Microfluidic Digestive Systems for Drug Analysis

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Chapter 4

A microfluidic model for infantile *in vitro* digestions: Characterization of lactoferrin digestion

Abstract

We present a miniaturized, flow-through model for infantile *in vitro* digestions. Microfluidic 'chaotic' mixers were employed as microreactors to help emulate the biochemical processing going on in the infantile stomach and intestine. Simulated digestive fluids were introduced into these micromixers, and the mixtures were incubated for 60 min after both the gastric phase and the intestinal phase. The pH of the infantile stomach was set at 5.3, which is higher than that of adults. This leads to entirely different patterns of digestion for the milk protein, lactoferrin, used in our study as a model compound. It was found that lactoferrin remained undigested as it passed through the gastric phase and reached the intestinal phase intact, unlike in adult digestions. In the intestinal phase, lactoferrin was rapidly digested. Our miniaturized infantile *in vitro* digestive system requires much less labor and chemicals than standard approaches, and shows great potential for future automation.

Manuscript in Preparation

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4.1. Introduction

The effects of processes in the gastrointestinal (GI) tract are emulated by exposing samples to simulated digestive juices during *in vitro* digestions, one of the most commonly used techniques to study digestive processes in the nutritional sciences. Experiments are done in a batch-wise fashion in test tubes by sequentially adding juices to the sample and inverting the test tube repeatedly to achieve mixing after each addition. We recently published the development of a flow-through microfluidic system to recreate the adult digestive tract [1], inspired by these larger-scale *in vitro* digestive systems described in the literature. There are three reasons why our concept of a continuously flowing, digestive microsystem is entirely different from conventional batch digestion in test tubes [2,3], as discussed below.

First, in a continuously flowing system, all reactions are occurring all the time. However, the extent to which any given reaction has taken place depends on the location within the device at which the reaction is being observed. In batch chemistry, on the other hand, all reactions occur at the same location (a single test tube). The extent to which any given reaction has progressed depends on the time elapsed since the start of that reaction. At any given time in our microfluidic system, then, we have access to all the stages of progression of the ongoing digestion reactions. In other words, we have a complete overview of these reactions from beginning to end. This facilitates sampling at any intermediate time point with a (theoretically) infinitely large time resolution.

Second, the distance traveled in the flow system can be transposed to reaction time, and the reaction mixture at any given distance travelled will have a fixed composition. This means that a sample collected, for instance, at the halfway point of a 60-min incubation loop in our system will always have been exposed to the digestive juice it is in for exactly 30 min. Our flow approach facilitates precise sampling at exact time points throughout the digestion.

Third, a different approach to mixing is necessary in a microsystem, since flows are generally laminar, which leads to impaired mixing and thus delayed onset of reactions. The tubes used in batch *in vitro* digestions are usually actively mixed by inversion (*e.g.* by rotary mixers), causing complete mixing within minutes. We chose to employ ‘chaotic’ micromixers [4]. These micromixers comprise microchannels containing arrays of grooves in one surface of the microchannel. These grooves are oriented at an oblique angle with respect to the channel

sidewalls, and thus redirect solution flows to create helical flows inside the microchannel. This causes the different liquids to be mixed to fold over one another, thereby greatly enlarging the contact area at the interface between the liquid streams. The distances that molecules need to diffuse to reach the neighboring stream and be mixed is significantly reduced. The mixing behavior inside these devices depends on the flow rate but is generally very fast. In fact, mixing of two side-by-side streams may be complete in milliseconds [5]. The versatility of this continuous-flow system makes it perfectly suited for mimicking different *in vitro* digestion conditions. These conditions can easily be changed by the user, making this approach one of great potential for the automation of digestive processing.

In this work, we describe the development of such a flow-through system to emulate the digestive functions of infants. It is especially important to have a good model for infantile digestion, since it is cumbersome to perform *in vivo* experiments with infants. Even though the anatomy of the digestive tract of infants is very similar to that of adults, the digestive processes in infants differ from those in adults in a number of key aspects. First of all, since infants take their nutrition in the form of liquids, these materials are swallowed immediately, and there is not much digestion that takes place in the oral phase as there is no mastication. The ingested materials are rapidly passed down to the stomach, where the local pH is around ~5, instead of being as low as 1–3 in adults [6]. Furthermore, the gastric enzymatic load is lower in infants than in adults, and a shorter residence time is reported in the infant stomach. Lastly, the digestive mixture, known as chyme, reaches the small intestine, where the local enzymatic load is lower than in adults as well, and the residence time varies greatly. In a recently published *in vitro* infantile digestion model [7], the authors compared several models for infantile digestion to define a consensus model, based on a compromise between the different conditions (e.g. pH ranges, residence times) adopted in the infantile digestive tract in the different models. These parameters vary greatly, depending on a number of factors such as the age of the infant and whether it was born preterm or not. Ménard *et al*. chose a residence time of 60 min in the gastric phase, at a pH of 5.3, derived from *in vivo* measurements of infantile gastric acidity. Intestinal phase digestion also took 60 min at a pH of 6.6. The enzymatic loads in both stages were determined in earlier work [8], and were applied in the consensus model using sample solution, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) in volume ratios of 39 to 23 to 38 (Figure 4-1).

