Great escape: how infectious SARS-CoV-2 avoids inactivation by gastric acidity and intestinal bile

The study by Lee *et al*[1] showed that the short-term current use of proton pump inhibitors (PPIs) for less than 1 month was associated with severe clinical outcomes for patients with COVID-19. The authors speculated that individuals taking PPIs had increased gastric pH, leading to higher SARS-CoV-2 viral loads associated with a severe course of COVID-19. Many studies suggested that a proportion of patients with COVID-19 experiencing GI symptoms such as diarrhoea, nausea and vomiting had overall more severe disease.[2] However, it is not clearly understood how SARS-CoV-2 could survive the passage through the harsh gastric acidity and persist through the intestinal contents to infect the intestinal epithelia. It is known that the gastric pH varies greatly, depending on whether the individual is in a fasting or feeding state (between 1.23 and 6.7, respectively).[3] Similarly, bile concentrations in the small intestine can fluctuate from as low as 2.6 mM in fasted state to over 15 mM in the fed state.[4] Whether changes in gastric pH and bile allow the virus to escape gastric and intestinal inactivation to infect the intestine is not well understood. Therefore, to understand the effect of stomach acidity, digestive components and meals on the infectivity of swallowed SARS-CoV-2, the virus ~6 log 50% tissue infective dose ($TCID_{50}$)/mL was incubated at 37°C for 60 min in simulated gastric fluid of different pH (1.5–6.0), pepsin (0–8 mg/mL), pancreatin (0–5 mg/mL) or bile (0–15 mM) solutions and for up to 120 min in simulated biorelevant gastric and intestinal fluids supplemented with digestive enzymes that represent either fasting or feeding states (see online supplemental materials for details). SARS-CoV-2 was highly inactivated by gastric pH of ≤2.5 (~5.8 log reduction), showed a~3.2 log reduction at pH 3 and was less affected by pH between 3.5 and 6.0 (~1 log reduction) (figure 1A). Pepsin had no significant effect on SARS-CoV-2 infectivity (figure 1B). The virus infectivity was significantly reduced by pancreatin in a dose-dependent manner and by bile in an inversely proportional manner (figure 1C,D). Furthermore, under fasting, gastric followed by intestinal fluids highly reduced SARS-CoV-2 infectivity by ~4 log

