (Bio-Rad, Rishon LeZion, Israel) and imaged using a Microtek 9800XL Plus scanner (Microtek, Carson, CA). All other chemicals used for SDS-PAGE analysis were from Bio-Rad Laboratories (Rishon LeZion, Israel).

2.2 Materials

Bovine lactoferrin (LF) (Vivinal lactoferrin FD, 95.6% protein) was kindly donated by DMV International (Delhi, NY, USA), food grade β-lactoglobulin (β-lg) (BioPURE Betalactoglobulin, 97.6% protein) and α-lactalbumin (α-lac) (Alpha-lactalbumin, 97.3% protein) were provided by Davisco Food International Inc. (Le Sueur, MN, USA). Pepsin (920 units/mg protein, cat. P7000), Trypsin (15008 U/mg protein, cat. T0303) and α-chymotrypsin (65.622 U/mg protein, cat. C4129) from porcine, Sodium glycodeoxycholate (cat. G9910), Taurocholic acid sodium salt hydrate (cat. T4009) and phenylmethylsulfonyl fluoride (PMSF, cat. P7626) were purchased from Sigma-Aldrich (Rehovot, Israel). All other chemicals used were of analytical grade and were used as received.

Simulated digestive fluids. This study used simulated gastric fluid (SGF), simulated duodenal fluid (SDF) and simulated bile fluid (SBF) which were made in DDW from stock solutions as described in detail in **Table 2**. These fluids were also adjusted to meet physiological ionic concentrations of the elderly (as detailed in **Table 2**)[13, 32]. Acid and alkali bottles were filled with 0.2M HCl and 0.5M NH_4HCO_3 and pumped into the respective bioreactors through peristaltic pumps located in the corresponding bioreactor controllers. Pepsin was dissolved in SGF, Trypsin and α-chymotrypsin were dissolved in SDF and kept on ice until use. Bile salts (Sodium glycodeoxycholate and Taurocholic acid sodium salt hydrate) were dissolved in 4.5 ml of SBF, 4M $CaCl_2$ was added according to physiological concentrations (detailed in **Table 2**) and this simulated bile secretion was pumped into the duodenal bioreactor through a designated peristaltic pump.

3. Results and discussion

In light of the growing need for foods and oral formulations that can meet geriatric needs and physiological capabilities, this work sought to develop a highly bio-relevant yet generic *in vitro* digestion system simulating the aged gut. First, a new bi-compartmental computer controlled set up was established based on the extensive knowledge reported in the literature on *in vitro* models recreating the healthy adult gut[5, 7, 8, 21]. This advanced system was made up of commercially available bioreactors and is described in detail herein as well as in the supplementary material. Based on this open and accessible platform, a comprehensive literature review was pursued to gain detailed quantitative information regarding the physicochemical parameters of the aged gut[9]. Thus, the distinct gastric pH gradients, enzymatic levels of pepsin, gastric mixing and gastric emptying found in the elderly were programmed into the control software. This model was also adjusted to address the duodenal pH, bile composition and secretion profiles as well as pancreatic composition in timed bursts which were all taken into account in the control of the second bioreactor mimicking the duodenum.

Once the *in vitro* elderly model was set up, the proteolytic breakdown of whey protein isolate, as a realistic product, was evaluated and outcomes of adult and elderly digestion (findings provided in the supplementary material) enabled determining protein dissipation during digestion alongside monitoring the breakdown patterns formed therein. These findings demonstrated that the continuous stirred tank reactor (CSTR) design of the model enabled portions of intact proteins to be introduced into the second CSTR mimicking the duodenum. This is believed to be a more realistic representation of digestion than common batch models in which gastric emptying is unaccounted for. The findings also substantiated that differences in protein breakdown and resistance occur between adults and the elderly; showing high similarity to the differences in the digestibility of whey proteins in adults versus infants[21]. Moreover, one could infer from these findings that fast-digesting or pre-