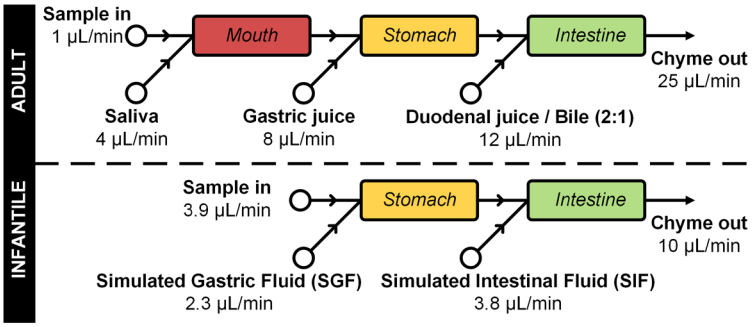

Figure 4-1 Comparison of the continuous-flow infantile digestive system with the miniaturized adult digestive system [1]. Each colored rectangle represents a grooved micromixer. Inlet pumps are shown as circles, with flow rates selected to obtain the physiological ratios of the respective digestive juices. The output of these systems is digested sample in a mixture of simulated digestive juices, referred to as chyme (as it is called in the *in vivo* situation). The chyme may be used for further downstream analysis. Note that the infantile system is a simplified version of the adult system, since infants don't need to chew their food. A "mouth" is thus not required.

The established *in vitro* models for infantile digestion described above require skilled technicians and allow for ample time to reproduce digestive processes. Our flow-through microfluidic digestive system is much easier to use, as it enables continuous operation of the device in a hands-off manner. pH values are determined by the mineral composition of the artificial digestive juices, and residence times are determined by the length of the incubation loops and the respective flow rates. Here, we demonstrate the digestive functions of our system by digesting lactoferrin, a 78 kDa, iron-containing protein that naturally occurs in both human and bovine milk. This protein has functions relevant to infant nutrition as well as the infantile immune system [9]. The digestion of lactoferrin is markedly different in infants than in adults, which leads to a higher availability of intact lactoferrin in the infantile gut, where it may have an antimicrobial effect and may be taken up into the body [10]. Lactoferrin is thus a very suitable model compound for comparing the performance of our *in vitro* infantile system with our previously published adult system (de Haan *et al.*, 2019).

4.2. Materials and Methods

Chemicals were obtained from Sigma-Aldrich/Merck (Zwijndrecht, the Netherlands), unless stated otherwise. Ultrapure water (18.2 MΩ·cm) was used to prepare solutions for digestion and aqueous eluents for chromatography.

4.2.1. Fabrication of Microfluidic Devices

Two 'chaotic' micromixers (Figure 4-2) were employed to mix samples with SGF and SIF, respectively [4,5]. The fabrication of these micromixers and the assembly of devices has been reported elsewhere [1]. In short, each micromixer consisted of a 300-µm-wide, 51.5-mm-long mixing channel (1.38 µL volume), with two inlets and one common outlet. The channel was 60 µm deep, and contained an array of 16 consecutive, closely spaced groups of twelve 50-µm-deep, 110-µm-wide herringbone-shaped grooves embedded in the channel ceiling to cause perturbations of the laminar flow in the channel. Mixing in the channel is greatly enhanced by the groove arrays, as the resulting flow pattern "folds" the two liquids into one another. The channel structures were patterned in poly(dimethylsiloxane) (PDMS) by SU-8 micromolding, and were sealed by bonding to a plain glass microscope slide, yielding hybrid PDMS-glass micromixers. The inlets of the channels were connected to a custom-built, pressure-driven flow control system[11] (Bronkhorst High-Tech B.V. Ruurlo, the Netherlands) using poly(tetrafluoroethylene) (PTFE) tubing (0.8/1.6 mm inner/outer diameter, Polyfluor Plastics, Breda, the Netherlands) and blunt needles (Fine-Ject 21G, HenkeSassWolf, Tuttlingen, Germany). Containers filled with the samples and simulated digestive fluids were pressurized to propel fluids from these containers via tubing to the microfluidic devices at precisely controlled flow rates. A specially designed, 3D-printed interface on the flow control system allowed connections using fingertight connectors (BGB Analytik, Böckten, Switzerland) from the containers to the flow sensors, and from the flow sensors to the inlet tubing of the microfluidic device. The microfluidic set-up was placed in an incubator kept at 37°C. A 74-cm-long piece of PTFE tubing was placed after the gastric micromixer, corresponding to a 1-h