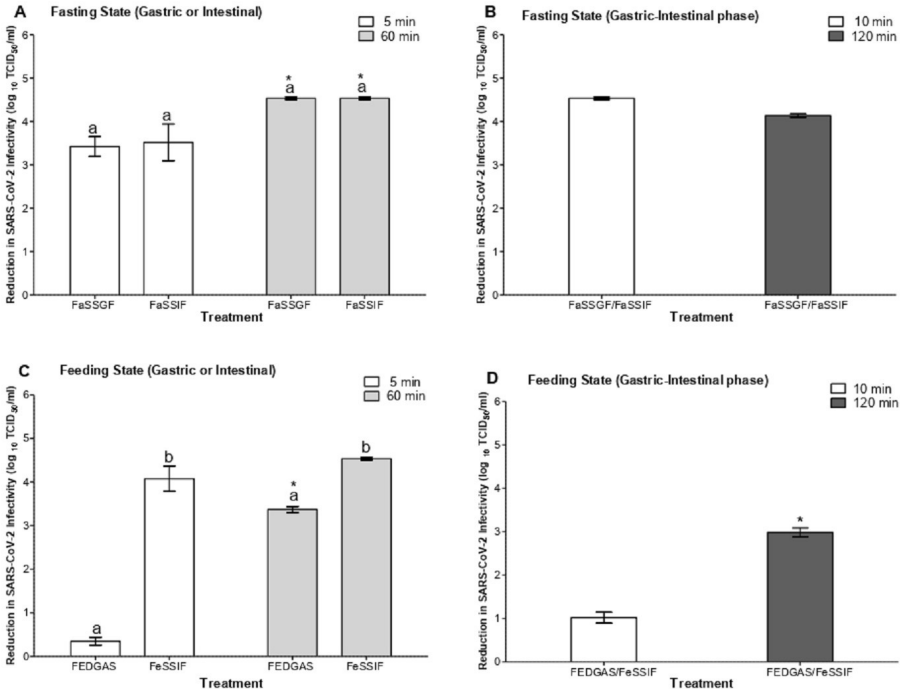

Figure 2 Reductions in SARS-CoV-2 infectivity titres (log $TCID_{50}$/mL) when the virus was tested under in vitro digestion simulating fasting state (A) with gastric FaSSGF (pH 1.6 and bile at 0.08 mM) or intestinal FaSSIF (pH 6.5 and bile at 3 mM) or (B) with gastric followed by intestinal fluids and under feeding state with (C) gastric FEDGAS (pH 3 and bile at 0.3 g/L) or intestinal FeSSIF (pH 5 and bile at 15 mM) or (D) with gastric followed by intestinal fluids. Comparing treatments within a time point: means with different letters differ significantly ($p< 0.05$). Comparing corresponding treatments among time points: significant differences are denoted with asterisks. FaSSGF, fasting state simulating gastric fluids; FaSSIF, fasting state simulating intestinal fluids; FEDGAS, feeding gastric fluid; FeSSIF: feeding state simulating intestinal fluids.

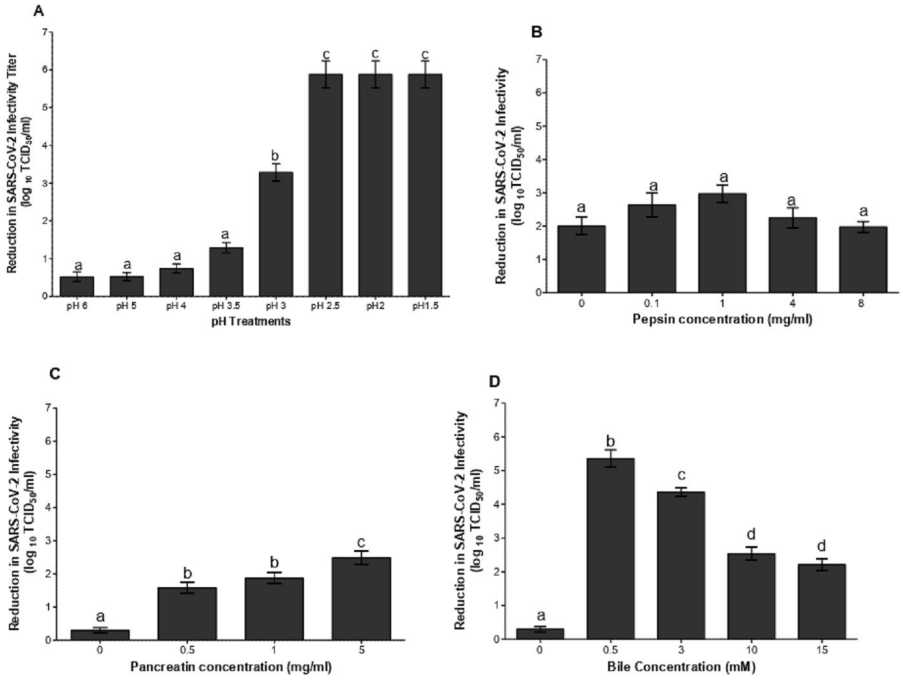

Figure 1 Reduction in SARS-CoV-2 infectivity titre (log $TCID_{50}$/mL) when the virus was incubated for 1 hour at 37°C in (A) gastric fluid with different pH, (B) pepsin prepared in standard simulated gastric fluid (pH 3), (C) pancreatin prepared in standard simulated intestinal fluid (pH 7) and (D) bile prepared in standard simulated intestinal fluid (pH 7). Means with different letters differ significantly ($p<0.05$).