digested proteins would have better bioaccessibility and consequently could show improved performance in providing the elderly with amino acids. This notion is also supported by a recent study in which a diet containing fast-digesting, i.e. highly bioacessible and bioavialbaleproteins, improved the uptake of essential amino acids in elderly people aged over 70 years [33]. Therefore, further work sought to deepen our understanding of the comparative digestive fate of individual whey proteins, namely of β-lg, α-lac and LF in an adult versus an elderly model, with corresponding results given in **Figure 4**. Briefly, α-lac and LF were found to be fast-digesting in the adult model compared to β-lg. This concurs with previous *in vitro* and *in vivo* studies which found such proteins to have similar susceptibility to gastric proteolysis[21, 34]. Yet, in the elderly model β-lg was found to be more readily digested than both proteins which were found to endure even three hours of digestion. In addition, high MW bands (Mw>70kDa) were observed to appear in the elderly model, both in the gastric vessel and in the duodenal vessel. These protein bands could be attributed to some protein aggregates which are expected to be formed, as the gastric vessel pH values were initialized at 6.2 and dwell values around the pI of β-lg and α-lac (4.5<pH<5.5). In LF, such aggregates could be formed due to the combination of ionic strength and pH, which have been reported to alter LF's pI to about 6.0 [35]. The notion of protein aggregation is also supported by the dissipation of the high MW bands in the duodenal vessel (in which pH was constant and above 6.0).

Previous reports indicate the β-lg shows low enzymatic degradation under adult digestion conditions[21, 36]. β-lg duodenal proteolysis has also been shown to be retarded by physiological phospholipids such as phosphatidylcholine[37]. The low digestive breakdown of β-lg was also corroborated in this study which showed β-lg indeed survives gastric digestion and starts significant degradation only in the adult duodenum (**Figure 4A**). Further experiments are needed to increase the bio-relevance of these experiments through the use of phospholipids, as non-standard yet bio-relevant digestive components[7]. Application of the gentler elderly digestive conditions revealed that

β-lg susceptibility actually increased under these conditions (**Figure 4**B). This was found to be contrary to the trends observed for α-lac and LF (**Figures 4C, 4D, 4E** and **4F**) which were found to exhibit a sustained proteolysis under elderly GIT conditions. In respect to lactoferrin, it was also noted to generate distinct peptide bands during its breakdown in the elderly model. This could be of importance as LF has been identified as a precursor for some bioactive peptides [17, 18]. This study confirms that protein digestibility does vary with age due to the collection of irreversible changes in GIT function; however, susceptibility to proteolysis does not exhibit a generic trend for the whey proteins that were tested.

4. Conclusions

This study sought to generate a new bi-compartmental digestion model which could be used as a generic research tool in interrogating the digestive fate of liquid formulations. The detailed explanations of the system have been made readily available herein and as supplementary material in the hope that such a tool for controlled, systematic and mechanistic studies could prove highly useful in future attempts to develop age-tailored foods. The bio-relevance of this bi-compartmental model could be further increased and the versatility of the bioreactors offers many possibilities to do so, for example gradual pancreatic secretion or the incorporation of phospholipids. Such further modifications and improvements should carefully rely on human physiological data and take to mind complexity of experiments versus the scientific relevance of the modifications and their compatibility to the investigation at hand. The supplementary material also provides some other relevant information which was not included in this study, such as activity of lipases and amylases in the elderly as well as saliva composition. This information could prove useful in future studies of food digestion. Further, the application of this model to study protein digestibility enabled gathering of data suggesting the breakdown and bioaccessibility of proteins identified through adult digestion models do not necessarily maintain these traits under elderly GIT conditions. Overall, this study

highlights the need to extend and enhance the use of highly bio-relevant *in vitro* digestion systems to help put the development of age-tailored liquid formulations on a scientific basis.

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Table and Figure Captions

Table 1. Parameters of *in vitro* gastro-duodenal conditions for adult or elderly models.

Table 2. Composition of Simulated Gastric, Duodenal or Bile solutions (SGF, SDF and SBF, respectively) made up to 1000 ml solutions.

Figure 1. Schematic illustration of the bi-compartmental digestion model**,** highlighting computer and operator controlled parameters enabling recreating digestion dynamic events.