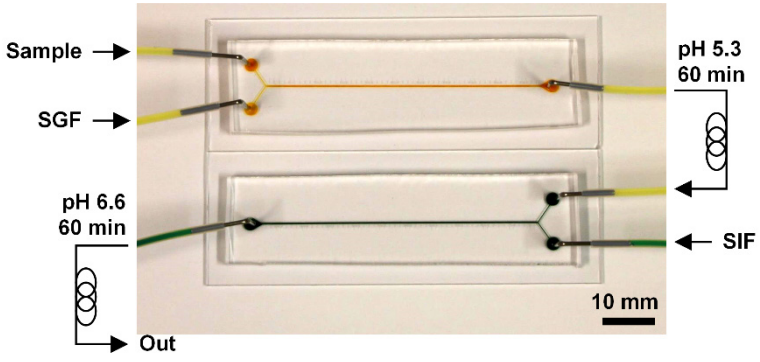

Figure 4-2 Photograph of the miniaturized system, consisting of two micromixers with a volume of 1.38 µL each. Samples are mixed with simulated gastric fluid (SGF) in the first microreactor (channel filled with yellow dye), after which the gastric mixture is incubated under continuous flow (6.2 µL/min) in the incubation loop at pH 5.3 for 60 min. Then, simulated intestinal fluid (SIF) is added in the second microreactor (green dye) and the mixture is incubated at 10.0 µL/min and pH 6.6 for another 60 min.

sample incubation time at a flow rate of 6.2 µL/min before flowing into the next micromixer (Figure 4-2). After the intestinal micromixer, a 119.4-cm-long piece of PTFE tubing was used to incubate the mixture for 1 h at a flow rate of 10 µL/min.

4.2.2. Device Operation

Infantile versions of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to a modified procedure stemming from the literature [7]. In conventional batch-type reactions, the pH value changes when juices are mixed together by adding one juice to the other, and the resulting pH must be adjusted by adding HCl or NaOH. This type of intermediate pH adjustment is not possible in microfluidic devices. Therefore, we have optimized the pH value of the gastric juices and the intestinal juice so that when they are mixed at the appropriate ratio, the resulting pH is the correct one (Table 4-1). SIF was centrifuged at 2,000 rcf for 5 min to remove a fine precipitate that had formed during preparation (possibly calcium carbonate). Juices were pre-warmed before usage. The microfluidic system was operated continuously, and was run for 2 h before samples were collected. Samples were obtained at different locations within the system, starting from the outlet of the intestinal loop and working backwards upstream. By doing so, all samples could be collected consecutively without interfering with the digestive process upstream. The incubation loops were pre-cut (and reconnected with a blunt needle) to facilitate sampling at tubing lengths corresponding to 1, 5, 10, 15, 30, 45, and 60 min of incubation in each phase (see S.I., Table S 4-1). The control experiments were done in a 15-mL test tube (Greiner Bio-One, Frickenhausen, Germany), by mixing 3.9 mL lactoferrin (50 mg/mL in ultrapure water, Vivinal, FrieslandCampina, Amersfoort, the Netherlands) with 2.3 mL SGF and incubating in a rotator (24 rpm) at 37 °C for 60 min. Then, 3.8 mL of SIF was added, and the mixture was incubated for another 60 min. Samples were collected manually at the same timepoints as for the microfluidic system. All samples were immediately diluted to achieve a lactoferrin concentration of 2.0 mg/mL lactoferrin, and then mixed 1:1 with a denaturation buffer (0.1 M BIS-TRIS, 6 M guanidine HCl, 5.37 mM citric acid, 19.5 mM dithiothreitol in ultrapure water, pH 7.0). After 1 h of incubation, the samples were mixed 1:1 with Eluent A (see Section 4.2.3 below) and analyzed by RP-HPLC [12].

Table 4-1 Composition of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) as used in this study. These compositions have been adopted from an earlier study [7], but with the pH values pre-adjusted for optimal digestive conditions.

