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## Colloidal transport of lipid digesta in human and porcine small intestinal mucus

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### Abbreviations:

CLSM, confocal laser scanning microscopy; D, diffusion coefficient; DG, diglyceride; FFA, free fatty acid; GI, gastrointestinal; MG, monoglyceride; TG, triglyceride.

34 **Abstract**

35

36 Small intestinal mucus transport of food-derived particulates has not been extensively studied, despite mucus  
37 being a barrier nutrients need to cross before absorption. We used complex dispersions of digesta obtained  
38 from simulated, dynamic gastrointestinal digestion of yogurt to examine the penetrability of human and  
39 porcine mucus to the particles formed of lipolysis products. Quantitative, time-lapse confocal microscopy  
40 revealed a sieve-like behaviour of the pig jejunal and ileal mucus. The digesta diffusivity decreased significantly  
41 over the first 30 min of mucus penetration, and then remained constant at ca.  $5 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$  (approx. 70%  
42 decrease from initial values). A non-significantly different penetrability was recorded for the ileal mucus of  
43 adult humans. The digesta diffusion rates in neonatal, jejunal mucus of 2 week old piglets were 5–8 times  
44 higher than in the three different types of adult mucus. This is the first report that validates the mucus of fully-  
45 grown pigs as a human-relevant substitute for mucus permeation studies of nutrients/bio-actives and/or  
46 complex colloidal dispersions (e.g., post-digestion food particulates, orally-administrated delivery systems).

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48

49

50 **Keywords**

51 Nutrient transport; Dynamic digestion model; Mucus barrier; Lipolysis; Proteolysis; Particle diffusion.

52

## 53 1. Introduction

54

55 The small intestine is the part of the alimentary tract where most of food digestion and nutrient  
56 absorption take place. In order to get absorbed by the body, any nutrients released from food during digestion  
57 in the intestinal lumen need to penetrate through the mucus layer that separates the underlying epithelium  
58 from luminal contents (Johansson, Sjövall, & Hansson, 2013). The mucus layer is a selective barrier. It allows  
59 the passage of molecules (e.g., water-soluble nutrients, bioactives, pharmaceuticals, etc.) and small particles  
60 (e.g., lipid/bile salt mixed micelles, etc.), but prevents the epithelium from direct exposure to luminal  
61 microorganisms, including pathogens (Cone, 2009). It also lubricates the mucosal epithelium, which is crucial in  
62 protecting the tissue from abrasions that can be caused by the peristaltic movement of luminal contents.

63 The small intestinal mucus is a viscoelastic hydrogel made from a number of components, amongst  
64 which the MUC2 mucin glycoprotein is the major gel-forming macromolecule responsible for the porous  
65 structure and rheological properties of the mucus (Corfield, Carroll, Myerscough, & Probert, 2001), (Round et  
66 al., 2012). Several studies have also pointed out at the importance of extracellular DNA in maintaining the  
67 microstructural organisation and high viscosity of the mucus as well as its barrier properties with regard to  
68 penetrability to particles and bacteria (Macierzanka et al., 2014), (Lock et al., 2020).

69 Despite the importance of small intestinal mucus in regulating the access of nutrients to the  
70 epithelium, there is a limited number of studies looking at the penetrability of the mucus layer by digested  
71 food. So far, most of the research in this area has either focused on monitoring the local diffusivity of  
72 molecules (e.g., polyphenols, alginates) inside the mucus matrix (Gonzales et al., 2015), (Mackie et al., 2016), or  
73 on characterising the mucus structure by a combination of qualitative and quantitative microscopy methods,  
4 often with the use of synthetic particles simulating digesta. In the latter case, one of regularly used methods is  
5 multiple-particle tracking (MPT), which relies on simultaneous real-time monitoring of the displacement of  
6 hundreds or thousands of individual particles (S. K. Lai, Wang, Wirtz, & Hanes, 2009). Evaluation of the  
7 diffusivity of identical-in-size nano- or micro-particles allows for probing the permeability, microrheology, and

78 heterogeneity in microstructural organisation of mucus (S. K. Lai, Wang, Hida, Cone, & Hanes, 2010),  
79 (Macierzanka, Mackie, & Krupa, 2019), (Lock et al., 2020). This method has been used to determine the role of  
80 the size and surface chemistry of particles on their transport in mucus (Yildiz, McKelvey, Marsac, & Carrier,  
81 2015), (Maisel, Ensign, Reddy, Cone, & Hanes, 2015). For instance, it was shown that a high negative charge of  
82 the surface of polystyrene beads, caused by adsorption of anionic intestinal surfactants – bile salts (BS),  
83 significantly enhanced the ability of the beads to diffuse in the small intestinal mucus (Macierzanka et al.,  
84 2011). It was suggested this was due to the BS adsorption preventing mucoadhesion of the beads to the  
85 negatively-charged mucus matrix. MPT has also been used to assess the intestinal mucus diffusion of post-  
86 digestion emulsion droplets (i.e., the droplets obtained after *in vitro* gastro-duodenal proteolysis of protein-  
87 stabilised oil-in-water emulsions, (Macierzanka et al., 2011), (Macierzanka et al., 2012)). However, these  
88 measurements were only conducted using size-narrowed monomodal fractions of droplets with well-defined  
89 diameters. Whilst monitoring the diffusion of spherical particles with known, uniform sizes does allow for  
90 calculating microrheological parameters of the mucus, the application of such particles can be of limited  
91 physiological relevance, as gastrointestinal (GI) digestion of food emulsions usually yields a broad distribution  
92 of particles in the gut lumen – from nano-size mixed micelles of bile salts and the lipolysis products to larger,  
93 micro-size droplets of partially digested lipids (Armand et al., 1999). Under physiological conditions of the small  
94 intestine, it is these complex dispersions of food-derived particles that interact simultaneously with the mucus  
95 layer. Therefore, studies looking at the intestinal bioaccessibility and transport rates of nutrients should use  
96 such physiologically relevant dispersions.

97 Another potential limitation of most scientific studies that have been conducted over the recent years  
98 in this area is that they used animal mucus, usually obtained from mice, rats or pigs, to simulate a difficult-to-  
9 obtain human mucus. Even for porcine mucus, its human-relevance cannot be assumed without scientific  
0 validation, despite the human GI physiology being historically considered most similar to that of pigs (Zhang,  
1 Widmer, & Tzipori, 2013). This might be especially true for studying interactions with digesta produced from

102 real foods, where colloidal complexity of such dispersions can highlight even small differences in  
103 microstructural organisation and penetrability of the human mucus relative to mucus of animal origin.

104           This study had more than one goal. Firstly, we used digesta samples obtained from semi-liquid food  
105 emulsion (natural yogurt was used), following different digestion times, to look at their ability to penetrate the  
106 small intestinal mucus and whether the transport characteristics change in time. By investigating this, we also  
107 intended to give an example of how *in vitro* digestion models can be complemented with simulating the  
108 passive transport of nutrients/particles through the mucus layer under intestinal conditions. Secondly, we  
109 wanted to compare the permeability of porcine and human small intestinal mucus to complex digesta  
110 produced from real food. This aimed to examine the hypothesis of a similar barrier function of the porcine and  
111 human mucus, and, if confirmed, would validate the use of porcine mucus for future investigations on the  
112 mucus transport of post-digestion food particulates under simulated human small intestinal conditions.

113

## 114 **2. Materials and methods**

115

### 116 **2.1. Human small intestinal mucus**

117 The collection and studies of the human small intestinal mucus were approved by the ethics committee  
118 of the Regional Medical Chamber in Rzeszów, Poland (certificate no. 4/B/2015). All methods were planned and  
119 conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Aspiration of mucus  
120 from the terminal ileum was done during diagnostic colonoscopy performed at the Teaching Hospital No 1 in  
121 Rzeszów. The procedure duration was extended for a maximum of 5 min to obtain mucus samples. A typical  
122 indication for colonoscopy was either occasional lower-GI bleeding, persistent unexplained abdominal pain, or  
123 screening and surveillance of colorectal polyps, and 51 individuals (31 men and 20 women), aged 34–67 years,  
124 were initially included in the study. Any evidence of inflammatory changes to the mucosa of the colon and/or  
125 the terminal ileum confirmed during the procedure disqualified a subject from the study. All individuals who  
126 agreed to take part in the study were clearly informed about the procedure and instructed by a clinician  
127 regarding the bowel preparation. Informed consent was provided by all participants prior to examination.  
128 Personal information of the volunteers was de-identified.