Figure 2 : Postprandial pH gradients (A) and gastric emptying (B) in the adult and elderly models

Figure 3 : Bile salts flow to V2 in the adult and elderly models

Figure 4. SDS-PAGE analyses of digesta samples collected during adult digestion [A] β-lactoglobulin, [C] α-lactalbumin and [E] lactoferrin or during elderly digestion of [B] β-lactoglobulin, [D] α-lactalbumin and [F] lactoferrin.

414 **List of Tables and Figures**

415 **Table 1**

Bioreactor conditions		Adult	Elderly	Justifications
Gastric bioreactor conditions				
Initial volume of SGF + Sample [ml]		100	100	
Stirrer rate [RPM] 20 sec pulses, 1-2 times per min in average		200	100	Pulsatile nature intended to recreate gastric constrictions, based on relevant reports[26, 27, 38].
pH gradient [t_0-t_{end}]		4.5-1.2	6.2-2	Adult values based on past reports[8, 23]. Elderly values based on a study of 79 healthy elderly people[12].
Enzyme levels	Pepsin [U/ml]	1000	750	Elderly values based on a past study[14] defined through percentage of activity and compared to healthy adult subjects[39].
Gastric empting [Elashoff equation[28] parameters] Beginning emptying after 5 min	$t_{1/2}$ [min] β	80.5 0.7	80.5 0.4	Based on a study comparing GI transit between elderly and young adults[10]
Intestinal bioreactor conditions				
Initial volume of pure SDF [ml] Volume before initiation of gastric emptying		10	10	
pH stat		6.1	6.5	Elderly values based on a study of 79 healthy elderly people[12].
Pancreatic enzymes	Trypsin [U/ml]	100	46	Added at two bursts. The first (10%) after 10 minutes and second (90%) after 50 minutes from the beginning of the experiment. Values derived from two human studies [11, 13]
	α- chymotrypsin [U/ml]	50	23	
Bile salts	Sodium glycodeoxycholate [mM]	4	2.67	Values derived from human studies [11, 13].
	Taurocholic acid sodium salt hydrate	4	2.67	
Bi-phasic bile secretion rate [mL/min] Initiated after initiation of gastric emptying	Phase 1: 0-5min	0.67	0.67	
	Phase 2: 5-end of experiment	0.022	$8.7*10^{-3}$	
Total bile salts volume [ml]		3	2	
Total experiment time [min]		120	180	Duration defined based on a human study[12]

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417 **Table 2**

Compound	Stock Solutions [g/l]	Volumes to add from stock solutions		
		SGF [ml]	SDF [ml]	SBF [ml]
KCl	46.72	56	10.8	10.8
KH_2PO_4	68	1.8	1.6	35.8
$NaHCO_3$	84	13	85	19
NaCl	120	20	16	16
$MgCl_2(H_2O)_6$	30	4	2.2	2.2
NH_4Cl	27.28	2	--------	--------
$NaH_2PO_4(H_2O)_2$	166	------- -	--------	20
Urea	22.5	0.6	4.8	10.4
pH adjustment		0.3	8.1	8.2
NaOH 1M			1	
HCl 1M			0.6	2
HCl 32%		6		
NaOH 5M				8
To add directly into V1 or V2 before digestion				
$CaCl_2(H2O)_2$ 4M [µl/ml]	Adult	0.15	0.15	0.925
	Elderly	0.15	0.3	1.85