Simulated Gastric Fluid (SGF)		**Simulated Intestinal Fluid (SIF)**	
Compound	**Concentration**	**Compound**	**Concentration**
NaCl	94 mM	NaCl	164 mM
KCl	13 mM	KCl	10 mM
Pepsin (porcine)†	724 U/mL (7.1 mg/mL)	$NaHCO_3$	85 mM
Lipase	51 U/mL (1.0 mg/mL)	$CaCl_2$	3 mM
(*Rhizopus oryzae*)†		Bile (bovine)†	2 mg/mL
		Pancreatin (porcine)†	237 U/mL (lipase); 42 U/mL (protease); (24.5 mg/mL)
NaOH	0.55 mM	HCl	53 mM
pH	5.3	pH	6.6
Ultrapure water		Ultrapure water	

†: Concentrations of digestive enzymes are those present in the digestive juice, before mixing with samples. For the determination of enzyme activities, we refer to the INFOGEST standardized model for adult digestions [2].

4.2.3. Monitoring Lactoferrin Digestion Progress by Reversed-Phase High Performance Liquid Chromatography (RP–HPLC)

The progress of digestion of lactoferrin in the microfluidic system and in test tubes was analyzed by reversed-phase high performance liquid chromatography (RP–HPLC) according to a modified literature procedure [12]. The HPLC system (VWR-Hitachi Elite LaChrom, with pump L-2130, autosampler L-2200, column oven L-2300, and UV detector L-2400) was equipped with a C4 column (250 × 2.1 mm, 5 µm particles, 300 Å pores, Grace Vydac, Columbia, MD, USA) kept at 30 °C. Samples of 10 µL were injected onto the column and UV absorbance of effluents was monitored at 220 nm (Supporting Information, S.I., Figure S 4-1). Two eluents, Eluent A (10% acetonitrile, 0.1% trifluoroacetic acid, ultrapure water) and Eluent B (90% acetonitrile, 0.07% trifluoroacetic acid, ultrapure water), were used in the following gradient elution profile at a total flow rate of 0.250 mL/min: 0% B from 0–5 min; increasing to 60% B from 5–20 min; further increasing from 60% to 90% B from 20–23 min; held at 90% B from 23–28 min; decreasing from 90% to 0% B from 28–30 min; held at 0% B from 30–35 min.

4.2.4. Digestion Fragment Profiling using Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Samples containing 5 µg of (digested) lactoferrin were denatured by lithium dodecyl sulfate (NuPAGE LDS, Carlsbad, CA, USA) and separated on a 4–12% Bis-Tris gel (1× MES buffer,

200 V, 22 min), stained with Coomassie Blue, and de-stained in ultrapure water. Photographs (S.I., Figure S 4-2) were analyzed with ImageJ software and converted to intensity vs. molecular weight plots, using the marker lane (PageRuler Plus, ThermoFisher, Waltham, MA, USA) for calculation of the molecular weight (S.I., Figure S 4-3).

4.3. Results and Discussion

The micromixers that were used for this infantile continuous-flow, miniaturized digestive system have been used for a plethora of applications [13]. The herringbone-shaped grooves in the channel ceiling greatly enhance the mixing process in the laminar flow inside these channels, with nearly complete mixing (depending on parameters such as flow rate and viscosity) taking place within the first millimeters of the mixing channel, in times as short as milliseconds [1,5]. The low internal volume of the mixing channel (1.38 µL) results in a low dead volume inside the system, which limits loss or dispersion of sample (lactoferrin) molecules and fragment peptides before analysis by HPLC or SDS-PAGE. The artificial digestive fluids used were optimized from earlier studies to exhibit the correct pH for each stage, eliminating the need for pH adjustments during *in vitro* digestion. This, combined with the continuous flow in the system, makes continuous operation possible without requiring a skilled technician, allowing for future automation.

Figure 4-3 shows the digestion of model protein, lactoferrin, in our miniaturized digestive system. As a control, lactoferrin was digested in a batch-wise digestion in test tubes in parallel. No breakdown of lactoferrin is visible in the first 60 min, corresponding to the gastric phase. Since the infantile gastric pH of 5.3 is higher than in adults, the pH is non-optimal for the enzymatic activity of pepsin, making this enzyme less active than in the adult stomach (pH 1–3). Digestion progresses rapidly after addition of the SIF at 60 min. This is because SIF contains pancreatin, an extract from the pancreas that contains several different proteases. The pH of 6.6 ensures that these enzymes are catalytically active and lactoferrin is digested rapidly. When the tube-based and microfluidic digestions in Figure 4-3 are compared, no difference in the percentage of remaining lactoferrin is observed in either the gastric phase (0-60 min) or intestinal phase (60-120 min). We can conclude that the miniaturized system exhibits equal digestive performance, but requires less hands-on time and significantly smaller amounts of sample and digestive juices (a net total volume of 1.2 mL was used per assay in the flow system, and 12 mL per batch-wise test tube assay – a tenfold reduction).