$TCID_{50}$/mL within 10 min (figure 2B). In contrast, under feeding state, there was only ~1 log $TCID_{50}$/mL reduction in SARS-CoV-2 infectivity (figure 2D). To our knowledge, this is the first report that examines the effect of individual digestive enzymes on SARS-CoV-2 infectivity and shows that infectious SARS-CoV-2 can escape the stomach and intestinal inactivation during feeding. During meal consumption, the pH of the stomach rises to ~6, and the meal's effect on gastric pH may still be apparent for over 3 hours, after which the pH decreases to ~2.5.[3] The feeding gastric fluid used in our study represented the acidity (pH 3) of the stomach fluids when the stomach is 75% empty within 3–6 hours of consuming a high-fat Food and Drug Administration (FDA) FDA meal, suggesting that at a higher pH during early hours of digestion, there would be even lesser inactivation effect on SARS-CoV-2. The PPIs are known to raise the pH of the stomach, allowing microbes to escape the gastric pH barrier, which leads to increased acute gastroenteritis and community-acquired pneumonia.[5 6] In fact, Middle East respiratory syndrome coronavirus(MERS-CoV) injected intragastrically in mice showed worse outcomes when the mice were pretreated with antiacid drugs.[7] Previous studies that assessed the effect of pH on SARS-CoV-2 infectivity are unreliable as they did not simulate the temperature or physiological fluids of the stomach as well as studies using biorelevant fluids because the authors did not supplement the fluids with digestive enzymes nor did they test the consecutive effect of the fluids[8 9] (see online supplemental material). The inverse proportional effect of bile on SARS-CoV-2 might be explained by the fact that bile salts form primary micelles at lower concentrations (representing fasting state) which can be better at solubilising the lipid bilayer in the SARS-CoV-2's envelope than stabilised micelles formed at higher bile concentrations (representing fed state).[10]

Taken together, higher stomach pH and higher bile concentrations allow ingested SARS-CoV-2 to escape the GI inactivation, which would give the virus a higher chance to infect the intestine, supporting Lee *et al*'s speculations. Further studies using dynamic in vitro digestion models, animal models and human biopsies from patients with COVID-19 are needed to understand the various factors affecting the infectivity of SARS-CoV-2 as it passes through the GI tract.

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Materials and Methods

Preparation of viral stocks: The US reference strain SARS-CoV-2 USA-WA 1/2020 (BEI resources NR-52281) was propagated in African green monkey kidney cells (Vero E6 ATCC CRL-1586) originally at Dr. RJ Hogan's BSL3 laboratory (College of Veterinary Medicine, University of Georgia, Athens, GA) as described previously [1] and gifted to our laboratory. Handling of SARS-CoV-2 was done under strict BSL3 biosafety protocols at the Center for Food Safety BSL3 laboratory. Vero E6 cells were propagated in DMEM +10% FBS. All cell culture media were supplemented with 1% antibiotic-antimycotic cocktail. One or two-day-old 90% confluent cells were used to prepare virus stocks using a multiplicity of infection of 0.01. Harvesting the virus was done between 62-72 h of incubation. Infected cells were collected from the flasks and centrifuged at low speed (300 x *g* for 10 minutes at 4 °C) to pellet the cell debris, while supernatants containing the virus, were aliquoted (100-200 µl) and stored at -80 °C. SARS-CoV-2 was ultra-filtered through an Amicon® 100K Ultra-15 (Millipore) immediately after harvest to remove virus-interfering cell culture debris and to exchange the virus cell culture media with water which is a more relevant matrix [2]. An aliquot of the virus was immediately titrated as described below.

Effect of gastric pH on SARS-CoV-2 infectivity: Previous studies that assessed the effect of pH on SARS-CoV-2 infectivity are unreliable as they did not simulate the temperature or physiologic fluids of the stomach [3, 4]. Furthermore, a previous simulation study showed that the main Protease for SARS-CoV-2 remains stable at pH 3 [5]. Also, it is predicted that the variation of pH is of relevance to the virus cell entry and exit, because it affects the folding and unfolding state of the S protein and its affinity to ACE2 [6, 7]. Therefore, a gastric fluid with various pH was prepared following the protocol published previously [8]. Specifically, 8.3 g proteose peptone, 3.5 g D-