129 All participants included in the study followed a low residue diet protocol for 5 days prior to  
130 colonoscopy in order to improve bowel preparation. Individuals listed for colonoscopy received bowel  
131 preparation with 4L of Fortrans® (Ipsen Pharma) aqueous solution (1 sachet dissolved in 1L of water). One  
132 sachet of Fortrans® powder contained Macrogol 4000 (64 g), anhydrous sodium sulphate (5.7 g), sodium  
133 bicarbonate (1.68 g), sodium chloride (1.46 g), and potassium chloride (0.75 g). The timing of bowel  
134 preparation prior to colonoscopy has a very significant impact on the preparation quality. It has been reported  
5 that the optimal time for the colonoscopy procedure after completion of bowel preparation is 3-4 h, and  
6 should be less than 8 h after completion of the preparation (Seo et al., 2012). This concept has led to a split-  
7 dose preparation, which requires taking a portion of the bowel preparation solution the night prior to  
8 colonoscopy (50% of the total dose) and the remaining portion on the day of colonoscopy (Bucci et al., 2014).

139 The split-dose preparation was used in this study. The participants received 2 sachets of Fortrans® diluted in 2L  
140 of water between 6:00–8:00 pm on the day before colonoscopy and the remaining 2L of solution between  
141 5:00–6:00 am on the day of the procedure.

142 As a routine part of colonoscopy examination, the intubation of the terminal ileum is performed (Meral  
143 et al., 2018). This part of the small bowel contains a relatively thin layer of the mucus which is distributed on  
144 the mucosal surface of the bowel wall. We only performed aspiration of the mucus samples from the terminal  
145 ileum in participants who had bowel preparation rated as excellent (i.e., rate 9 of the Boston Scale (E. J. Lai,  
146 Calderwood, Doros, Fix, & Jacobson, 2009)). Once the terminal ileum was intubated with the colonoscope, the  
147 mucosal layer was inspected and an optimal area for aspiration of mucus chosen (i.e., an area free of residual  
148 liquid, etc.). At the same time, an assistant endoscopist inserted a disposable plastic catheter through the  
149 biopsy channel of the colonoscope. While inserting the catheter, air was being constantly insufflated with a  
150 syringe attached to one end of the catheter in order to prevent incidental aspiration of any material that might  
151 have resided in the biopsy channel and would potentially lead to the contamination of a sample. Once the  
152 catheter was in the lumen of the terminal ileum, the principal endoscopist was in charge of the control of the  
153 colonoscope tip and the catheter. The assistant endoscopist performed aspiration of the mucus by applying a  
154 gentle suction with a syringe. Mucus was only aspirated into the tip of the catheter in order to limit disturbing  
155 of the sampled mucus, and from the mucosal surface of the terminal ileum located between 5 cm and 25 cm  
156 from the ileocecal valve. Typically, no more than 0.5 mL mucus was aspirated from one subject over the limited  
157 time that was allowed for mucus collection. Immediately after aspiration, the plastic catheter was removed and  
158 the sample gently transferred into 0.5 mL plastic test tubes. The tubes were sealed and instantly immersed in  
159 liquid nitrogen for snap freezing. Samples were stored at -80 °C prior to further examination.

0 Mucus samples collected from five individuals (three women and two men; 51-56 years old) were used  
1 in the experiments described below in order to narrow down the age range of adult humans.

2

3

### **2.2. Porcine small intestinal mucus**

164 The collection of porcine mucus, including the *ex vivo* handling of intestinal tissue, was done as  
165 described previously (Macierzanka et al., 2014), (Macierzanka, Mackie, et al., 2019). In brief, mucus was gently  
166 removed with a soft-rubber scraper from the mucosal surface of freshly excised and cleaned small intestines of  
167 (i) 6–8-month old pigs (this being referred to as the ‘adult pig mucus’ or the ‘mucus from fully-grown pigs’  
168 throughout the paper) and (ii) 2 week old piglets (this being referred to as the ‘piglet mucus’). Pig mucus was  
169 collected from the most proximal, 1-meter-long jejunal segment and/or the most distal, 1-metre-long ileal  
170 segment. The last 5 cm of the ileum before the ileocecal valve was discarded. Piglet mucus was only collected  
171 from the most proximal jejunum (0.5–0.7-meter-long segment). Aliquots of collected mucus were immediately  
172 transferred to 0.5 mL plastic screw-cap tubes for snap freezing in liquid nitrogen, and then stored at -80 °C. The  
173 collection was carried out within 20 min from animal slaughter. Mucus was incubated for 10 min at RT before  
174 use. As reported previously (Macierzanka et al., 2011), (Macierzanka, Mackie, et al., 2019), the freezing, storing  
175 and thawing cause no significant differences in the macro- and microrheological properties of small intestinal  
176 mucus.

### 177

### 178 **2.3. Yogurt *in vitro* dynamic digestion and characterisation of digesta samples**

179 A commercially available, natural yogurt (pH 4.2) was used. The yogurt was obtained from cow's milk  
180 and contained 3.5 wt% fat, 4.5 wt% protein and 6.6 wt% carbohydrates, according to the information provided  
181 by the producer (Danone). The yogurt (120 g) was put through the dynamic *in vitro* gastrointestinal digestion  
182 simulator DIDGI® (Fig 1A, (Ménard et al., 2014)), mimicking the adult human gastric and small intestinal  
183 conditions. The digestion was done in triplicate, following the procedure described before (Ménard, Famelart,  
184 et al., 2018), with some modifications. The specific gastrointestinal digestion parameters applied have been  
5 summarised in Table 1. The flow of enzyme secretions, prepared with either simulated gastric fluid (SGF) or  
6 simulated intestinal fluid (SIF; Table 1), as well as the emptying rates and pH, were continuously monitored  
7 using the STORM® software of the DIDGI® system (INRAE, France). Gastric and intestinal emptying rates  
8 followed an exponential equation as described by (Elashoff, Reedy, & Meyer, 1982). The gastric and intestinal



189 half-times ( $t_{1/2}$ ) of 70 min and 160 min, respectively, and  $\beta$  coefficient of 2 and 1.6, respectively, were applied  
190 according to the values reported by Minekus et al. (Minekus, Marteau, Havenaar, & Huisintveld, 1995) for  
191 yoghurt digestion in adults. A gastric acidification curve was obtained after modelling data from adult humans  
192 (Malagelada, Longstreth, Summerskill, & Go, 1976). Evolutions in the volumes of gastric and small intestinal  
193 contents, as well as in pH values, have been shown in Supplementary Figs. S1 and S2. In the gastric  
194 compartment of digestion, porcine pepsin (Sigma-Aldrich, P6887) was used at 2000 U/mL of gastric content  
195 (Minekus et al., 2014), and lipase A12 from *Aspergillus niger* (Amano) at 18 U/mL of gastric content. The  
196 intestinal conditions were set up as reported by Minekus et al. (Minekus et al., 1995), using pancreatin from  
197 porcine pancreas (Sigma-Aldrich, P7545) and porcine bile extract (Sigma-Aldrich, B8631). Intestinal pH was  
198 maintained at 6.6.

199 The gastrointestinal digestion was carried out continuously for 4.5h at 37°C. Samples of digesta were  
200 taken periodically from the gastric compartment (after 0.25, 0.5, 1, and 2h from the beginning of the digestion  
201 procedure) and the intestinal compartment (after 1, 2, 3 and 4h from the beginning of the procedure) and  
202 analysed using the methods described below. The enzymatic activity in digesta samples, that were withdrawn  
203 for subsequent analysis of the proteolysis progress, was immediately inhibited by using protease inhibitors,  
204 pepstatin A (0.72 mM) for gastric samples and phenylmethanesulfonyl fluoride (0.37 mg/mL; Sigma-Aldrich,  
205 93482) for intestinal samples. The extent of proteolysis in digesta samples was assessed by SDS-PAGE using the  
206 procedure described elsewhere (Ménard, Famelart, et al., 2018), (Böttger et al., 2019). The intestinal digesta  
207 samples that were used in the mucus penetration experiments (section 2.4) were additionally treated with  
208 Orlistat (Sigma-Aldrich, O4139) at the final concentration of 200  $\mu$ M. Lipolysis in the digesta samples  
209 withdrawn for the purpose of analysing changes in the lipid profiles was inhibited by the addition of 0.1 M 4-  
0 bromophenylboronic acid solution in methanol (Sigma-Aldrich, B75956; 50  $\mu$ L of solution per 1 mL of digesta).  
1 A direct extraction of lipids from digesta was performed after sampling, and was based on the Folch method  
2 (Ménard, Bourlieu, et al., 2018). Thin layer chromatography (TLC) was used to assess the overall lipid profiles of  
3 digesta samples, according to the method described previously (Ménard, Bourlieu, et al., 2018). The TLC

214 allowed for monitoring the disappearance of triglycerides (TGs) present in the yogurt and the appearance of  
215 lipolysis products during digestion.