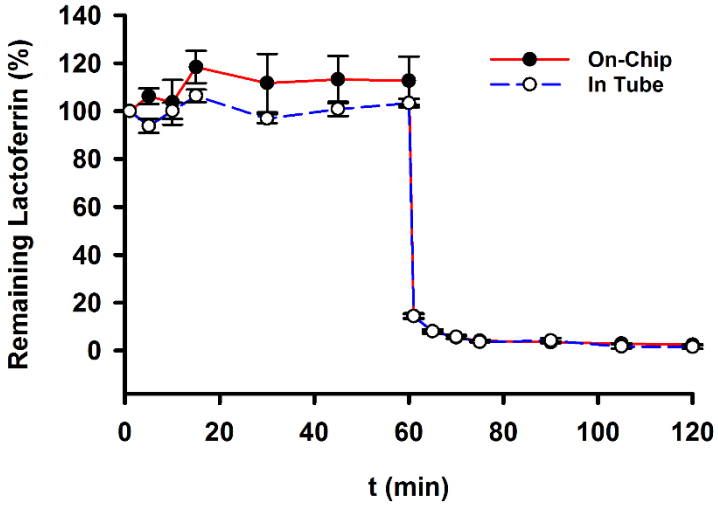

Figure 4-3 The percentage of lactoferrin remaining as digestion progresses under infantile-digestion conditions was determined quantitatively by RP-HPLC (n = 3, mean ± standard error of the mean). Lactoferrin concentrations remained unchanged in the gastric phase (0–60 min), and dropped sharply in the intestinal phase (60–120 min). Samples from the digestion in the miniaturized system ("On-Chip") were compared to a standard *in vitro* digestion in test tubes ("In Tube"), in which tubes were rotated head-over-heels and digestive juices were added sequentially by hand. No difference in the digestion process taking place in these two systems was found.

Even though the separation of proteins and peptides by HPLC is good, it can only be used to quantify the percentage of remaining lactoferrin in the digestion solutions. The peptide fragments of lactoferrin that arise upon digestion all show short retention on the HPLC column and elute as one cluster of peaks (see S.I., Figure S 4-1); fragments of different sizes cannot be resolved. Moreover, not all the peptides formed may contain sufficient, UV-absorbing, aromatic amino-acid residues or peptide bonds to be detectable at 220 nm. Therefore, SDS–PAGE analysis (size-based separation of peptides and proteins) was used as an alternative analytical approach to compare lactoferrin fragmentation in the adult and infantile microfluidic digestion systems. The sample lanes corresponding to 30-min lactoferrin digestion in the gastric and intestinal phases (S.I., Figure S 4-2) were converted to intensity plots (Figure 4-4) to compare on-chip and in-tube experiments of infantile digestion with the miniaturized adult digestive system reported previously [1].

From these plots, we can see that no digestion of lactoferrin has taken place in the infantile gastric phase after 30 min, since the lactoferrin peak appears equally large as that for the pure lactoferrin control and there are virtually no peptide fragments with a lower molecular weight. In the adult gastric samples, however, we see the lactoferrin peak has decreased in size compared to the pure lactoferrin sample. There is also an accumulation of several peptide fragments having sizes between 4 and 32 kDa. There is no difference between the infantile and

adult systems when looking at the 30 min intestinal samples: all lactoferrin content has been converted into smaller peptides or amino acids, such that no trace of lactoferrin remains in these gels. Therefore, this infantile digestive system differs from the adult system with respect to lactoferrin digestion. Unlike the adult system, this protein was not digested in the infantile