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glucose (anhydrous), 2.05 g sodium chloride, 0.6 g potassium dihydrogen phosphate, 0.11 g calcium chloride, 0.37 g potassium chloride, 0.05 g porcine bile extract, 0.1 g lysozyme (from hen egg white), and 0.0133 g pepsin were mixed in 1L water. All chemicals were purchased from Sigma-Aldrich. The fluid was then divided into aliquots and the pH was adjusted to 1.5, 2, 2.5, 3, 3.5, 4, 5 and 6 using 0.5 M HCl. The various aliquots were filter sterilized (0.45 µm), saved at 4 °C, and used within one day. The volume of 2.5 M NaOH required to neutralize each of these different pH adjusted buffers was pre-determined before testing on SARS-CoV-2. Three technical replicates were tested for each buffer. The whole experiment was repeated two more times with freshly made buffers. Averages and standard errors were calculated from the nine technical replicates

Effect of pepsin on SARS-CoV-2 infectivity: Porcine pepsin (Sigma) was prepared at 10% (100 mg/ml) in a gastric fluid (pH 3) which was prepared as described previously [9]. In the literature, pepsin was reported to be used at various concentrations, specifically 0.01 mg/ml [8], 0.1 mg/ml [10], 3.5 mg/ml [11], and 8 mg/ml [9]. Therefore, pepsin was tested at a range of concentrations (0.1, 1, 4 and 8 mg/ml) to determine its effect on SARS-CoV-2 infectivity.

Effect of pancreatin on SARS-CoV-2 infectivity: Porcine pancreatin (8X USP, Sigma) was prepared at 5% (50 mg/ml) in an intestinal fluid (pH 7) as described previously [9]. Pancreatin was used previously at a concentration of 5 mg/ml to test its effect when combined with bile on influenza A [11], therefore, pancreatin was tested at a range of concentrations up to 5 mg/ml for its effect on SARS-CoV-2 infectivity.

Effect of bile on SARS-CoV-2 infectivity: Porcine bile extract (Sigma) was prepared at 2% in an intestinal fluid (pH 7) as described previously [9]. The concentration of fresh bile salts in the 2% extract was measured using the total bile acid assay kit (Cell Biolabs, Inc) and determined to be

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38 mM. Bile concentrations in the small intestine can fluctuate from as low as 2.6 mM in fasted state to over 15 mM in the fed state [12]. Therefore, bile was tested for its effects on SARS-CoV-2 infectivity at 0.5, 3, 10 and 15 mM.

All preparations were filter sterilized (0.45 µm) and used immediately. Three technical replicates were tested for each digestive enzyme and bile. The whole experiment was repeated three more times from freshly made preparations. Averages and standard errors were calculated from the twelve technical replicates.

Effect of in vitro simulated digestion on SARS-CoV-2 infectivity: To test the effect of digestion on SARS-CoV-2 infectivity, the commercially available and commonly used [13] gastric (FaSSGF or FEDGAS supplemented with pepsin at a final concentration of 8 mg/ml) and intestinal (FaSSIF or FeSSIF supplemented with pancreatin at final concentration of 5 mg/ml) fluids simulating fasting and feeding states were prepared as per manufacturer's instructions (Biorelevant, UK). For fasting state, the **Fa**sting **S**tate **S**imulating **G**astric **F**luids (FaSSGF, pH 1.6, containing 0.08 mM taurocholate a bile salt, 0.02 mM phospholipids, 34 mM sodium and 59 mM chloride) and the **Fa**sting **S**tate **S**imulating **I**ntestinal **F**luids (FaSSIF, pH 6.5, containing 3 mM taurocholate, 0.75 mM phospholipids, 148 mM sodium, 106 mM chloride and 29 mM phosphate) were used. For feeding state, the gastric fluid FEDGAS (pH 3) representing a high-fat FDA meal and containing fats, carbohydrates, dietary fibers and bile salts at 62.5, 62.5, 3.1, 0.3 g/900 ml, respectively was used. FEDGAS pH 3 represents a high-fat FDA meal when the stomach is 75% empty within 3-6 hours after a meal. The intestinal fluid simulating feeding was FeSSIF (pH 5) containing 15 mM taurocholate, 3.75 mM phospholipids, 319 mM sodium, 203 mM chloride and 144 mM acetic acid.