216 The particle size distribution of yogurt, digesta samples, as well as some dispersions of materials used  
217 in the digestion model (i.e., insoluble particles in the bile extract and pancreatin preparations) was obtained  
218 from dynamic light scattering (Mastersizer 2000, Malvern Instruments, Malvern, UK) using the method  
219 described before (Ménard, Bourlieu, et al., 2018). Samples were measured either in their original state or after  
220 treatment with 0.5 % (w/v) sodium dodecyl sulfate (SDS) to evaluate the size distribution of deflocculated  
221 particles. The zeta-potential ( $\zeta$ ) of the gastrointestinal digesta samples was measured using the method  
222 described previously (Macierzanka et al., 2011), (Macierzanka et al., 2012). The gastrointestinal digesta  
223 samples were used in experiments looking at the diffusion of partially digested lipids through human and  
224 porcine small intestinal mucus.

225

#### 226 ***2.4. Diffusion of gastrointestinal digesta in human and porcine small intestinal mucus***

227 The ability of the gastrointestinal digesta (i.e., the digesta samples withdrawn from the intestinal  
228 compartment, after the yogurt had been exposed to the gastric followed by the small intestinal digestion for 1,  
229 2, 3 and 4 h, Fig. 1A,B) to penetrate into the mucus layer was assessed using time-lapse confocal laser scanning  
230 microscopy (CLSM). The mucus was stained for mucins with WGA-Oregon Green (Invitrogen, W6748; 1 mg/mL  
231 in PBS buffer (Sigma-Aldrich, P4417), pH 7.4, containing 2% (w/v) sodium azide). The mucus was gently mixed  
232 with the dye stock at a high mucus to stock ratio, 99: 1 (v/v), in order to minimise the dilution of mucus. This  
233 produced the final dye concentration of 10  $\mu\text{g}/\text{mL}$  and sodium azide concentration of 0.02 % (w/v) in mucus.  
234 Separately, the digesta was stained for lipids with Nile Red (Sigma-Aldrich, 72485) at a final dye concentration  
5 of 8  $\mu\text{g}/\text{mL}$ . The mucus was placed in a custom-made optical cell developed for this study. The cell consisted of  
6 a chamber (6×6×1 mm) for mucus and an adjacent channel (luminal space) for introducing digesta (Fig. 2A).  
7 After the chamber was filled with mucus to 60-80% of its volume, the cell was covered with a coverslip, and the  
8 mucus boundary localised under the microscope. The cell with mucus was incubated on the microscope stage

239 at  $37 \pm 0.1$  °C for 3 min. Subsequently, the liquid digesta (preconditioned at RT) was gently introduced through  
240 the channel inlet to the luminal space (Fig. 2A; ca. 100  $\mu$ L of digesta was required), and finally the channel  
241 sealed to prevent water evaporation during the experiment. This part of the procedure was done in less than  
242 60 s, which was sufficient to bring the temperature of the introduced digesta to ca. 37 °C. The introduction of  
243 digesta was done after the cell with mucus had been securely positioned on the microscope stage, in order to  
244 start monitoring the penetration of mucus with digesta immediately after the two components were brought  
245 into contact with each other. The transport progress was recorded at  $37 \pm 0.1$  °C with a Leica TCS SP upright  
246 confocal microscope (Leica Microsystems (UK) Ltd, UK). The penetration was followed for up to 90 min, and  
247 assessed using the procedure adopted from previous study (Mackie et al., 2016). Briefly, linear fluorescent  
248 intensity profiles of the Nile Red stained lipids in digesta were generated from the time-lapse images (500 $\times$ 500  
249  $\mu$ m, 1024 $\times$ 1024 pixel, 30 s time intervals) using Image-Pro Analyzer 7.0 software (Media Cybernetics Inc., Silver  
250 Spring, MD, USA). The diffusion coefficient was calculated from the fluorescence profiles using the following  
251 equation,  $F(x,t) = a \times \text{erfc}(x/(4Dt)^{1/2})$ , where  $F$  is the fluorescence of digesta stained for lipids described as a  
252 function of distance from the starting boundary  $x$  and time  $t$ . Here,  $a$  is an arbitrary scalar,  $\text{erfc}$  is the  
253 complimentary error function and  $D$  is the diffusion coefficient of the fluorescent lipids.

254 All experiments were performed for mucus samples obtained from 3–5 individual pigs, piglets or  
255 humans, and at least three times (i.e., for three separate mucus specimens) for each individual mucus source.  
256 Results are presented as the mean  $\pm$  SD and/or distributions of data from measurements, for each condition  
257 used. Statistical comparisons between two groups were made using a Student's  $t$ -test, and three (or more)  
258 groups were evaluated using 1-way ANOVA (significance level,  $\alpha = 0.05$ ).

259



## 260 3. Results and discussion

261

### 262 3.1. Yogurt *in vitro* digesta

263 The first step of the study was to obtain digesta from real food. Natural yogurt was selected as it is an  
264 example of a semi-liquid dairy product that is consumed worldwide and contains substantial quantities of all  
265 macronutrients. It was not the aim of this study to investigate in detail the gastrointestinal digestion of yogurt,  
266 but rather to produce *in vitro* a selection of liquid digesta samples that could be used to investigate the  
267 transport of partially digested food particulate matter through small intestinal mucus of various origins. The  
268 dynamic *in vitro* model of human gastrointestinal digestion was chosen to accurately mimic the digestive  
269 conditions in adult humans and to produce digesta.

270 Consequently, we have only carried out qualitative analyses of the proteolysis progress using SDS-PAGE  
271 (Fig. 1C). The protein bands corresponding to milk caseins showed a progressive and substantial reduction in  
272 intensity, which implied a rapid hydrolysis of the caseins by pepsin. After 0.5h of gastric digestion, there were  
273 no casein bands observed. The pepsinolysis of whey proteins,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin, seemed to be  
274 largely completed after 2h of the gastric digestion. The *in vitro* gastrointestinal proteolysis of yogurt has been  
275 studied before. Ménard et al. (Ménard, Famelart, et al., 2018) reported on relatively quick gastric degradation  
276 of caseins in several isocaloric yogurts that differed in the original viscosity (0.3 – 2.2 Pa s) and milk protein  
277 content (3.1 – 8.1 wt%). In that study, the amount of caseins in the yogurts was reduced to only 4 wt% of the  
278 original amount after 2h of the simulated dynamic gastric digestion with pepsin. The amounts of residual  $\beta$ -  
279 lactoglobulin and  $\alpha$ -lactalbumin at the end of the gastric digestion were more dependent on the original  
280 protein concentration and yogurt viscosity. However, for conventional yogurt (i.e., with a total protein content  
1 of 3.1 wt%; viscosity, 1.3 Pa s),  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin were reduced to only 3.1 wt% and 6.4 wt% of  
2 the original amounts, respectively. The digestion of both caseins and whey proteins was finalised in the  
3 intestinal compartment, and resulted in complete disappearance of the protein SDS-PAGE bands after 3h of the  
4 dynamic gastrointestinal digestion. A similar, rapid proteolysis of caseins and whey proteins was observed

285 under static gastrointestinal conditions for yogurts prepared with different casein:whey protein ratios (4.5:1,  
286 2.8:1 and 1.5:1) and 3.70 – 3.75 % total protein contents (Rioux & Turgeon, 2012). For all types of yogurt, the  
287 vast majority of the original amounts of caseins and whey proteins was hydrolysed during the gastric phase of  
288 digestion. Overall, the finding of the two studies described above are in good agreement with our results  
289 shown in Fig. 1C.