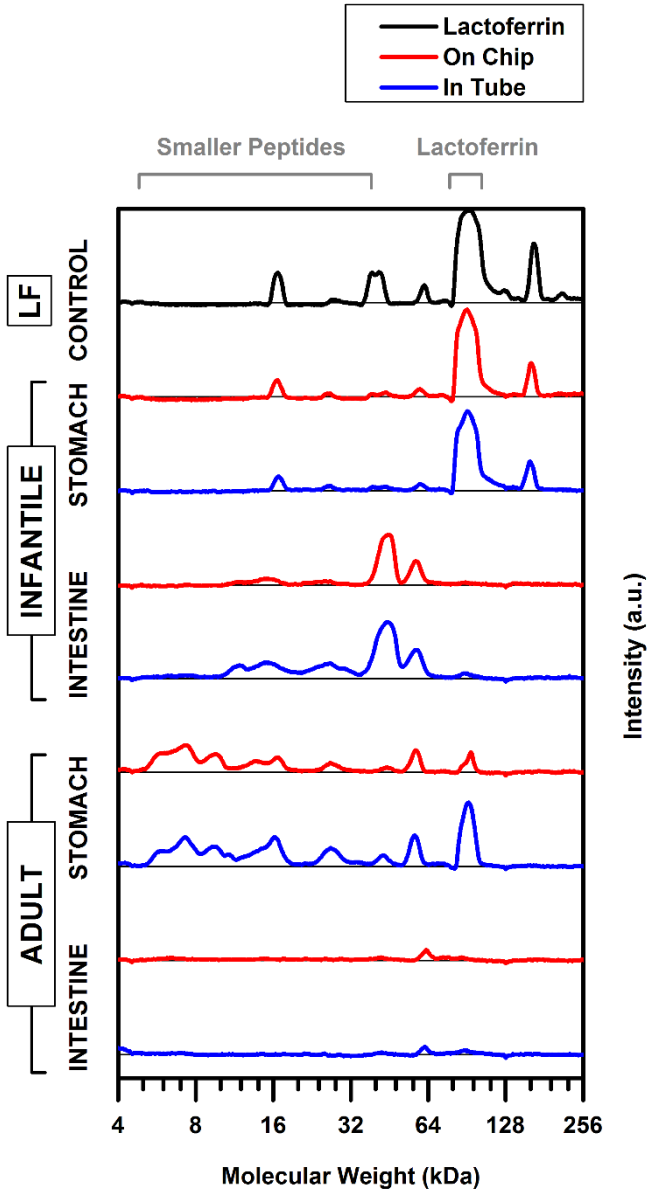

Figure 4-4 In order to make a comparison with the digestion of lactoferrin (LF) in the adult digestive system, samples were analyzed by SDS-PAGE, to separate proteins and peptides by size. The gel plots (see E.S.I. Fig. S2) were converted to the intensity plots shown above for facile comparison. The plots represent non-digested lactoferrin (black), samples taken from chip experiments (red), and samples taken from experiments in test tubes (blue). Samples were collected after 30 min of digestion in the stomach or intestine. The lactoferrin peak is visible at ~78 kDa. Upon digestion, this protein is hydrolyzed to smaller peptides, visible in the 4–32 kDa range. No peptides are visible in the gastric plots of the infantile system, indicating that no digestion has taken place (in accordance with Figure 4-3). In the adult system, a clear series of smaller peptides is visible in the gastric samples. No peptides are visible in the intestinal samples, since proteases rapidly hydrolyze peptides to very small peptides (too small to be visible in these plots).

gastric phase and so reached the intestinal phase intact, to be rapidly digested into smaller peptides in the intestinal phase. Further analysis with mass spectrometric techniques could elucidate the exact patterns of protein digestion, to yield deeper insights into the *in vitro* and *in vivo* characteristics of lactoferrin digestion.

4.4. Conclusion

We have developed a miniaturized, flow-through model for infantile digestion, that has potential application in nutritional and pharmaceutical science, for instance in the formulation of nutritional products and drugs for infants. To demonstrate its functions, we used a model milk protein, lactoferrin, known to have several roles in the nutrition and immunological development of infants. Complete digestion of lactoferrin took place in the intestinal phase, after it passed the gastric phase unmodified. This was confirmed by control experiments in a traditional batch-wise digestion. This is completely different from digestion in the adult digestive tract, where lactoferrin was broken down to smaller peptide fragments in the gastric phase, and hardly any unmodified lactoferrin reached the intestinal phase. In future work, we will look deeper into how digestive processes proceed, by using mass spectrometric techniques to study digestion patterns. For this study, we adopted the same one-hour incubation times as used in larger-scale infantile digestion models, which are based on average physiological times *in vivo*. It is very well possible, however, that these relatively long incubation times are not necessary in a microfluidic setting. We believe that the extremely thorough mixing process in our flow-through system could significantly shorten the incubation times required to achieve physiologically relevant digestion *in vitro*, as digestive processes can start immediately. Optimization of incubation times should lead to significantly shorter sample digestion times, offering a route to more efficient *in vitro* testing of infantile food and drug products with our system. In short, the miniaturized, infantile digestive system described in this work represents an innovative tool for nutritional and pharmaceutical studies in the future, since it requires less labor, saves on chemical consumption, and has great potential for automation.