SARS-CoV-2 was incubated with each gastric and intestinal fluid separately for 5 or 60 min and with the gastric fluid (5 min) followed by intestinal fluids (5 min) for a total of 10 or 120 min

(named gastric-intestinal phase). All incubations were performed at 37°C and neutralized before testing on Vero Cells. Also, for gastric-intestinal digestion experiments the gastric fluid samples were neutralized before proceeding to the intestinal digestion phase. In addition, before testing on Vero cells, all samples were centrifuged at 18,000 rpm for 10 min at 4°C to remove the fat content. The detection limit for the samples from fasting state fluids on Vero cells was 1.72 log $TCID_{50}$/ml whereas those from feeding state was 2.72 log $TCID_{50}$/ml. The whole experiment was repeated one more time from freshly made fluids. Averages and standard errors were calculated from the six technical replicates.

While these biorelevant fluids have been used in two previous studies assessing their effect on SARS-CoV-2, however, the authors did not supplement the fluids with digestive enzymes nor did they test the consecutive effect of the fluids [14, 15]. For example, Zang et al 2020 study tested only simulated fasting fluids for gastric, small intestine and colonic fluid (FaSSGF, pH 1.6 and FaSSIF-Ve, pH 6.5) and FaSSCoF, pH 7.8, respectively) and showed that SARS-CoV-2 was inactivated within 10 to 60 min by ~95 to 99% and 20 to 60% in gastric and intestinal fluids, respectively. However, our results for the same types of fluids during fasting state showed much higher inactivation (>3 log i.e. 99.9%) within 5 min. The difference with our study maybe due to their use of a recombinant virus (a recombinant SARS-CoV-2 virus containing mNeonGreen) and the lack of digestive enzymes in their fluids. The other study by Lee et al 2020 tested digestion under both fasting and feeding states and reported similar results with our gastric fasting and intestinal feeding fluids showing a 99.99% (i.e. 4 log) reduction of SARS-CoV-2 infectivity but within 30 min. However, their data regarding gastric feeding fluids and intestinal fasting fluid showing no significant inactivation for SARS-CoV-2 infectivity, contrasted with our data for both of these fluids and that of Zang et al 2020 for the fasting fluid. This is likely because Lee et al (2020), used

gastric feeding buffer at a much higher pH than ours (pH 5 versus pH3, respectively) and our data showed that at pH 5 there was little inactivation for SARS-CoV-2 in gastric buffer at 37 °C for 60 min. Again, the authors did not supplement their gastric or intestinal fluids with digestive enzymes. Notably, these two previous studies reported performing one experiment with three technical replicas, while our data represent all three independent experiments with three replicates for each experiment.

Determination of virus infectivity: The $TCID_{50}$ assay was performed as described in our previous research [2]. Briefly, 1-2 day-old 90% confluent cell monolayers in 96-well plates were infected in quadruplet with serially diluted samples (1:10) in cell culture media supplemented with 2% FBS and 1% anti-anti and incubated at 37 °C. The plates were inspected for cytopathic effects (CPE) between day 4-5 post-infection. Viral titers were estimated following the Reed-Muench equation for the calculation of $TCID_{50}$ [16]. In addition to experimental controls, control positive (virus with known titer) and control negative (cell culture media) were included in each experiment.

Statistics: GraphPad Prism version 5 (GraphPad Software, USA) was used for all statistical analyses. The entire data set was transformed to $\log_{10}$. The log reductions in infectivity were calculated based on SARS-CoV-2 infectivity in untreated samples incubated under the same conditions. One way or two-way analysis of variance (ANOVA) followed by Tukey or Bonferroni post-tests, respectively were used to determine significant differences in mean infectivity titers. The factors analyzed included time and treatment. Differences in means were considered significant when the *P* value was less than 0.05 and are denoted in the figures by either alphabets or asterisks. Data are expressed as the mean ± standard error (SE).

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