290 In the present study, we have also looked at changes in the lipid profile of the milk fat fraction of  
291 yogurt (Fig. 1D). Unsurprisingly, the TLC showed triglycerides (TGs) as a major lipid class in the yogurt before  
292 digestion. They were hydrolysed to some extent to free fatty acids (FFAs) and partial glycerides – di- and  
293 monoglycerides (DGs, MGs) – during gastric digestion, and after 2h TGs coexisted with the lipolysis products.  
294 The complete conversion of remaining TGs seemed to largely take place during the first 1h of the intestinal  
295 lipolysis with pancreatin (Fig. 1D). The intestinal phase of digestion is where chyme is exposed to highly  
296 surface-active bile salts (Macierzanka, Torcello-Gómez, Jungnickel, & Maldonado-Valderrama, 2019). These  
297 physiological surfactants aid in effective intestinal lipolysis by emulsifying ingested lipids, and by solubilising  
298 and removing the lipolysis products from the oil–water interface. All these processes can lead to the reduction  
299 in the size of original TG droplets and in the formation of mixed micelles, composed of the lipolysis products,  
300 bile salts, as well as other polar lipids delivered with bile or ingested food (e.g., phospholipids). The resulting  
301 gastrointestinal digesta is usually a complex mixture of dispersed/solubilised and surface-active lipids, including  
302 FFAs, DGs, MGs, bile salts and other lipids (e.g., phospholipids, cholesterol) (Armand et al., 1999), (Fatouros,  
303 Walrand, Bergenstahl, & Müllertz, 2009). In our study, these types of lipids were observed in digesta produced  
304 after 1–4h of the gastrointestinal digestion (Fig. 1D).

305 The  $\zeta$ -potential values did not differ significantly ( $P>.05$ ) between the four gastrointestinal digesta  
6 samples (i.e., GI-1h,  $-49.8 \pm 1.1$  mV; GI-2h,  $-48.6 \pm 2.1$  mV; GI-3h,  $-49.0 \pm 2.3$  mV; GI-4h,  $-49.2 \pm 1.8$  mV), and  
7 were comparable to the results obtained for other post-digestion emulsions that had been digested in the  
8 presence of bile salts (Macierzanka et al., 2011), (Macierzanka et al., 2012). It was also noticed that the original  
9 size of lipid droplets in yogurt was reduced due to lipolysis and interfacial activity of bile salts (Fig. 1E). This

310 behaviour is consistent with the results of other studies (Bonnaire et al., 2008), (Armand et al., 1999). However,  
311 it was difficult to compare the mean particle size between the gastrointestinal digesta and the original yogurt,  
312 as the digesta samples revealed multimodal size distributions (Fig. 1E). Treating samples with SDS before  
313 measurement did not change the size profiles (data not shown), suggesting the polydispersity was not the  
314 effect of particle flocculation. The particle size distributions of the digesta samples seemed to be obscured to  
315 some extent by a particulate matter introduced to the intestine digestion mix with the pancreatin and the bile  
316 salt extract. These insoluble particles were likely to be cellular debris remaining in the preparations after  
317 extraction of pancreatic enzymes and bile salts from animal materials by the producer (Sigma-Aldrich). When  
318 measured separately, the pancreatin and the bile salt extract showed an existence of insoluble particles  
319 ranging in size from ca. 1  $\mu\text{m}$  to  $>1000 \mu\text{m}$ , with most particles being well over 20  $\mu\text{m}$  in diameter (Fig. 1E).  
320 Those particles contributed to the volume size distributions of the digesta samples withdrawn during digestion.  
321 However, the majority were much larger than the particles of digested lipids and did not dominate the  
322 distribution profiles of digesta samples (Fig. 1E). This meant they could not greatly contribute to the overall  
323 number of particles, the major fraction of which was represented by considerably smaller lipid particles. The  
324 negligible contribution of the insoluble particulate matter that derived from pancreatin and bile extract was  
325 confirmed by converting the volume distributions of the four gastrointestinal digesta samples to number  
326 distributions (Fig. 1F). The gastrointestinal digesta was further used in the major part of the study looking at  
327 the effect of the type of small intestinal mucus on its penetrability to the post-digestion lipid particles.

### 329 **3.2. Diffusion of gastrointestinal digesta in the small intestinal mucus**

#### 330 **3.2.1. Sieve-like barrier properties of mucus**

2 We have developed a simple optical cell that allowed for convenient and reproducible monitoring of  
3 interactions between mucus and colloidal dispersions. The cell was used to bring the liquid digesta samples  
4 into contact with the small intestinal mucus without compromising the integrity of the mucus gel. This way, the

335 penetration of the mucus layer by the lipid particles undergoing Brownian motion in the digesta could be  
336 monitored immediately after the digesta and mucus were brought together (Fig. 2A,B), mimicking the passage  
337 of dispersed/solubilised lipids from the small intestinal lumen into the mucus overlaying the intestinal  
338 epithelium. Fluorescent intensity profiles of stained lipids were generated from the time-lapse CLSM images  
339 (Fig. 2C) and used to assess the diffusion of lipid digesta in the mucus layer as explained in section 2.4.

340 By applying this approach, we have initially evaluated an influence of the digestion time on the  
341 diffusion rate of digesta in mucus. The mucus scraped from the jejunal segments of the small intestine in adult  
342 pigs was chosen for experiments as this type of *ex vivo* mucus had previously been shown to produce relatively  
343 little individual variability regarding rheological properties (Macierzanka et al., 2011). It has also been shown,  
344 by multiple-particle tracking of 500 nm latex beads, that porcine mucus collected *ex vivo* maintains the same  
345 microstructural characteristics and permeability to particles as the native mucus overlaying the jejunal mucosa,  
346 even if the *ex vivo* mucus was subjected to freezing and thawing after collection (Macierzanka, Mackie, et al.,  
347 2019).

348 Figure 3A shows the comparison of diffusion coefficients of the digesta samples obtained after 1, 2, 3  
349 and 4h of gastrointestinal digestion. For each mucus penetration time, there was a subtle inverse correlation  
350 between the mean diffusivity value and the digestion time. However, the correlations were not statistically  
351 significant ( $P > .05$ ) within the same penetration times, even during the first several minutes after the start of  
352 mucus penetration when the effect was most noticeable (Fig. 3A). This might have been due to the relatively  
353 small differences in the size distribution profiles between the four time-point digesta samples collected during  
354 the course of digestion (Fig. 1F). Much more profound was a progressive decrease in the apparent diffusion of  
355 digesta over the time of mucus penetration (Fig. 3A). This trend was consistent for all four types of  
6 gastrointestinal digesta. A significant drop in diffusion coefficient was observed during the first 30 min after the  
7 initial penetration of mucus, and then the diffusivity remained fairly constant until the end of experiment. The  
8 ratios of diffusion coefficients from 5 min to 90 min after the start of penetration into the mucus ( $D_{90}/D_5$ )  
9 showed consistently for each digesta that there was a nearly 70% reduction of the initial diffusivity after 90 min

360 (Fig. 3B). An explanation for this phenomenon may lie in the way the small intestinal mucus is organised at the  
361 microscale. The gel-forming MUC2 mucin oligomers are able to form a porous network in the mucus, which can  
362 limit movement only to particles not restricted by the mesh size. In murine jejunal mucus, an average pore size  
363 of 200–220 nm was found (Bajka, Rigby, Cross, Macierzanka, & Mackie, 2015). However, the reported sizes  
364 ranged broadly, from 75 nm to approx. 500 nm. Furthermore, the transport of particles can be affected by the  
365 spatial organisation of mucin microscale domains in the mucus. A hierarchical model of colloidal transport  
366 through the small intestinal mucus was postulated by Round and co-workers (Round et al., 2012). It involves an  
367 existence of individual lamellae of MUC2 mucin network rather than a continuous three-dimensional network  
368 of the mucin polymer. According to the model, the weak interactions between the lamellae allow for the  
369 passage of particles larger than the pores in lamellae as those particles are able to diffuse along transient  
370 channels between lamellae. Particles smaller than the pore size can traverse directly through the lamellae as  
371 long as they are not sterically trapped. More recently, Meldrum et al. (Meldrum et al., 2018) reported on a  
372 similar mechanism of a network assembly of MUC2 mucin in which viscoelastic microscale domains of mucin  
373 oligomers were proposed to form via hydrogen bonding and  $\text{Ca}^{2+}$ -mediated links. Individual domains were then  
374 assembled in a yield stress, gel-like fluid. In the previous studies on multiple-particle tracking in mucus  
375 (Macierzanka et al., 2011), (Macierzanka et al., 2014), (Bajka et al., 2015), a broad diffusivity distribution was  
376 observed for non-mucoadhesive, monodisperse nano- and micro-beads that were used to probe the  
377 microviscosity of the porcine small intestinal mucus. Those results confirmed a high degree of mucus  
378 heterogeneity and might also support the postulated hierarchical organisation of the mucus microstructure.