4.5. Acknowledgments

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4.6. CRediT Author Contributions

Pim de Haan: Conceptualization, Data curation, Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing. **Daigo Natsuhara:** Investigation; Validation; Writing – review & editing. **Vassilis Triantis:** Methodology; Resources; Validation; Writing – review & editing. **Takayuki Shibata:** Supervision; Writing – review & editing. **Elisabeth Verpoorte:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – original draft; Writing – review & editing.

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4.8. Supplementary Information

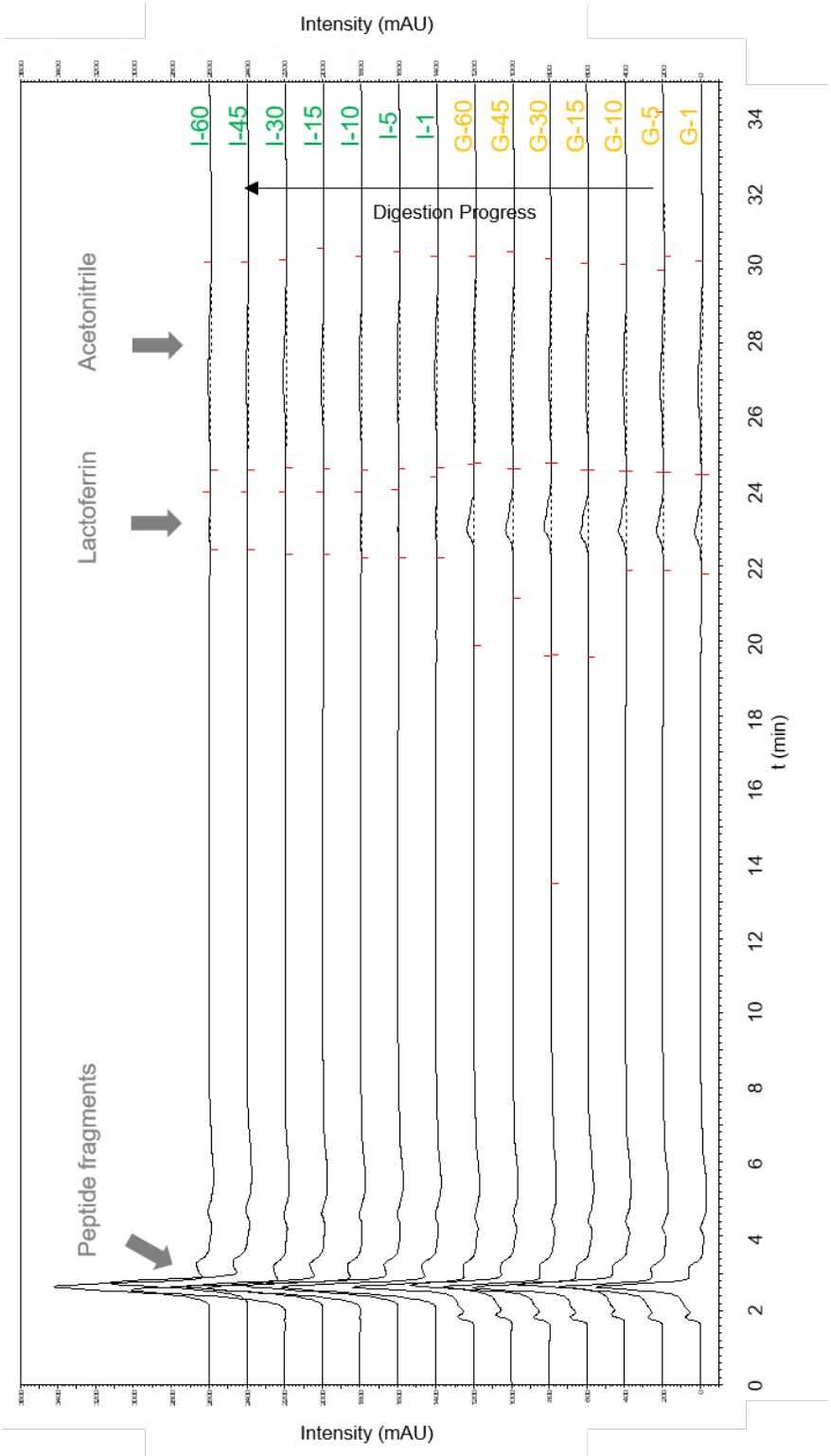

Figure S 4-1 Overlay of chromatograms obtained for samples taken during the digestion of lactoferrin in the miniaturized system. From the bottom up: after 1–5–10–15–30–45–60 min in the gastric phase (labeled G-1, etc.), and after 1–5–10–15–30–45–60 min in the intestinal phase (labeled I-1, etc.). The lactoferrin peak is visible at a retention time of around 23 min, and both the peak height and area are decreasing as the digestion progresses. Smaller peptides (generated by digestion) elute earlier and cause peaks at 1–5 min. Note that the broad peak at 25–30 min is an artefact of the gradient elution, caused by acetonitrile.