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420 **Figure 1**

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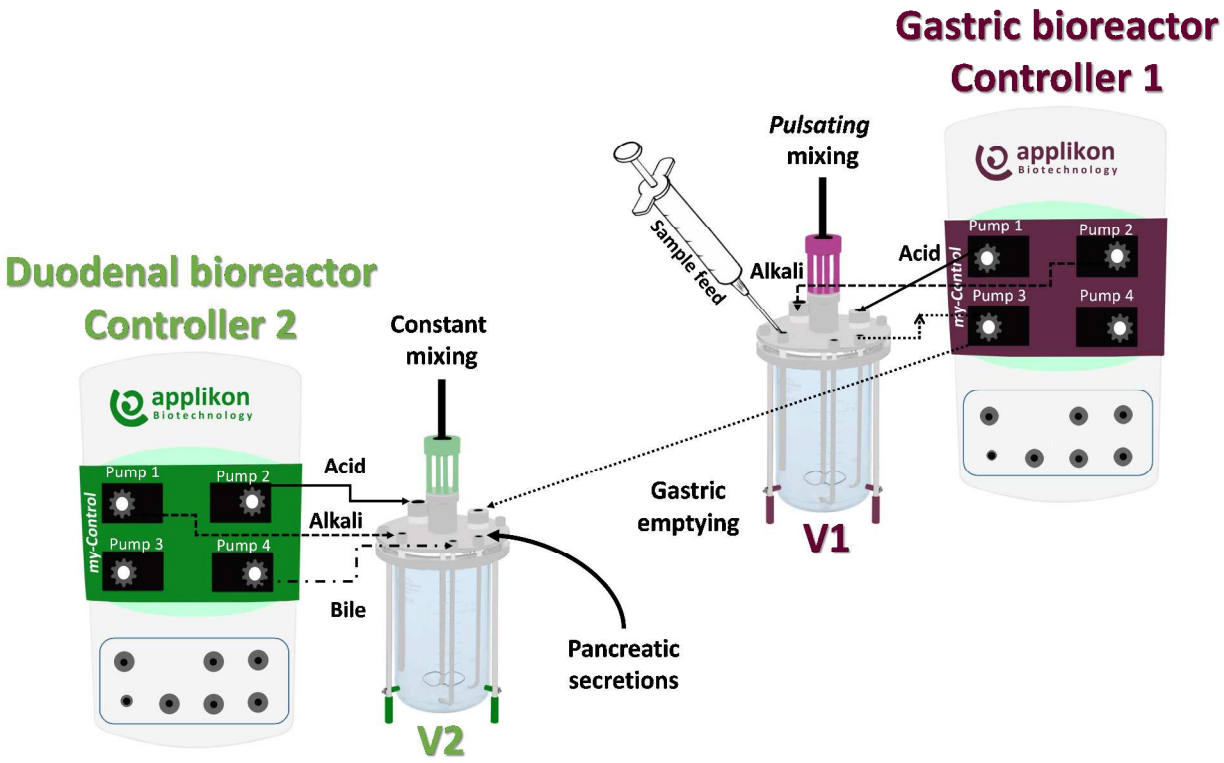

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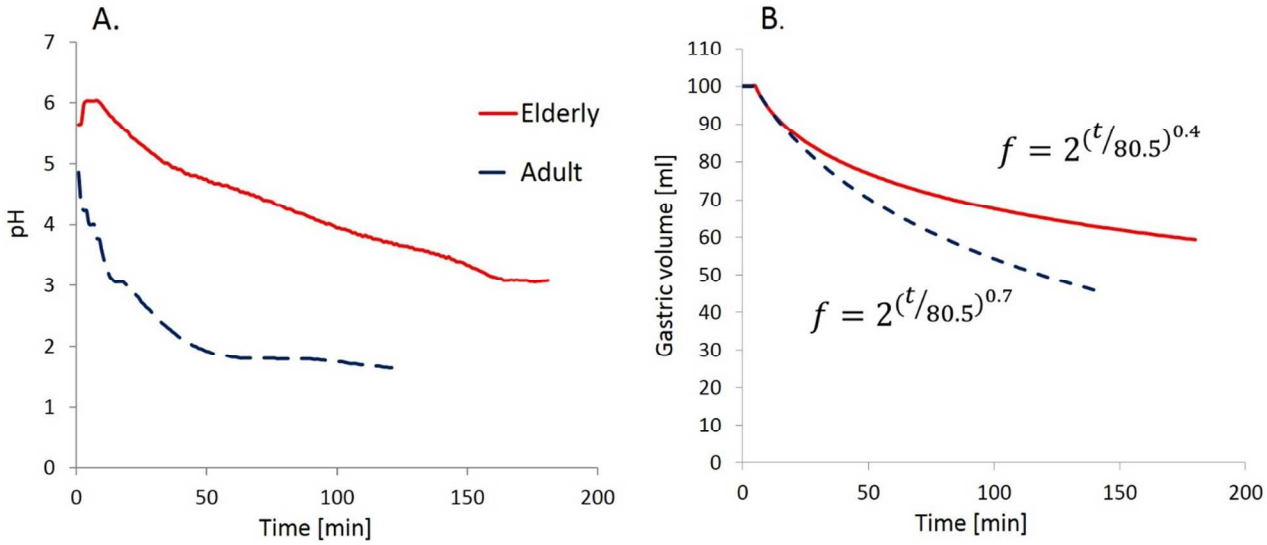
433 **Figure 2**