379 Taking into account these previous findings as well as the results of the present study (Fig. 3), the small  
380 intestinal mucus may be considered to act as a sieve to a polydisperse digesta through the network of channels  
1 and pores of various sizes. The concept of size filtering has been previously introduced for different types of  
2 mucus (Lieleg, Vladescu, & Ribbeck, 2010); it assumes that particles smaller than mucus mesh size are allowed  
3 to pass while larger particles are rejected. The contrasting concept of interaction filtering is based on the  
4 strength of interaction between particles and the mucus polymer network, so the weakly interacting particles



385 are not retained in the mucus and allowed to diffuse. In this study, the gastrointestinal digesta was produced in  
386 the presence of bile extract containing bile salts, which must have been responsible for the high negative  
387 charge of the digesta dispersions ( $\zeta$ -potential ca. -49 mV, see section 3.1.). The adsorption of anionic bile salts  
388 has been shown previously to hugely enhance the diffusivity of particles in small intestinal mucus by  
389 strengthening their negative surface charge and thus preventing mucoadhesion (Macierzanka et al., 2011),  
390 (Macierzanka et al., 2012). Therefore, the size filtering mechanism seems to be the most likely explanation in  
391 the present study for the sieve-like behaviour of the mucus confronted with the yogurt digesta. It is plausible  
392 that the largest lipid particles in digesta were immobilised within a relatively short time after penetrating into  
393 the mucus layer, whereas smaller particles were able to diffuse deeper into the mucus. The former was  
394 suggested by an increase in red fluorescence in the boundary region of the mucus over the fluorescence  
395 recorded for digesta in the luminal space (Fig. 2C). Consequently, it was only the smallest particles that were  
396 likely to penetrate through the surface region of mucus clogged with the lipid droplets larger than the mucus  
397 mesh size (i.e., the size of channels and pores), and continued to diffuse freely in the mucus at a constant rate  
398 until the end of the penetration experiment (90 min, Fig. 3A). This may suggest that, under physiological  
399 conditions of the gut, lipolysis continues in the intestinal mucus after partially digested fat droplets leave the  
400 intestinal lumen and get entangled in the mucus. The diffusion through the mucus and towards the underlying  
401 epithelium would then only continue after they become gradually reduced to smaller droplets and/or particles  
402 of assembled lipolysis products due to enzymatic hydrolysis and assistance of the surface-active bile salts.

403 A similar clogging of intestinal mucus was observed after it was exposed to the soluble dietary fibre  
404 sodium alginate (Mackie et al., 2016). As a result, the apparent diffusivity of that high molecular weight  
405 polymer was reduced in the function of mucus penetration time, despite the lack of interaction between the  
6 mucin and the alginate. The local diffusivity of lipid digesta inside the small intestinal mucus matrix has been  
7 studied before (Mackie, Rigby, Harvey, & Bajka, 2016). However, our present report is the first time the sieve-  
8 like behaviour of mucus has been shown to affect the transport rates of a complex, polydisperse mixture of  
9 post-digestion lipid particles during the penetration into the mucus layer from a simulated luminal space.

410

### 411 3.2.2. Penetration of human and porcine mucus layer

412 Having analysed the diffusion of various digesta samples in the pig jejunal mucus, we looked at how the  
413 permeation of digesta may be affected by the type of small intestinal mucus. The penetrability was compared  
414 between mucus samples obtained from the jejunum of adult pigs, and the ileum of adult pigs and humans. We  
415 also included jejunal mucus from 2 week old piglets in the study. This aimed to investigate the following,  
416 individual comparisons of mucus types: (i) age-specific (i.e., piglet vs. adult pig; both for jejunal mucus), (ii)  
417 intestinal location-specific (i.e., jejunal mucus vs. ileal mucus; both for adult pig), and (iii) species-specific (i.e.,  
418 adult pig vs. adult human; both for ileal mucus). The yogurt digesta produced after 2h of gastrointestinal  
419 digestion (GI-2h) was used for analysing penetrability of different types of mucus. As for the initial experiments  
420 shown in Figs. 2 and 3, mucus samples were exposed to the digesta at 37°C, and diffusion of fluorescently-  
421 labelled lipids into the mucus monitored in the function of time (Fig. 4).

422 The fluorescence profiles revealed a striking difference in penetration rates between the two jejunal  
423 types of mucus analysed (Fig. 4A). The piglet mucus was significantly more permeable to the diffusing digesta  
424 than the pig mucus, and after 5 min from the start of penetration the respective mean diffusion coefficients  
425 were  $6.56 \times 10^{-11} \text{ m}^2\text{s}^{-1}$  and  $1.29 \times 10^{-11} \text{ m}^2\text{s}^{-1}$  ( $P < .05$ , Fig. 4B). This indicates the piglet mucus presents a less  
426 effective barrier to passively diffusing lipid particles. The microstructural organisation of the pig and the piglet  
427 jejunal mucus has been compared previously, using direct tracking of the movement of 500 nm latex beads in  
428 mucus (Macierzanka et al., 2014). The two mucus secretions used in that study were pre-treated with DNase in  
429 order to reduce the impact of extracellular DNA on the viscosity experienced by the probe particles. The study  
430 revealed a substantial difference in the diffusivity of beads, which was largely due to contrasting ways the  
1 mucin matrix was organised between the two mucus types. In the mucus of fully-grown pigs, the mucin  
2 produced a coherent porous network, whereas in the piglet mucus the network was more heterogeneous and  
3 fragmented. It consisted of large aggregates of mucin polymer surrounded by regions with lower local  
4 concentrations of the glycoprotein. The most rapid diffusion of the tracer particles was observed in those

435 regions. According to the same report, the piglet mucus also contained approx. 40% less extracellular DNA than  
436 the pig mucus. The differences in both the mucin organisation and the DNA concentration were suggested to  
437 be caused by disparities in the distribution of mucin-producing goblet cells and in the epithelial cell turnover  
438 during neonatal development compared to adulthood. Because of this, the mean microviscosity experienced  
439 by the latex beads diffusing in the piglet mucus was roughly half the value recorded for the pig counterpart  
440 (Macierzanka et al., 2014). The impact of developmental age on differences in chemical composition, structural  
441 properties and permeability to particles of small intestinal mucus was studied recently in rats (Lock et al.,  
442 2020). The gut immaturity was suggested to be responsible for significantly lower concentrations of mucin and  
443 DNA in the ileal mucus of 5 day old rat pups relative to 21 day old rats. Particle tracking experiments, using 200  
444 nm PEG-, carboxyl- and amine-modified polystyrene particles, showed the mucus from early developmental  
445 age rats (5 day old) was substantially more permeable to diffusing particle compared to 21 day old rats. The  
446 mucus of the former age group was also significantly more penetrable to flagellated bacteria (*E. coli*),  
447 suggesting immaturity may contribute to enhanced exposure of the intestinal epithelium to microbes.

448           Since the structural barrier properties of mucus appear to develop during infancy, it is not surprising  
449 that in the present study we observed a higher diffusivity of digesta in the 2 week old piglet mucus than in the  
450 adult pig mucus. The broad size distribution of lipid particles in the digesta (Fig. 1E) does not allow for using  
451 them to probe the microviscosity of mucus, as the diffusion of an individual particle greatly depends on its  
452 diameter (S. K. Lai et al., 2010). However, results of the time-lapse measurements with digesta produced from  
453 a real dairy product may provide new insights into how the permeability of jejunal mucus to lipids can change  
454 postneonatally. Because of the relatively fast penetration of digesta into the piglet mucus, the experiment was  
455 terminated after 50 min from the initial penetration of this type of mucus (Fig. 4B). Although the diffusivity of  
6 digesta was reduced progressively over the first 30 min in the piglet mucus, the difference between the  
7 diffusion coefficients after 5 min and 30 min was not significant ( $P>.05$ ). This suggests the channels and pores  
8 in the mucus matrix were not substantially clogged with large lipid particles, and allowed fast transport. This  
9 also supports the previous findings of a loose and easy-to-penetrate structure of neonatal mucus (Macierzanka



460 et al., 2014), (Lock et al., 2020). The diffusivity in piglet mucus remained unchanged for the remaining 20 min.  
461 This is in contrast to what was observed for the pig jejunal mucus. There was a significant drop in the rate of  
462 digesta diffusion measured between 5 min and 30 min ( $P < .05$ ) from the start of mucus penetration. The  
463 diffusion coefficient remained constant after 30 min and until the end of experiment. The difference in  
464 penetrability of the two types of jejunal mucus was further emphasised by comparing the ratios of diffusion  
465 coefficients from 5 min to 50 min ( $D_{50}/D_5$ , Fig. 4C). For the piglet mucus, the ratio was almost twice as high as  
466 for the pig mucus, meaning that the apparent diffusivity of digesta was reduced far more substantially by the  
467 pig mucus.