Table S 4-1 Overview of places where incubation tubing was pre-cut and reconnected with a blunt needle to facilitate sampling. The tubing had an inner diameter of 0.8 mm, with a volume of 5.03 µL/cm. Note that the tubing lengths required for a given sample incubation are larger for the intestine than the stomach. This is because the total flow rate after the stomach-micromixer is smaller than after the addition of SIF to the solution flow entering the intestine-microreactor.

Sample time (min)	After Stomach (cm)	After Intestine (cm)
1	1.2	2.0
5	6.2	10.0
10	12.3	19.9
15	18.5	29.9
30	37.0	59.7
45	55.5	89.6
60	74.0	119.4

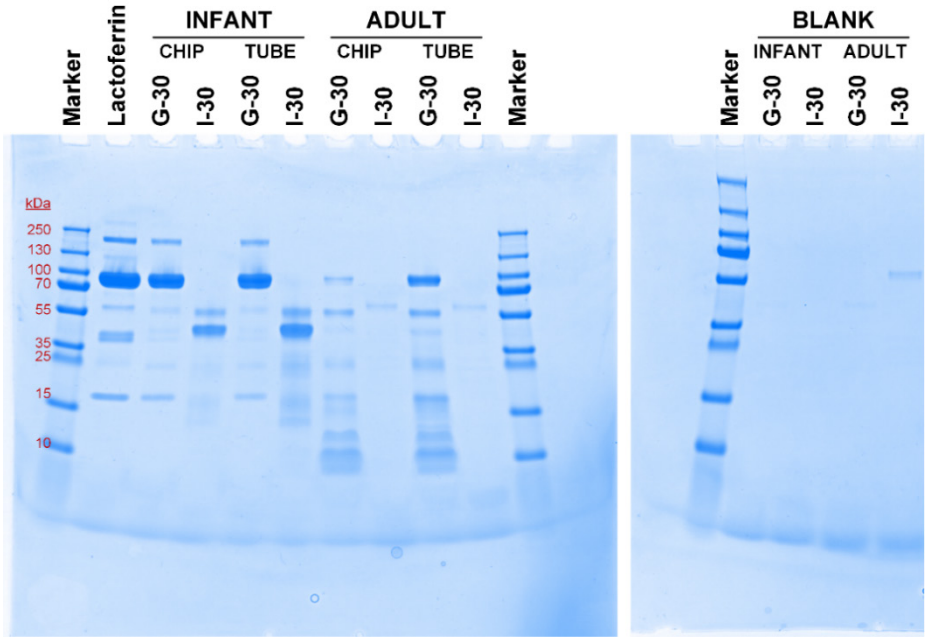

Figure S 4-2 Photographs of the SDS–PAGE gels used as the basis for the intensity plots to analyze the lactoferrin fragmentation patterns in Figure 4-4 of the main text. G-30: sample collected after 30 min in the gastric (stomach) phase. I-30: sample after 30 min in the intestine.

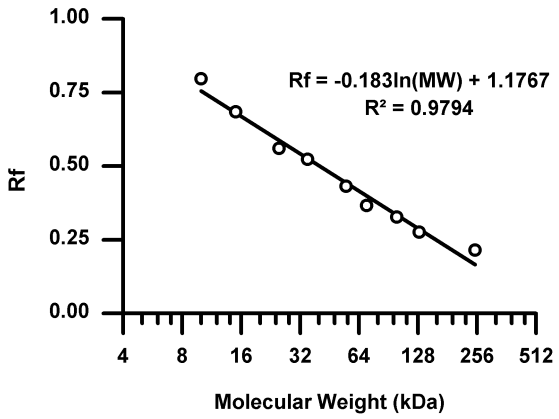

Figure S 4-3 Conversion of values for the relative migration distances (Rf) of proteins to molecular weight using the protein marker lane in Figure S 4-2.