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435 **Figure 3**

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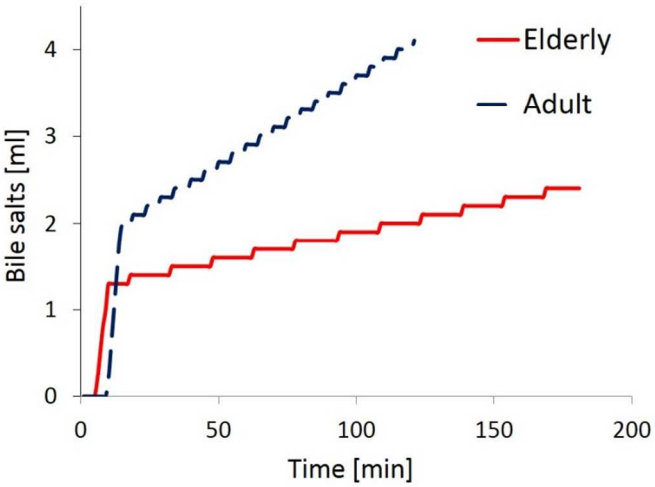

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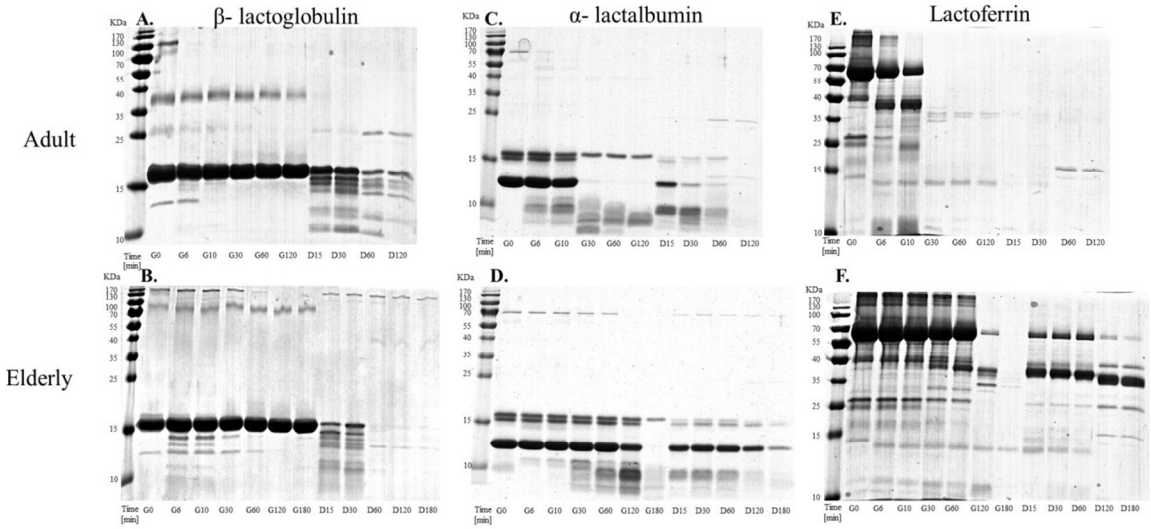
443 **Figure 4**



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