468 The above suggest that the transmucus transport of lipids in a neonatal small intestine might be much  
469 faster than in a mature gut. It can potentially involve the arrival of lipid droplets/particles with a large spectrum  
470 of sizes in close proximity to the epithelium, which may enhance the rate of intestinal absorption of lipolysis  
471 products that are eventually released from hydrolysed fat droplets. This, however, can be limited by low  
472 activity/concentration of pancreatic lipase in the neonatal small intestine, as reported for the human pre-term  
473 and full-term infants relative to adults (Bourlieu et al., 2014). Further studies would be required to assess the  
474 impact of mucus structure on the rate of intestinal lipid absorption.

475 Comparing the permeability of mucus from different segments of the small intestine has not attracted  
476 much scientific attention so far despite the fact that the mucosal architecture varies longitudinally as the villi  
477 length decreases from the duodenum to the ileum, which may affect the structural properties of the mucus  
478 layer. In our study, the sieve-like barrier behaviour, characteristic of the pig jejunal mucus, was also observed  
479 for the ileal mucus obtained from fully-grown pigs. The time evolution in the fluorescence intensity profiles of  
480 digesta, and thus in the diffusivity observed in the ileal mucus mirrored that recorded for the jejunal mucus  
1 (Fig. 4A,B). The  $D_{50}/D_5$  ratios for both types of pig mucus were very similar, and showed that there was an  
2 almost 70% reduction in the apparent diffusion of digesta after 50 min of mucus penetration regardless of the  
3 original, anatomical location of the mucus (Fig. 4C). The ratios did not change significantly after extending the  
4 penetration to 90 min (Supplementary Fig. S3). The reduction of the diffusion rate in the function of

485 penetration time, over the initial 50 min, meant that for both types of mucus some lipid particles could  
486 penetrate as deep as 100–150  $\mu\text{m}$  into the mucus layer during the first 5–10 min, whereas the penetration  
487 depth could only increase to ca. 300  $\mu\text{m}$  after 50 min (Fig. 4A). The fact that the diffusion coefficients were very  
488 similar for both types of mucus (Fig. 4B) suggests that *in vivo* the transmucosal transport times might depend  
489 on variations in the thickness of the mucus layer between the jejunum and the ileum, rather than on  
490 differences in mucus microstructure. However, the effect of varying thickness on transport times might be  
491 difficult to account for. To our knowledge, there are no studies looking at how these variations might look in  
492 humans. In rats, the ileal mucus layer was reported to be significantly thicker than the duodenal and jejunal  
493 mucus gel layers ( $480 \pm 47 \mu\text{m}$ ,  $170 \pm 38 \mu\text{m}$  and  $123 \pm 4 \mu\text{m}$ , respectively, (Atuma, Strugala, Allen, & Holm,  
494 2001)). It was also observed that the mean mucus thickness can be reduced approx. 7-fold in the rat ileum and  
495 even >20-fold in the jejunum after feeding relative to the animals fasted for 48h (Szentkuti & Lorenz, 1995).  
496 However, in the mouse, no substantial difference was found between the thickness of jejunal and ileal mucus  
497 (Ermund, Schütte, Johansson, Gustafsson, & Hansson, 2013). There were no significant differences observed  
498 for the mean pore size of mucus and the size distribution profiles between the two intestinal segments either  
499 (Bajka et al., 2015). The latter mouse study supports our finding of the very comparable penetrability of jejunal  
500 and ileal mucus to digesta (Fig. 4B), and implies a similar lack of difference in microstructural organisation of  
501 mucus in proximal and distal regions of the pig small intestine.

502 Importantly, we also looked at how the human mucus was penetrated by the lipid particles of digesta.  
503 The results showed a very similar behaviour of the human ileal mucus and the pig jejunal and ileal mucus (Fig.  
504 4B). Analogically to the pig mucus, the diffusion coefficient of digesta decreased significantly ( $P < .05$ ) over the  
505 first 30 min after bringing the digesta into contact with human mucus, and remained largely unchanged after  
6 that time at ca.  $5 \times 10^{-12} \text{m}^2\text{s}^{-1}$ . There was no significant difference in how the diffusivity evolved in time  
7 between the human mucus and the pig mucus, which has been shown by comparing the actual values of  
8 diffusion coefficient (Fig. 4B) as well as the ratios of diffusivities at various stages of mucus penetration (Fig. 4C,  
9 Supplementary Fig. S3). All these results imply a very similar microstructural organisation of the adult human

510 and pig small intestinal mucus with regard to penetrability to polydisperse colloidal particles. These data bring  
511 about the proof for the human-relevance of adult pig small intestinal mucus in terms of selective barrier  
512 properties to post-digestion luminal lipids.

513

#### 514 **4. Conclusions**

515

516 We compared *ex vivo* the penetrability of several types of small intestinal mucus to digesta obtained  
517 after simulated dynamic gastrointestinal digestion of yogurt. The overall apparent diffusivity of digesta in adult  
518 pig mucus decreased significantly during the first 30 min of penetration, suggesting a sieve-like response of the  
519 mucus matrix to the post-digestion lipid particles varying in size. The rate of digesta diffusion in the mucus of 2  
520 week old piglets was several times faster. This suggests that neonatal mucus is a less effective barrier to  
521 passively diffusing particles relative to adult mucus.

522 Most importantly, we showed that the evolution in penetrability of the adult human small intestinal  
523 mucus to diffusing lipids was analogous to the penetration profiles of the mucus obtained from adult pigs. To  
524 our knowledge, it is the first time such analogies have been reported for the transport of gastrointestinal  
525 digesta. This validates the use of small intestinal mucus from fully-grown pigs as a human-relevant substitute in  
526 studies focusing on the transmucous transports of molecules and complex colloidal dispersions (e.g.,  
527 nutrients/bioactives, digested foods, orally-administrated drug delivery systems) under simulated conditions of  
528 the adult human small intestine. The close resemblance in penetrability to diffusing entities of the adult pig  
529 mucus and the adult human mucus suggests that some analogy might also be expected between the examined  
530 piglet mucus and the mucus of human infants, which was not studied. However, confirming any such similarity  
1 would certainly require further studies that, for obvious ethical reasons, might be problematic.

2

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539

#### 540 **Supplementary Material**

541 Additional characterisation of methods and data.

542

#### 543 **Declarations of interest**

544 None.

545

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547

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671 **Tables**

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673 Table 1. Gastrointestinal parameters of *in vitro* dynamic digestion of yogurt

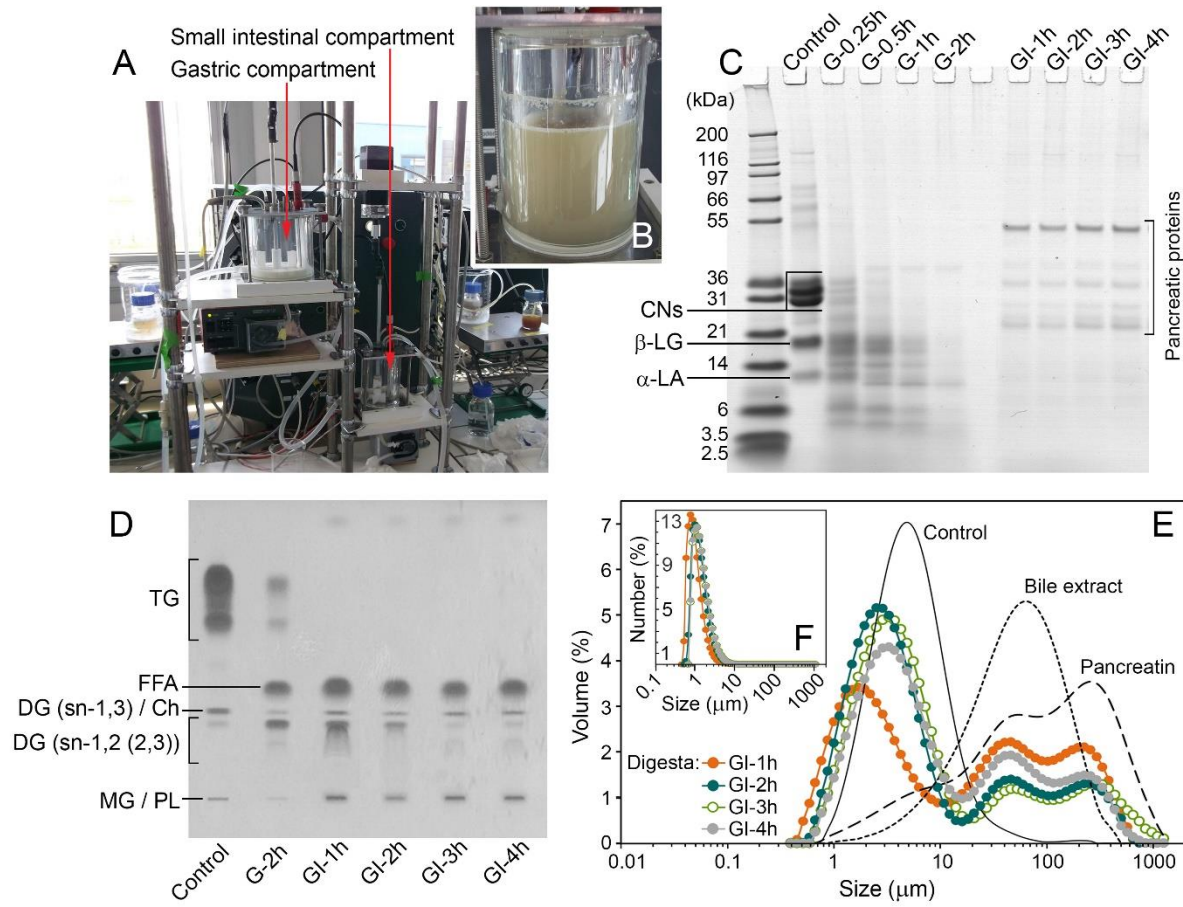
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<b>Gastric conditions (37°C)</b>		
Simulated Gastric Fluid (SGF)	Na <sup>+</sup>	100 mmol/L
(stock solution adjusted at pH 6.5)	Ca <sup>2+</sup>	1 mmol/L
Yogurt	Ingested amount	120 g
Gastric pH (acidification curve) (using 1M HCl)	pH = 1.68 + 3.82 <sup>(-t/42)</sup> (t, time after ingestion in min)	
SGF + pepsin (porcine)	Pepsin	2000 U/mL of gastric content
	Flow rate	0.75 mL/min from 0 to 10 min
	Flow rate	0.25 mL/min from 10 min to the end of the gastric phase
SGF + lipase (fungal)	Lipase	18 U/mL of gastric content
	Flow rate	0.75 mL/min from 0 to 10 min
	Flow rate	0.25 mL/min from 10 min to the end of the gastric phase
Gastric emptying (Elashoff fitting)	t <sub>1/2</sub>	70 min
	β	2
<b>Intestinal conditions (37°C)</b>		
Simulated Intestinal Fluid (SIF)	Na <sup>+</sup>	100 mmol/L
(stock solution adjusted at pH 6.2)	Ca <sup>2+</sup>	1 mmol/L
Intestinal pH (using 1M NaHCO <sub>3</sub> )	pH	6.6
SIF + bile extract (porcine)	Bile	4% from 0 to 30 min
	Bile	2% from 30 min to the end
	Flow rate	0.5 mL/min from 0 min to the end
SIF + pancreatin (porcine)	Pancreatin	7%
	Flow rate	0.25 mL/min from 0 min to the end
Intestinal emptying (Elashoff fitting)	t <sub>1/2</sub>	160 min
	β	1.6

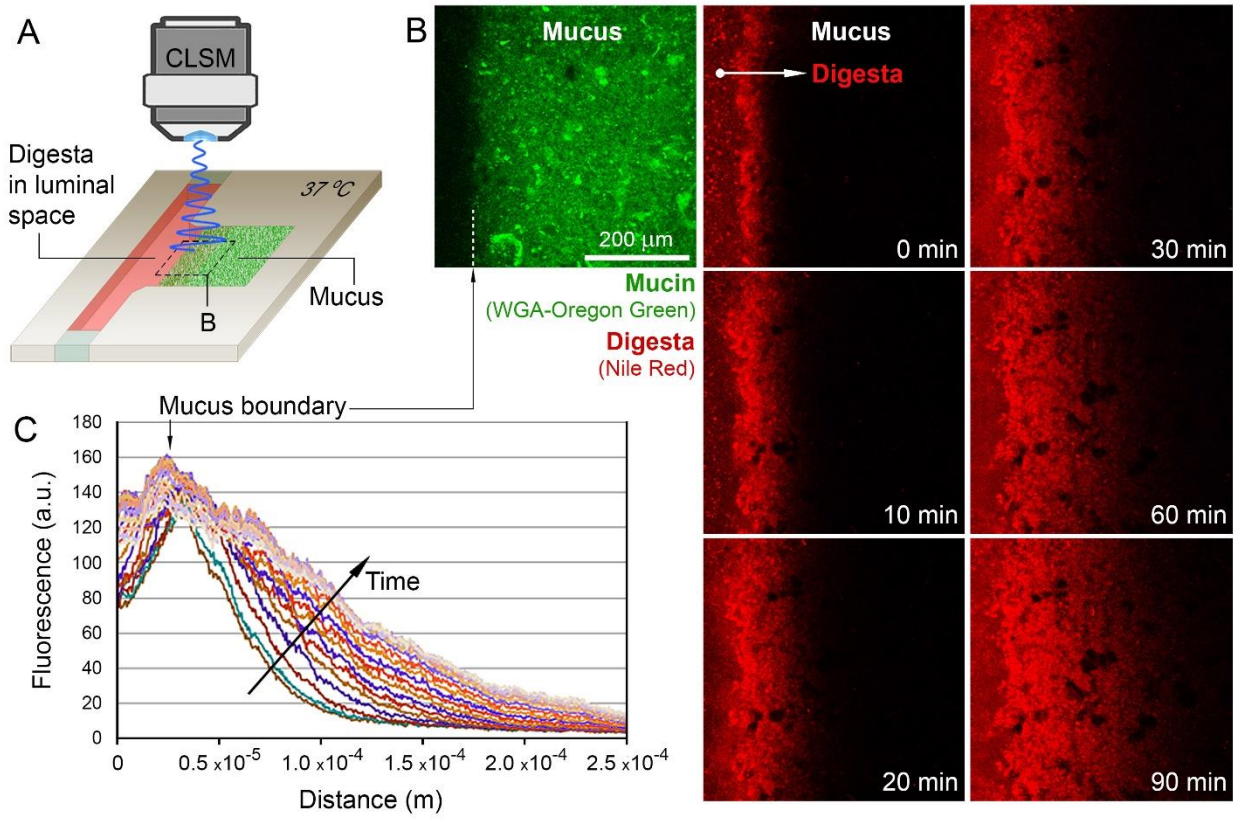
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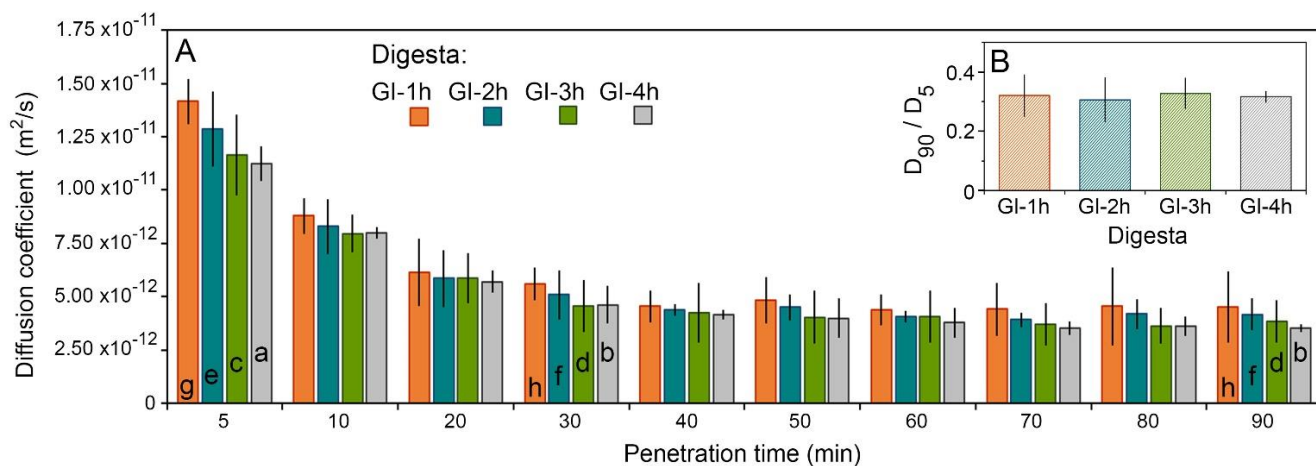


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683 **Fig. 1. *In vitro* dynamic digestion of yogurt and characterisation of digesta samples.** (A) The DIDGI® dynamic *in vitro* gastrointestinal (GI) digestion system comprising interconnected gastric and small intestinal compartments. (B) The small intestinal compartment with GI digesta produced after 4h of yogurt digestion. (C) SDS-PAGE, (D) TLC, and (E,F) particle size distributions ((E) volume distribution and (F) number distribution) of digestion time-point samples. Samples withdrawn from the gastric and the small intestinal compartments are marked with 'G' and 'GI', respectively, followed by the time from the beginning of the digestion experiment. The graph (E) also shows the size distribution profiles for particles in the bile extract and pancreatin preparations used in digestion. The 'Control' (in C-E) refers to the yogurt before digestion. Abbreviations: CNs, caseins; β-LG, β-lactoglobulin; α-LA, α-lactalbumin; TG, triglyceride; DG, diglyceride; MG, monoglyceride; PL, phospholipid; Ch, cholesterol; FFA, free fatty acid.



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**Fig. 2. The time-lapse CLSM experimental set-up for tracking the extent of digesta penetration into the small intestinal mucus.** (A) Schematic representation of a custom-made optical cell used for monitoring the boundary between mucus and digesta (i.e., the area enclosed by the dashed line). (B) Representative CLSM images showing a layer of pig jejunal mucus stained with WGA-Oregon Green for mucin (green channel) and positioned in the optical cell. The mucus layer was exposed for up to 90 min to digesta (red channel) produced from yogurt after 3h of dynamic *in vitro* gastrointestinal digestion (GI-3h). The red fluorescence shows progressive penetration of the mucus layer by the digesta stained for lipids with Nile Red. (C) Evolution in the red fluorescence intensity profiles of digesta in the function of the distance of penetration into the mucus. The profiles were generated at different times over a period of 90 min after the initial penetration of the mucus layer.



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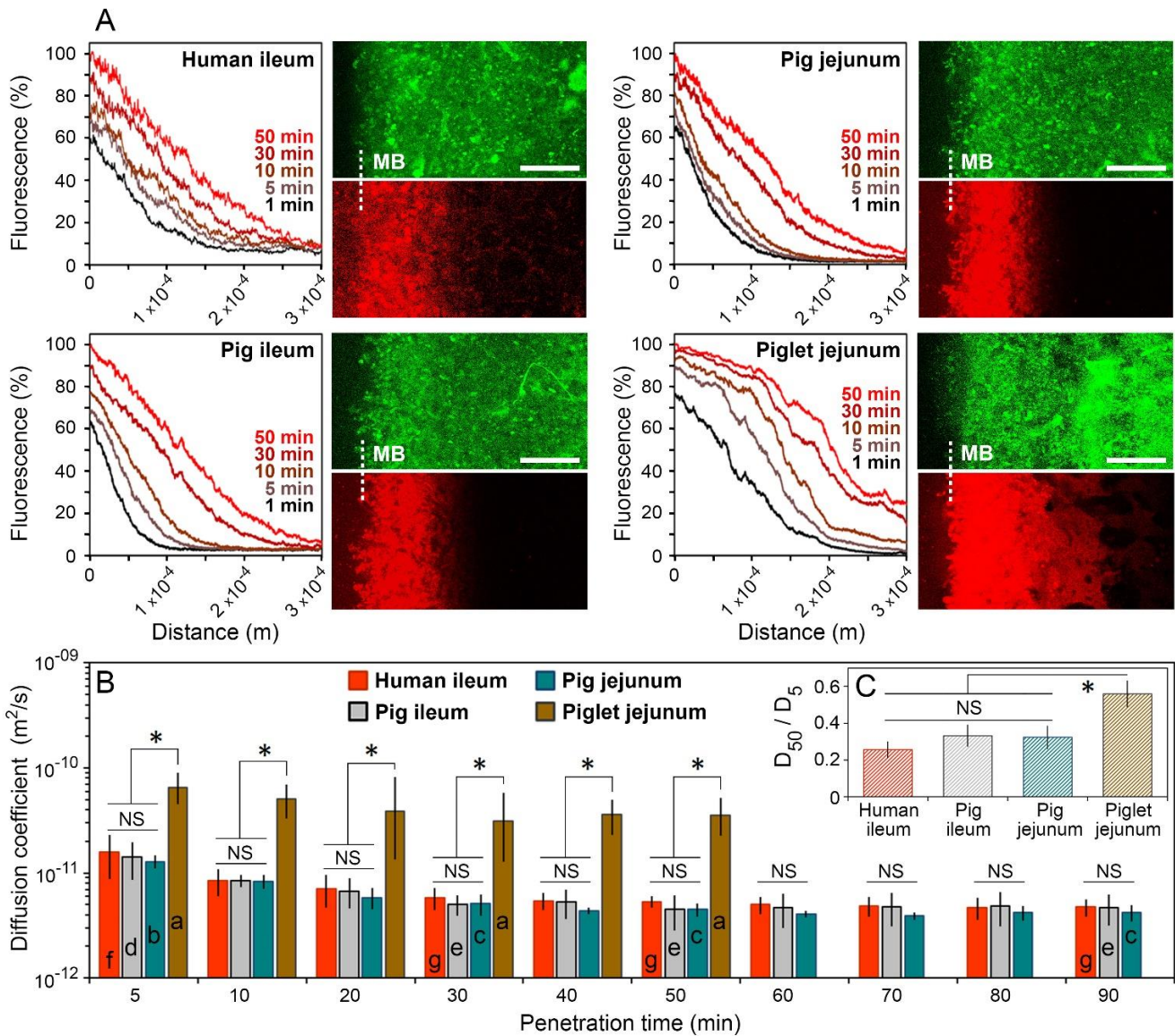
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**Fig. 3. Diffusion of yogurt digesta in the pig jejunal mucus. The effect of gastrointestinal digestion time on the diffusivity of digesta in the mucus.** (A) Evolution of the diffusion coefficients of digesta samples as measured from the fluorescence intensity profiles of lipids in digesta over a period of 90 min after the initial penetration of the mucus layer. The digesta samples were obtained after either 1, 2, 3 or 4h of dynamic *in vitro* gastrointestinal digestion of yogurt (GI-1h, GI-2h, GI-3h or GI-4h), stained fluorescently for lipids, and exposed *ex vivo* to the mucus (Fig. 2). Bars marked with identical letters within the same type of digesta are not significantly different at  $\alpha = 0.05$ . (B) The ratio of diffusion coefficients of the digesta at 5 min and 90 min ( $D_{90}/D_5$ ) after initial penetration of the mucus layer. All measurements were conducted at  $37 \pm 0.1$  °C. Mean  $\pm$  SD ( $n = 3$ ).



**Fig. 4. Comparison of the penetration rates of gastrointestinal yogurt digesta into the human and porcine small intestinal mucus.** The digesta was obtained after 2h of dynamic *in vitro* gastrointestinal digestion of yogurt (GI-2h) and exposed *ex vivo* to the mucus collected from the human ileum, the pig ileum, the pig jejunum, and the piglet jejunum. (A) Representative fluorescence intensity profiles (normal to the mucus boundary) of digesta stained for lipids in the function of the distance of penetration into the mucus. The fluorescence profiles were recorded at 1, 5, 10, 30 and 50 min after the initial penetration of the mucus layer. Each fluorescence graph is accompanied by CLSM images showing the mucus boundary (MB; mucus is shown in green channel) and the extent of digesta penetration (red channel) into mucus after 10 min. The scale bars correspond to 100  $\mu m$ . (B) Evolution of the diffusion coefficient of the yogurt digesta in the four mucus types over a period of up to 90 min after the initial penetration of mucus, as measured from the fluorescence intensity profiles of lipids in digesta. Bars marked with identical letters within the same type of mucus are not significantly different at  $\alpha = 0.05$ . (C) The ratio of diffusion coefficients of the digesta at 5 min and 50 min ( $D_{50}/D_5$ ) after initial penetration of the mucus layer. All measurements were conducted at  $37 \pm 0.1$  °C. Mean  $\pm$  SD ( $n = 3-5$ ); \*  $P < .05$ ; NS, not significant ( $P > .05$ ).